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Study of the Growth-Promoting Effect of Insulin in Relation to Carbohydrate Metabolism in Organ Culture of Rat Mammary Carcinoma*

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INTRODUCTION

IN A PREVIOUS paper [1], it was shown that insulin stimulated DNA synthesis and mitotic activity in explants of carcinogen-induced, hormone-dependent mammary carcinomas of the rat in organ culture. Preliminary data suggested that insulin had little effect on glucose utilization by the explants and thereby raised the question as to whether the growth-promoting effect of insulin was mediated through an effect on carbohydrate metabolism. It is the purpose of this paper to extend the study of the growth-promoting effect of insulin in relation to carbohydrate metabolism.

MATERIAL AND METHODS

Mammary tumors

Mammary carcinomas were induced in Sprague–Dawley female rats by a single feeding of 7,12-dimethylbenz(a)anthracene [2].

Culture technique

The cultures were carried out essentially as described previously [1]. The explants, composed each of twelve tumor-tissue fragments arranged on a disc of cellulose-acetate fabric, were maintained at the surface of 2 ml of medium 199 (B.B.L., Baltimore) in Petri dishes. The explants were cultured for 2 days in an atmosphere of 95% O_2 and 5% CO_2 at 37°C.

Assay for DNA synthesis and glucose consumption

Cell proliferation was estimated by measuring thymidine-^{14}C incorporation into DNA. Twenty-four hours before the end of culture, the explants were exposed to fresh medium containing thymidine-^{14}C (Calbiochem, sp. act. 17·1 mc/mM), 25 mμc/ml. The specific radioactivity of DNA was measured at the end of culture and expressed as count/min/μg DNA-P [1].

The glucose concentration in the culture medium was measured by the glucose-oxidase method using Glucostat reagents (Worthington Biochem. Corp., N.J.). Glucose consumption was defined as the amount of glucose consumed during the last 24-hr period of culture per unit DNA-P in the explant.

Additions to the culture medium

Insulin (Calbiochem, 24·3 iu/mg) was added at a concentration of 40 μg/ml. The concentrations of glucose and fructose are indicated in the text. Pyruvate (Na salt) was added at a

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concentration of 308 mg% (equivalent to 250 mg% glucose with respect to carbon units); an equivalent amount of Na ions was subtracted from the salt solution.

Experimental design

Each experiment was carried out with tissue fragments from a single tumor. Each experimental condition was applied to four replicate explants, composed each of twelve tissue fragments, as stated above. The results were expressed as the means of the four replications. Only the results pertaining to tumors with insulin-sensitive growth *in vitro*, as defined by a significant ($p<0{\cdot}01$) increase in thymidine-^{14}C incorporation caused by insulin, are presented here. Results from insulin-independent tumors [1] are not included.

RESULTS

Relative effects of insulin on thymidine-^{14}C incorporation and glucose consumption

The relative effects of insulin on 24-hr thymidine-^{14}C incorporation and glucose consumption were studied in 2-day cultures of sixteen mammary tumors. Glucose concentration in the culture medium was 250 mg%. The results are plotted in Fig. 1. The marked stimulation of thymidine-^{14}C incorporation effected by insulin contrasts with the small and inconstant effect on glucose consumption. Increase in glucose consumption was less than 25% in all but four tumors and was absent in about half of them. There was no obvious correlation between these two variables.

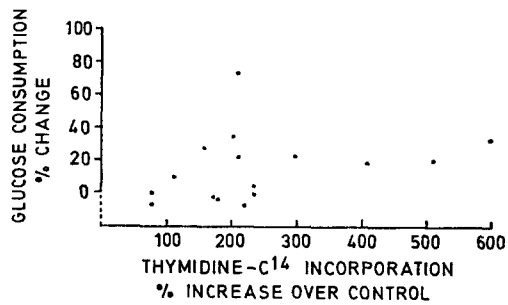


Fig. 1. Relative effects of insulin on thymidine-^{14}C incorporation and glucose consumption in sixteen mammary tumors. Glucose concentration, 250 mg %. Each point represents the mean value for four replicate explants.

Effect of increasing glucose concentration on thymidine-^{14}C incorporation

The experimental findings are given in Table 1. Regardless of the presence or absence of insulin, increasing glucose concentration up to 1000 mg% and 2000 mg%, resulted in no increase, but rather in a small decrease, in thymidine-^{14}C incorporation.

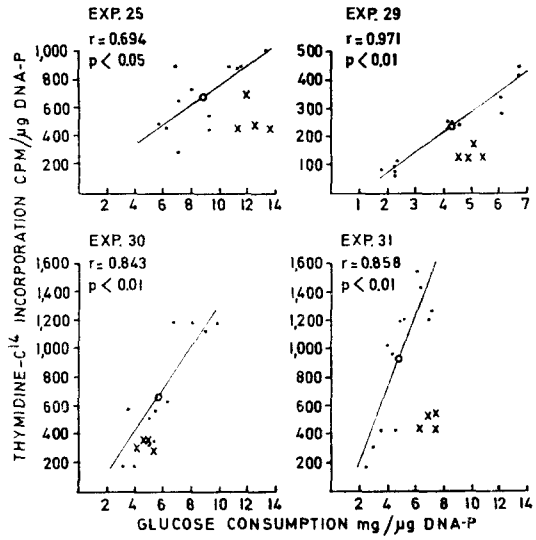


Fig. 2. Correlation between thymidine-^{14}C incorporation and glucose consumption, as measured in the absence of pyruvate in experiments no. 25, 29, 30 and 31 of Table 3. The black dots (●) represent individual values for explants cultured in the presence of insulin, at glucose concentrations of 50 mg %, 100 mg % and 250 mg %. The lines represent the regression of thymidine-^{14}C incorporation on glucose consumption for these explants. r is the correlation coefficient between these values and p estimates its statistical significance. The crosses (x) designate the values found for the explants cultured in the absence of insulin, at glucose concentration of 250 mg %.

Table 1. Effect of increasing glucose concentration in the culture medium on thymidine-^{14}C incorporation into DNA (count/min/µg DNA-P)

[Mean results for four replicate explants]

Exp. no.		Glucose concentration (mg %)			
		250	500	1000	2000
2	Control	130			49
	Insulin	860			552
4	Control	204	152	135	
	Insulin	682	674	575	

Table 2. Comparison of glucose and fructose as substrates

[Thymidine-^{14}C incorporation (count/min/μg DNA-P). Mean results for four replicate explants]

Exp. no. 2		Glucose (mg %)	Fructose (mg %)				
		250	250	500	750	1000	1500
6	Control	176	151				
	Insulin	648	208				
7	Control	120	124				
	Insulin	613	208				
12	Control	199	158	233	210		
	Insulin	1332	268	894	1069		
23	Control	209		234		208	154
	Insulin	820		794		786	777

Fructose vs. glucose

Experiments were carried out to compare fructose, at different concentrations, and glucose as substrates. The results are given in Table 2. In the absence of insulin, thymidine-^{14}C incorporation was the same whether glucose, 250 mg%, or fructose, at concentrations ranging from 250 mg% to 1500 mg%, was used as the substrate. In the presence of insulin, higher concentrations of fructose than of glucose were needed to reach similar levels of thymidine-^{14}C incorporation.

Reduction of glucose concentration vs. omission of insulin. Addition of pyruvate

The foregoing data did not seem to fit the simple interpretation whereby insulin would stimulate thymidine-^{14}C incorporation merely by increasing the amount of glucose transferred into the cells. The following experiments were designed to further challenge this interpretation. According to it, sufficient reduction of glucose concentration in the presence of insulin should bring about exactly the same situation as the lack of insulin. In both of these presumably

Table 3. Comparison of two types of experimental conditions: (a) *reduction of glucose concentration in the presence of insulin;* (b) *omission of insulin at glucose concentration of* 250 mg %. *Effect of addition of pyruvate under these conditions*

[Thymidine-^{14}C incorporation (count/min/μg DNA-P). Mean results for four replicate explants]

Exp. no.	No insulin	Insulin added							
Glucose concentration (mg %)	250	0		50		100		250	
Pyruvate (308 mg %)	0 +	0	+	0	+	0	+	0	+
10	167 138 n.s.*							608 621 n.s.	
11	192 207 n.s.							522 450 n.s.	
25	525	128		423 717 $p<0{\cdot}01$		707 770 n.s.		919 840 n.s.	
29	145 140 n.s.	18		87 246 $p<0{\cdot}01$		247 406 n.s.		376 500 $p<0{\cdot}05$	
30	388 284 n.s.	10		254 618 $p<0{\cdot}025$		575 640 n.s.		1198 722 $p<0{\cdot}01$	
31	493 409 n.s.	10		332 634 $p<0{\cdot}025$		1104 968 n.s.		1366 939 $p<0{\cdot}01$	

*The differences were tested for statistical significance by Student's *t*- test.
n.s. = no significant difference ($p>0{\cdot}05$).

identical situations, the addition of a substrate not dependent upon insulin for entry into cells, such as pyruvate, should raise the depressed level of thymidine-^{14}C incorporation to the same extent. The experimental data are presented in Table 3 and Fig. 2.

Reduction of glucose concentration to 50 mg% in the presence of insulin depressed thymidine-^{14}C incorporation below the level observed in the absence of insulin (Table 3). This depression was clearly dependent upon a corresponding decrease in glucose consumption, as shown in the graphs of Fig. 2. Correlation between these variables was significant in all four experiments. In contrast, when depression of thymidine-^{14}C incorporation was effected by omission of insulin, it could not be accounted for by a corresponding decrease in glucose consumption. This is clearly seen in the graphs of Fig. 2 where all points representing the data obtained in the absence of insulin (crosses) fall below the regression lines constructed with the glucose-concentration data. The constant location of all sixteen experimental points below the lines indicates a highly significant difference.

The effect of adding pyruvate on the depressed level of thymidine-^{14}C incorporation was next studied. The results are presented in Table 3. In the presence of insulin at glucose concentration of 50 mg%, addition of pyruvate always produced a marked and significant increase in thymidine-^{14}C incorporation. In contrast, stimulation of thymidine-^{14}C incorporation by pyruvate never occurred in the absence of insulin. Under the other experimental conditions of Table 3 (with insulin, glucose 100 mg % and 250 mg%), addition of pyruvate did not produce consistent changes. Significant effects were observed in only three cases out of ten: thymidine-^{14}C incorporation was increased in one case (Exp. no. 29). and decreased in two cases (Exp. no. 30 and no. 31).

DISCUSSION

The growth-promoting activity of insulin has been demonstrated in several tissues *in vitro*. It was originally reported in primary explant cultures [3]. It was also demonstrated in strains of continuously cultivated cells [4] and in the ear epidermis of the mouse [5]. In organ culture, insulin has been considered by Trowell [6] as a growth-promoting factor for a variety of tissues and was routinely incorporated in his culture media. Recently, extensive experiments have been conducted on the mammary gland in organ culture. It was found that insulin was required for the maintenance of explants from the mammary gland of several animal species [7, 8]. In addition, it produced a marked ductal hyperplasia in the mammary gland of the mouse [9, 10] and in the human breast [11]. Finally, it was shown to stimulate DNA synthesis and mitotic activity in organ cultures of the normal mammary gland of the mouse [12] and of carcinogen-induced, hormone-dependent mammary carcinomas of the rat [1].

The mechanism of the growth-promoting action of insulin on tissues *in vitro* has been little studied as yet. In a recent review of this problem, Lasnitzki [13] made the following statement: "the promotion of growth is associated with an increase of glucose uptake and utilization. It is not certain which is the primary effect: stimulation of cell proliferation followed by a rise in glucose consumption or an increased glucose utilization which makes a rise in cell multiplication possible". Although there is little doubt that insulin affects certain metabolic reactions in the absence of glucose in different tissues [14, 15], it is generally agreed that the principal physiological action of insulin is to increase glucose transfer across the cell membrane [16]. In view of these considerations, the finding reported here that the growth-promoting effect of insulin on the rat mammary carcinoma was accomplished with little or no effect on glucose consumption was therefore of interest.

Moretti and DeOme [17] have shown that insulin failed to increase glucose consumption in explants of a mammary carcinoma of the mouse while a marked increase occurred in the normal mammary gland. In the rat mammary tumor studied here, the lack of effect of insulin on glucose metabolism was not as constant as in the mouse tumor. This may be related to the fact that, unlike the mouse tumor, the rat mammary carcinoma has retained to a variable extent some of the properties of the normal gland, as reflected by its hormone dependence *in vivo*.

The point of interest is that, whereas the effect of insulin on glucose consumption was partially or completely lost in the rat tumor, its stimulating effect on cell multiplication was retained. This observation makes it difficult to accept the simple view that insulin stimulated cell proliferation by merely accelerating glucose transfer into the cell.

Additional evidence against the simple glucose-transfer hypothesis was derived from the study of cell proliferation as influenced by changes in carbohydrate substrates. Failure to increase thymidine incorporation by raising

glucose concentration fourfold and eightfold suggests that glucose availability was not rate limiting, unless the transport mechanism was already saturated. Clarification of this point by measuring glucose uptake was not attempted because, at high glucose concentration, the percentage utilized was too small to be accurately determined. Testing fructose in a wide range of concentrations as a substitute for glucose seemed justified because fructose in the absence of insulin is metabolized faster by several tissues *in vitro*, and thereby duplicates, at least partly, the effect of insulin with glucose as substrate. This is so in adipose tissue [18], in mouse epidermis [5] and in some strains of continuously cultivated cells [4]. However, in our experiments, fructose proved no more effective than glucose in sustaining thymidine incorporation in the absence of insulin. In the presence of insulin, higher concentrations of fructose than of glucose were required to produce comparable effects.

The most significant evidence against the simple glucose-transfer hypothesis in our system was provided by the experiments involving reduction of glucose concentration as well as pyruvate addition. In accordance with this hypothesis, the reduction of glucose concentration in the presence of insulin to levels where glucose availability becomes rate limiting should realize exactly the same situation as the omission of insulin. In both cases, glucose availability inside the cell would be the only rate-limiting factor. That these situations in fact are not the same is clearly demonstrated by two observations. First, reduction of glucose concentration in the culture medium resulted in a much sharper decrease in glucose consumption than did the omission of insulin. Secondly, the addition of pyruvate to rate-limiting concentrations of glucose in the presence of insulin markedly increased thymidine-^{14}C incorporation. In contrast, when omission of insulin was the rate-limiting factor, the addition of pyruvate, a substrate not dependent upon insulin for entry into cells, never displayed any stimulating effect. It would therefore appear that omission of insulin did not depress thymidine-^{14}C incorporation by simply limiting glucose availability inside the cell.

It is concluded that two main properties of insulin which are usually associated in tissue and organ culture, namely stimulation of glucose metabolism and promotion of growth, are dissociated in explants of the rat mammary carcinoma, possibly by virtue of incomplete and selective dedifferentiation. In addition, concordant evidence is presented which suggests that promotion of growth by insulin in these explants is not mediated through a stimulating effect on glucose uptake and utilization.

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RESUME

Dans un travail précédent, nous avons décrit l'action stimulante de l'insuline, en culture organotypique, sur la croissance d'un carcinome mammaire hormono-dépendant du rat, induit par administration d'un carcinogène. Nous avons étudié ici cet effet de l'insuline en relation avec le métabolisme des hydrates de carbone.

Nous avons trouvé que la stimulation de croissance par l'insuline, mesurée par l'incorporation de la thymidine-^{14}C dans l'ADN, s'accompagne d'un effet faible ou nul sur la consommation du glucose. L'accroissement de la concentration du glucose, jusqu'à une valeur 4 ou 8 fois supérieure à celle habituellement utilisée, n'augmente pas le niveau de la prolifération cellulaire. Le remplacement du glucose par du fructose, à diverses concentrations, ne permet pas de reproduire, même partiellement, l'effet de l'insuline. A faible concentration, en présence d'insuline, le glucose s'avère un facteur limitatif. Dans ce cas, sa consommation s'abaisse à des valeurs inférieures à celles observées en l'absence d'insuline; à ce moment, l'addition de pyruvate relève fortement le niveau de prolifération cellulaire. En revanche, en l'absence d'insuline, le pyruvate ne manifeste jamais d'effet stimulant sur la prolifération cellulaire.

Ces données permettent de conclure que deux propriétés importantes de l'insuline, qui sont habituellement associées en culture d'organe et de tissu, à savoir la stimulation du métabolisme du glucose et l'induction de la croissance, apparaissent en fait dissociées dans

les explants des carcinomes mammaires du rat; ce fait s'explique peut-être par une dédifférenciation sélective. D'autre part, ces données semblent bien montrer que l'induction de la croissance cellulaire de ces explants par l'insuline ne s'explique pas par un effet stimulant sur la consommation du glucose.

SUMMARY

The previously reported growth-promoting effect of insulin on explants of carcinogen-induced, hormone-dependent mammary carcinomas of the rat in organ culture has been studied in relation to carbohydrate metabolism.

It was found that the growth-promoting effect of insulin, as measured by thymidine-^{14}C incorporation into DNA, was accomplished with little or no effect on glucose consumption. Raising the concentration of glucose four- or eightfold above the usual level failed to increase cell proliferation. Substituting fructose, in a wide range of concentrations, for glucose failed to duplicate, even partly, the effect of insulin. At low concentration, in the presence of insulin, glucose became a rate-limiting factor. In this case, glucose consumption decreased to lower values than those observed in the absence of insulin; moreover, addition of pyruvate markedly raised the depressed level of cell proliferation. In contrast, in the absence of insulin, pyruvate always failed to stimulate cell proliferation.

These data led to the conclusion that two main properties of insulin which are usually associated in tissue and organ culture, namely stimulation of glucose metabolism and promotion of growth, are dissociated in explants of the rat mammary carcinoma, possibly by virtue of selective dedifferentiation. In addition, these data strongly suggest that promotion of growth by insulin in these explants was not mediated through a stimulating effect on glucose uptake and utilization.

ZUSAMMENFASSUNG

Die in einem früheren Artikel beschriebene wachstumsfördernde Wirkung des Insulins auf Gewebskulturen eines durch Karzinogene erzeugten, hormonabhängigen Mamma-Ca der Ratte, wurde jetzt in Organkulturen in Abhängigkeit zum Kohlehydratstoffwechsel studiert.

Wir fanden, dass die wachstumsfördernde Wirkung des Insulins, gemessen am Finbau von C^{14} markiertem Thymidin in die DNS, nur eine sehr schwache oder gar keine Wirkung auf den Glukosestoffwechsel hat. Auch die Steigerung der Glukosekonzentration auf das 4 bis 8 fache vermehrt die Zellproliferation nicht. Ersetzt man Glukose durch Fructose (in verschiedenen Konzentrationen) so bleibt die Insulinwirkung völlig aus. Eine niedrigere Glukosekonzentration begrenzt bei Anwesenheit von Insulin die Wachstumsrate. In diesem Fall sinkt der Glukoseverbrauch auf tiefere Werte als wenn kein Insulin vorhanden ist. Gleichzeitig erhöht der Zusatz von Pyruvat die Zellproliferation beträchtlich. Ist kein Insulin vorhanden, so kann Pyruvat die Proliferation nicht stimulieren.

Diese Versuche erlauben folgende Schlussfolgerungen: die zwei Haupteigenschaften von Insulin, die Stimulierung des Glukosestoffwechsels und die Wachstumssteigerung, die Organ- und Zellkulturen gemeinsam auftreten, verhalten sich in der Gewebekultur von Rattenbrustkrebs verschieden. Diese Tatsache könnte eventl.- durch eine selektive Differenzierung erklärt werden. Weiterhin kann man aus diesen Versuchen ableiten, dass die Wachstumsförderung durch Insulin in diesen Gewebskulturen nicht durch die Stimulierung der Glukoseaufnahme und Verwertung durch das Insulin bedingt ist.

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The Proliferative Activity of Plasma Cells from Plasmocytoma *in vitro**

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THE IMPLICATION of the term "myeloproliferative disease" has been debated in recent years. Clinical and experimental data have shown that the mitotic activity of a population of malignant blood cells (that is: the proliferative, non-proliferative and expanding compartments) is not as has been previously assumed. On the other hand, the evaluation of the proliferative activity of the pathological cells involved in these diseases cannot be precisely defined, due to

(1) the variable extent of the proliferative compartment of the haematopoietic tissue and organs;

(2) the mitotic activity of malignant blood cells is not always comparable to that of their normal counterparts.

Stathmokinetic tests [1, 2] have shown that the plasma cells from cases of plasmacytoma (multiple myeloma) have a low rate of proliferation [3]; similar behaviour was observed in acute leukaemia [4]. In order to obtain further data on the proliferative capacity of plasma cells from plasmocytoma we have studied their incorporation of tritiated thymidine, by incubating bone marrow samples with the isotope *in vitro*.

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METHODS AND MATERIALS

Thirteen patients with plasmocytoma and one case of Waldenstrom's macroglobulinaemia were studied.

The plasma proteins in 9 of these cases were identified by electrophoresis, in 4 other cases and in the patient with Waldenstrom's macro globulinaemia, the proteins were identified immunologically. The first group of 9 patients were studied by the addition of ^{3}H-TdR to a culture of bone marrow cells; the isotope was added 2 hours after commencing the culture and the incubation continued for a further 2 hours. The second group of 4 patients and the case of Waldenstrom's macroglobulinaemia were studied by adding the ^{3}H-TdR for 1 hr, for it has been shown that labelling occurs within that time [5]; this labelling was made immediately after taking the sample and after 4 hr and 8 hr in culture.

In all the experiments ^{3}H-TdR (specific activity 1·9 c/mMole), obtained from the Radiochemical Centre, Amersham, Bucks, England, was added to the culture in a concentration of 2 μc/ml. The cells were cultured in Hanks solution with heparin. Cell smears were methanol fixed, coated with Kodak AR-10 stripping film, exposed for 1 week at 4°C, developed and stained by the May Grünwald-Giemsa method. The differential cell count, percentage of labelled cells, and mean grain counts were determined. Cell counts were on 1000 or 2000 cells.

RESULTS

1st group of patients

Table 1 shows the percentage of plasma cells (mature and immature) in the bone marrow and the labelling index (labelling index=no. of labelled cells/1000 cells [5]). It can be seen that there was a low frequency of plasma cells incorporating ^{3}H-TdR. Oen patient, no. 3, had a high labelling index, 270; the plasma of this patient contained α 2-β myeloma protein, and was the only one of this type in the series. The plasma cells in Case No. 2 had large swollen cytoplasm, suggesting the storage of intracellular material.

Table 1. Plasmocytoma–labelling pattern (1st group of cases)

Case	Plasma cell °/₀₀ in total marrow cells	Labelling index
1	480	60
2	400	20
3	610	270
4	100	30
5	950	40
6	700	125
7	950	50
8	500	40
9	610	25

2nd group of patients

Immunological techniques showed 3 of them to have plasmocytomas producing gamma-G (IgG), one gamma-A (IgA) and one Waldenstrom's macroglobulinaemia (IgM). All these patients had skeletal involvement and the plasma cells were mainly of the mature type. In the case of Waldenstrom's macroglobulinaemia, the pathological cells in the bone marrow were lympho-reticular elements. These were distributed unevenly through the haemopoietic tissue, as is usual in this disease.

In this group of cases "flash" labelling with ^{3}H-TdR was made at different times during the period of culture, immediately after taking the marrow, after 4 hr and after 8 hr incubation. These experiments were made to see if the proliferative activity was altered by incubation *in vitro*. The results are shown in Table 2. There is a marked contrast in the labelling index of the plasma cells and the other proliferative cells in the bone marrow. It can be seen that both the erythroblasts, and the dividing leucocytes have an index of labelling higher than that of plasma cells. No incorporation of ^{3}H-TdR was observed in metamyelocytes or polymorphonuclear granulocytes. The cells from patients nos. 2 and 4 showed a considerable variation of labelling index as the duration of the culture increased, the frequency of normal blood forming cells was low in these samples. On the other hand, marrow from patients nos. 1 and 3 had fairly constant labelling indices; here there was still a considerable proportion of normal cells in the marrow. The results of these experiments are summarised in Fig. 1.

There is some evidence to suggest that the more immature cells have a higher uptake of ^{3}H-TdR than mature plasma cells, this has been observed in other cases of plasmocytoma [6], and a similar observation has been made on the plasma cells in normal bone marrow [7]. This would indicate a higher rate of DNA synthesis by these well labelled cells. The mean grain count/cell of the few labelled plasma cells was similar to that of the granulocyte precursors. The basophilic erythroblasts had a higher grain count than the granulocyte precursors, which occur in normal bone marrow [8, 10].

Table 3 shows the results obtained by investigating the bone marrow from the patient with Waldenstrom's macroglobulinaemia. The particular feature in this case is that the pathological cells show almost no evidence of labelling. On the contrary, the erythroblasts and granuloblasts showed normal labelling indices.

Table 2. Plasmocytoma-labelling pattern (2nd group of cases)

Differential count	°/₀₀				Labelling index				Mean grain count/cell			
Cases:	1	2	3	4	1	2	3	4	1	2	3	4
Erythroblasts	140	90	170	20	285	550	235	500	29	34	32	32
Myeloblasts and myelocytes	290	50	250	60	241	600	320	500	15	15	18·5	12
Metamyelocytes and polys	220	60	260	10	—	—	—	—	—	—	—	—
Plasmocytes	350	800	320	910	27	50	62·4	21	10·5	13·75	17·5	11·5

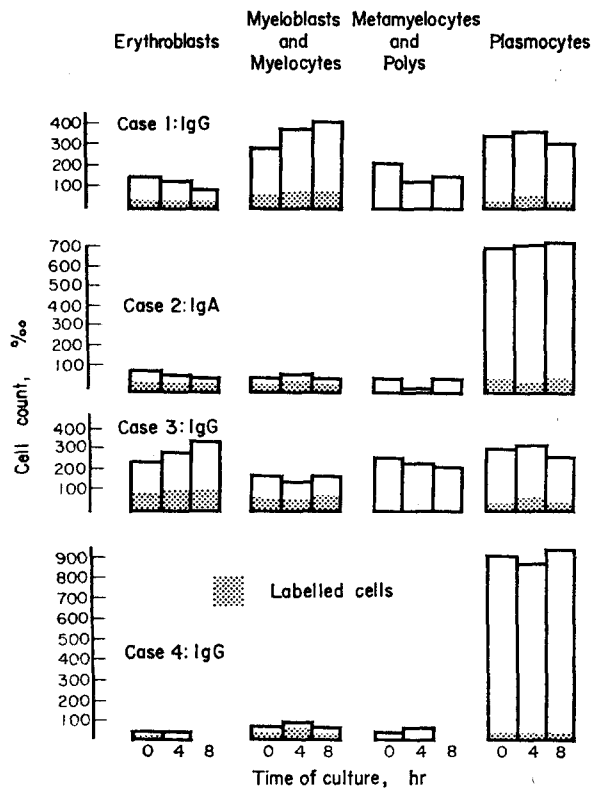


Fig. 1. *Effects of incubation on the labelling index.*

DISCUSSION AND CONCLUSIONS

The first point to be considered is the evaluation of the methods used to judge the proliferative activity of bone marrow in tissue and cell culture. This argument has been extensively reviewed both for the mitotic and stathmokinetic indices [9], and for the autoradiographic technique [10,11]. As both methods adopted have been proved reliable, and as both of them gave corresponding results showing a low proliferation in plasma cells from plasmocytoma bone marrow, it may be inferred that these cells are endowed with a low division rate. Such evaluation takes into account the mitotic rate of the other cells in human bone marrow, with particular regard to the precursors of erythrocytes and granulocytes. A similar conclusion was also drawn by "flash" labelling experiments *in vivo* [12].

The question is still open as to whether plasma cells and plasmoblasts from plasmocytoma are endowed with lower, equal or higher division rates than the corresponding cells from normal marrow. The same problem exists for the cells in acute leukaemia, and other forms of leukaemia. As far as this matter is concerned, some investigators [13, 14] obtained almost no evidence of ^{3}H-TdR incorporation into plasma cells from normal bone marrow, while plasma cells from plasmocytoma marrow showed some uptake. In a few experiments (not reported here) on plasma cells from normal bone marrow, we have obtained similar results. However, a comparison between plasma cells from plasmocytoma, and equivalent cells in normal marrow is very difficult, owing to:

(1) the relative paucity of plasma cells in normal bone marrow and,

(2) the prevalence in normal bone marrow of mature plasma cells, while in plasmocytoma immature cells are often prevalent.

On the other hand, it has been shown in the present experiments, as in previous ones [15], that the progressive cell maturation leads to a decrease in proliferative activity until a complete cessation of cell division is reached. The same behaviour was observed by investigating the proliferative activity of lymphocytes and plasma cells in experimental animals [16]. In our experiments this pattern is also seen in the granulocytic line, where the labelling index was rather high in myeloblasts and myelocytes, and no labelling was found in metamyelocytes and polymorphonuclears. In conclusion, it is hard to decide whether the differences between the normal bone marrow plasma cells, and the plasma cells from plasmocytoma bone marrow are due to different physiopathological conditions, or to different maturation stage.

The patient with Waldenstrom's macroglobulinaemia merits special mention as a particularly low proliferative activity was observed in the pathological cell series. While a definite interpretation of this phenomenon is not yet possible, the hypothesis is suggested

Table 3. Waldenstrom's macroglobulinemia

Differential count	‰			Labelling index			Mean grain count/cell		
	0h	4h	8h	0h	4h	8h	0h	4h	8h
Erythroblasts	270	260	280	148	269	143	29·5	33	37·5
Myeloblasts and Myelocytes	440	500	500	136	220	180	26·6	22	20
Metamyelocytes and polys	180	120	140	—	—	—	—	—	—
Lympho-reticular cells	110	120	80	—	—	—	—	—	—

that most of these cells (lympho-reticular elements) have become old when still in the undifferentiated stage, owing to a complete block of their maturation and differentiation. As a result, a proliferative defect of the cell population, taken as a whole, occurred. Such interpretation is also supported by the analogous phenomena observed in acute leukaemias of different degrees of differentiation: in undifferentiated acute leukaemia, the proliferative activity of the cell population, taken as a whole, is much lower than in the partially differentiated forms, like acute promyelocytic leukaemia.

RESUME

L'activité proliférative des plasmocytes et des plasmoblastes de la moelle de patients atteints de plasmocytome fut déterminée par marquage à la thymidine tritiée in vitro. *On observa une faible incorporation de l'isotope, indiquant une activité proliférative réduite des plasmocytes anormaux, similaire à celle qui a été observée précédemment par l'étude des index mitotiques et stathmocinétiques. Cependant, les indices de prolifération étaient plus élevés dans les cellules les plus immatures, de sorte qu'il est nécessaire que la maturité des cellules soit prise en considération.*

SUMMARY

The proliferative activity of plasma cells and plasmoblast marrow of subjects with plasmocytoma was determined by labelling with ^3H-TdR in vitro. *A low incorporation of the isotope was observed, indicating a poor proliferative activity of the abnormal plasma cell population, similar to that previously by observation of mitotic and stathmokinetic indices. However, the proliferative indices were higher in the more immature cells, so that cell maturity should be taken into account.*

ZUSAMMENFASSUNG

Die Proliferation von Knochenmarksplasmazellen und plasmoblasten von Plasmozytompatienten wurde mittels ^3H-TdR-Markierung in vitro *bestimmt. Der Markierungsindex war niedrig, was auf eine geringe proliferative Aktivität der pathologischen Plasmazellenpopulation hinweist. Dies entspricht früheren Beobachtungen über den Mitosen- und statmokinetischen Index. Es ist bemerkenswert, dass der Proliferationindex bei den unreifen Zellen höher war. Bei der Auswertung muss deshalb das Reifestadium der Zellen beachten werden.*

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Viral Factors in Human Carcinogenesis*

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THE NUMBER of known oncogenic viruses is steadily increasing. (Table 1). The list includes representatives of all of the four major DNA virus groups and two of seven RNA groups. So far no virus of helical symmetry (rhabdo-, paramyxo- and myxovirus) has been associated with tumor production nor any member of the picornavirus group, the only animal viruses that cause a true lysis of the host cell. The exceedingly large arbovirus group, not yet systematically studied in this respect, may still prove to include some members with oncogenic properties.

Even with typically oncogenic viruses, tumor induction *in vivo* or host cell transformation *in vitro* seem to be rare events. In most infected cells the virus enters into a lytic cycle leading to degeneration and death of the host cell; transformation occurs only at a rate of about one in a million.

Some, like polyomavirus, multiply in and transform any type of cell. Others like leukosis may multiply in practically all types of cells but preferably transform only one or a very restricted number of types. Papilloma virus, finally, has a very limited target range, the germinative epithelium of the skin and certain mucous membranes. These and other facts indicate that not only the virus but also host factors are instrumental in tumor induction and transformation. Interferon production may be one mechanism of importance in this respect, but otherwise the field is largely open for speculation.

Very often a transformed cell ceases to produce and release virus. It is generally assumed that the virus in such cases has become integrated into the genome of the host cell, a process presumably analogous to lysogenization of a bacterium. This assumption, although very likely, has yet to be experimentally proven. Immunity, in the sense used in bacteriophage research, i.e. resistance to superinfection and repression of superinfecting virus of the same type, is only seldom found in transformed animal cells. Another characteristic of a lysogenic bacterium is its sensitivity to induction with u.v. light, mutagenic or cancerogenic chemicals by which the prophage is derepressed and enters into the vegetative phase. Attempts by such methods to induce virus synthesis in non-releasing transformed animal cells have so far failed.

Nevertheless, since some degree of structural analogy between virus and host cell DNA would be a prequisite for integration, several authors have looked for similarities in their composition by means of hybridization experiments, base composition analysis, and recently 'nearest neighbor' analysis. The implications of the results so far obtained are not yet clear. It is indeed doubtful whether such studies *a priori* could be expected to yield any useful information. What is actually needed for integration —through crossing over—is a structural analogy of only minute parts of the genomes, corresponding to one locus of each of them. No methods of sufficient sensitivity are at present available for detection of such minor similarities

An important feature of virus-induced transformation is the acquisition by the cell of specific immunological characteristics. All cells transformed by the same virus irrespective of their origin carry common antigenic markers, and they differ antigenically from those transformed by other viruses. Some of the new antigens are apparently located in the cell surface with the character of transplantation

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Table 1. Viruses of known oncogenic capacity

Group	Type	Host
DNA-viruses		
Poxvirus	Molluscum contagiosum	Man
	Yaba	Monkey
	Fibroma	Monkey
Herpesvirus	Bovine rhinotracheitis	Cattle
	Lucké	Frog
Adenovirus	8 human	Hamster, Mouse
	6 simian	
	1 bovine	
	1 avian	
Papovavirus	Papilloma subgroup	Man, several species of mammals
	Polyoma	Mouse and other rodents
	SV 40	Hamster
RNA-viruses		
Paramyxovirus	———	———
Rhabdovirus	———	———
Myxovirus	———	———
Leukosis virus	Fowl leukosis several immunological types	Chicken and other birds
	Rous sarcoma	Birds, several mammals
	Murine leukosis, several immunological types	Mouse, Rat
	Bittner	Mouse
	Cat leukemia	Cat
Reovirus	Wound tumor	Plants
Arbovirus	———	———
Picornavirus	———	———

antigens. Certain mechanisms regulating cell growth, such as contact inhibition, are obviously triggered from the cell surface and structural changes in this region might therefore offer important clues to the problem of the nature of malignancy. For this reason the immunological aspects of the process of transformation deserve particular attention.

Generally speaking virus-induced antigens fall into three main classes.

(a) *early proteins*; mainly enzymes needed for synthesis of virus nucleic acid; not incorporated into the mature virion.

(b) *structural proteins*; synthesis initiated after appearance of virus nucleic acid; represent structural elements of the virus particle.

(c) *cell surface antigens*; are identical with the proteinaceous projections on the surface of enveloped virions and are transferred to the virion in the process of maturation by 'budding'. Also some naked viruses (adeno- and papovavirus) induce synthesis of specific cell surface antigens which, however, in these cases are not incorporated in the virion.

The DNA virus genome seems to contain two operons coding for the early enzymes and for the structural proteins, respectively, and coupled in a switch mechanism. Thus, when DNA synthesis is initiated, production of early proteins is switched off and that of structural proteins switched on. The enzymes formed in the first phase then gradually disappear and are no longer demonstrable at the time of virus maturation. They are thus not released from a cell undergoing a so-called lytic cycle* of infection, they do not appear extracellularly and consequently do not normally induce antibody formation in an infected animal. If DNA synthesis is blocked with FUDR, the switch mechanism does not operate: early protein is continuously synthesized but structural proteins are not. The same seems to be true of tumor transformed, non-releasing cells in which no virus DNA is being formed. Such cells produce early protein and apparently release it; tumorbearing animals produce antibodies to these antigens.

The specific antigens of virus-induced tumors, demonstrable in fluorescent antibody, complement fixation or cytotoxicity tests or by

*Among animal viruses only those of the picornavirus group seem to cause a true lysis of the host cell and to be released in a burst. All the others are set free through shedding (budding) or in a slow process of secretion with a concomitant gradual degeneration of the host cell.

means of graft reactions, are variously called neo-, tumor (T) or tumor specific transplantation (TST) antigens. The original assumption that they were distinct from the antigens appearing in the course of a 'normal' lytic cycle of virus reproduction is probably not correct. Thus, the fluorescent staining 'neo-antigens' are demonstrable in the early phase of the lytic cycle as well and the specific Gross lymphoma transplantation antigen seems to be indistinguishable from the virus envelope (V) antigen.

We have to assume, then, that all of the new antigens are coded for in the viral genome and that they are synthesized in lytic infections as well as in transformed cells. Whether or not infection will result in emergence of a tumor cell is at least partly dependent upon to what extent virus synthesis, maturation and degeneration of the host cell might be controlled. In some cases (Rous sarcoma virus) the virus is apparently genetically defective, unable to induce formation of mature virions without genetic supplementation by a helper virus. In other cases interferon production might help to establish an equilibrium between virus and host cell. Finally, integration of the virus genome could be one of the possible defense mechanisms by which control of virus synthesis might be achieved.

The phenomenon of immunological transformation which transgresses the species borders has made a screening of human tumors for a viral aetiology possible. The attempts so far made in this direction have not yielded very encouraging results. With one possible exception (see below) none of the viruses of known oncogenic capacity which have been tested seems to have been responsible for any of the human tumors examined. An approach to the problem along different lines, however, has yielded some evidence to indicate that at least one human malignancy, Burkitt's African lymphoma [1], might have a viral etiology.

As is now well known the epidemiology of the Burkitt lymphoma is clearly suggestive of a mechanism of insect transmission and thus of an infectious etiology. The search for possible etiologic agents has yielded several candidates. Many of these findings are most probably incidental and insignificant but two of them seem to deserve more serious discussion

In 1964 Bell *et al.* [2] described the isolation from biopsy material of a strain of reovirus type 3 and later [3] reported positive findings in 7 out of 31 cases. Stanley and his group in Australia established a transplantable lymphoma by passage of spleen cells from a mouse nine months earlier infected with reovirus type 3 [4]. According to these authors the mouse tumor displays many similarities to Burkitt's lymphoma as regards anatomical distribution, histopathology and behavior in tissue culture. No infective virus is formed but the tumor cells are said to contain a reovirus specific CF antigen. Stanley has assumed the virus to have become integrated in the host cell thereby causing malignant transformation [5, 6].

As indicated in Table 1, one reovirus, the wound tumor virus, is capable of inducing tumor formation in plants [7]. This virus is also one of the relatively few types capable of multiplying in insect vectors (leaf-hoppers) as well as in the host plant. Animal reoviruses have also been reported to be carried by arthropods, namely several species of culicine mosquitos [8, 9].

The evidence for reovirus as a cause of human lymphoma is thus mainly circumstantial. It should be remembered that reoviruses are widely distributed among warm-blooded animals, usually without causing overt disease and thus likely to be frequently encountered as passenger viruses. It would be somewhat surprising to find a virus outside the leukosis virus group associated with typical lymphoma. Several of the findings mentioned above seem to need confirmation, and unless further studies strengthen the candidacy of reoviruses as lymphoma agents a certain scepticism is justified.

Considerably more promising are studies initated in 1964 by Epstein *et al.* [10]. Several cell lines have been derived from Burkitt tumor material. In almost all of these, intracellular particles of an umistakable virus character have been observed. In a positive culture up to 20% of the cells may carry particles, more frequently when the culture has been exposed to suboptimal conditions. Particles are found in the nucleus but appear mainly in the cytoplasm where they seem to assume a more mature state [11]. No virus seems to be released from the cells and unequivocal, successful transfer of infection to other cells has not yet been reported.

Particle-carrying cells stained with fluorescent labeled serum from all of the 30 lymphoma patients so far tested. Of sera from healthy controls 54% of those from African children, 35% from American adults likewise gave positive reactions. Antisera against certain known viruses, mainly of the Herpes group, did not react.

Particles were set free by sonication of the cells, purified and concentrated by means of

density gradient centrifugation. Such preparations were examined in the electron microscope for antibody coating and particle agglutination. Again a reaction of a specific nature was observed [12].

Most authors describe these particles as *herpesvirus-like* and have presented electron micrographs of purified material prepared by the negative staining technique. On the other hand, leukosis viruses are often referred to as *myxovirus-like*. In both cases, the terminology is based on rather superficial similarities and is unfortunate, because it might contribute to obscure some important facts.

Like myxoviruses, leukosis viruses are supplied with an envelope but this is the only similarity between them. Myxoviruses have convoluted helical nucleocapsids, fitting tightly into the envelope. Their bouyant density is about 1·25. As to the architecture of leukosis viruses reference is made to a recent paper by Padgett and Levine [13] who studied the effect of snake venom on the Rauscher mouse leukemia virus. According to these authors the nucleic acid is enclosed in an inner shell supporting capsomers shaped as hollow cylinders. On top of these is layered a possibly lipid substance giving the nucleocapsid a smooth outer surface. The nucleocapsid is separated from the envelope by an apparently structureless space, the inter-membraneous space (Beard) or viroplasm.* The presence of the viroplasm may account for the low bouyant density of leukosis viruses, 1·16 to 1·19.

If the particles discovered by Epstein *et al.* and purified by Henle *et al.* are compared on one hand to herpesvirus, on the other to the structures revealed by snake venom digestion of the Rauscher virus, there seems to be little doubt in which group the Burkitt particles really belong. Thus, their diameter was consistently found to be smaller than that of the herpesvirus capsid, by about 20% [11], whereas it tallies very well with the measurements of the naked Rauscher capsid. The negatively stained preparations shown in the papers by Henle *et al.* and by Padgett and Levine look remarkably alike. Although the exact number of capsomers can hardly be determined in either capsid, they cannot be very different.

*The term viroplasm has been applied in several connotations. Here it is used as an analogy to the term cytoplasm.

In my opinion, therefore, there are good reasons for the assumption that the Burkitt particles represent naked leukosis virus capsids. The question why maturation does not seem to proceed beyond a certain point cannot yet be answered. The virus might be defective like the Rous virus or a block has been established at some late stage of the maturation process. A search for possible helper viruses or chemical inducers would seem to be indicated.

Whatever the significance of the Burkitt agent—it can hardly be doubted that we are dealing with a virus—the serological studies indicate that it is widely distributed. This by itself is not incompatible with the assumption of its etiological importance. In fact this would duplicate experiences gained in the field of animal oncology according to which infections with oncogenic viruses in most cases run subclinical courses, tumor induction occurring only under special conditions, in hereditarily predisposed animals, under influence of hormonal stimulation, through failure of the normal immunity mechanisms, after exposure to co-carcinogens, or in conjunction with other as yet unknown contributing factors.

It should be pointed out that the discovery of the Burkitt agent is not the first indication of a possible viral etiology of human leukemia. Several observations on virus-like particles in leukemic material have been reported before 1964 and summarized in several reviews [14, 15]. The findings here described were singled out because they seem to have prepared a firmer basis for interpretation and for further experimental work than was previously available. Only one additional report will be mentioned, that of Fink *et al.* [16] who claim that 68% of human lymphomas studied by them gave cross reactions with Rauscher mouse leukemia in indirect fluorescent antibody tests. This finding, if confirmed, might prove to be of great practical and theoretical interest.

If in the future the Burkitt agent is shown to be a leukosis agent and a cause of human leukemia, will this provide the final solution to the etiological problems? Most probably not. The experience gained in studies of avian and murine leukemia has taught us to expect to find a whole group of more or less related human viruses and maybe some types shared by other mammals or birds. A virologist must look to the future with great expectations.

RESUME

Certains mécanismes réglant la croissance cellulaire semblent être étroitement associés à des structures spécifiques de la membrane cellulaire. Il est, dès lors, intéressant de constater que les infections virales semblent être régulièrement associées à des modifications immunologiques de la surface des cellules infectées. Les nouveaux antigenes sont certainement codés dans le génome du virus et sont probablement formés aussi dans les cellules en voie de lyse. Des antigènes "de tumeur" ne sont donc pas, en eux-mêmes, une preuve évidente de la transformation tumorale.

Pour qu'il y ait transformation, il faut que la synthèse intracellulaire du virus et l'effet cytopathique de l'infection soient controlés. Trois possibilités se présentent: (a) le virus pouttait être défectif, incapable de multiplication, sauf en présence d'un virus aidant; (b) une réaction de défense, par production d'interféron, ou par un mécanisme analogue, une réaction de défense, par production d'interféron, ou par un mécanisme analogue, pourrait réduire la vitesse de synthèse du virus et permettre la poursuite de l'activité cellulaire; (c) le virus pourrait être intégré dans le génome de la cellule par un processus semblable à la lysogénie des bactéries.

L'apparition d'antigènes spécifiques offre la possibilité d'évaluer le rôle étiologique possible, dans les cancers humains, de certains virus reconnus comme cancérigènes chez l'animal. Jusqu' à présent, les études dans ce sens n'ont pas apporté de résultats spécifiques.

Actuellement la leucémie humaine, et surtout le syndrome de Burkitt, semblent être des exemples particulièrement prometteurs d'affections malignes dans lesquelles des virus pourraient jouer un rôle étiologique. Deux types de structures, ayant l'apparence de virus, mises en evidence dans des cellules leucémiques, semblent avoir un intérêt particulier, puisque ces structrues sont morphologiquement semblables à des virus connus pour leur action leucémogène chez l'animal (les particules de "type C" et les particules "herpes virus-like"). A l'heure actuelle, la transmission de l'infection à des celles normales n'a pas été obtenue de façon certaine, et les structures observées peuvent correspondre à des virus défectifs.

L'isolement d'autres types de virus, à partir de matériel leucémique, a aussi été décrit, mais la possibilité qu'il s'agisse de virus de passage, sans relation avec la leucémie, n'a pas été exclue.

SUMMARY

Some of the mechanisms regulating cell growth seem to be intimately associated with specific structures in the cell surface. Therefore, the fact that virus infection seems to be regularly associated with immunological changes in the surface of the host cell deserves particular attention. The new antigens are undoubtedly coded in the virus genome and are probably formed also in cells undergoing lytic infections. "Tumor" antigens are therefore not by themselves conclusive evidence of tumor transformation.

One requirement for transformation is that the intracellular virus synthesis and the cytopathic effect of infection have to be brought under control. Three possibilities present themselves: (a) the virus might be defective, incapable of replication except in the presence of a helper virus; (b) a defense reaction by interferon production or the like might reduce the rate of virus synthesis and permit continued cell activity; (c) the virus might be integrated in the cell genome in analogy to lysogeny in bacteria.

The appearance of specific tumor antigens has provided an opportunity for evaluation of the possible etiological role in human malignancies of a number of viruses known to be oncogenic in animals. So far, extensive studies along such lines have failed to produce any significant results.

At present, human leukemia and particularly Burkitt's African lymphoma seem to be the most promising examples of possibly virus-induced human tumours. Two types of virus-like structures found to be associated with leukemic cells or tissue cultures derived from leukemic material are of particular interest, since they are morphologically similar to viruses known to produce leukemia in animals ("C-type" and "herpes virus-like" particles). So far, transmission of infection to normal cells has not been unequivocally achieved and the structures observed may therefore represent defective viruses.

Isolation from leukemic material of other types of viruses has been reported. The possibility of pick-ups of unrelated passenger viruses has yet to be excluded.

ZUSAMMENFASSUNG

Gewisse Mechanismen, die das Zellwachstum regulieren, scheinen an sepzifische Strukturen der Zelloberfläche gebunden zu sein. Virusinfektionen scheinen regelmässig mit immunologischen Veränderung der Zellmembran verbunden zu sein. Die neu auftretenden Antigene entsprechen wahrscheinlich bestimmten Virusgenen und entstehen auch in solchen Zellen, die infolge einer lytischen Infektion untergehen. "Tumorantigene" sind daher nicht notwendigerweise ein eindeutiger Beweis für Tumortransformation.

Eine Bedingung für Tumortransformation ist die Aufhebung der zytopathogenen Wirkung des Virus. Drei verschiedene Mechanismen wären denkbar: (a) das Virus ist defekt und kann sich nur mittels eines Helfervirus vermehren; (b) eine Abwehrreaktion (z.B. Interferon) hemmt die Vermehrung des Virus; (c) das Virus wird in das Zellgenom integriert, wie es von lysogenen Bakterien bekannt ist.

Die Bildung spezifischer Tumorantigene ermöglichte die Untersuchung einer Anzahl im Tierversuch oncogener Viren in bezug auf ihre ätiologische Bedeutung bei menschlichen Tumoren. Umfangreiche Untersuchungen haben bis heute kein entscheidendes Resultat gebracht.

Menschliche Leukämien und das als Burkitt-Tumor bekannte afrikanische Lymphon sind vorläufig die am ehesten virusbedingten menschlichen Malignome. In leukämischen Zellen oder Gewebekultur sind virusähnliche Strukturen beschrieben worden, die Aehnlichkeiten haben mit Viren, die bei Tieren Leukämienhervorrufen ("C"-Typus und "herpesvirusähnliche" Körperchen). Die Uebertragung auf normale Zellen ist noch nicht einwandfrei gelungen; bei den gefundenen Strukturen könnte es sich deshalb um defekte Viren handlen.

Ueber die Isolierung verschiedener anderer Viren aus leukämischem Material wurde auch berichtet. Möglicherweise handelt es sich aber um belanglose sog. Passageviren.

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Studies on Cell Viability in Aspirates from Human Lymph Nodes*

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ASPIRATION biopsy, as a cytologic technique, is becoming increasingly popular for the diagnosis of human tumours in many organs (prostrate and salivary glands, lung, breast, etc.). There is also an extensive literature on the use of aspiration biopsy cytology in diagnosing disorders of lymph nodes [1, 2].

A review of 1200 consecutive aspiration biopsies of lymph nodes of the neck, which were performed at Radiumhemmet during the period 1957–1959 [3], showed that a cytologic diagnosis of malignancy can readily be made from study of aspirate from nodes with metastatic carcinoma, reticulum cell sarcoma or Hodgkin's disease. In lymphocyte–lymphoblast lymphoma, however, the smears may be difficult to distinguish from those in chronic benign lymphadenitis. Such diagnostic uncertainty points to a need for alternative methods of analyzing the aspirate.

In 1960, Nowell [4] reported blastocytic transformation of human lymphocytes from peripheral blood when these cells were cultured in the presence of phytohaemagglutinin (P.H.A.). The stimulating action of P.H.A. has been observed also in lymphocytes from other sources, such as the thoracic duct [5]. Many recent studies have shown that lymphocytes sampled from peripheral blood in cases of chronic lymphocytic leukaemia do not respond in this way to P.H.A. [6–10]. In contrast to normal leukocytes, few or none of these cells undergo blastocytic or mitotic changes when stimulated with P.H.A. It is therefore conceivable that the P.H.A. test on lymph node aspirate could assist the differential diagnosis in cases of suspected lymphocyte–lymphoblast lymphoma.

Use of aspiration biopsy for sampling lymphocytes in biologic analyses, however, raises the question of how far the aspiration procedure affects the viability of the sampled cells.

Aspiration biopsy can be defined as a procedure in which negative pressure is created in the syringe, and, as a result, cellular material is drawn from the tissue into the needle [11]. *In vitro* investigations on the influence of such pressure differences on cell viability showed that negative pressures similar to those arising during aspiration biopsy had no apparent effect on the viability of He-La and bone-marrow cells [12]. The cells thus remained impermeable to eosin and lissamine green, dyes which are commonly used in testing the viability of mammalian cells. The proliferation rates of the cells were likewise unchanged by the negative pressures: proliferation of He-La cells was studied in tissue cultures and proliferation of bone-marrow cells in transplantation studies on lethally irradiated mice.

In vivo, however, subjection of cells to equivalent negative pressures involves some additional factors. Forces of adhesion and, especially as regards epithelial cells, intercellular cohesion forces then maintain the

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position of the cells in relation to their surroundings. We must therefore consider how far the breakdown of the adhesion and cohesion forces during aspiration biopsy affects the viability of the sampled cells.

This question was investigated in aspiration biopsies of rabbit lymph nodes and rat spleens, using various negative pressures. The viability of the sampled cells was determined with the trypan blue permeability test and the trypsin digestion test [13]. The results are presented in Fig. 1. When the pressure difference in the needle during the aspiration was about 100 mm Hg, the mean score of viable cells in lymph node and spleen aspirates was 85% in the trypan blue test and 76% in the trypsin digestion test. Increase of the negative pressure to 740 mm Hg had only a slight effect on the cell viability scores.

These observations suggest that the recorded

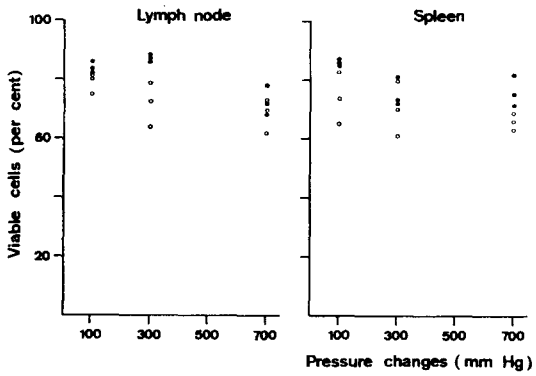


Fig. 1. Relationship between negative pressure during aspiration biopsy and cell viability in aspirates from lymph nodes (rabbit) and spleen (rat).
● *dye permeability test*
O *trypsin digestion test*

cell damage occurred when the cells were detached from their surrounding tissues by needling and/or the negative pressure. Once within the needle, the cells apparently were unaffected by the prevailing pressure.

The results of P.H.A. tests on aspirated lymphocytes are reported in Table 1. The blastocytic transformation was studied in short-term tissue cultures according to a previously described technique [14]. The numbers of sampled lymphocytes from each node were in the range $2{\cdot}3\times10^6$ to $9{\cdot}8\times10^6$, and the trypan blue test indicated that 75–93 per cent of these cells were viable.

When cultured in the presence of P.H.A., 70–90% of the then apparently intact cells from nodes with chronic benign lymphadenitis, sarcoidosis or Hodgkin's disease displayed blastocytic transformation (Figs. 2a and b). Only occasionally did transformation occur in cells aspirated from nodes with lymphoblastic lymphoma. In control runs without added P.H.A., lymphocytes from the same sources showed no or only occasional blastocytic change.

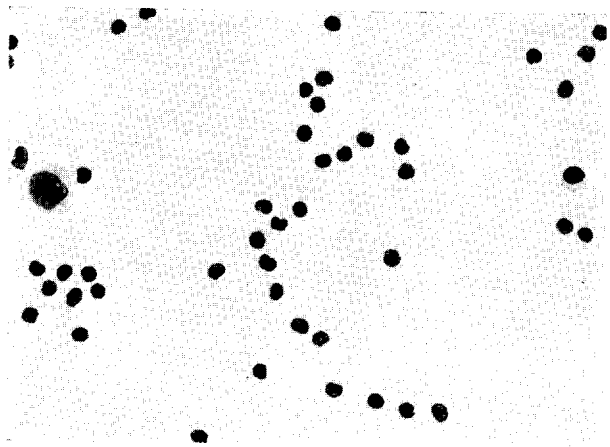
The blastocytic transformation of lymphocytes aspirated from human lymph nodes also indicated that the biologic activity of these cells was unimpaired by the needling and the negative pressures used in the aspiration.

Future investigations should show if and to what extent P.H.A.-induced blastocytic transformation of lymphocytes aspirated from palpable lymph nodes can be used in diagnosis, particularly in differentiating between benign conditions and lymphocyte–lymphoblast lymphoma.

Table 1. Blastocytic transformation of lymphatic cells aspirated from human lymph nodes

Case No.	Diagnosis	Initial cell count ($\times10^6$)	Cell viability trypan blue test (%)	Blastocytic transformation: Cultures with P.H.A. (%)	Blastocytic transformation: Controls without P.H.A. (%)
1.	Benign lymphadenitis	4·5		90	5
2.	Benign lymphadenitis	6·7	78·4	70	2
3.	Benign lymphadenitis	7·3	89·0	80	0
4.	Sarcoidosis	6·3	93·0	90	0
5.	Hodgkin's disease	6·5		70	0
6.	Hodgkin's disease	5·0	84·0	80	0
7.	Hodgkin's disease	2·3	75·0	80	0
8.	Lymphocyte–lymphoblast lymphoma	9·8	91·2	occasional cells	0
9.	Lymphocyte–lymphoblast lymphoma	2·5	89·0	0	0
10.	Lymphocyte–lymphoblast lymphoma	9·8	91·2	occasional cells	0

(a)



(b)

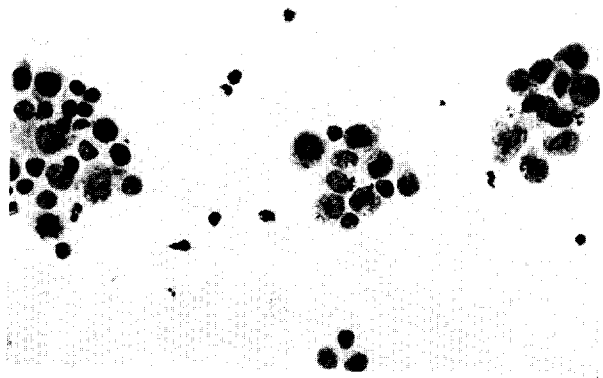


Fig. 2. Aspirate from a lymph node with Hodgkin's disease.

(a) *Lymphocytes, a Reed–Sternberg cell in its early stage (left) and a plasma cell (right) are shown.*

(b) *The same aspirate after incubation with P.H.A. for* 72 *hr. Most of the intact lymphocytes show blastocytic transformation. Two mitotic figures are also seen.*
Harris haematoxylin × 380.

(to face p. 24)

RESUME

La ponction-biopsie, par aiguille fine, des ganglions lymphatiques, est très aisément supportée par les patients. Lorsque la pression négative est d'environ 100 *mm Hg, pendant l'aspiration,* 76 *à* 85% *des cellules aspirées sont viables, comme le démontrent les tests de perméabilité aux colorants et la digestion par la trypsine. Un tel matériel est donc favorable pour l'investigation biologique.*

Les lymphocytes provenant de ganglions humains pathologiques (lymphadénite bénigne, maladie de Hodgkin, lymphoblasto- et lymphocytosarcome) furent cultivés, pendant de courtes périodes, en présence de phytohémagglutinine. Dans la lymphadénite bénigne et dans la maladie de Hodgkin, 80% *environ des lymphocytes cultivés présentaient une transformation blastique, tandis qu'une telle transformation était exceptionnelle dans le lymphoblasto- et le lymphosarcome.*

SUMMARY

Fine-needle aspiration biopsy of human lymph nodes causes little discomfort. When the negative pressure during the aspiration was about 100 *mm Hg,* 76–85% *of the aspirated cells were viable in dye permeability or trypsin digestion tests. Such aspirate is therefore suitable for biologic work.*

Lymphocytes aspirated from human lymph nodes with various pathologic conditions (benign lymphadenitis, Hodgkin's disease, lymphocyte–lymphoblast lymphoma) were submitted to short-term tissue culture in the presence of P.H.A. In benign lymphadenitis and Hodgkin's disease about 80% *of the intact cultured lymphocytes exhibited blastocytic transformation. In lymphocyte–lymphoblast lymphoma, transformation occurred only occasionally.*

ZUSAMMENFASSUNG

Lymphknotenbiopsien durch Punktion mit feiner Nadel verursachen den Patienten kaum Beschwerden. Die Autoren stellten fest, dass 76–85% *der so gewonnenen Zellen durch einen negativen Aspirationsdruck von ca.* 100 *mm Hg nicht geschädigt werden, was sie anhand der Farbstoffpermeabilität oder dem Trypsintest nachwiesen.*

Lymphozyten wurden aus Lymphknoten von Patienten mit gutartiger Lymphadenitis und mit Hodgkin'scher Krankheit aspiriert und unter Zusatz von PHA kurzfristig in vitro kultiviert. Ca. 80% *der intakten Lymphozyten von Patienten beider Krankheitsgruppen wandelten sich in Gewebekultur blastocytär um. Eine solche Umwandlung wurde dagegen bei dem Lymphoblasto- und dem Lymphosarcom eine Ausnahme.*

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Progress in Treatment of Cancer of Uterine Cervix*

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INTRODUCTION

THE TREATMENT of carcinoma of the cervix is a twentieth century epic that is still incomplete. Over 13,000 women die of the disease annually in the U.S.A. and no race nor country is immune. The story begins at the turn of the century with the publication [1] of the first logical surgical attack, while at much the same time Pierre and Marie Curie had announced the result of their work on radioactivity. Both methods of treatment were adopted and have been used ever since.

Their rival merits were summarized 40 years later [2] by the statement that, "Medical opinion the world over appears to be more or less agreed that the Wertheim operation has been superceded by radium and deep X-rays". This was based upon reports from sixteen radiotherapy centres of a 27 per cent 5-year apparent cure rate of 7958 patients, representing 88% of all cases seen in the areas concerned. For comparison Victor Bonney [3] had just published 5-year figures for his first 500 Wertheim operations with apparent cure of 40 and 63% operability, but a primary mortality of 15%. These comparable figures were achieved by a single surgeon of great skill and experience. The radical vaginal or Schauta operation (which makes no attempt remove pelvic lymphnodes) gave a similar cure rate with a lower primary mortality [4].

The end of the second world war brought great improvements in technique both for surgery and irradiation but it became less easy to compare results. Surgical centres, especially in America, introduced more radical operations and with the facilities of modern transport a high degree of selection of cases became inevitable. New diagnostic techniques revealed a hitherto untapped supply of early cases of doubtful invasion, and workers became increasingly dissatisfied with the classification of the disease into its four traditional stages prior to treatment. The picture to-day is one of transition with surgery and chemotherapy making inroads into the established position of radiotherapy.

EARLY DISEASE

Modern diagnostic techniques of exfoliative cytology, colpo-microscopy and serial section biopsy have increased the recognition of early disease, with the introduction of Stage 0 for apparently pre-invasive cancer. Furthermore the histological finding of early stromal invasion in biopsy specimens from patients free from clinical signs of invasive cancer has suggested that a modification of Stage I (Stage IA) be applied to these cases. Difficulties arise in interpretation of the histological picture by different pathologists (even granted adequate biopsy material) and a recent survey [5] has emphasised this and attempted to standardize the criteria for diagnosis of dysplasia, pre-invasion and micro-invasion.

The pre-invasive lesion appears to spread in three ways. It may flow off the cervix onto the vaginal fornix, pass up the endocervical canal even beyond the internal os, or creep up the ducts of the cervical racemose glands before invading the stroma. Wide surgical extirpation of most of the cervix would seem necessary to

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ensure removal of sufficient tissue to establish the exact diagnosis, and this cone biopsy is now widely practised: rather than multiple punches or a superficial ring of squamo-columnar junction. The advantage of the large cone is that further treatment may be unnecessary, the procedure being curative as well as fully diagnostic [6]. Recent work [7] suggests that the pre-invasive lesion may in fact be cured by removal of lesser amounts of tissue insufficient to excise the entire lesion and that this may be followed by further pregnancy without undue risk.

The treatment of micro-invasion (Stage IA) has not yet been established and the difficulty of defining this condition adds to the uncertainty of the clinician. Most would treat it surgically, although individual preference may vary between a conservative attitude (when the cervix has already been amputated), a simple hysterectomy (abdominal or vaginal) and more radical operations of the Wertheim type. In this connection it should be emphasised that radiotherapy is potentially more radical than surgery as ovarian function is generally destroyed. The surgeon operating for an early invasive growth in a young woman will often preserve an ovary.

ROUTINE TREATMENT

"Radiotherapy is generally acknowledged to be the preferred method of treatment: it has enjoyed this position from the time of the first world war to the present" [8]. The author of a standard American textbook justifies this claim by quoting apparent cure rates of 50% in long series of unselected cases, emphasising that no unselected surgical series is comparable. He points to the undoubted fact that almost all cases can receive this form of treatment with its low morbidity and mortality and he relegates surgery to the secondary role of treating irradiation failures.

This view however is not universally held. It is admitted that despite easy access to the cervix for irradiation some 25% of cases have an unhealed cervix at the end of treatment and the question of radioresistance has received much attention. The grading of histological types of tumour regarding degree of cellular differentiation has some significance in this respect. The oxygenation of the tumour bed seems to be of importance, so that preliminary treatment of anaemia and use of high oxygen tension may be helpful. Medication with Vitamin K, testosterone and tocopherol have been suggested but the exact reason for radio-resistance is unknown and its very existence is still in dispute.

The other theoretical disadvantage of radiotherapy is the decrease in effective dosage with increasing distance from a source of radium. Unfortunately cervical cancer will metastasize to pelvic lymphatic nodes in approximately 25% of all cases and the radiotherapist has no effective means of telling whether this has occurred. Thus his scheme for adequate dosage must include maximum irradiation of the centre of the pelvis with supplementary concentration as necessary at the side walls. It has been disputed as to whether irradiation has the power to destroy cancer in these pelvic lymph-nodes but the work of Kottmeier in Stockholm is strongly suggestive that it can be done.

The failure of radiotherapy to cure all cases of apparently early disease has led to a re-evaluation of radical surgery. The tradition that this would have a high primary mortality was abolished when Meigs [9] published his series of 100 consecutive Wertheim operations without a death. The surgery of the Boston school used a more radical and elaborate technique than that traditionally employed in Britain and when this was combined with radio-therapy it produced a high rate of urinary fistulas. That this was not inevitable was shown by Schlink [10] who was the first to start a large series of selected cases treated by radical abdominal surgery after pre-operative treatment with radium. Two series from Britain have recently been published, treated in this way. Currie [11] reports 400 such operations with 5 deaths and 7 fistulas. His overall 5-year cure rate was 75% with figures as high as 84 per cent for Stage I (including 62 per cent for the 15% of Stage I cases with cancer in the excised pelvic lymph-nodes). Stallworthy [12] uses a higher initial dose of radium but takes exceptional care to preserve the blood supply to the ureter and bladder. His report on 285 cases (2 deaths and 7 fistulas) showed a 71% apparent cure rate with operability of 68% of all cases seen.

There are many other surgical techniques. The radical vaginal or Schauta operation is still performed and it is claimed that more parametric and paravaginal tissue can be removed by this approach. It is sometimes combined with an abdominal operation to dissect the pelvic lymphatic nodes. A synchronous combined approach has been used [13] which allows a complete vaginectomy to be included more easily with the abdominal operation, but these modifications do not affect the essential issue. Would overall results be

improved if cases could be selected for either irradiation or surgery according as to degree of radio-resistance and lymphnode involvement? The answer to this question is still unknown but research continues in an attempt to evaluate the patient more completely before treatment. Pelvic phlebography may show distortion and filling defects of the external iliac vein due to malignant lymphatic nodes. Pelvic lymphography [14] may show filling defects in hypogastric lymphnodes suggestive of malignant deposits. Serial biopsies from the cervix during treatment [15] may indicate the degree of radio-sensitivity. Vaginal exfoliative cytology techniques have indicated two further possible aids. The Sensitisation Response (S.R.), an index of some unusual cells in the pre-radiation smear was described by the Grahams [16], while the Radiation Response (R.R.) had been observed formerly [17]. These have been intensively studied as prognostic aids but the most recent evaluation [18] was unenthusiastic. It may well be that some cases can be cured with equal efficiency by either surgery or irradiation while others are inevitably incurable. Nevertheless, it would appear that combined therapy for selected operable cases carried out by surgeons and therapists of experience may well salvage some cases that would otherwise be lost.

THE ADVANCED CASE

So long as the overall cure rate remains at no better than 50% there will be many cases of advanced disease. They represent the sum of those presenting too late for hopeful treatment and those whose therapy has proved to be inadequate. On rare occasions, especially in the elderly, the disease may seem to become spontaneously arrested, or some unconventional form of treatment, either physical or emotional, may lead to an apparent cure. Generally, however, progress is inexorable with the miseries of urinary and faecal fistulas, visceral and nerve root pain, and perpetual offensive discharge until merciful uraemia supervenes. Palliative procedures such as diversion of urine or faeces, neurosurgical injections or operations on the spinal theca and ligation of the main anterior trunk of the internal iliac arteries may be justified for the few. The terminal care may impose great physical and emotional demands upon those who nurse and care for these unhappy sufferers.

It was against this background of advanced lethal disease, frequently isolated in the pelvis, that Brunschwig [19] propounded the concept of surgical exenteration. The operation entailed the removal of all the pelvic viscera with lymphnode dissection and transplanation of ureters into a terminal "wet" colostomy. Later modifications have preferred an isolated loop of ileum as a urinary conduit, while in some cases it has proved possible to save either rectum or bladder. The complete operation imposes a considerable strain upon both patient and surgeon and most clinics would advise it only in cases with a reasonable chance of permanent cure. It is most applicable when the disease is centrally placed, involving viscera rather than the pelvic wall and lymphnodes are not expected to be heavily involved. The selection of suitable cases poses great difficulty. If used for terminal disease there will be little salvage, while in less advanced cases there will always be the feeling that a more conservative operation might have sufficed.

The use of cytotoxic chemotherapy against cervical cancer gave little promise of success until Sullivan [20] suggested that intra-arterial therapy might surround the cancer cell with a locally lethal concentration of chemotherapeutic agent. The first report of apparent success was 2 years later when Trussell [21] reported from Uganda (where radiotherapy was not available) the use of intra-arterial methotrexate as primary treatment for 15 advanced cases. There was spectacular reduction in the size of most tumours and one Stage III case might have been cured but was lost to follow up. Subsequent experience elsewhere has given mixed results and some authorities would not advise it after the blood supply to the tumour has been impaired by radiotherapy. Nevertheless, Hodgkinson [22] reports experience of 19 cases and believes it to be a promising palliative measure that is temporarily effective, dangerous and not completely understood.

CONCLUSION

Further progress may come about in a number of ways. It is possible (though unlikely) that universal screening of women by vaginal cytology or other techniques will eliminate the problem of invasive cervical cancer, allowing cases to be treated early by local surgery. It may be that the three methods of treatment currently available, surgery, radiotherapy and intra-arterial drug infusion, may be used in some new combination or sequence with results superior to those already recorded. Or maybe some new biochemical or immunological technique will suddenly out date our present efforts and drive this female scourge from the face of the globe.

RESUME

Les résultats du traitement du cancer invasif du col utérin ne produisaient jadis que des taux de guérison inférieurs à 25%, que l'on utilisât la chirurgie ou la radiothérapie. Les progrès les plus importants ont été obtenus par le diagnostic précoce, notamment grâce au dépistage. Une chirurgie limitée, dans des ces pré-invasifs, ou micro-invasifs, n'est pas mutilante et donne un haut pourcentage de guérisons.

La radiothérapie est actuellement capable d'obtenir des guérisons dans 40 à 50% des cas, mais les problèmes de la radio-résistance et de l'invasion ganglionnaire demeurent. Il serait utile de déceler ces facteurs péjoratifs avant le traitement. La chirurgie radicale peut produire des résultats remarquables dans les mains d'experts, surtout si elle est précédée de radiothérapie, mais l'inévitable sélection des cas rend une comparaison valable difficile.

La maladie à un stade avancé constitute un sérieux problème. Les interventions d'exentération pelvienne peuvent sauver quelques vies. Les agents cytostatiques aideront parfois des malades ayant des métastases à distance, et, administrés par perfusions intra-artérielle continue, peuvent provoquer une régression de la tumeur primitive. Les progrès de l'avenir viendront probablement de techniques biochimiques et immunologiques entièrement nouvelles.

SUMMARY

Results for treatment of invasive cancer formerly showed apparent cure rates below 25% for all cases seen, whether irradiation or radical surgery was used. The most important progress has been in the technique of early diagnosis, including the screening of apparently healthy women. Limited surgery in cases of pre-invasion and micro-invasion may preserve function and give a high percentage of cures.

Radiotherapy to-day may give cure rates of 40–50% but the problems of radio-resistance and lymph node involvement remain. Diagnosis of these adverse factors prior to treatment would be beneficial. Radical surgery by experts will produce fine results especially if preceded by radiotherapy, but the inevitable selection of cases makes valid comparison dubious.

Advanced disease presents a challenge. Exenteration procedures will save a few lives. Cytotoxic drugs will sometimes help the case with remote metastases, but when given by continuous intra-arterial perfusion they may produce regression of the primary tumour. Future progress should come from some entirely new biochemical or immunological procedure.

ZUSAMMENFASSUNG

Die Behandlung der invasiven Cervixkarzinome ergab früher eine Heilungsrate von unter 25% für alle Fälle, gleichgültig ob mit Bestrahlung oder Radikaloperation behandelt. Der wichtigste Fortschritt wurde auf dem Gebiete der Früherkennung und der Reihenuntersuchung symptomloser Frauen gemacht.

Mit Radiotherapie allein kann heute eine Heilungsrate von 40–50% erwartet werden. Ungelöst ist das Problem der Strahlenresistenz und der Lymphknotenmetastasierung. Die Abklärung dieser Parameter vor der Therapie wäre wünschenswert. Radikaloperation durch einen Experten gibt sehr gute Resultate, besonders wenn kombiniert mit Vorbestrahlung. Die unvermeidliche Selektion der Fälle verunmöglicht aber eine statistische Auswertung der Resultate.

Die fortgeschrittenen Fälle sind immer noch ein groses Problem. Radikale Ausräumung kann in einzelnen Fällen helfen. Cytostatica können Fernmetastasen günstig beeinflusen, und durch intraarterielle Perfusion kann der Primärtumor direkt angegangen werden. Der weitere Fortschritt wird wahrscheinlich nur möglich sein durch die Entwicklung von vollkommen neuen biochemischen und immunologischen Methoden.

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Printer's Note:
We regret the error in author's initial.
F. SUGÁR *should read* J. SUGÁR

An Electron Microscopic Study of Early Invasive Growth in Human Skin Tumours and Laryngeal Carcinoma*

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INTRODUCTION

THE MORPHOLOGICAL features accompanying carcinomatous growth and invasion were discussed in a previous paper [1], in which our observations were based on experimentally-induced skin carcinomata of mice. The ultrastructure of skin and laryngeal carcinogenesis was described in two subsequent papers [2, 3], and, in a further paper, it has been argued that the degeneration of tumour cells plays an important role in the progressive development of invasive and infiltrative growth [4].

In this study, the fine structural changes, as seen at the earliest stages of invasive growth, are investigated, and an attempt made to determine whether cell degeneration, and the loosening of intercellular contacts, observed in preneoplastic conditions [1, 2], might prove to be of significance as the predisposing cause of the commencement of invasive growth.

Human material was studied, and the ultrastructure of both skin and laryngeal precancers and cancers investigated, some of the latter being in the incipient phase. The relevant surgical pathology and histology are summarized in Venkei-Sugár's [5] and Leicher's monographs [6].

MATERIAL AND METHOD

Eleven biopsy specimens of normal skin, 6 of senile keratoma, 3 of leukoplakia of the lip, 12 of keratoacanthoma and 40 of squamous cell carcinoma of the skin, were studied by electron microscopy. Laryngeal material was divided as follows: 11 specimens of normal mucous membrane, 7 of pachydermia, 6 of chronic laryngitis and 4 of carcinoma.

In all cases, paraffin embedded sections were made for routine histology. For electron microscopy, each specimen was divided into 2 different parts, one of which was fixed in (*a*): 4·5% glutaraldehyde in cacodylate buffer at pH 7·3 for 2 hr, washed overnight in cacodylate buffer and finally embedded in glycol-methacrylate—GMA [7, 8], and the other portion was fixed in (*b*): 4·5% glutaraldehyde, and postfixed with 2% osmium tetroxide in veronal buffer, at pH 7·3 for 2 hr and then embedded in Araldite [9].

Embedding the material in glycol-methacrylate, a water-soluble epoxy-type plastic, makes it possible to apply enzymic digestion procedures to ultra-thin sections. Prior to staining, the sections were treated with the following enzyme solutions [10, 11]: 0·1% ribonuclease in distilled water adjusted to pH 6·8 for 1 hr at 37°C 0·1% and deoxyribonuclease in distilled water at pH 6·6 for 5 hr at 37°C.

The contrast of the thin sections was enhanced with uranyl acetate followed by lead plumbise (Karnovsky's method A) [12]. The sections were studied with a JEM 6C electron microscope.

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RESULTS

A fundamental structural property of squamous epithelium undergoing alteration in the course of carcinogenesis was found to be the loosening up of cell connections in all layers. As a result of the loss of cohesion, intercellular spaces became widely enlarged and villiform processes were extruded from the cytoplasm into these spaces. Because of the considerable dilatation of the spaces, and the formation of microvilli and cytoplasmic processes, the basement membrane could no longer run parallel with the cytoplasmic membrane of the basal cells. In this way, spaces were formed between the basement membrane and the cells, and these parts of the basement membrane became areas of predilection for gap formation.

Invasive growth is generally thought to begin with an epithelial penetration through the basement membrane, and yet, in the present work we found what appeared to be gaps in the membrane already established in examples of laryngeal papilloma, and pachydermia, leukoplakia, senile keratoma, and keratoacanthoma (Fig. 1). These gaps measured 0·05–0·01mμ and cytoplasmic processes were observed to protrude through them into the underlying connective tissue; none of these processes being enveloped by a basement membrane, even in these precancerous conditions (Figs. 2, 3). This observation would appear to be conclusive proof that most of the gaps were true gaps, and not artefacts resulting from the plane of sectioning. Moreover, in normal skin, and in normal mucous membrane any discontinuity of the basement membrane was very rarely found, whereas, in preneoplastic states, such as those mentioned above, the basement membrane was frequently seem to ramify, sending branches into the superficial layer of the dermis.

Massive destruction of the basement membrane occurred only at a later stage of invasive growth, but even at this stage there were some areas of tumour in which intact membrane could be found. However, usually, no basement membrane could be detected around the undifferentiated carcinoma cell masses, and projections of invasive growth.

The extensive cell degeneration, localized to single cells, or to cell groups, which is going on in the epithelial cells, may be the cause of the loosening of cell contacts. This would explain why the intercellular spaces contained varying quantities of ribosomes, remnants of membranes and other cellular debris, to which we shall refer as intercellular exudate.

In the hyperplastic epithelium, light (electron lucent) and dark, (electron opaque) cells were seen (Fig. 4). In an attempt to determine one of the factors leading to cell degeneration, the particles containing nucleoproteins were studied.

As well as the nuclear chromatin, chromatin associated with nucleoli was also found. The interchromatinic matter of the karyoplasma consisted mainly of other proteins, among which tiny interchromatinic granules were seen. Their sizes ranged from 200 to 250Å.

In normal human skin and in precancers as well as in tumours, perichromatinic granules, described by Watson [13], could be demonstrated. Their sizes varied between 300–350Å. The nucleoli were enlarged and their number was increased. Deoxyribonuclease did not affect material fixed in glutaraldehyde, but the digestive action of ribonuclease could be clearly demonstrated. Neither deoxyribonuclease nor ribonuclease could make either inter- or perichromatinic granules disappear (Fig. 5 a, b, c,). The number of interchromatinic granules in the cells of precancer was strikingly high (Fig. 6). Pari-passu with the progress of carcinogenesis chromatin was scattered in the form of crude lumps throughout the nucleoplasm.

DISCUSSION

The ultrastructure of the basement membrane has been the object of detailed studies resulting in the identification of several zones. Observations related to the ultrastructure of the dermoepidermal junction were summarized by Swift and Saxton [14]. It was made clear that the electron opaque membrane revealed by electron microscopy represents a much thinner layer than the membrane detected by silver impregnation or PAS reaction used in light microscopy. According to Swift and Saxton [14], the positive PAS reaction is due to the thick reticulin layer underlying the electron opaque membrane. This is why electron microscopy of the basement membrane cannot be simply compared with the results obtained by light microscopy. In preneoplastic conditions preceding squamous cell carcinoma, the continuity of the basement membrane can in general be demonstrated. The part played by the basement membrane in invasive growth is controversial even in these days. Its passive insignificant role is often mentioned in the literature. It has been proved by immunohistochemical procedures that the basement membrane, rather than being a product of the

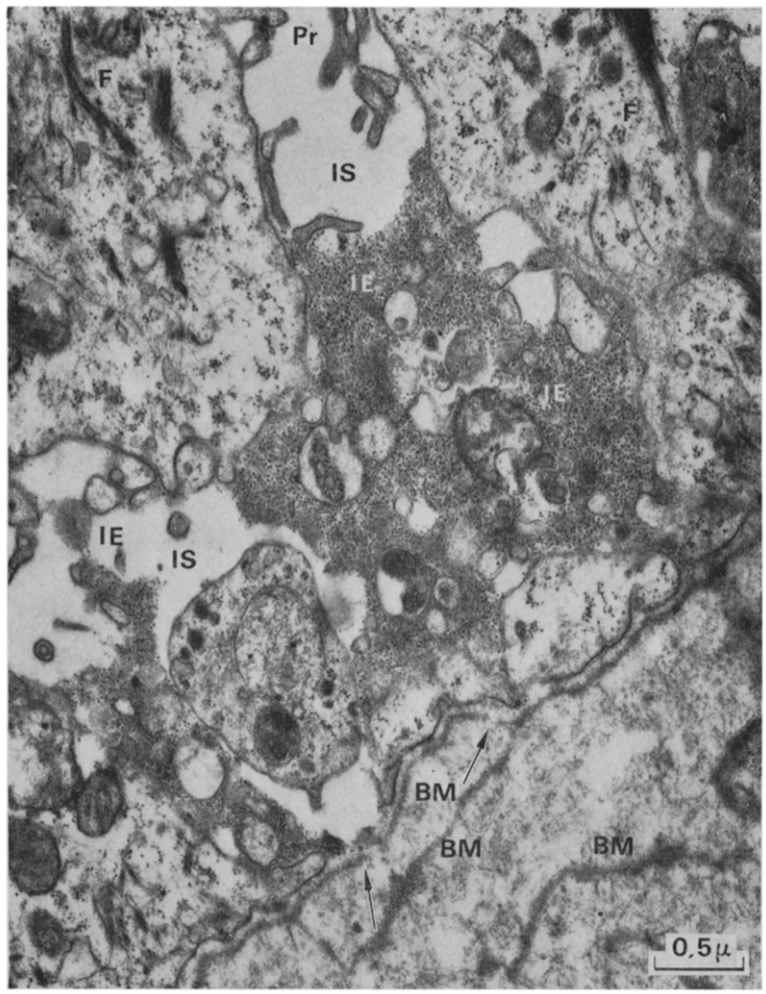


Fig. 1. *Senile keratoma. At the dermo-epidermal junction, cytoplasmic processes and debris of cellular origin, mainly ribosomes, can be seen in the dilated intercellular spaces. In places the basement membrane has thinned out, and it even seems as if its continuity were broken (see arrows). There is also branching of the basement membrane into the superficial layers of the connective tissue.* BM = *basement membrane,* IS = *intercellular spaces,* Pr = *cytoplasmic processes,* F = *fibrils,* IE = *intercellular exudate.* 7900 × 3· 5.

(to face p. 34)



Fig. 2. *Laryngeal papilloma. The cytoplasmic processes of the epithelial cell are protruding into the connective tissue: their continuity with the epithelial cell may be clearly seen. In the connective tissue these processes are not surrounded by electron-opaque basement membrane* (see arrow). (BM=*basement membrane*, F=*fibrils*, Pr=*cytoplasmic processes*) 11,500×2·5.

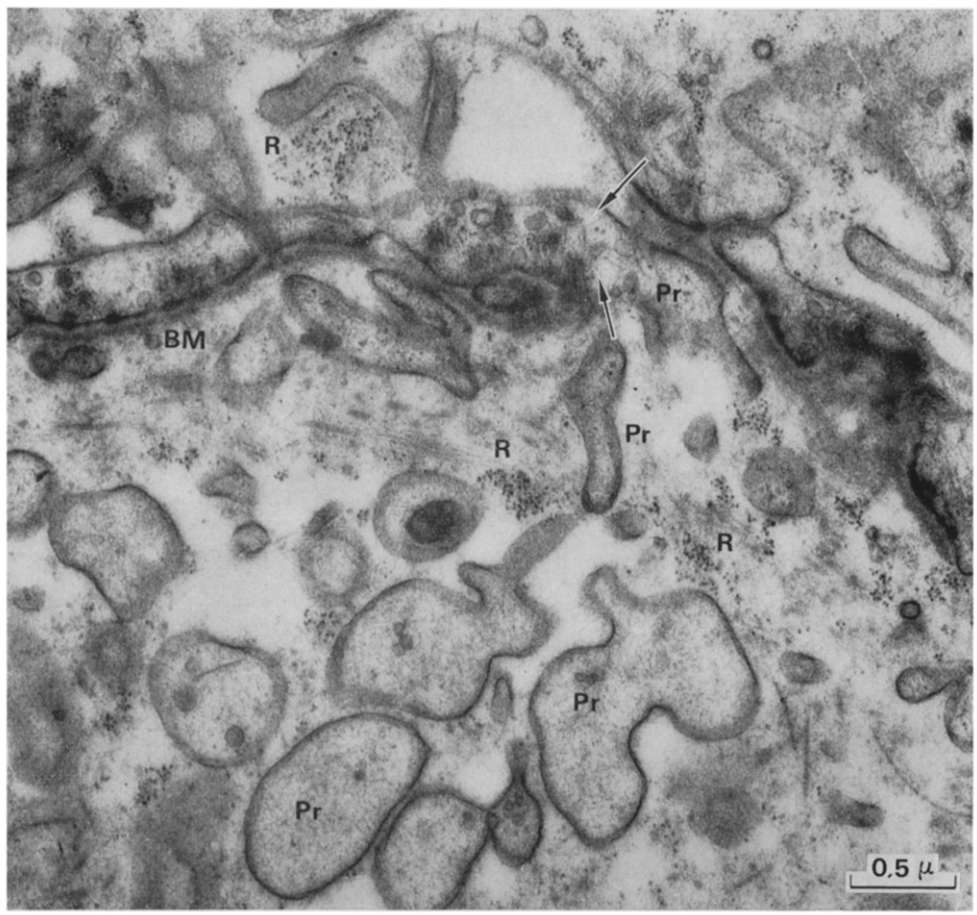


Fig. 3. Senile keratoma. The continuity of the basement membrane is broken (see arrows). Cytoplasmic processes are protruding towards the dermis. No tonofibrils can be demonstrated in the processes. In the connective tissue free ribosomes granules are visible which have been released into the connective tissue from the intercellular spaces. (BM = *basement membrane*, Pr = *cytoplasmic prosesses*, *R* = *ribosomes*) 11,000 × 3.

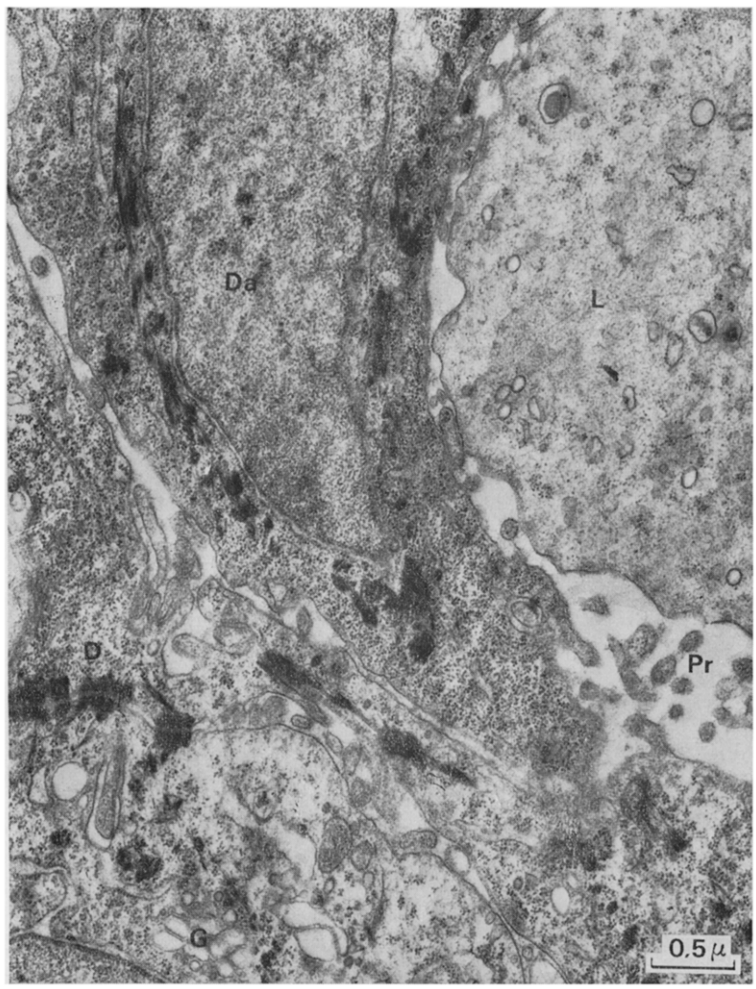
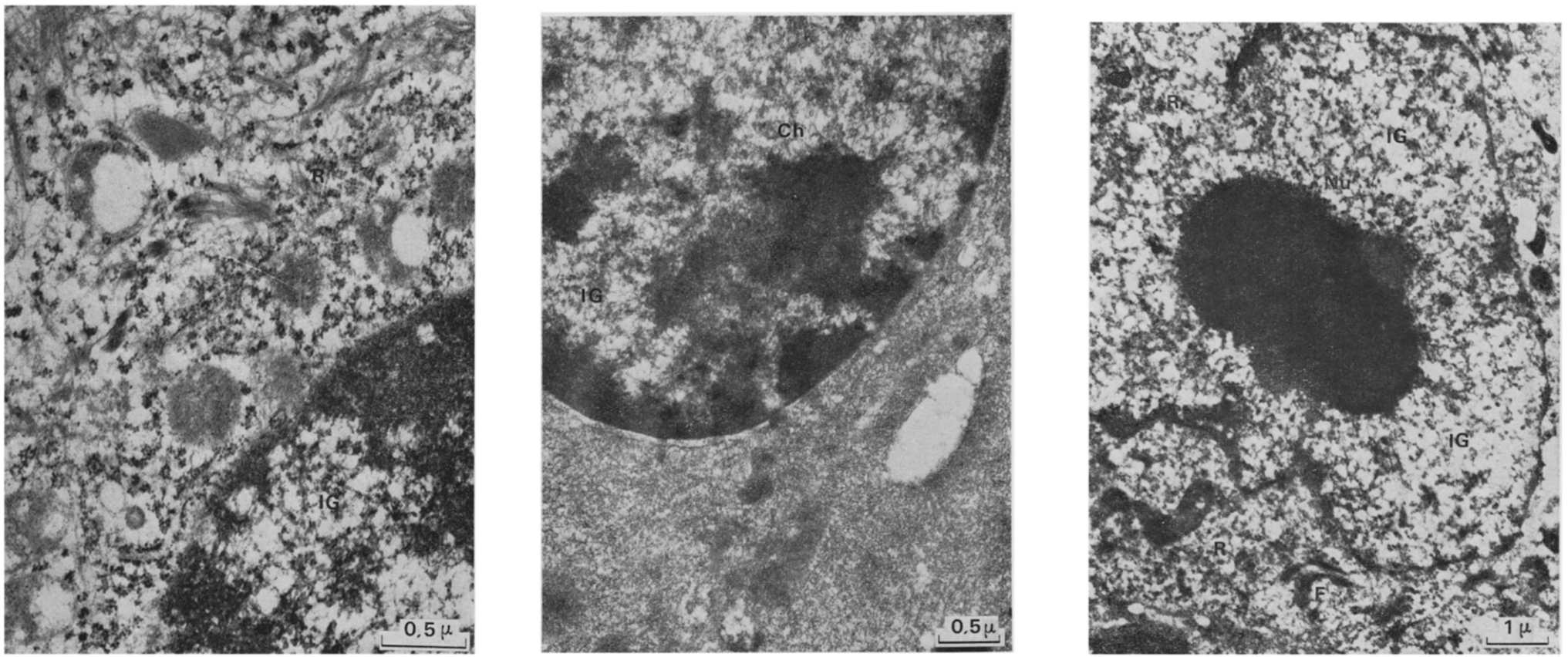


Fig. 4. *Pachydermia with cellular atypia of the left vocal cord. As well as 'dark' cells containing large numbers of free ribosomes, a 'light' cell may also be seen with scanty ribosomes. This cell has etiolated filaments, and no desosomes are seen. In the intercellular spaces, villiform processes are visible.* (D = *desmosomes*, G = *Golgi zone*, Da = *a dark cell*, L = *a light cell*, Pr = *cytoplasmic processes*). 10,100 × 2·5.



a b c

Fig. 5. Enzyme digestion in the intact human epidermis. (a) *cell without enzyme digestion Intact embedded in glycolmethacrylate (GMA),* 10,100+3·5; (b) 1 *hr digestion with* 0·1% *ribonuclease, embedded in GMA,* 7050×3·5; (c) 4 *hr digestion with* 0·1% *deoxiribonuclease, embedded in GMA.* 3700×3·5.

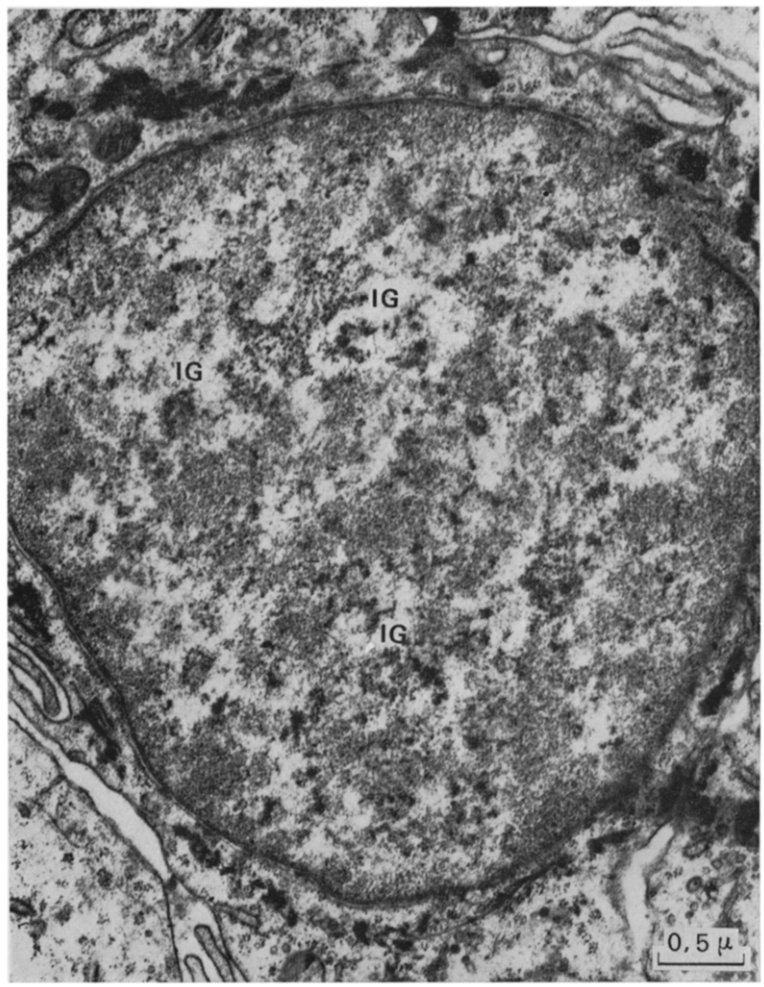


Fig. 6. *Keratoacanthoma of the skin. RNase and DNase resistant, interchromatmic granules (Ig) in great numbers can he seen in the nucleus.* 10,800 × 2 · 5.

connective tissue, is, in fact, produced by the epithelial tissue [15].

According to our observations, the dilatation of intercellular spaces and cell generation play an important part in starting the initial stages of invasive growth. The destruction of single cells results in loosening of the epithelial tissues and cell debris appears in the intercellular spaces. All this promotes the release of single cells and the destruction of the basement membrane. A property of the epithelial cells, which is most readily observed by electron microscopy, is that a great number of cytoplasmic processes are formed when intercellular spaces become dilated. Great number of such cytoplasmic processes are formed at places where there are gaps in the basement membrane. This phenomenon may be referred to as *micro-invasion at the electron microscopic level.* This can be observed not only in cancer, but also in precancerous lesions.

Early invasive growth is associated not only with the breakdown of the basement membrane, but also apparently with biological changes [16, 17], such as the production of toxic metabolites [18, 19] and of enzymes [20–22], changes in immune response [23–25], absence of contact inhibition, [26] and changing bioelectrical and chemical cell-surface properties [27–32]. In light and electron microscopic studies of tumours, gaps have been observed in the basement membrane through which cytoplasm protrudes into the connective tissue [33–35]. It would appear that we have been the first to observe this phenomenon in precancers. In our opinion, its significance in early invasive growth needs evaluation. Various theories have been put forward to explain cancer-cell release: viz detached tumour emboli [36–38], disintegration due to calcium deficiency [39] and amoeboid motility of cells [40, 41]. From electronmicrographs of cytoplasmic pseudopodes and villiform processes, evidence for these movements can also be obtained. Oberling and Bernhardt [42] have classified carcinomatous cells as 'light' and 'dark' cells according to their electron opacity. These 'light' and 'dark' cells may be readily identified in regenerating tissue following partial hepatectomy [43], during carcinogenesis due to thioacetamide [44], and following administration of alpha-naphthyl-isothiocyanate [45] and after carbon tetrachloride intoxication [46]. These 'dark' and 'light' cells also appear during the development of squamous cell carcinoma [2]. The electron opacity of the cytoplasm is largely controlled by the water content of the cells, along with the number of ribosomes and other organelles.

The marked increase in the number of ribosomes and interchromatinic granules in the 'dark' cells, should be considered as indicative of a disturbance of nucleic acid synthesis. This phenomenon can be explained by Cohen's [47] 'thymineless death' hypothesis, according to which DNA synthesis stops, while RNA synthesis continues for some time longer, thus producing cell death. As regards the 'light' cells, increased electron lucidity, may be taken as an ultrastructural sign of degeneration of these forms also.

Acknowledgements—The author is indebted to Dr. Tibor Venkei and Dr. László Faragó for giving access to the clinical material.

RESUME

Les études, au microscope electronique, du carcinome épidermoïde ont révélé la dissolution des contacts entre les cellules, au cours de la carcinogénèse.

La dégénérescence des cellules joue un rôle dans le développement qui conduit à la perte de la cohésion des cellules et au début de la croissance invasive.

Même avant l'irruption frontale à travers la membrane basale, pendant la phase paranéoplasique, des pseudopodes cytoplasmiques, provenant des cellules épithéliales, pénètrent, à travers les fissures de la membrane basale, dans le tissu connectif. Ce phénomène a été considéré comme une micro-invasion, à l'échelle du microscope électronique.

La structure fine des particules des cellules contenant des nucléoprotéines, c'est-à-dire de la chromatine et des granules interchromatiniques, subit des changements considérables au cours de la carcinogénèse.

SUMMARY

Electron microscopic studies of the morphogenesis of squamous cell carcinoma revealed the loosening up of cell contacts in the course of carcinogenesis. Cell degeneration plays a part in the development of the loss of cell cohesion and in commencement of invasive growth.

In the preneoplastic phase, before any massive destruction of the basement membrane

takes place, cytoplasmic processes originating from epithelial cells, penetrate into the connective tissue through gaps in the basement membrane. This phenomenon is referred to as micro-invasion at the electron microscopic level.

The fine structure of cell particles containing nucleo-proteins, i.e. chromatin particles and also interchromatinic granules, undergoes considerable modification in the course of carcinogenesis.

ZUSAMMENFASSUNG

Die elektronenmikroskopische Erforschung der Morphogenese von Plattenepithelkarzinom ergab eine Lockerung der Zellkontakte im Verlaufe der Karzinogenese. Zelldegeneration spielt eine Rolle beim Verlust der Zellkohäsion und beim Beginn des invasiven Wachstums.

In der präneoplastischen Phase, vor jeder massiven Zerstörung der Basalmembran, dringen Zytoplasmafortsätze von Epithelialzellen her durch Lücken in der Basalmembran in das Bindegewebe. Diese Erscheinung wird als Mikroinvasion auf Elektronenmikroskop-Ebene bezeichnet.

Die Feinstruktur der Zellpartikel, die Nukleoproteine enthalten, also der Chromatinpartikel und der Interchromatingranula, erfährt im Verlaufe der Karzinogenese erhebliche Veränderungen.

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Effect of Chemotherapeutic Agents and Hormones on Organ Cultures of Human Tumours

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A NUMBER of systems have been developed for assessing the effects of chemotherapeutic agent on tumour cells *in vitro* (reviewed by Lazarus *et al.*) [1]. In general, for solid human tumours, organ cultures can be considered to be closer to the *in vivo* situation than monolayer cultures [2].

In monolayer cultures, only a small and not necessarily representative proportion of the original cell population of the tissue gives rise to the final culture. Algard [3] has observed that the hamster kidney tumour, induced by stilboestrol, will retain dependence on stilboestrol when cultured in organ-culture, but as monolayer cultures, the cells proliferate independent of stilboestrol, thus specific sensitivities and functions tend to be lost in monolayer culture.

One obstacle to the routine use of organ cultures for an assay system compared to monolayers is the greater difficulty of assessing the effects of the drugs. Measurements of cell number and viability are much more difficult in organ culture than in monolayer culture, and most assessments of viability of the tissue after cultures have been made on the basis of histological examination of sections of the organ culture, which is extremely difficult to quantitate.

The technique of measuring the uptake of an essential metabolite, labelled with a radioactive isotope, such as phosphate, amino acids and nucleosides, has been used for quantitative measurement of the viability of cells in culture [4, 5]. To demonstrate the possibility of using the tracer technique to estimate cell viability in organ culture, we have used ^{32}P-phosphate, correlating cell viability with ^{32}P-uptake in organ cultures of mouse sarcoma 180. We found that the toxicity of melphalan can be expressed by the inhibition of ^{32}P-incorporation into the nucleic acid fraction [6].

In the present series of studies, we have used ^{3}H-Thymidine because it is a specific DNA label and a more sensitive system. Using ^{3}H-Thymidine, we are also able to obtain discrete autoradiographs of the tissues after culture.

The method of culture we have adopted utilizes agar-gel nutrient medium containing TC109 [6]. On the agar, small grafts of Millipore filters are placed, carrying the specimens which are fragments of the tissue cut into 1–2 mm^3. The petri dishes carrying the cultures are placed in a sealed perspex box and gassed with warmed moistened gas consisting of 95% O_2 and 5% CO_2.

The cytotoxic drugs or hormones are usually added to the medium at the beginning of the culture at concentrations of 10^{-5}–10^{-6} g/ml. The cultures are terminated between 3–5 days. Several specimens are removed for ^{3}H extraction, first with cold TCA then with hot

TCA. The extracts are counted in a scintillation counter. At the same time, several specimens are fixed for histological examination. We have found that duplicate samples of the same tissue may incorporate widely differing amounts of ^{3}H-TdR/mg. of tissue. Pieces of the same tissue often vary in their degree of cellularity; sometimes one piece would be highly cellular and the adjacent one would contain only a few tumour cells interspersed between large areas of fibrous stroma. At times, both pieces would be highly cellular, but one would contain much larger areas of clearly dead or dying cells.

We have, therefore, attempted to eliminate these variations between duplicate samples by expressing the results as

$$\frac{\text{^{3}H activity incorporated into DNA}}{\text{Total ^{3}H activity in tissue}} \% = \% \text{ Ratio}$$

As a first approximation this eliminates the difference in cellularity between duplicate specimens. The usefulness of this ratio in expressing the results can be illustrated in Table 1, where 4 replicate cultures of an ovarian

Table 1. *Expt. no.* 122 *carcinoma ovary, in culture for* 3 *days*

dis/min	% ratio	
7390	77	Controls
1415	75	
2455	70	
1355	52	

carcinoma were cultured for 3 days, and then extracted for ^{3}H activity. The radioactivity expressed as dis/min in DNA/mg dry wt. varies by a factor of 5 among the tissues. But when it is expressed as per cent ratio of total uptake, the results agree reasonably well.

As the variation in the duplicate results is a property of the tissue, we are using this ratio expression empirically. Its use reduced the differences between duplicates without reducing the differences arising from the action of different chemotherapeutic agents on the tissue. A representative experiment is shown in Table 2; the effect of the toxicity of the chemotherapeutic agents on a culture of a carcinoma of the maxilla is clearly seen by expressing the results as per cent ratio method.

In order to prevent the danger of relying entirely on the tracer results to express the drug action, the histology of the specimen after culture was always examined and we are able to get good correlation between these two methods. Fig. 1 (a, b, c, d, e) illustrates the histology of the samples in Table 2.

Although there is generally good agreement between the histological appearance of the tissue and the incorporation studies, there are fundamental differences between the two methods. The tracer technique summates the effect of the drug on the whole specimen, whereas histology, by examining the cells at different regions could reveal interesting results such as illustrated in Fig. 2, an autoradiograph of a 4-day culture of a mammary tumour. The epithelial cells of the ducts did not take up ^{3}H-TdR uniformly. In some regions, the cells are apparently quite healthy and yet they do not incorporate ^{3}H-TdR.

From a number of cultures of a variety of tumours, we have observed that no individual tumour treated with various chemotherapeutic agents including alkylating agents such as melphalan, and an antimetabolite such as methotrexate, was specifically sensitive to any

Table 2. *Illustrating the effect of expressing the results of ^{3}H-thymidine uptake as the* % *ratio* $\frac{^3H \text{ in DNA}}{\text{Total } ^3H \text{ in tissue}}$ %

[*Expt. no.* 69 *carcinoma of the maxilla, in culture for* 5 *days*]

Duplicate results dis/min in DNA/mg dry wt.	% Ratio	Drug
2063	96	Control
2668	83	
2877	83	ThioTEPA 10^{-6} g/ml
2083	80	
4440	81	Methotrexate, 10^{-6} g/ml
5060	87	
893	27	Melphalan, 10^{-6} g/ml
1012	34	

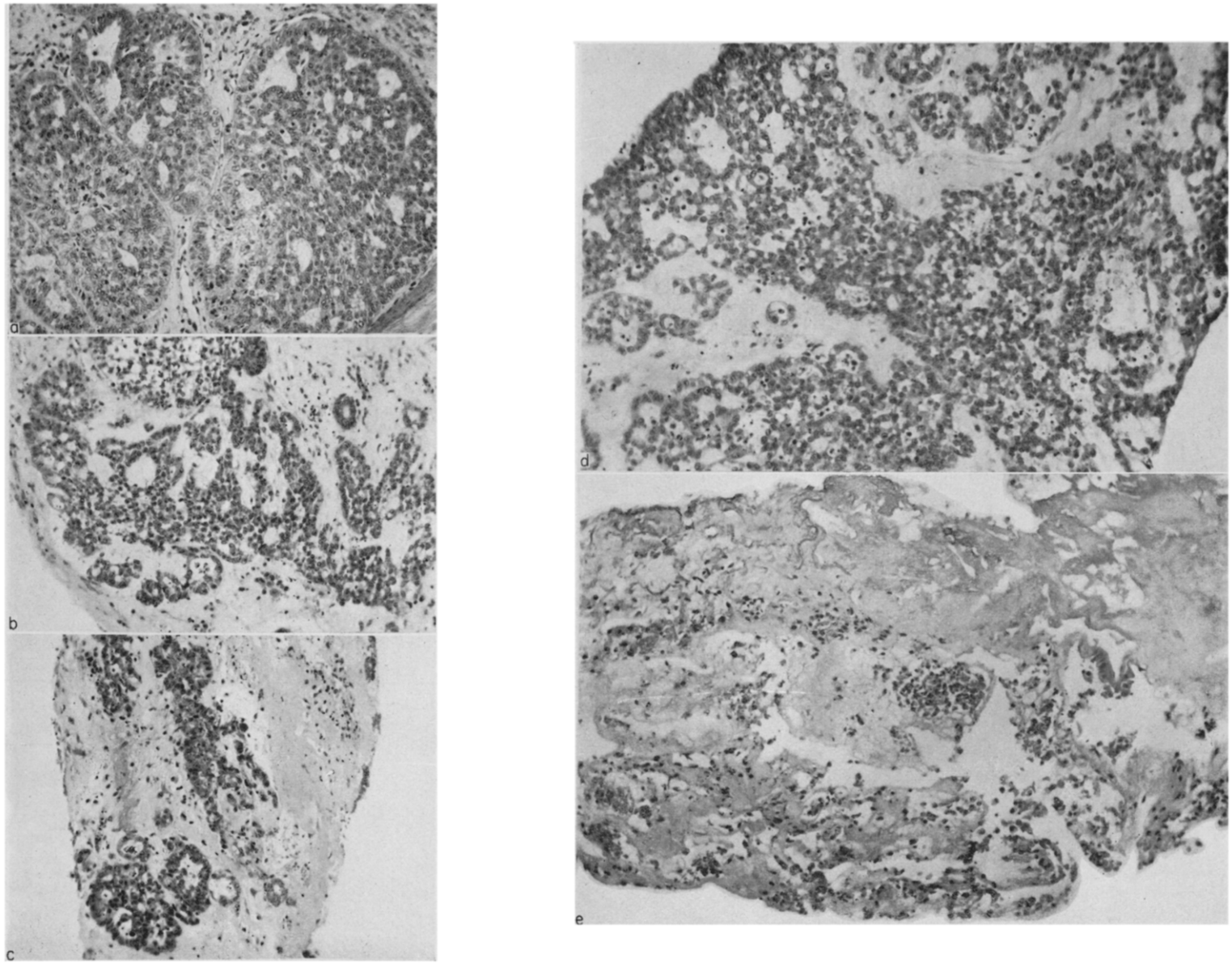


Fig. 1. *Specimen of a Ca. Maxilla. Mag.* ×200. (a) *Original uncultured tissue.* (b) *Cultured* 5 *days, Control tissue.* (c) *Cultured* 5 *days* + *Thiotepa* 10^{-6} *g/ml.* (d) *Cultured* 5 *days* + *Methotrexate* 10^{-6} *g/ml.* (e) *Cultured* 5 *days* + *Melphalan* 10^{-6} *g/ml.*

(to face p. 40)

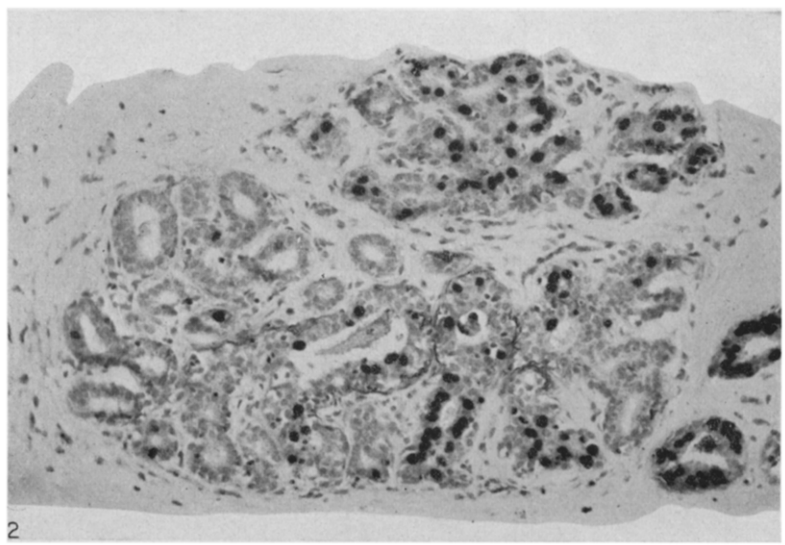


Fig. 2. Autoradiograph of a specimen of breast tumour cultured for 4 days in the presence of ^{3}H-thymidine

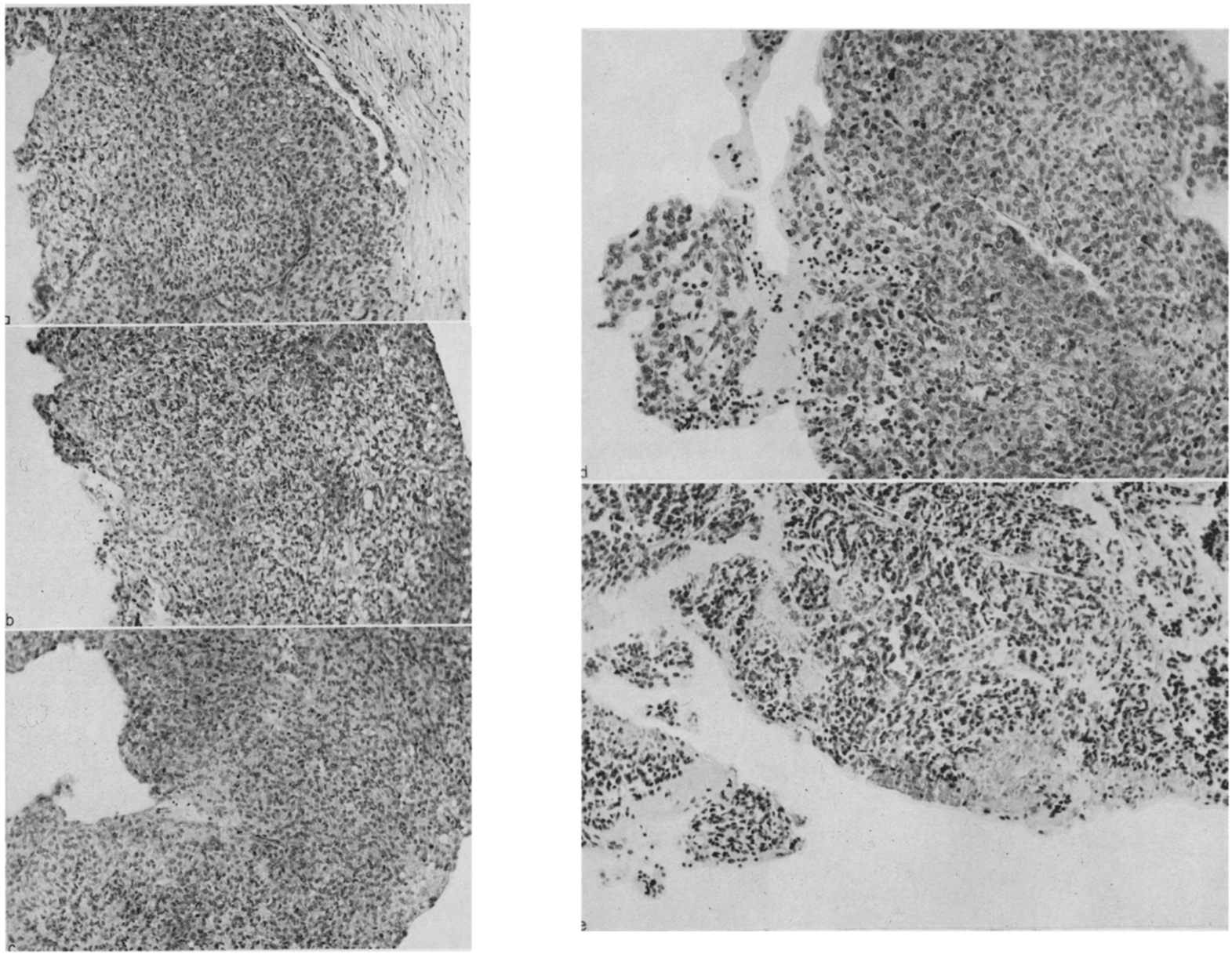


Fig. 3. Specimen of a Ca. kidney. (a) *Original un-cultured tissue.* (b) *Cultured 4 days, Control tissue.* (c) *Cultured 4 days+Hydrocortisone* 10^{-6} *g/ml.* (*d*) *Cultured 4 days+stilboestrol* 10^{-6} *g/ml.* (e) *Cultured 4 days+Provera* 10^{-6} *g/ml.*

Table 3. Summary of results of ^{3}H-thymidine incorporation expressed as % ratios

Specimen	Length of culture (days)	Control (%)	Melphalan at 10^{-6} g/ml (%)	Thio-PETA at 10^{-6} g/ml (%)	Methotrexate at 10^{-6} g/ml (%)	Mannitol-myleran at 10^{-6} g/ml (%)
Melanoma 1	3	90	9	46	89	—
Melanoma 2	3	92	35	45	92	—
Melanoma 3	3	50	28	46	52	20
Melanoma 4	5	41	45	37	49	29
Melanoma 5	3	68	58	62	80	65
Ca ovary	5	60	53	42	41	26
Lymph node, melanoma metastasis	6	36	10	10	58	—
Lymph node, chronic lymphocytic leukaemia	2	52	48	53	72	54
Glioma	2	66	—	—	98 97% at 5×10^{-5} g/ml	—
Carcinoma of the maxilla	5	90	30	82	84	—
Retroperitoneal sarcoma	4	58	68	82	68	—

one drug. The order of effectiveness of these drugs is mannitol myleran $\geq$ melphalan $>$ ThioTEPA $>$ methotrexate. Thus, if a tumour is particularly sensitive to one drug, it is sensitive to the others in that order. A representative group of results is shown in Table 3. Whereas there is a definite difference in the degree of sensitivity of various tumours towards a particular drug, such as melphalan, there is no evidence of specificity of any of the four drugs tested. For example, several melanomas have been studied in this system and, as shown in Table 3, melanoma 1, 2 and 3 show definite sensitivity towards melphalan to different extents, but the cytotoxic drugs maintain the order of toxicity as mentioned above. Melanomas 4 and 5 are relatively insensitive to melphalan, and similarly the other drugs have no effect on these cultures.

The correlation of *in vitro* experiments with the *in vivo* observation has been hampered by the difficulty of assessing the effect of melphalan clinically. However, in the several cases in which the specimens showed sensitivity towards melphalan *in vitro*, a prolongation of the expected life of the patients was obtained when the patients were treated with melphalan. The detailed analysis by Ariel and Pack [7] on the results of treatment of 117 patients with malignant melanomas has shown definite sensitivity to melphalan, and we hope to accumulate more *in vitro* results to establish correlation between the *in vitro* and *in vivo* effect of melphalan.

A direct relationship between gonadal and corticosteroid hormones and renal neoplasia has been established in the experimental field [8–10]. This knowledge has been applied to the treatment of patients with advanced metastatic renal cell carcinoma (Bloom and Wallace [11]). A definite objective response was obtained in 8 of 38 cases treated with various steroid hormones. Seven patients showed improvement with a progestational compound, Provera, and the remaining case with testosterone. In 2 cases treated with Provera, acceleration of tumour growth appeared to occur, associated with more rapid deterioration in the patient's general condition. One of these patients subsequently showed marked response with testosterone and lived for 3 years free from clinical evidence of active disease.

With the objective of establishing an *in vitro*

Table 4. The effect of hormones on the incorporation of ^{3}H-thymidine by cultures of 'normal' kidney
[*Expt. no.* 100 *in culture for* 3 *days*]

Duplicate values dis/min in DNA/mg dry wt.	% Ratio		Hormone
926	51	50	Control
346			
441	49	49	Hydrocortisone, 5×10^{-6} g/ml
443	49		
838	48	55	Progesterone, 5×10^{-6} g/ml
461	61		
415	37	43	Stilboestrol, 5×10^{-6} g/ml
862	49		
622	51	53	Testosterone, 5×10^{-6} g/ml
1358	54		

screening of hormone agents on human renal cell carcinomas, we have cultured several specimens of the carcinoma as well as the normal kidney tissue. The hormones used are: Provera, testosterone propionate, hydrocortisone, and stilboestrol all at 10^{-6} to 5×10^{-6} g/ml.

Five normal kidney specimens were received and have survived in cultures for 3–5 days.

The normal tissues were insensitive to the hormones as assessed by histological appearance. Tracer incorporation technique was applied to one specimen, and the results are shown in Table 4. In each case the histology of treated tissue is indistinguishable from that of the control cultures. Table 5 shows the results of normal tissues cultured in the presence of various hormones. Slight stimulatia by Provera and testosterone, and slight inhibition by hydrocortisone was observed for one specimen. The remaining 4 specimens showed no effect with hydrocortisone, Provera, and testosterone. Stilboestrol was consistently without effect on the cultures.

In contrast, most of the kidney tumours showed toxic effects on exposure to the hormones. Thirteen specimens were received, out of which 4 specimens were unsuitable for culture because the tissue either consisted of a fibrous mass, or was necrotic. Three cultures out of the remaining 9 specimens were poorly maintained and the results were not analysable.

The remaining 6 specimens maintained well in culture and the results are summarised in Table 5. Hydrocortisone frequently improved the appearance of the tumour cultures relative to the controls, whereas stilboestrol was toxic

Table 5. Summary of histological assessment of the effect of hormones on cultures of 'normal' and malignant kidney tissue

		Hydrocortisone	Provera	Testosterone	Stilboestrol
Tumours	Enhancement	4	0	1	0
6	No effect	1	3	2	1
specimens	Inhibition	1	3	3	5

		Hydrocortisone	Provera	Testosterone	Stilboestrol
Normal	Enhancement	0	1	1	0
5	No effect	4	4	4	5
specimens	Inhibition	1	0	0	0

in varying degree to 5 out of tumours.

The histological appearance of a culture of renal carcinomas in the presence of various hormones is illustrated in Fig. 3 (a–e).

Unlike the lack of specificity encountered with the testing of individual chemotherapeutic drugs on organ cultures of various human tumours, the renal cell carcinoma specimens showed some degree of specific sensitivity towards individual hormones. For example, 2 tumours were sensitive to both Provera and testosterone, while 2 specimens were insensitive to these hormones, but sensitive to stilboestrol. One specimen was unaffected by Provera, but was strongly inhibited to testosterone.

At present there have not been sufficient cases to enable a correlation to be drawn between the *in vitro* results of hormones and the clinical results of hormone treatment, but further experiments are continuing.

In summary, two chief points have emerged from the studies on renal tumours.

(1) The clear contrast between the lack of harmful effect of the hormones on "normal" kidney tissue compared with the frequent toxic or enhancing effect of hormones on human renal tumour tissue.

(2) The apparent hormone-specificity of the effects on some specimens in marked contrast with the lack of specificity observed with the chemotherapeutic agents.

RESUME

L'effet inhibiteur d'agents chimiothérapiques sur diverses tumeurs humaines, maintenues en culture d'organe, a été déterminé en mesurant l'inhibition de l'incorporation de la thymidine tritiée dans l'ADN, et par des examens histologiques. On obtint une bonne corrélation entre ces deux méthodes.

Aucun agent chimiothérapique, y compris des agents alkylants et des antimétabolites, ne montrait une spécificité d'action pour les tumeurs. La sensibilité de certaines tumeurs à une même substance variait de façon considérable, mais l'ordre de sensibilité d'une tumeur donnée à la série des agents étudiés restait constant. Ce test in vitro *est, dès lors, utile pour déterminer la sensibilité relative d'une tumeur aux différentes substances, et ceci est, jusqu'à présent, confirmé par l'expérience clinique.*

Contrastant avec le manque de spécificité des agents chimiothérapiques, certaines hormones ont manifesté, en culture d'organe, des effets spécifiques dans certaines tumeurs rénales. Le tissu rénal normal, cultivé dans les memes conditions, n'était pas influencé par ces hormones. Trop peu de patients ont été traités pour permettre, dès à présent, une comparaison entre l'effet in vivo *et l'effet* in vitro *de ces hormones.*

SUMMARY

The inhibitory effects of chemotherapeutic drugs on a variety of human tumours maintained as organcultures have been assessed by measuring the inhibition of the amount of incorporation of tritiated thymidine into the DNA present in the cultures, and by histological examination. Good agreement between both criteria was obtained.

None of the chemotherapeutic agents, including alkylating agents and an antimetabolite, showed any specificity of action on the tumours. Some tumours varied considerably in their sensitivity to any single drug, but the order of sensitivity of any tumour to the series of drugs was constant. This in vitro *test, therefore, is of use in determining the relative sensitivity of any individual tumour to these agents in general, and so far the clinical results have demonstrated the usefulness of this test system.*

In contrast with the lack of specificity of the chemotherapeutic drugs, some hormones have given specific effects with some sample of human renal tumours in organ culture. Organ cultures of normal human kidney tissue have not been significantly affected by any of the hormones. Insufficient patients have received hormone treatment to permit any comparison of the in vitro *and clinical effects of these hormones to be made yet.*

ZUSAMMENFASSUNG

Die Hemmwirkung chemotherapeutischer Substanzen auf verschiedene menschliche Tumoren in Organkultur wurde erstens durch Messung des H-3-Thymidineinbaus in die DNS und zweitens durch histologische Untersuchung erfasst. Beide Methoden stimmten gut überein.

Für keines der Chemotherapeutica, einschliesslich alkylierende Substanzen und Antimetaboliten, wurde eine spezifische Tumorwirkung gefunden. Einige Tumoren zeigten eine recht unterschiedliche Empfindlichkeit gegenüber einzelnen Substanzen, doch war der Empfindlichkeitsgrad eines gegebenen Tumors gegenüber der Serie der geprüften Substanzen konstant. Dieser in vitro *Test kann deshalb zur Bestimmung der relativen Empfindlichkeit eines bestimmten Tumors gegenüber chemotherapeutischen Substanzen im allgemeinen verwendet werden; er hat sich bereits in der Klinik bewährt.*

Im Gegensatz zu der fehlenden Spezifizität der chemotherapeutischen Substanzen zeigten einige Hormone eine spezifische Wirkung auf menschliche Nierentumoren in Organkultur. Normales menschliches Nierengewebe in Organkultur wurde jedoch von diesen Hormonen nicht wesentlich beeinflusst. Die Zahl der Patienten, die bisher eine Hormontherapie erhalten haben, ist zu klein, um einen Vergleich der in vitro *Resultate mit der klinischen Wirkung zu erlauben.*

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Action of Ibenzmethyzin (N-isopropyl-α-(2-methyl-hydrazino)-*p*-toluamid-hydrochlorid, Natulan^R, IMTH) on Protein Synthesis in the Rat Liver

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In vitro, under aerobic conditions, Ibenzmethyzin (IMTH), a methylhydrazine derivate, leads to a decrease in viscosity of aqueous solutions of DNA‡. This effect is a consequence of the formation of H_2O_2 during auto-oxydation of methylhydrazine derivatives. DNa is split into fragments, whereby the double helical structure within the fragments is preserved [1, 2]. A summary of what is known about the mode of action and the therapeutic effects has been recently presented [3]. The *biological* mode of action has not been elucidated. On the basis of *in vitro* studies the possibility was put forward that methylhydrazine derivatives spontaneously give compounds that act *in vivo* as alkylating agents [4]. It has been shown by incorporation experiments *in vivo* that pre-existing DNA remains unchanged, but that the rate of formation of *new* DNA is depressed under the influence of IMTH [5].

The results of the above mentioned *in vitro* experiments with IMTH and DNA in aqueous solutions allows one to formulate the following working hypothesis:

(a) IMTH acts *in vivo* also on DNA, whereby the biosynthesis of (*m*–) RNA is blocked;
(b) As a consequence of lack of continuous formation of *m*-RNA, the rate of protein biosynthesis becomes more and more depressed.

In the presetn investigation, this hypothesis was tested:

(a) by measuring the rate of protein biosynthesis in the isolated perfused rat liver,

(b) by examining some aspects of the physicochemical state of DNA of rat liver and

(c) by using systematically all known factors for protein biosynthesis in cell free amino acid incorporating rat liver systems. Evidence is presented that in the rat liver IMTH alters in some way the ribosomes, e.g. by peripheral methylation of ribosomal RNA.

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‡Abbreviations to be used in this paper: ATP—adenosine-5′-triphosphate; CTP—cytidine-5′-triphosphate; DNA—deoxyribonucleic acid; DOC—deoxycholate; GTP—guanosine-5′-triphosphate; ip—intraperitoneal; O.D.-U.—optical density unit; PEP—phosphoenolpyruvate, potassium salt; Poly-U—polyuridylic acid; PVS—polyvinyl sulfate; *m*-RNA—messenger RNA; *r*-RNA—ribosomal RNA; *s*-RNA—soluble RNA; RNA'ase—(pancreatic) ribonuclease; SDS—sodium dodecyl sulfate; SSC—standard sodium-citrate (1 × SSC = 0·15 M-NaCl, 0·015 M-trisodium citrate, pH 7); Tris—Tris (hydroxymethyl)-aminomethane.

METHODS

1. *Animals and IMTH application*

Experimental animals were female, 3–5 months old, 150–250 g white rats of our own inbred strain. The animals had an Altromin-standard diet and water *ad libitum*. They were fasted during one night before perfusion experiments. Before perfusion experiments IMTH was given 1–4 times; before other types of experiments, 3–4 times, every second day, intraperitoneally, in a single dose of 200 mg/kg. Rat livers were used in all experiments.

2. *Perfusion of the isolated rat liver*

The preparation of the perfusion solution (Tyrode solution) with washed bovine erythrocytes but without antibiotics, the perfusion machine, and the technique of operation, are described in full detail [6–8]. The volume of the perfusion medium was 200 ml. Livers were perfused for 2 hr.

Additions—Glycine-1-C^{14} (Radiochemical Center, Amersham) was added in a total dose of 1·5 mg and a total activity of 20μC. In some experiments, IMTH was added with a final concentration of 5 mM.

Determinations. $C^{14}O_2$ was collected in NaOH every 15 min. $BaC^{14}O_3$ was prepared as described [9] and counted in a Friesecke and Hoepfner window counter with methane flow. Activities were accumulated over 2 hr and are indicated as cpm/l g liver/2 hr.

At the end of the perfusion, livers were fractionated to prepare *nuclei*, *microsomal fraction* and *soluble fraction*. Livers were perfused and washed with ice cold 0·15 *M* KCl, cut with scissors and homogenized in a precooled all-glass-homogenizer at low speed for 30 sec with 1 part of buffer (KH_2PO_4–Na_2HPO_4 0·02 *M*, pH 7·6; KCl 0·025 *M*, $KHCO_3$ 0·03 *M*, Sucrose 0·25 *M*, $MgCl_2$ 0·01 *M*). Isolation of nuclei was achieved according to Begg and Munro [10].

Postnuclear supernatants were centrifuged at 20,000 **g** for 20 min, and the pellets were discarded.

Postmitochondrial supernatants were centrifuged in an Omega 1 ultracentrifuge W 60 rotor at 50,000 rev/min for 2 hr. Resulting pellets were the microsomal fraction, and the supernatants the soluble fraction.

Nuclear, microsomal and soluble fractions were precipitated with 1 part 10% $HClO_4$, incubated at 90° C for 15 min, and prepared further as described elsewhere [11].

Preparations were counted as described, corrected as usual and are indicated as cpm/mg protein/2 hr unless otherwise stated.

3. *Isolation of DNA*

DNA was isolated from rat livers pretreated 3–4 times with IMTH *in vivo*, and from control livers according to Marmur [12], except that the isopropanol step was omitted in order to save possible DNA-fragments.

4. *Denaturation of DNA* [13]

DNA preparations in 1 × SSC (0·15 *M* NaCl, 0·015 *M* trisodium citrate, pH7, were heated at 100° C for 15 min and rapidly cooled to 0°C. The increase of extinction was measured in a Zeiss spectrophotometer at 260 mμ at 0°C.

5. *Density gradient ultracentrifugation of DNA* [14]

3 ml of optical grade CsCl (Merck, Darmstadt, Germany) in 1 × SSC, pH 7, were mixed with 70–90 γ DNA. The density of the solution was adjusted to 1·71 g/cm³, and was checked by reading the refractive index. The solutions, in 5 ml tubes of the SW 40 rotor of the Omega 1 ultracentrifuge, were overlayered with liquid paraffin and centrifuged at 35,500 rev/min and 25°C for 40–50 hr. At the end, the bottoms of the tubes were punctured by a needle and two-drop fractions were collected. Optical densities and refractive indices for calculation of the densities were determined.

Further details can be found in the legends.

6. *Isolation of total RNA* [15–19]

RNA was labelled with 200 μC P^{32} (Swiss) or 100 μC orotic acid-6-C^{14} (New England) by intraperitoneal injection into rats. 1–2 g of liver were homogenized at 60°C with 3 strokes and low speed in all-glass homogenizers in 5 ml of the following buffer: 0·01 *M* acetate buffer, pH 5·1; 0·005 *M* sucrose, 0·0001 *M* $MnCl_2$, 0·05 *M* NaCl, 0·5 % SDS, 25 γ/ml PVS (Siegfried, Switzerland and Eastman-Kodak, Rochester, N.Y., U.S.A.). Immediately 5 ml of 88% (v/v) fresh phenol (Merck, Darmstadt, Germany) in the above mentioned SDS buffer (60°C) were added and the mixture was extracted for 3 min at 60°C and immediately cooled to 0°C.

The suspension was centrifuged at 0°C and 20,000 **g** for 1 min. The bufferphase and the interphase were re-extracted twice with fresh phenol–SDS buffer in the same manner. Finally, the nucleic acids were precipitated with 1/10 vol. 20% K acetate, pH 5, and 2 vol ethanol for 2 hr at −15° C. RNA was dissolved in 0·01 *M* acetate buffer, 0·0001 *M* $MnCl_2$, 0·05 *M* NaCl, 20 γ/ml PVS, pH 5, and reprecipitated 2–3 times with ethanol.

7. *Isolation of soluble RNA* [20, 21]

Soluble RNA was extracted from the soluble fraction (described under *Methods*, 2, except that nuclei were rehomogenized and resedimented. This supernatant was additionally used for preparing the soluble fraction). The soluble fraction was cleared by a second run at 50,000 rev/min and 0°C for 3 hr. The two upper thirds of the supernatant were diluted with 2 volumes of distilled water and brought to pH 5·1 with 0·2 *N* acetic acid with constant stirring. After 30 min, the precipitate was centrifuged at 6000 rev/min and 0°C for 20 min. The sediment was then extracted with fresh 90% (v/v) phenol in 0·01 *M* acetate buffer, pH 5·1, as described elsewhere [20, 21].

Soluble RNA was stripped in 0·1 *M* Na_2CO_3, pH 10 at 37° C for 1 hr according to Chapeville *et al.* [22].

8. *Isolation of polysomes* [23, 24]

Microsomes as prepared under *Methods*, 2, were gently suspended in buffer (0·025 *M* KCL, 0·008 *M* $MgCl_2$, 0·1M Tris, 0·15 *M* sucrose, 1·3% DOC, pH 7·4) and centrifuged through layers of abruptly changing concentration of sucrose in the mentioned buffer (15% and 35% sucrose) as described by Wettstein *et al.* [24]. Pellets were gently suspended in the appropriate buffer and cleared at 20,000 **g** for 20 min.

9. *Isolation of ribosomes*

Ribosomes were isolated according to a method described elsewhere [25].

10. *Sucrose gradient analyses*

Polysomes—Polysomes were ultracentrifuged through

0·025 *M* KCL, 0·008 *M* $MgCl_2$, 0·1 *M* Tris, pH 7·4, 15%–35% sucrose (g/v) in the SW 40 rotor of the Omega 1 ultracentrifuge for 30 min at 0°C and 39,000 rev/min. Two drops per fraction were collected.

Ribosomes. Ribosomes, unless stated otherwise, were ultracentrifuged through 5–20% sucrose (g/v) in distilled water in the SW 40 swinging bucket rotor of the Omega ultracentrifuge for 30 min at 0°C and 39,000 rev/min. Two drops per fraction were collected.

Total RNA. 200–500 **y** RNA in 0·2 ml were centrifuged through 0.01 *M* acetate buffer, 0·0001 *M* $McCl_2$, 0·05 *M* NaCl, 20 γ/ml PVS, pH 5·1, 5–20% sucrose (g/v), in the SW 40 rotor for 3·3 hr at 0° C and 39,000 rpm. Two drops per fraction were collected.

Soluble RNA. 0·2 ml containing *s*-RNA were centrifuged through 0·01 *M* acetate buffer, pH 5·1, 5–20% sucrose (g/v), in the SW 40 rotor for 4–6 hr at 0°C and 39,000 rev/min. Two drops per fraction were collected. Further details are indicated in the legends.

11. *Amino-acyl-s-RNA-synthetases* [21]

The pH 5 precipitate was prepared according to *Methods*, 2 and 7. Sediments were washed with 0·05 *M* K acetate buffer, pH 5·1, and suspended in a minimal amount of 0·01 *M* Tris at pH 8·5. The pH was brought to 7·4 and 10% streptomycin-sulfate in 0·01 *M* Tris, pH 7·4, was added to a final concentration of 1% under constant stirring. The stirring was continued for 20 min. The precipitate was sedimented at 10,000 **g** and 0°C for 10 min. Supernatants containing the synthetases were dialyzed overnight at 4°C against 2 l of the buffer finally used in the assays, which contained 500 mg of reduced glutathione.

12. *"Transferases"* [21, 26]

The pH-5-supernatant was prepared according to Wettstein and Noll [21]. Finally, 1 ml of the appropriate buffer contained the material of 3 g of liver. Solutions were dialyzed over night with the synthetase fraction.

13. *Incorporation experiments of labelled amino acids in vitro into s-RNA or into proteins*

(a) *Loading of* s-*RNA with L-leucine-*1-C^{14} in vitro. *s*-RNA and amino-acyl-*s*-RNA synthetases of pretreated and control animals were permuted, in a systematic way, as indicated in the legends. Incubation mixtures (1·5 ml) contained in 1 ml: Tris 100 μM, KCl 25 μM, $MgCl_2$ 8 μM, pH 7·8; ATP 5 μM, CTP 0·5 μM, PEP 10μM, pyruvic kinase 10μg; synthetases 2 mg protein, *s*-RNA 370 γ, L-leucine-l-C^{14} 0·2 μM and 2·5 μC (1 mC/5·2 mg; 25 μC/1 μM; Calbiochem, Lucerne). Aliquots of 0·3 ml were taken after incubation for 0, 5, 10, 15 and 40 min at 37°C with aeration, added to 1 ml H_20 containing 2 mg of bovine albumine and precipitated with 1 ml cold 10 % $HClO_4$. Precipitates were washed 4 times with 2 ml 1% $HClO_4$, containing 5 mg/ml of unlabelled leucine. Precipitates were finally dissolved in NH_4OH and counted in planchets as described earlier.

(b) *Incorporation of L-leucine-*1-C^{14} *into polysomes* in vitro. Again, the various factors were permuted in a systematic way. Incubation mixtures contained in a total of 0·5 ml: Tris 50 μM, KCl 12·5 μM, $MgCl_2$ 1 μM, pH 7·8; ATP 2·5 μM, CTP 0·5 μM, GTP 0·5 μM, PEP 5 μM, reduced glutathione 1·5 μM, pyruvic kinase 5 μg, *s*-RNA (stripped) 500 γ, synthetases 500 γ protein, "transferases" together 500 γ protein, polysomes 5 O.D.-units, 20 amino acids, 15 mμM each, except L-Leucine-1-C^{14} 0·4 μM and 5μ C (25 μC/1 μM). These were incubated for 0 and 30 min at 37°C with aeration. Then 0·1 ml of a solution containing 1 mg/ml bovine albumin and 10 mg/ml cold leucine was added. Mixtures were precipitated with 0·6 ml cold 10% $HC10_4$ and incubated for 15 min at 90°C. The final procedure is as described above.

(c) *Incorporation of phenylalanine into polyphenylalanine using preincubated polysomes and polyuridylic acid.* 66 O.D.-units of control or pretreated liver polysomes were preincubated for 15 min at 37°C with aeration to use up endogenous messenger in a final volume of 1 ml, containing:

Tris 100 μM, KCl 25 μM, $MgCl_2$ 8 μM, pH 7·8; ATP 2·5 μM, CTP 0·5 μM, GTP 0·5 μM, glutathione 1·5 μM, PEP 5 μM, pyruvic kinase 5μg, soluble cell fraction 0·7 ml, *s*-RNA 1 mg, 20 cold amino acids 5 mμM each.

0·2 ml of this mixture were pipetted into 0·5 ml containing:

Tris 50 μM, KCl 12·5 μM, $MgCl_2$ 1 μM, pH 7·8; ATP 2·5 μM, CTP 0·5 μM, GTP 0·5 μM, glutathione 1·5 μM, PEP 5·0 μM, pyruvic kinase 5 μg, synthetases and transferases 500 μg protein each, of livers of control rats, *s*-RNA 500 γ, of livers of control rats, polyuridylic acid 125 γ and L-phenylalanine-2-C^{14} 5 μC and 2 μM (2·5 mC/mM) (Calbiochem).

Incubation was for 10 and 30 min at 37° C. Aliquots of 0·1 ml were precipitated with 10% $HC10_4$, incubated at 90°C for 15 min, washed, and counted as described above. Aliquots of 0·2 ml were directly ultracentrifuge in sucrose gradients (15–35% sucrose, 60 min 0° C, 25,000 rev/min).

14. *Determination of nucleic acids, protein, N and P*

DNA was measured quantitatively according to Schneider with the diphenylamine-reaction described by Dische. RNA was measured quantitatively with the orcine-reaction described by Mejbaum [27]. Protein was determined according to Lowry *et al.* [28] with bovine albumine as standard. Nitrogen and phosphorus were determined according to Kjeldahl and Fiske-Subbarow respectively [29].

15. *Binding of labelled IMTH to cellular components* in vivo

Female, 3 months old, 180 g control rats received 10–12 μC (1 mg) IMTH ip, labelled either in the terminal methylgroup with C^{14}, or the central carbonyl group with C^{14}. They were killed 10 min later and livers were thoroughly perfused with 0·15 *M* KCL. The livers were then homogenized in 0·002 *M* $CaCl_2$, 0·001 *M* $MgCl_2$, 0·25 *M* sucrose, and fractionated according to *Methods* 2. Every particulate fraction, including the mitochondrial fraction, was washed to constancy of specific activities, and finally plated on alumina planchets, dried, and counted.

16. *Binding of labelled IMTH to the microsomal fraction* in vitro

Postmitochondrial supernatants of control rats were prepared in Tris 0·1 *M*, $MgCl_2$ 0·008 *M*, KCl 0·025 *M*, sucrose 0·15 *M*, pH 7·4. One ml contained the microsomes of 1/20 liver. The postmitochondrial supernatant

was incubated with additions (see legend to Fig. 9) for 30 min at 37°C and pH 7·4 with aeration. Microsomes were then washed to constancy of radioactivity (3–5 times 2 hr at 50,000 rev/min and 0°C), plated on planchets, dried and counted.

RESULTS

1. *Weight decrease*

Weight decreases of rats and rat livers under IMTH- application are given in Table 1.

2. *Quantitative determination of DNA and RNA in liver tissue*

Quantitative determinations of DNA and RNA in liver tissue as related to units fresh liver, showed no significant differences between control and pretreated animals.

3. *Perfusion of the isolated rat liver*

(a) *$C^{14}O_2$ formation during the perfusion.* In Fig. 1, *A*, *a–e*, the $C^{14}O_2$ production, accumulated during the time of perfusion, is shown. Addition of IMTH to the perfusion solution was without influence. The $C^{14}O_2$ production, however, decreases in a regular fashion dependent upon the number of doses given *in vivo*.

(b) *C^{14} incorporation into proteins of various cell components during the perfusion.* In Fig. 1, *B–E*, *a–e*, the specific activities of proteins of various liver cell organellae after 2 hr perfusion with glycine-1-C^{14} are shown; addition of IMTH to the perfusion solution leads to an appreciable decrease of the specific activities. Pretreatment

*Table 1. Weight decreases of rats and rat livers under IMTH**

Doses	Number of animals	Average weight	Average decrease	Number of livers	Average weight	Average decrease	Total weight: liver weight
Controls	51	216·0	—	51	9·28	—	23·27
IMTH							
1×40mg	8	214·3	1·7	8	9·25	0·03	23·17
2×40 mg	8	206·7	9·3	8	9·15	0·13	22·59
3×40 mg	20	189·0	27·0	24	8·77	0·51	21·55
4×40 mg	14	181·1	34·9	14	8·61	0·67	21·03

*Weights are given in g; they were measured before the first, and one day after the last, IMTH injection.

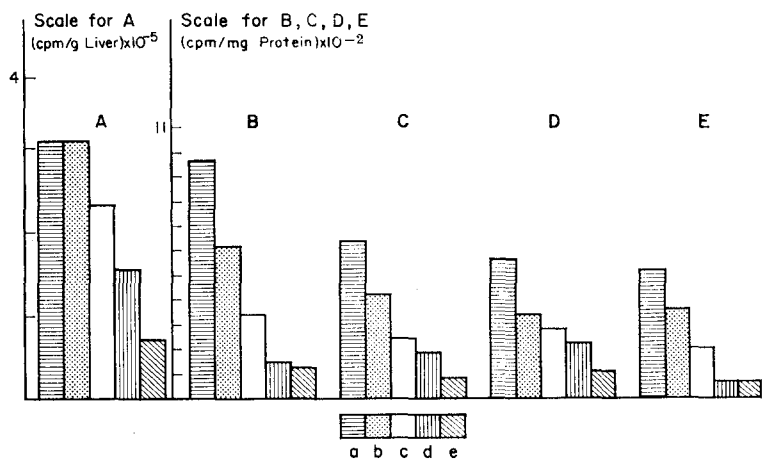


Fig. 1. Specific activities of different cell fractions and of $C^{14}O_2$ after 2 hr perfusion of isolated rat livers with glycine-1-C^{14}; dependence upon application and numbers of doses of IMTH.

Averages of at least 8 experiments per block.

A Total cpm in $C^{14}O_2$ as $BaC^{14}O_3$ per g liver, accumulated during 2 hr,
B Specific activities of microsomal liver proteins,
C Specific activities of proteins of total liver homogenates,
D Specific activities of proteins of liver cell nuclei,
E Specific activities of proteins of the soluble liver fraction.

a Control animals,
b Addition to the perfusion solution of 5 mM IMTH,
c Pretreatment *in vivo*: 1×40 mg/200 g body weight.
d Pretreatment *in vivo*: 2–3×40 mg/200 g body weight.
e Pretreatment *in vivo*: 4×40 mg/200 g body weight.

in vivo reduces the incorporation even more, eventually almost completely. The strongest influence is visible in the microsomal fraction (*B*, *a–e*).

4. *Experiments with isolated DNA*

(a) *Denaturation* was found to be the same in control and pretreated animals (liver–DNA samples).

(b) DNA's of livers of control and of pretreated animals show equal values of apparent density (1·685 g/cm³) (Figs. 2 and 3) in *density gradient centrifugation.* If DNA of *control livers* is incubated *in vitro* with *IMTH* (Figs. 2 and 3

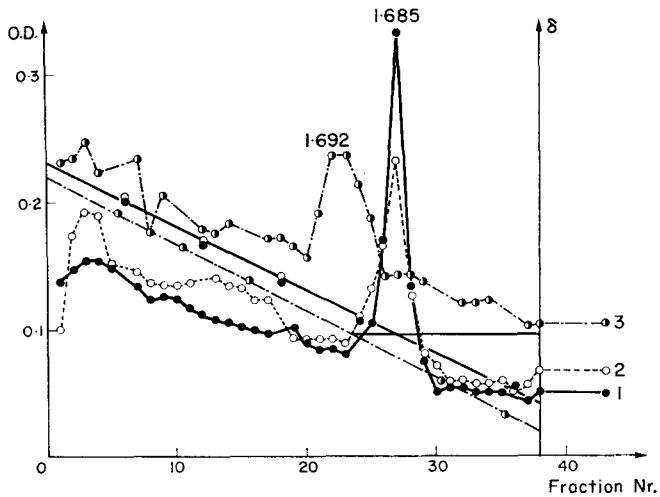


Fig. 2. Density gradient ultracentrifugation of DNA in CsCl.

Curve 1: DNA of livers of control rats, 90γ, in CsCl in 1×SSC, 50hr, 35,500 rev/min, 25°C. 38 fractions were collected. The density of 1·685 g/cm³ is an uncorrected value.

Curve 2: DNA of livers of 3 times pretreated animals, 73 γ, in CsCl in 1×SSC, 50 hr, 35,500 rev/min, 25°C. 38 fractions were collected.

Curve 3: 720γ DNA of livers of control rats in 0·2 ml 1×SSC were incubated during 25 hr at 37°C with 0·2 ml 1×SSC containing 0·002 *M* IMTH. 0·05 ml of this mixture (90γDNA) were then centrifuged in CsCl in 1×SSC for 42 hr at 35,500 rev/min and 25°C. 37 fractions were collected. The density of 1·692 is an uncorrected and questionable value.

The oblique straight lines represent the density values; direction of sedimentation is from right to left.

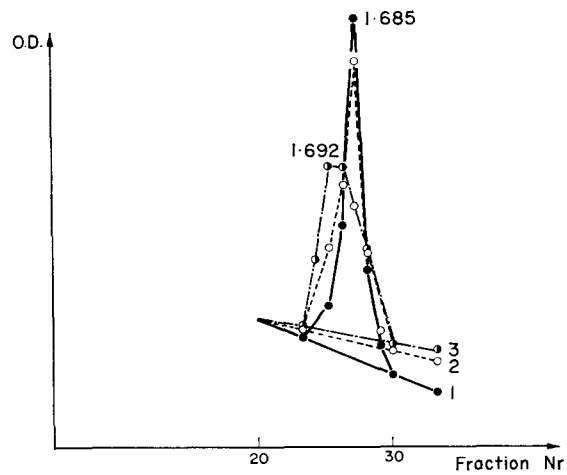


Fig. 3. Density gradient ultracentrifugation of DNA in CsCl.
Redrawing of Fig. 2.

The curves are the same as in Fig. 2. The basal points of the O.D.-peaks were connected and the areas were recalculated in such a fashion that areas 2 and 3 are equal to area 1. The densities are uncorrected. The oblique straight lines represent the connections between the basal points of the O.D.-peaks.

curves 3) the extinction curve is broadened and flattened. The amount represented by this curve is smaller than would be expected on the basis of the amount used for incubation with IMTH (90γ). In Fig. 3, the areas given by the extinction curve have been recalculated in such a way that all areas are equal. They then represent equal amounts of DNA. The change in shape of curve 3 (incubated *in vitro*) is apparent, whereas curves 1 and 2 match satisfactorily.

5. *Experiments with RNA*

(a) *Experiments with total RNA of different cell fractions.* If liver tissue of control and pretreated animals is homogenized, separated into nuclear, mitochondrial, microsomal and soluble fractions and precipitated at various times after ip injection of 180–200 μc of P^{32} into the rats, then specific activities (cpm/mg precipitate) increase in the same way. *The incorporation of P^{32}*, in vivo, *as a function of time, into total* RNA *of various cell organellae, is not changed by pretreatment* in vivo.

(b) *Experiments with different RNA's of total liver tissue.* In other experiments control and pretreated rats received, according to weight, either 180–200 μC P^{32} in phys. saline, or 90–100 μC orotic acid-6-C^{14} (4·9 mC/mM, New England Corporation) ip. They were killed 30 min, 45 min, 1 hr, 2 hr, 3 hr, 5 hr, and 7 hr later. Parts of total liver were extracted with hot phenol, and RNA was analyzed in sucrose gradients. *The incorporation of P^{32} or C^{14}*, in vivo, *as a function of time, into various fractions of RNA of total liver tissue is not changed by pretreatment* in vivo.

(c) *Sucrose gradients with stripped liver* s-*RNA.* In Fig. 4, zonal centrifugation patterns of stripped liver *s*-RNA from control and pretreated rats are shown. No differences of sedimentation behaviour can be seen.

The sedimentation characteristics are changed however, if *s*-RNA of control animals is incubated *in vitro* with IMTH (Fig. 5, curve 2). As all incubation mixtures contained the same amount of *s*-RNA, parts of *s*-RNA must have been eliminated by dialysis, as the O.D.-profile in curve 2 is appreciably lower than in curve 1. Furthermore, the O.D.-profile in Fig. 5, curve 2, is distorted with material sedimenting below 4 *s*.

6. *Experiments with polysomes*

(a) *Zonal centrifugation of polysomes.* Sucrose gradient analysis of polysomes of livers of control and of pretreated rats gives, in both cases, the familiar profiles, without increase of the monomeric 78 *s* component in comparison to the polymeric larger fractions. Furthermore, there is no difference in the rate of breakdown of the large fractions or the appearance of the products when polysomes are incubated with RNA'ase (2 γ/ml, 20°C, 1, 5, 30 min).

(b) *Binding of polyuridylic acid to ribosomes.* The unchanged profiles of pretreated polysomes favor the idea, that *m*-RNA is not only synthesized, but also bound, in a regular fashion to ribosomes, despite the presence of IMTH. Experiments to bind the artificial

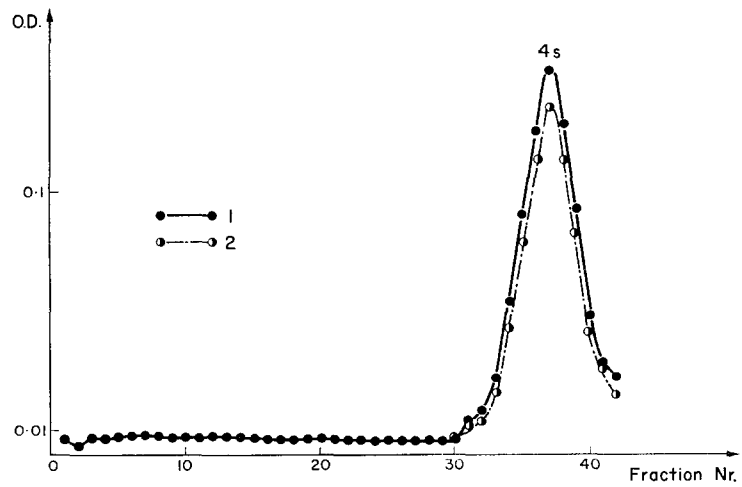


Fig. 4. Zonal centrifugation of stripped liver s-*RNA of control and pretreated rats in sucrose gradients.*
Curve 1: *s*-RNA of livers of control rats (800 γ in 0·2 ml acetate buffer).
Curve 2: *s*-RNA mixture of livers of control rats and pretreated rats (together about 700 γ in 0·2 ml acetate buffer).
Direction of sedimentation is from right and left.

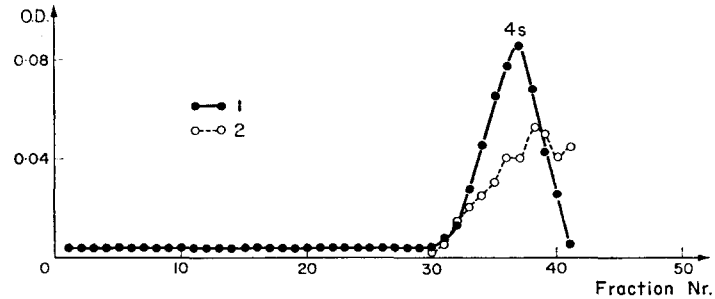


Fig. 5. *Zonal centrifugation of stripped liver* s-*RNA of control rats, incubated* in vitro *with IMTH, in sucrose gradients.*

Curve 1: *s*-RNA of livers of control rats, incubated and dialyzed as described under Curve 2, but without addition of IMTH.

Curve 2: *s*-RNA of livers of control rats, incubated *in vitro* with IMTH (1200 γ *s*-RNA in 1·2 ml 1/15 *M* phosphate buffer, pH 7, containing 0·002 *M* IMTH; 37° C, 24 hr with aeration; dialyzed overnight against 2×2 L of 1/15 *M* phosphate buffer, pH 7; 0·2 ml were centrifuged).

Gradients: 1/15 *M* phosphate buffer, pH 7, 5–20% sucrose, 4·6 hr., 39,000 rev/min, 0°C. Direction of the sedimentation is from the right to the left.

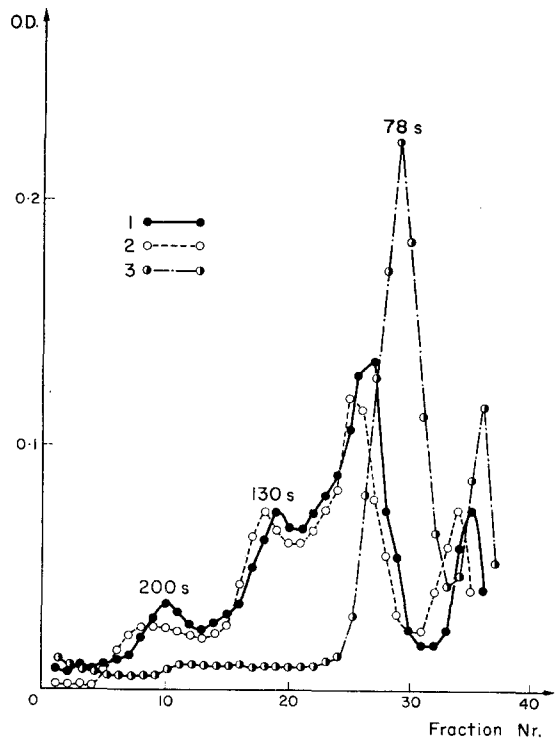


Fig. 6. *Zonal centrifugation of ribosomes in sucrose gradients and binding of polyuridylic acid* in vitro.

Curve 1: 460 γ control liver ribosomes +30 γ polyuridylic acid in 0·2 ml buffer (Tris 0·01 *M*, $MgCl_2$ 0·003 *M*, pH 7·5 [30]), ultracentrifuged for 30 min at 35,000 rev/min and 0°C through 5–20% sucrose in the same buffer; 36 fractions.

Curve 2: 400 γ pretreated liver ribosomes+25·7 γ polyuridylic acid in 0·2 ml of the above mentioned buffer, ultracentrifuged in the same way as stated under curve 1; 34 fractions.

Curve 3: Control. 460 γ ribosomes of control livers +30 γ polyuridylic acid in 0·2 ml distilled water, ultracentrifuged as described above through 5–20% sucrose in H_2O; 37 fractions.

O.D. peak to the right side: free polyuridylic acid.

O.D. peak to the left side: free 78 s ribosomes.

In our hands, rat liver ribosomes in water dissociate only very slowly in their two components [31]. Apparently there is no binding of ribosomes to polyuridylic acid in the absence of Mg^{2+}.

Direction of sedimentation is from the right to the left.

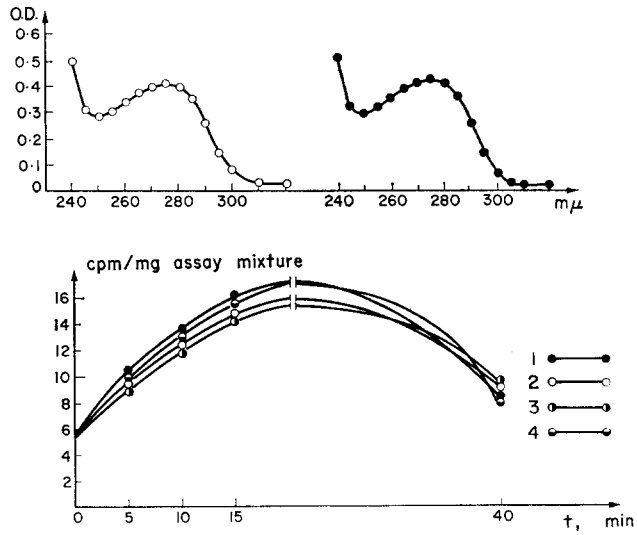


Fig. 7. Amino-acyl-s-RNA synthetases; incorporation of L-leucine-1-C^{14} into s-RNA in vitro

Above: *Spectra of the amino-acyl-s-RNA synthetases fraction*
left side: synthetases of livers of pretreated animals after the streptomycin sulfate step,
right side: synthetases of livers of control rats after the streptomycin sulfate step.

Below: *Incorporation of L-Leucine-1-C^{14} into s-RNA* in vitro
Curve 1: *s*-RNA+synthetases, both of livers of control rats.
Curve 2: pretreated *s*-RNA+control synthetases.
Curve 3: *s*-RNA+synthetases, both of livers of pretreated animals.
Curve 4: control *s*-RNA + pretreated synthetases.

messenger polyuridylic acid to ribosomes give further evidence for this assumption. Again, no differences in the sedimentation behaviour in sucrose gradients can be found (Fig. 6).

7. *Biological activity of different fractions in* in vitro *systems*

(a) *Loading of* s-*RNA* in vitro. In Fig. 7, the spectra of the synthetases after precipitation of

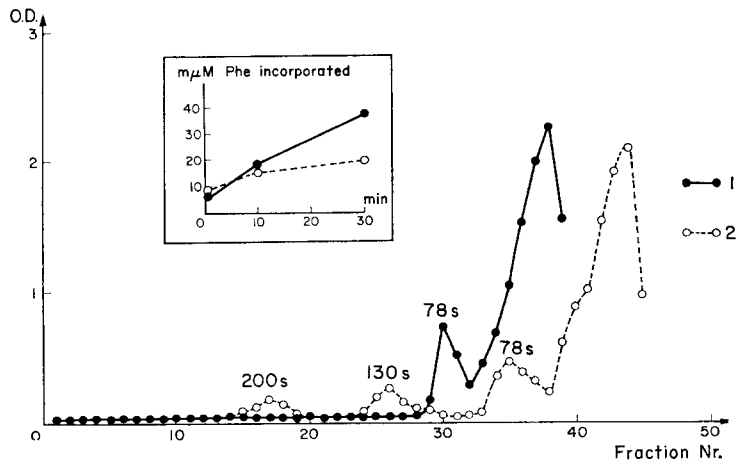


Fig. 8. Inhibition of polyphenylalanine formation in vitro *by IMTH given* in vivo.

Inset: mμM phenylalanine-2-C^{14} incorporated into acid precipitable material, calculated for 20 O.D.-units polysomes, in 30 min.

Large drawing Sucrose gradient of the whole incubation mixture. The large peaks at the right side represent free polyuridylic acid and *s*-RNA.
Direction of sedimentation is from right to the left.

Curve 1: Polysomes of control rat liver.

Curve 2: Polysomes of pretreated rat liver.

*Table 2. Radioactivity incorporated into polypeptides after 30 min incubation**

Additions	Polysomes of livers of control rats+synthetases of livers of control rats†	Polysomes of livers of pretreated rags+synthetases of livers of control rats†
Control transferases Control *s*-RNA	473	158
Control transferases Pretreated *s*-RNA	451	161
Pretreated transferases Control *s*-RNA	483	176
Pretreated transferases Pretreated *s*-RNA	460	132

*Results are given in cpm/10 O.D.-units of polysomes, corrected for background, self-absorption and zero value. Zero values were 120 cpm/10 O.D.-units.
†On basis of the results of incorporation into *s*-RNA.

s-RNA with streptomycin sulfate from the pH-5-precipitate are shown (Fig. 7; upper part). Such preparations were used for incorporation experiments as shown in Fig. 7, lower part, Table 2 and Fig. 8.

Within experimental error, the incorporation rate into *s*-RNA is the same in all cases of permutation.

(b) *Incorporation of L-leucine-1-C^{14} into polypeptides* in vitro. Incorporation values are shown in Table 2. They show that *incorporation is significantly decreased in all cases, where polysomes of livers of pretreated animals are used* for incubation. Furthermore, the results indicate that *s*-RNA of livers of pretreated animals transfers amino acids correctly into control polysomes, at least in the case of leucine and also that the activity of the transferase fractions of pretreated livers seems to be normal under our conditions. However, the exact site of IMTH—action is still not known, as *m*-RNA or ribosomal components might be damaged in the polysomal complex. Therefore, in the following experiments, the artificial messenger polyuridylic acid was used.

(c) *Incorporation of phenylalanine-C^{14} into (artificial) polysomes* in vitro, *using polyuridyclic acid.* In Fig. 8, the incorporation of phenylalanine-C^{14} into polyphenylalanine and the sedimentation behavior of the (artificial) polysomes is shown.

Again, when using *pretreated polysomes, less radioactivity* is *incorporated,* despite the presence of an artificial, presumably intact messenger (Fig. 8, inset). On the other hand, the *polysomal nature* of material used for incubation is *still visible* in this case (Fig. 8, large drawing, curve 2), whereas the polysomal peaks have completely disappeared, if control polysomes are used. There remains a single peak in the region of the 80 *s* monomeric component (Fig. 8, large drawing, curve 1).

8. *Binding of C^{14}-labelled IMTH to cellular components* in vivo

Table 3 shows the distribution of radioactivity 10 min after a single ip injection of labelled IMTH. $2{\cdot}9 \cdot 10_3$ ($=1{\cdot}3\gamma$) out of $2{\cdot}2 \cdot 10^6$ cpm injected ($=1$ mg) are found in the microsomal fraction of a whole liver.

There is only a small percentage of radio-

*Table 3. Distribution of Radioactivity (C^{14}) in various liver cell organellae, 10 min after ip injection of labelled IMTH**

	IMTH, C^{14}-labelled in the terminal methylgroup	IMTH, C^{14} labelled in the central carbonyl group
Soluble fraction	5000	7000
Microsomal fraction	2900	1300
Mitochondrial fraction	2200	1600
Nuclear fraction	30	110
Total	10,100	10,000

*Values are calculated for a total 10 g liver, free of blood, and corrected for background.

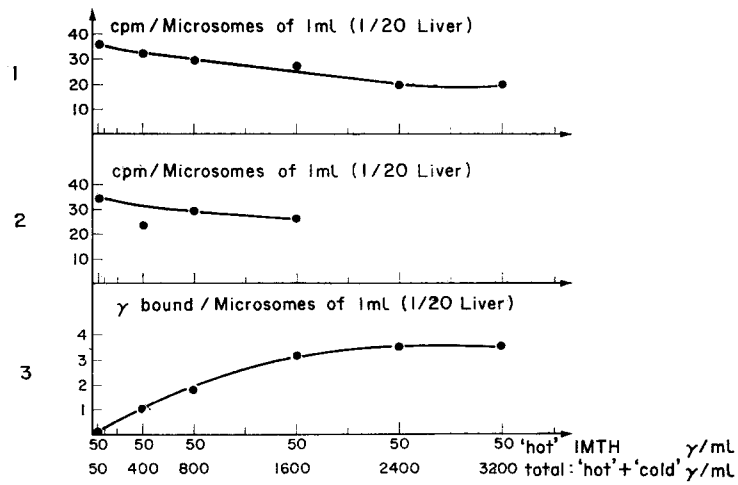


Fig. 9. Binding of labelled IMTH to microsomes in vitro. *Dependence upon concentration of IMTH.*

Additions: 1. IMTH, totally 10μC and 1 mg, 0·5 μC/ml, C^{14}-labelled in the terminal methyl group, and cold IMTH as indicated.
2. IMTH, totally 10μC and 1 mg, 0·5 μC/ml, C^{14}-labelled in the central carbonyl group, and cold IMTH as indicated.

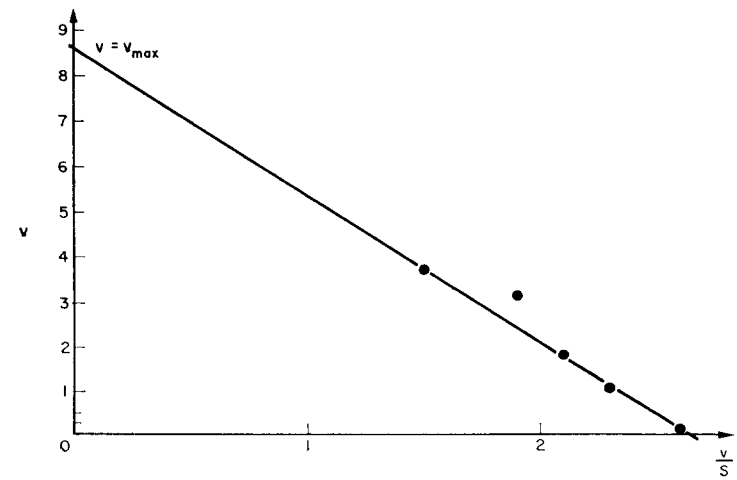
Curve 3: Redrawing of Curve 1:
For every point in curve 1, specific activities were calculated: cpm/mg (hot+cold) IMTH/ml incubation mixture. As *radioactivity* was the same in all tubes, whereas the *amount* was increasing as indicated at the abscissa, the *specific activity* decreases from point to point. Thus, the counts per minute *fixed* to the microsomes of a given tube correspond to a certain *amount* of IMTH. In curve 3, *amounts* are plotted against *concentration* in the incubation mixture.

activity in the nuclear fraction, under our conditions, 10 min after the injection of the labelled IMTH.

9. *Binding of C^{14}-labelled IMTH to the microsomal fraction* in vitro

There is evidence for a saturation curve with increasing substrate (IMTH) concentrations. Under our conditions, the curves are independent of the site of labelling (Fig. 9). The curves can be transformed into a straight line according to Eadie and Hofstee [32, 33], whereby v_{max}=170 γ/microsomes of 1 liver/30 min (Fig. 10).

Other experiments showed that, dependent upon *temperature*, a binding curve results which



10. *Binding of labelled IMTH to microsomes* in vitro. *Dependence upon concentration of IMTH.*
Redrawing of No. 1 and 3 of Fig. 9.
Values were computed according to Eadie and Hofstee [32, 33]. v=reaction rate, γ IMTH bound to microsomes of 500 mg liver during 30 min.; v_{max}=maximal reaction rate at optimal concentration of substrate=172 γ/microsomes of 1 liver/30 min; K_m=constant of Michaelis =3000 γ/ml.

corresponds to the combined activation–inactivation curves (maximum at 60° C), and that there is no clear *pH-optimum* between pH 4 and 9. There is a linear increase dependent upon *time* to 120 min incubation. There seems to be binding not only to the microsomal, but also to the polysomal fraction, as judged by sucrose layer centrifugation of DOC treated microsomes [24].

DISCUSSION

Our questions were:

(a) Does application of IMTH result in an impairment of the biosynthesis of proteins *in vivo* and/or *in vitro*?

(b) If (a) is answered positively, can an interaction of IMTH with DNA *in vivo* be found?

Question (a) is answered positively in view of the results of liver perfusion.

Question (b) can only tentatively be answered negatively despite the results of the physical examination of DNA and of RNA biosynthesis. Alkylation of DNA is not ruled out and even massive alkylation must not affect necessarily RNA synthesis. Yet, the *negative* results favour a *peripheral* action of IMTH on protein biosynthesis. The *positive* findings are:

(a) impairment of protein biosynthesis in the isolated perfused rat liver;

(b) impairment of leucine incorporation into polysomes of pretreated livers;

(c) impairment of phenylalanine incorporation into a system composed of pretreated ribosomes and polyuridylic acid;

(d) Binding of labelled IMTH to microsomes and polysomes respectively.

The exchange experiments show clearly, that *only pretreated polysomes* are responsible for the lowering of the incorporation rate of leucine. The experiment of polyphenylalanine synthesis directed by commercial, intact, polyuridylic acid would indicate furthermore that *ribosomes* are the damaged component. As the rate of incorporation is lower than in the controls, while the polysomal structure remains visible, it may be possible that the rate of movement of ribosomes along the messenger strand is lower; less peptide bonds would then be formed per unit time, even if *m*-RNA and *s*-RNA are bound to ribosomes. This explanation is not certain. As possibly pretreated polysomes do *not disintegrate* normally during *pre-incubation*, the fast sedimenting fractions would then be pre-existent polysomes, and not newly formed Poly-U-polysomes.

Every explanation for the IMTH action *in vivo*, based solely on the assumption of a disturbance of energy metabolism or substrate concentration, is insufficient. In the cell free systems used, substrates and energy are added after the dialysis of the components.

It can not be decided as yet, whether *in vivo* this effect on protein biosynthesis is the only effect. For example, an additional mitochondrial disturbance could hamper the ATP formation, and in consequence, the activation of amino acids.

On the other hand, the explanation given here for the decrease in speed of protein biosynthesis could easily be related to a methylation of bases by IMTH *after* formation of nucleic acids *in vivo* [3, 34]; this methylation leads, for example, to the formation of methylguanine in *RNA*. This, as alkylation of *DNA*, would not have easily been detected by our methods. Our findings of a binding of IMTH to microsomes and polysomes, not in the nucleus, but in the *periphery* of the liver cell would also fit this concept. Indeed, the radioactivity found in nuclei is small, but relatively large in the *microsomal* fraction, and is here, *in vitro*, dependent upon *time* and *concentration*. The fact that increasing the concentration of IMTH *in vitro* results in a plateau of radioactivity in the microsomal fraction and that a constant radioactivity is remaining independent of the number of washings of the fraction favours the idea, that there is true binding and not mere contamination. It can not be decided, however, by these experiments, whether there is binding of the *whole* molecule, or of a derivative of IMTH or whether methylation of *r*-RNA comes into play, or both. The fact that binding was independent of the site of labelling of IMTH would favour a binding of the intact molecule, at least as a first step. Then demethylation and transmethylation by a microsomal enzyme system might occur. Such enzyme systems can be found in the microsomal fraction and are *inducible* by IMTH [35].

If DNA or *s*-RNA are incubated *in vitro* with IMTH there is good evidence that both nucleic acids are partially split into small, dialyzable, fragments. It can be assumed that this splitting *in vitro* by IMTH is a general effect, and is applicable to all nucleic acids. It is questionable however, whether this effect of *splitting* can be found at all *in vivo* under the conditions used. *Discrete* effects of this kind may occur *in vivo* and might eventually explain long term changes of DNA represented

by teratogenesis, mutagenesis and cancerogenesis.

The mechanism proposed to explain the decrease of the rate of protein biosynthesis is not unknown. Wettstein *et al.* [36] described a similar effect of cycloheximide in rat liver, HeLa cells, and yeast *in vitro*.

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RESUME

On sait que l'IMTH dégrade l'ADN en solution. Les étapes de la biosynthèse protéique in vivo *et* in vitro *ont donc été étudiées chez des rats prétraités* in vivo *avec l'IMTH* (3×40 *mg i.p., tous les deux jours*). *Nos résultats sont les suivants:* 1. *L'arrêt de la biosynthèse protéique dans le foie de rat isolé et perfusé est presque total, mais il n'est pas évident que l'IMTH agisse* in vivo *sur l'ADN ou sur la biosynthèse des ARN messager, ribosomal ou soluble. Le nombre des polysomes semble être normal. Les ribosomes acceptent l'acide polyuridylique.* 2. *L'activité enzymatique dite pH* 5, *ainsi que la capacité de l'ARN soluble d'accepter des acides aminés marqués, paraît normales. L'ARN soluble, chargé, des rats prétraités, pénètre normalement dans les polysomes des animaux témoins.* 3. *Il a été démontré que l'incorporation de* C^{14} in vitro *est plus basse que la normale avec des polysomes d'animaux prétraités. Cet effet est couplé à une inhibition de la dégradation des polysomes.* 4. *L'IMTH marquée est liée en quantités réduites à la fraction microsomiale: le temps, la température, la concentration interviennent, et lep H, dans une moindre mesure.* 5. *Ces expériences n'excluent pas des effets limités sur les acides nucléiques, ou des effets additionnels sur les mitochondries.*

SUMMARY

IMTH has been reported to degrade DNA in solution. Therefore steps of protein biosynthesis in vivo *and* in vitro *were investigated after pretreating rats* in vivo *with IMTH* (3×40 *mg ip, every 2nd day*). *Our results are as follows:* 1. *In the isolated perfused rat liver, protein biosynthesis comes nearly to a full stop, but there is no clear evidence whether IMTH acts* in vivo *on DNA or the biosynthesis of* m-*RNA*, r-*RNA or* s-*RNA. The number of polysomes seems to be normal; ribosomes can accept polyuridylic acid.* 2. *The ability of pH*-5-*enzymes to load* s-*RNA and the capacity of* s-*RNA to accept labelled amino acids is normal; loaded* s-*RNA of pretreated animals can enter polysomes of control rats.* 3. *It has been shown that incorporation of* C^{14} in vitro *is lower than normal when polysomes of pretreated animals are used; this effect is coupled with an inhibited polysome breakdown.* 4. *Labelled IMTH is bound in small amounts to the microsomal fraction, dependent upon time, temperature and concentration, but barely upon pH.* 5. *Small effects on nucleic acids or additional effects on mitochondria are not excluded by these experiments.*

ZUSAMMENFASSUNG

IMTH spaltet DNA in Lösung. Deshalb wurden die Schritte der Proteinsynthese in vivo *und* in vitro *nach Vorbehandlung von Ratten* in vivo *mit IMTH* (3×40 *mg ip., jeden* 2. *Tag*) *verfolgt.*

Folgende Resultate wurden gefunden: 1. *In der isolierten perfundierten Rattenleber wird die Proteinbiosynthese nahezu völlig blockiert. Wir haben aber keine Anhaltspunkte dafür gefunden, dass IMTH auch* in vivo *auf DNA einwirkt oder die Synthese von* m-*RNA*, r-*RNA oder* s-*RNA hemmt. Die Zahl der Polysomen scheint normal zu sein; Ribosomen binden Poly-U.* 2. *Die Aktivität der pH*-5-*Enzyme und die Kapazität löslicher RNA, markierte Aminosäuren aufzunehmen, erscheinen normal. Beladene*

lösliche RNA vorbehandelter Tiere tritt normal in Polysomen von Kontrollratten ein. 3. Der Einbau von C^{14} aus Aminosäuren in säurepräzipitierbares Material ist nur dann niedriger als normal, wenn Polysomen vorbehandelter *Tiere verwendet werden. Dieser Effekt ist gekoppelt mit einem verzögerten Polysomenzusammenbruch. 4. Markiertes IMTH wird in kleinen Mengen an die Mikrosomenfraktion gebunden, in Abhängigkeit von Zeit, Temperatur und Konzentration, aber kaum in Abhängigkeit vom pH. 5. Geringfügige Veränderungen von Nukleinsäuren durch IMTH oder zusätzliche Störungen an den Mitochondrien sind auch im Lichte dieser Resultate möglich.*

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Dormancy by Dissociated Thyroid Cells in the Lungs of Mice

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INTRODUCTION

THE PRODUCTION of pulmonary growths by the intravenous inoculation of tumour cells has been investigated by many workers [1]. Experimental evidence suggests that only a small proportion of the injected tumour cells survive initially at the site of arrest and form metastases [2–4], the capillaries of the lung being a frequent site of lodgement by such cells [4, 5]. Similarly, the formation of other growths by the injection of tumour cells into the systemic circulation, appears to involve frequent lodgement in capillaries [6]. The subsequent penetration of the capillary endothelium and extension into the perivascular connective tissue [7, 8] is usually attributed to the invasive properties of the injected tumour cells.

The object of the present experiments was to test whether dissociated thyroid cells obtained from normal thyroid glands, and injected intravenously, will survive in the lungs of normal recipient mice. In addition, it seemed of interest to investigate the possible sites of such survival in relation to the endothelial barrier.

Previous work [9] has revealed that intravenous inoculation of dissociated cells from goitrogen-induced thyroid gland tumours in mice produced *extravascular* pulmonary lesions of thyroid tissue. Such pulmonary lesions seemed to occur only in recipient mice thyroidectomized by ^{131}I or rendered thyroxine deficient by short-term goitrogen treatment, and receiving no less than 10^5 thyroid cells. The short-term goitrogen treatment consisted of 0·05% methylthiouracil in their drinking water, beginning 2 days before the intravenous inoculation and lasting 6–12 weeks. It, then, became apparent that pulmonary lesions induced by the intravenous inoculation of thyroid-tumour cells were pituitary thyrotrophic hormone dependent, in conformity with the hormone dependence of goitrogen-induced tumours in rodents [10, 11]. Prolonged goitrogen treatment of mice injected with thyroid cells was avoided in view of the possibility of blood borne metastases of their own thyroid tissue to the lungs [12–14]. In the following experiments, all injected animals received 10^5 thyroid cells derived from normal thyroid glands. A preliminary communication of the present work has appeared elsewhere [15].

MATERIAL AND METHODS

Mice

A total of 267 female NZO/B1 mice, inbred for more than 65 generations and about 3 months of age, was used. After weaning, all mice were maintained on whole grain and tap water *ad libitum* with additional greens and a weekly ration of a mash composed of the following: bran 7, pollard 8, bone flour 1 and skim milk powder 4 parts by weight respectively. To each 1·5 kg of the mash, 32·5 ml. of cod liver oil and 60 g of iodized salt were added just before use. The weekly mash for mice treated with methylthiouracil (see below) was composed of equal parts of bran, pollard

and skim milk powder with additional milk pellets made from a water paste of skim milk powder.

Some mice provided the donor material. Others were injected iv with thyroid cells. A final group was not injected but was treated with methylthiouracil for varying periods.

Donor material

Suspensions of dissociated thyroid cells were prepared by trypsinization of thyroid glands (mean wt. 3·9 mg) from 228 mice. Twenty to thirty animals were killed by cervical dislocation and the excized thyroid glands were pooled in phosphate buffered saline (pH 7·3) at room temperature. In view of the small quantities of the donor material, the following trypsinization technique was found to yield the greatest number of dissociated thyroid cells. After pooling, the thyroid glands were transferred into a small sterile petri dish and were finely cut with iridectomy scissors. Ten ml of warm (37°) 0·25% trypsin (1:250 Difco) solution was then directly added and the mixture was gradually transferred, with a Pasteur pipette, into a 15-ml centrifuge tube. Further agitation of the mixture was carried out with the pipette, by suction and expulsion (about 20 times) before incubation in a water bath; and, at intervals of 15 min, during the incubation period (1 hr at 37°). After incubation the contents of the centrifuge tube were poured on to a stainless wire sieve (mesh 100 × 100 to the inch), resting on a petri dish, and allowed to strain by gravitation after an initial gentle suction–expulsion with a pipette. The strained trypsin solution was then centrifuged for 12 min at 1500 rev/min and, after decanting, the remaining minute sediment was washed once in phosphate buffered saline, suspended in 0·5 ml of the same medium and left for ¾ hr at room temperature for recovery from the traumatic effect of trypsin.

Viability Tests

Immediately before injection into the recipients, the following viability tests were made. (I) Phase contrast microscopic examination of a sample of cells from the suspension. Cells with haloes and well-defined edges were assumed to be viable, others to be degenerated. (II) The trypan blue staining-technique of Pappenheimer [16] or lissamine green 2% solution [17] was used. With either staining-technique it was found that about 90% of cells were impermeable to the dye. Cells were counted in a white cell counting chamber. The majority of counted cells appeared to be single with a very occasional group of 2 or 3 cells in contact.

Recipient mice

The dose of 10^5 thyroid cells was usually contained in about 0·2 ml of suspensions. All injections were given into either a dorsal or lateral tail vein with a tuberculin syringe and a 30 gauge needle.

Group A. Ten mice were injected with thyroid cells and had no further treatment until killed 6–26 weeks later. One died shortly after injection and was discarded.

Group B. Twelve mice were injected and were given 0·05% methylthiouracil (MTU) in their drinking water, starting at various intervals after injection (see Table 1).

Group C. This comprised 18 non-injected mice treated with MTU for varying periods (see Table 1).

The recipient mice were killed with coal gas at the end of required treatment. Intratracheal fixation was applied to all lungs before removal, by the injection of Helly's fluid through the tracheal wall. At necropsy, the lungs, thyroid gland with trachea, mediastinal tissue and any other organ of abnormal appearance was transferred and fixed into Helly's fluid for histological purposes. In view of the object of the present study, no histological investigation was made to find out whether thyroid tissue was present in bones or not. The lungs of Group A and Group C were sectioned serially, whereas serial sections from the lungs of animals of Group B were prepared when required. Sections were routinely stained by haematoxylin and eosin, and in selected cases also by PAS, Van Gieson, and Gomori's aldehyde–fuchsin [18].

RESULTS

Group A

Lungs. No evidence of thyroid tissues was seen in the lungs of these mice. Macroscopically and histologically the lungs appeared to be normal.

Thyroid glands. The thyroid gland sections in general showed large peripheral follicles rich in eosinophilic colloid lined by cuboidal epithelium; and, also, some smaller central follicles lined by slightly higher cuboidal epithelial cells.

Group B

Lungs. Macroscopically the lungs of six animals exhibited on the surface a few rounded pinkish-grey nodules mainly —1 mm dia. In one animal (Fig. 11), receiving MTU for 18

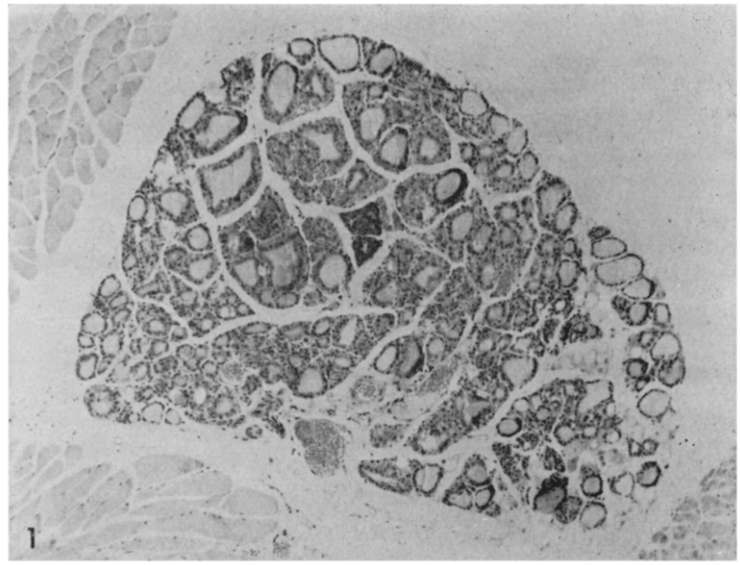


Fig. 1. *Thyroid gland from normal female NZO/B*1 *mouse, age* 26 *weeks. H & E*×78·4.

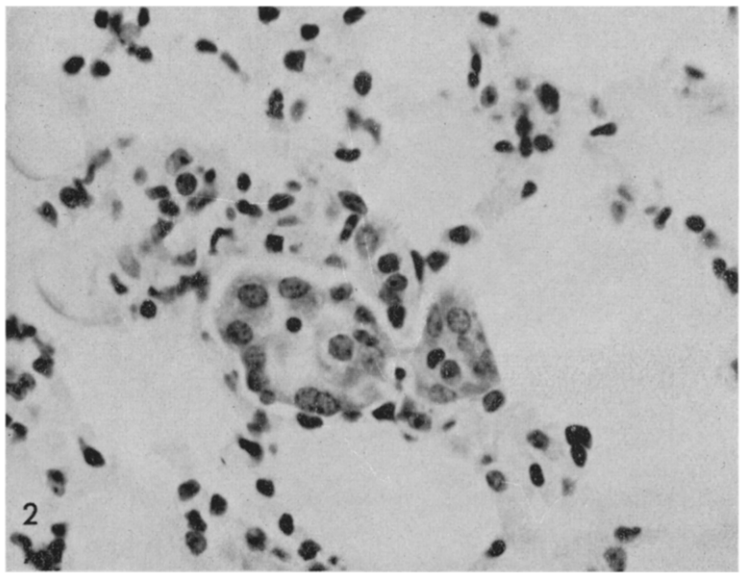


Fig. 2. *Lung from female NZO/B*1 *mouse, age* 22 *weeks, H & E*×610. *Six weeks previously this animal was injected intravenously with* 10^5 *thyroid cells. One hour following injection* 0·05% *methyl-thiouracil (MTU) was added in the drinking water. Note intra-alveolar proliferation of hyperplastic thyroid cells extending into an adjacent alveolus. Some colloid droplets are present.*

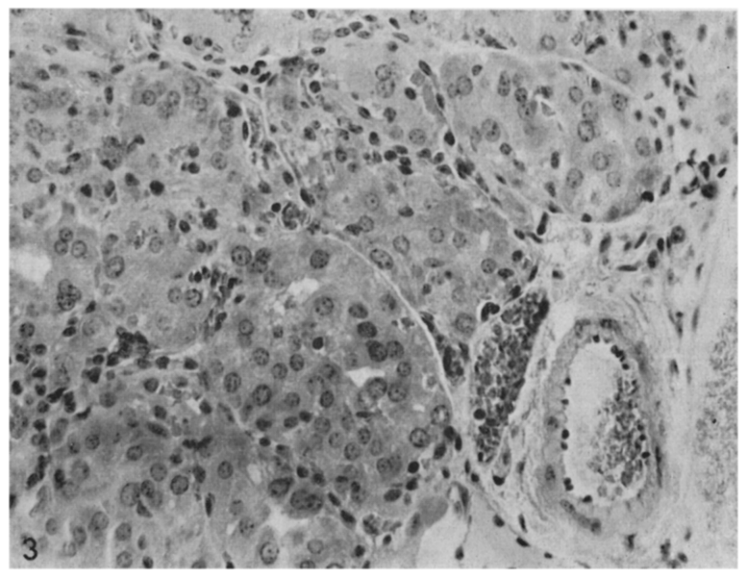


Fig. 3. *Same mouse as in Fig.* 2. *H & E*×329. *Thyroid gland. Typical effect of* 6 *weeks MTU treatment. Diffuse hyperplasia, epithelial hypertrophy and depletion of colloid.*

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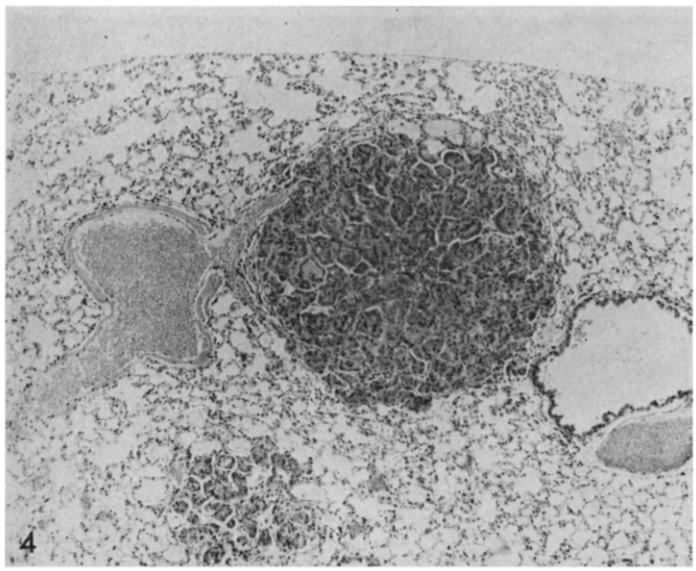


Fig. 4. *Lung from female NZO/Bl mouse, age* 47 *weeks, H & E* 88. *Injected intravenously with thyroid cells when* 12 *weeks old. Seventeen weeks later MTU was added in the drinking water. The animal was sacrificed after* 18 *weeks of MTU treatment. Two foci of hyperplastic thyroid tissue are seen, one in close proximity to a pulmonary artery.*

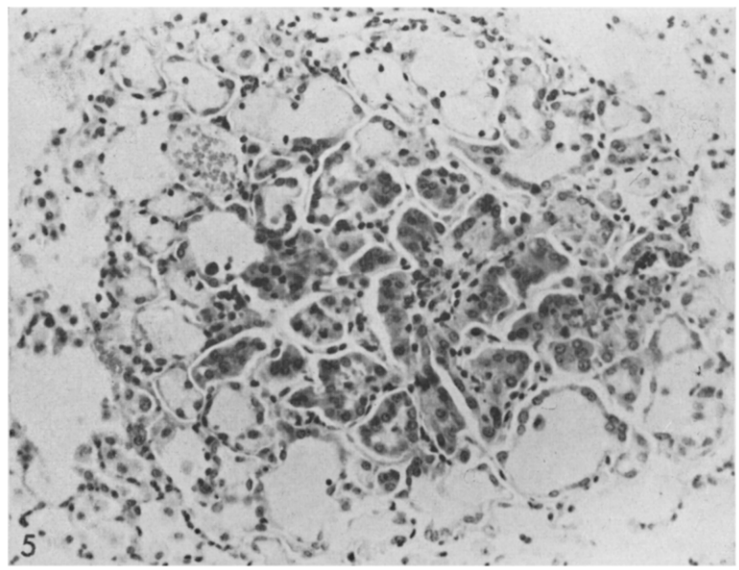


Fig. 5. *Same lung as in Fig.* 4. *H & E*×216. *Intra-alveolar proliferation of hyperplastic thyroid tissue and formation of peripheral follicles. The alveolar walls are clearly seen. Bizarre-shaped cell aggregates towards centre of lesion. Some free macrophages present at the periphery of lesion.*

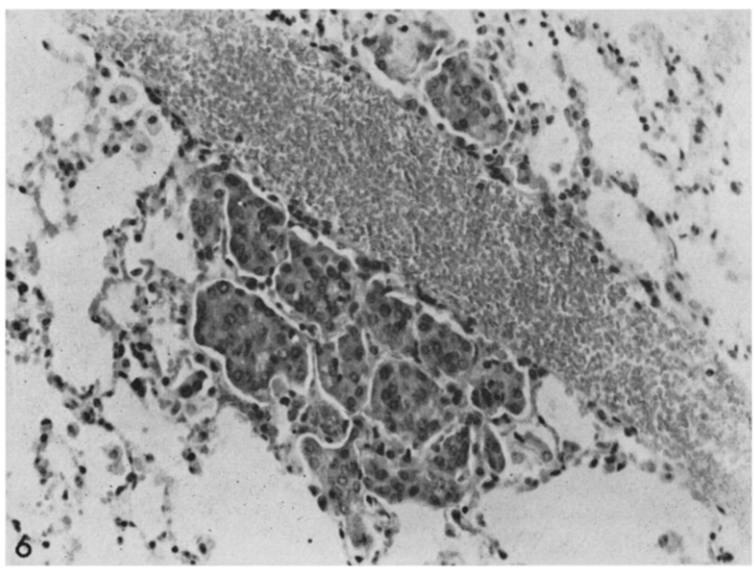


Fig. 6. *Same mouse as in Fig.* 4. *H & E*×216. *Lung. Perivascular and intra-alveolar proliferation of hyperplastic thyroid cells. Serial sections of large vessels traversing thyroid-cell lesions failed to show any abnormality within the lumen or vascular wall.*

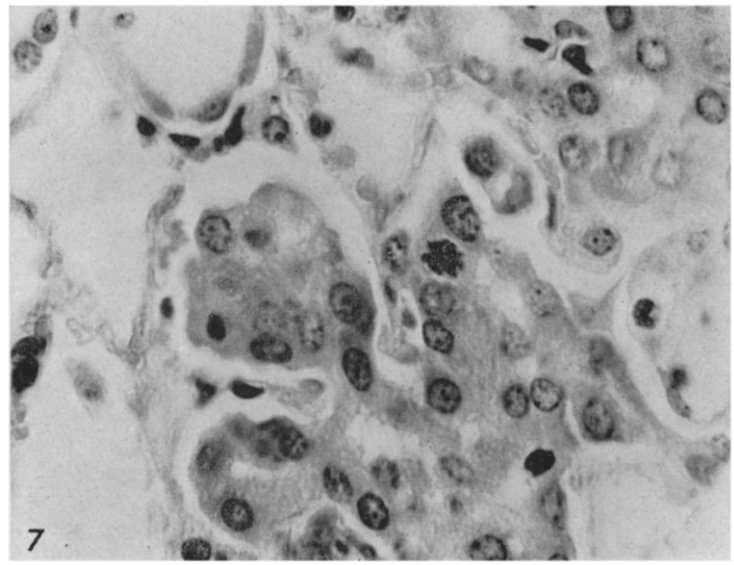


Fig. 7. Similarly treated mouse as in Fig. 4. H & E oil immersion × 952. *Lung. Proliferation of thyroid cells within alveolar spaces and extension to adjacent alveoli. Some colloid droplets and one mitotic figure are seen.*

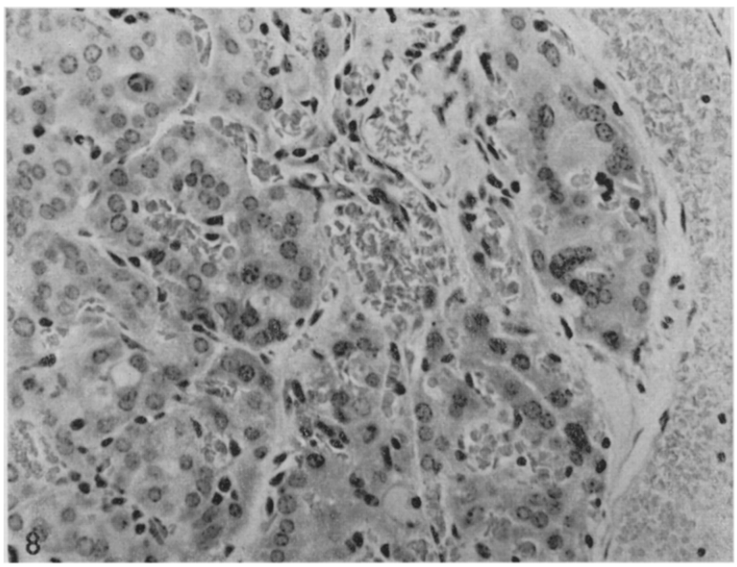


Fig. 8. Same mouse as in Fig. 4. H & E × 329. *Thyroid gland. Diffuse hyperplasia, depletion of colloid and formation of some multinucleated epithelial cells. To the right an enlarged venous sinusoid.*

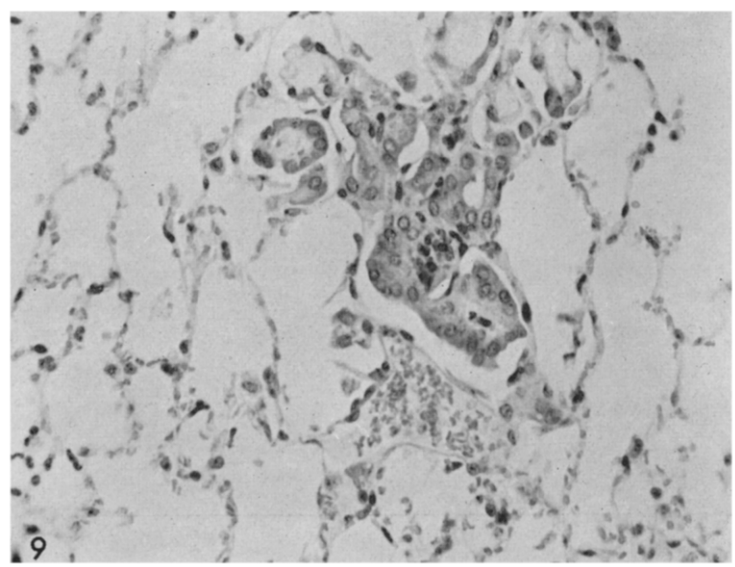


Fig. 9. Lung from female NZO/B1 mouse, age 76 weeks, H & E × 329. *Injected intravenously with thyroid cells when 12 weeks old. After 52 weeks on normal diet, MTU was added in the drinking water for a period of 12 weeks. Note intra-alveolar proliferation and follicle formation by thyroid cells.*

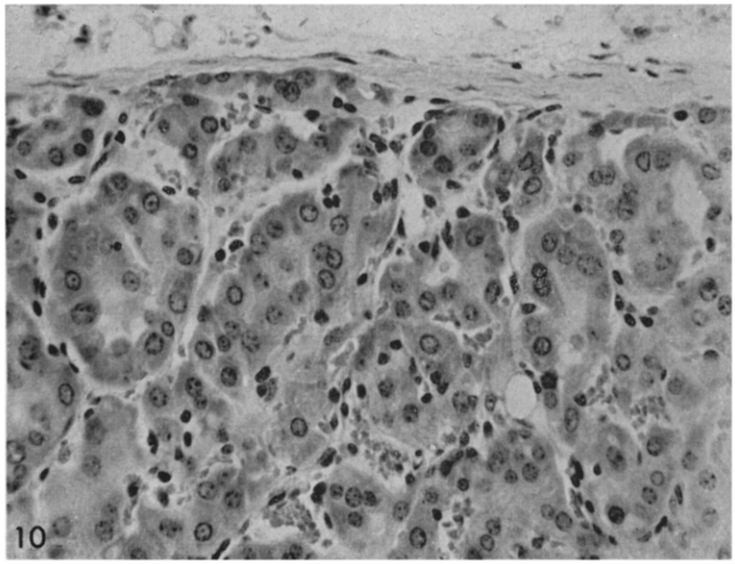


Fig. 10. *Same mouse and magnification as in Fig.* 9. *H & E. Thyroid gland. Effect of* 12 *weeks MTU treatment. Some colloid is still present within follicles. The hypertrophic thyroid cells are generally larger than those in Fig.* 9. *The thyroid capsule is intact.*



Fig. 11. *Similarly treated mouse as in Fig.* 4. *Gross appearance of thyroid gland trachea and lung.* $\times 3 \cdot 15$. *The thyroid gland is enlarged. The right upper pulmonary lobe has been slightly everted to demonstrate two thyroid-cell lesions. Such lesions are also seen on the surface of the left pulmonary lobe.*

Table 1. Summary of histological findings in the lungs of NZO mice

Mice injected intravenously with dissociated thyroid cells								Non-injected mice		
Group A			Group B					Group C		
No. of mice	Injected Sacrificed after following times (weeks)	Thyroid tissue in lungs* Micro‡	No. of mice	Injected & MTU† treated. MTU commenced at following times after injection	Duration MTU treatment (weeks)	Thyroid tissue in lungs Macro§	Micro‡	No. of mice	MTU treated Duration of treatment (weeks)	Thyroid tissue in lungs* Micro‡
2	6	—	1	1 hr	6	—	+	2	20	—
3	18	—	1	6 hr	17	—	+	2	21	—
4	26	—	1	24 hr	18	+	+	2	22	—
			1	48 hr	18	+	+	2	23	—
			1	72 hr	18	+	+	2	24	—
			3	17 weeks	18	+	+	2	25	—
			4	52 weeks	12	—	+	2	26	—
								2	27	—
								2	28	—
Total 9			12					18		

*Serially sectioned.
†0·05% methylthiouracil in drinking water.
‡Microscopically.
§Macroscopically.
—=absent. +=present.

weeks, some of these nodules measured 2 mm dia. each. All these nodules, histologically, consisted of hyperplastic thyroid tissue, similar to the tissue of other minute lesions within the lung parenchyma, as described below. Histologically the lungs of all animals in Group B contained small extravascular lesions of hyperplastic thyroid tissue. These lesions, some lying in close relation to an arteriole, varied in size and appearance and consisted of cuboidal or columnar epithelial cells forming focal areas of irregularly shaped, colloid-containing, follicles. The follicles were often situated at the periphery of the lesions. More centrally the lesions consisted of similar epithelial cells forming bizarre-shaped cell aggregates with much less pronounced follicle formation. In some other lesions, large follicles predominated throughout. All colloid present was pale eosinophilic and often PAS positive. There was slight variation in the size of nuclei and mitoses were occasionally seen. Some of the nuclei appeared to be moderately vesicular. Some striking features, however, were the proliferation of thyroid cells and the formation of follicles within the pulmonary alveolar spaces, and the extension from one alveolus to another through the antra or the pores of Kohn. The formation of an "hour-glass" constriction by interconnecting follicles, between adjacent pulmonary alveoli, was occasionally clearly seen. These intra-alveolar follicular structures were often avascular, their surface had no endothelial lining, and appeared to be separated from the surrounding alveolar walls. The latter persisted intact for some distance into the interior of some lesions, where they became less defined. In some other areas among thyroid cells, an occasional engorged capillary could be seen. Within smaller lesions the alveolar walls were evident throughout. Leucocytic reaction was absent or minimal whereas free vacuolated alveolar phagocytes were often seen at the periphery of lesions. In general, the mode of extension from one alveolus to another was similar in appearance to the intra-alveolar growth and extension of some human, metastatic lung-tumours. No thyroid cells, in either follicular or cluster form, were seen within blood vessels of the lung. No thrombosis was found.

Thyroid glands. The thyroid glands of animals in Group B were moderately hyperplastic. The enlargement appeared to be uniform throughout the gland. Sections showed a follicular hyperplasia, increased vascularity and some depletion of colloid. Focal variations in follicle appearance were also present. Epithelial hypertrophy was prominent and consisted of columnar epithelial cells lining the follicles. An occasional papillary infolding was seen. There was noticeable variation in the size of nuclei which appeared to have a decrease in the amount of chromatin and often were relatively vesicular. Some multinucleated epithelial cells were present possessing cytoplasm with no clear boundary. Such multinucleated cells were more numerous in animals treated with MTU for 18 weeks. Mitotic figures were occasionally seen. In all sections examined there was no evidence of vascular or capsular invasion. The trachealis and other muscle-tissue looked normal.

Group C

Lungs. Serial section of the lungs of all mice revealed no evidence of thyroid tissue. No abnormality was observed in the lungs or mediastinal lymph nodes.

Thyroid glands. The thyroid glands showed the classical effect of MTU treatment: diffuse hyperplasia, focal irregularities in follicle appearance, epithelial hypertrophy, reduction of colloid and formation of multinucleated epithelial cells. In animals treated from 26 to 28 weeks, follicles in close proximity to thin walled venous sinusoids failed to show any invasive changes. These juxta-venous follicles were similar in appearance to the follicle of the main mass of the gland. An occasional artery showed a slight hyperplasia of the muscular wall. There was no evidence of invasive growth in the gland, capsule or adjacent muscle tissue.

DISCUSSION

The absence of thyroid tissue in the lungs of mice in Group A was firstly interpreted as due to either failure of the injected cells to lodge in the lungs or failure to survive. Transpulmonary passage could not be excluded. Earlier studies by Zeidman and Buss [19] had demonstrated that intravenously injected *tumour* cells in suspension are capable of pulmonary transit. In addition, studies by other workers [20, 21] on the distribution of intravenously injected labelled *normal* cells pointed to the possibility of transpulmonary passage. Transplantability, as defined by Wallace [22], that is the ease of transplantation as measured by the number of cells required for a successful take, was also considered.

The affinity of thyroid tissue metastases to the lungs of rodents from goitrogen-induced thyroid tumours is well established. Furthermore, previous work has demonstrated that

dissociated thyroid cells from such tumours, injected intravenously, can survive and grow in the lungs of thyroxine deficient mice [9]. Therefore, in the present experiments, a third hypothesis was considered as a possible explanation of the absence of thyroid tissue in the lungs of Group A mice. Since the thyroid cells of the normal adult mouse very rarely divide [23, 24], the possibility remained that the injected cells may have lodged in the lungs and remained dormant. With this possibility in mind, the experiments of Group B were carried out, using MTU as a tool for the induction of proliferation in these cells.

The question arises as to what was the origin of the thyroid tissue seen in the lungs of mice in Group B. There is no reason to believe that these lesions were derived from the recipient's own thyroid gland as the result of MTU administration because in previous studies of NZO mice treated with MTU [25, 26], at least 30 weeks of treatment was required to produce the earliest metastases of thyroid tissue in the lungs; as a rule, such metastases occurred after 35–45 weeks of MTU treatment. In the present studies, shorter periods of treatment, even one of only 6 weeks, were employed. Furthermore, all animals in Group C failed to exhibit any thyroid tissue in their lungs although given longer periods of MTU treatment than the periods employed in the animals of Group B.

The possibility of vascular dissemination, in human beings, of thyroid cells from a normal thyroid gland to lymph nodes or bones has been discussed by a number of pathologists [27, 28]. However, the total number of cases of extra-thyroidal non-neoplastic thyroid tissue documented in the literature is small [29, 30]. Gricouroff [28] has suggested the possibility of migration of normal thyroid cells through lymphatics and their implantation into lymph nodes where they may remain dormant. Alternatively the implanted cells may proliferate and form a benign growth or may transform into malignant tumours. Whether spontaneous hematogenous autotransplantation of normal thyroid cells to the lungs of NZO mice may, occasionally, occur is not known. In the present experiments, the presence of hyperplastic thyroid tissue in the lungs of *all* animals in Group B and in *none* of the animals of Group C supports substantially the belief that the origin of the thyroid tissue seen in the lungs was the injected cells.

Theoretically, the site of pulmonary lodgement after the intravenous injection of dissociated thyroid cells may involve arterioles, capillaries or venules. Permanent, intravascular lodgement and survival in arterioles or venules, by adherence only to the endothelium, seems unlikely in the absence of an organized stroma and the presence of a flowing blood-medium. The noted absence of organized mural thrombi in arterioles or venules in all sections examined, as possible sites of thyroid-cell survival, points to either penetration of the endothelium of these vessels or, more likely, to lodgement in capillaries. Also, the histological findings in Group B in the present experiments indicate lodgement in capillaries. This is inferred by the frequent appearance of small thyroid-tissue foci in areas beneath the pleura or in areas some distance from larger vessels. Even when, occasionally, thyroid cells grew around thin-walled arterioles, serial sections of such areas demonstrated an intact arteriolar wall and a lumen containing only blood.

The question therefore arises whether thyroid cells arrested in the lung capillaries can survive intravascularly, for as long as a year, in a dormant state.

Human pathology provides many examples of emboli of normal tissue fragments found within pulmonary vessels after trauma, and containing viable cells. Also, in experimental studies, the intravenous injection of minced tissue from various normal organs has produced viable transplants within branches of the pulmonary artery [31–33].

The above studies, however, refer to successful transplantation, within large pulmonary vessels, of embolic tissue fragments rather than injection of single cells in suspension, devoid of stroma.

The structure of pulmonary alveolar walls, enmeshed by a capillary plexus, leaves three possible sites for survival of arrested thyroid cells: intracapillary, interstitial and intra-alveolar.

Although survival within the capillary lumen, for several months, in a single-cell state cannot be excluded, it seems unlikely. Serial sections of the lungs of Group A mice have failed to demonstrate even minute intravascular coagula as a possible nidus of prolonged survival. Also, the absence of intravascular thyroid cells in either follicular or cluster form in the lungs of Group B mice suggests extravascular survival.

Survival within pulmonary alveoli would have involved penetration and migration through endothelium, basement membrane and alveolar epithelium. In addition, the continuous renewal of the entire stock of alveolar phagocytic cells, about once a week [34], and

the constant migration of alveolar phagocytes towards bronchioles, makes it unlikely that thyroid cells could hibernate in the air sacs.

It seems, therefore, reasonable to believe that the thyroid cells remained dormant in the interstitial tissue of the lung.

A further point arises as to the effect of trypsin on the cells. Trypsin dissociated cells are altered cells. This alteration, apart from involving the disruption of the natural contacts between cells, may affect, at least, some of the surface properties of cells [35, 36].

According to Levan and Biesele [37] trypsinization can produce chromosome breaks and may have a mutagenic action. This was inferred from their studies in serial tissue cultures of mammalian cells. Edwards and Fogh [38], in electron-microscopic studies of human amnion cells, have reported that trypsinization produces micromorphological changes similar to those found in some malignant cells. In the present experiments, the use of adult thyroid cells, which were capable of remaining alive in a dormant state for several months, lends support to the belief that the replication mechanism was unlikely to be involved. If trypsinization was responsible for some malignant transformation then such transformation would have been expected, by the present definition of malignancy, to have led to thyroid-cell proliferation in the lungs of some animals in Group A.

As to the vascular endothelium, Marchesi [39] has shown by electronmicroscopy that, in inflammation, leucocytes leave the venules of the rat mesentery by inserting pseudopodia in inter-endothelial junctions, forming an "hour-glass" constriction and eventually passing through. Red cells were also found to pass through inter-endothelial spaces which were probably prepared by preceding leucocytes. A similar phenomenon of preceding leucocytic penetration, creating minute capillary endothelial defects, has been described by Wood [7] in his experiments *in vivo* with tumour cells in the rabbit ear chamber. Finally Marchesi and Gowans [40], in their electron microscopic studies, have demonstrated that, in the absence of inflammation, small lymphocytes emigrate out of the post-capillary venules of lymph-nodes by passing through the cytoplasm of the endothelial cells rather than through inter-endothelial junctions.

Whether the injected thyroid cells were capable of a behaviour similar to that of lymphocytes or whether the endothelium may have possibly formed temporary small openings assisting passage remains to be seen.

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RESUME

Des cellules thyroïdiennes dissociées, provenant de thyroïdes normales de souris de lignée pure, furent injectées, par voie intraveineuse, afin de déterminer leur possibilité de survie, dans les poumons des souris réceptrices.

Les données établies montrent que de telles cellules sont capables de demeurer dans le poumon, dans un état quiescent, durant des périodes pouvant atteindre un an, sans perdre leur capacité de proliférer et de former des follicules en réponse à une stimulation par l'hormone thyréotrope.

L'auteur discute l'hypothèse selon laquelle une telle survie nécessiterait la pénétration de la barrière endothéliale et la migration des cellules thyroïdiennes dans le tissu interstitiel du poumon.

SUMMARY

Dissociated thyroid cells obtained from normal thyroid glands of inbred mice were injected intravenously with a view to testing whether they were capable of prolonged survival in the lungs of normal recipient mice.

Evidence is adduced to show that such injected cells survive in a dormant state in the lung, even for as long as a year, without losing the capacity for proliferation and follicle formation in response to stimulation by thyrotrophic hormone.

The suggestion, that such survival may require penetration of the endothelial barrier and migration into the interstitial tissue of the lung, has been discussed.

ZUSAMMENFASSUNG

Dissoziierte Schilddrüsenzellen aus normalen Schilddrüsen von Inzuchtmäusen wurden gesunden Empfängermäusen intravenös injiziert, und ihre Fähigkeit, in der Lunge des Empfängertiers längere Zeit zu überleben, untersucht.

Es werden Anhaltspunkte dafür angeführt, dass solche injizierte Zellen in einem inaktiven Zustand ein volles Jahr überleben, ohne ihre Fähigkeit zur Proliferation und zur Follikelbildung bei Stimulierung durch thyreotropes Hormon zu verlieren.

Die Vermutung, solches Ueberleben erfordere die Durchdringung des Endothels und das Einwandern der Zellen in das Interstitialgewebe der Lunge, wird diskutiert.

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Comparative Studies of a Near-Tetraploid and a Near-Diploid Line of Ehrlich's Ascites Tumor Propagated *in vivo* and *in vitro* I. Metabolism and Growth

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INTRODUCTION

IN EXPERIMENTAL oncology ascites tumors have become widely used, mainly because they frequently offer better opportunities for quantitative studies than solid tumors. Thus, a large amount of information has been accumulated about growth, metabolism, cytogenetics, and immunogenetics of such tumors. Further information might be obtained by the study of ascites tumors grown in cultures, in which the environment can be better defined and better controlled than *in vivo*. However, the cultivation of ascites tumors *in vitro* has proved to be more difficult than one might expect, and only a small number of ascites tumors has been established as permanent cell lines in culture.

Ehrlich's ascites tumor has attracted the interest of more investigators than any other ascites tumor. Proliferation of this tumor *in vitro* was first obtained by Hull [1]. Since then a number of authors [2–14] have reported growth *in vitro* of Ehrlich's ascites tumor for varying periods of time. The result of these studies differ considerably as regards morphology, chromosome number, growth rate and tumor producing capacity. However, direct comparisons are hardly possible due to differences between the methods and the material used by the various authors.

The purpose of the present investigation was to elucidate the difference which may exist between the metabolism and growth of a near-tetraploid (ELT) and a near-diploid (ELD) line of Ehrlich's ascites tumor propagated *in vivo* and *in vitro*. In later publications, their cytological and immunological characteristics will be compared.

MATERIAL

The near-tetraploid ELT and the near-diploid ELD cells were originally obtained from Professor Klein at Karolinska Institutet in Stockholm. In our laboratory they have been carried as ascites tumors in inbred ST/a C_3H and DBA/2 mice. The ELT cells were explanted to cultures at the Fibiger laboratory in January 1964, and have since then been propagated *in vitro* in the medium described below. The ELD cells were adapted to *in vitro* conditions by Dr. Holmberg at the Research Division of Radiumhemmet in Stockholm in January 1962. We received them from Dr. Holmberg in June 1962, when they were grown in the same medium as the ELT cells with the exception that calf serum was used instead of fetal bovine serum. Since January 1964, the ELD cells have also received fetal bovine serum. The observations described in the present report were made during the period February 1964 to February 1966. In the following the designations ELTa and ELDa will be used to indicate the two cell lines carried *in*

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vivo, while the terms ELTc and ELDc will indicate the two cultured cell lines.

METHODS

Cultivation

The cells were grown in the so-called Fibiger flasks, which are a modification of the Carrel flask with a bottom surface area of 28 cm^2. They were grown directly on glass under a fluid medium (FIB 14b) composed of 20% fetal bovine serum and Eagle's MEM medium [15] containing twice the concentration of amino acids and four times the concentration of glutamine and vitamins recommended. Penicillin and streptomycin were added at concentrations of 150·000 i.u./l and 25 mg/l respectively. The medium was changed three times weekly and at the same time the cultures were gassed with a mixture (Dansk Ilt- og Brintfabrik) containing 5% CO_2, 20% O_2, and 75% N_2. The bicarbonate concentration was adjusted to yield a pH of 7·2.

The nutritional media were purchased from Microbiological Associates, Bethesda, Maryland, U.S.A. and from Grand Island Biological Company, N.Y., U.S.A. In some experiments, the medium was fortified with sodium succinate (British Drug Houses, Poole, England), lactate, oxalacetate, and ketoglutarate. The latter compounds were all purchased from Sigma, St. Louis, Mo., U.S.A.

For metabolic studies *in vivo* propagated cells were obtained from mice 4–6 days after the intraperitoneal transplantation. The cells were spun down at 500×**g** for 10 min and resuspended in Ringer–Locke's solution or the culture medium both fortified with tris (hydroxy-methyl)-aminomethane (Tris) at a concentration of 30 mM. *In vitro* propagated cells were harvested 24 hr after the last renewal of the medium. The medium was removed and after trypsinization with 0·2% trypsin, purchased from Difco, Detroit, Michigan, U.S.A., the cells were spun down at 500×**g** and resuspended in the above-mentioned reaction media.

Glycolysis was studied by measurements of glucose consumption and lactate accumulation in cell suspensions in Erlenmeyer flasks placed in a shaker in a water bath at 37°C. Glucose and lactate concentrations were measured every 30 min for 2–3 hr using specific enzymatic methods as described by Hugget and Nixon [16] and Horn and Bruns [17]. The reagents were purchased from Boehringer, Mannheim, Germany. Cellular respiration was studied with conventional Warburg technique.

The glycolytic and the respiratory activity was calculated per mg protein determined by a modification of Lowry's method as described by Oyama and Eagle [18].

Growth curves *in vivo* were obtained by daily counts of tumor cells from the different animals which were sacrificed by cervical dislocation. After aspiration of the ascites fluid the peritoneal cavity was washed with 5 ml Tyrode's solution. After dilution the cells were counted in a Bürker–Türk hemocytometer and the mean value was calculated. The mean doubling time *in vivo* was calculated with the standard formula

$$g=(T_2-T_1)\log 2/(\log N_2-\log N_1),$$

wherein T_2-T_1 is the growth time, N_1 the starting cell number per mouse, and N_2 the total cell number recovered at the end of the observation period, i.e. 2–3 days in the case of the ELTa cells and 3–4 days in the case of the ELDa cells. Experiments in which the final cell number per mouse exceeded 50×10^6 ELTa cells and 100×10^6 ELDa cells were disregarded, since these cells might no longer be in their exponential growth phase.

Growth curves *in vitro* were obtained by daily counts of ten cultures selected at random for 4–5 days. The cells were trypsinized with a 0·2% Difco trypsin solution and counted in a Bürker–Türk hemocytometer. The medium and the gas phase (5% CO_2, 20% O_2, and 75% N_2) were changed daily. The mean doubling time was calculated on the basis of ten such curves.

In some experiments, where the abscence of glucose from the medium or the presence of bicarbonate in varying concentrations were essential, fetal bovine serum dialyzed against running water for 2 hr and a balanced salt solution for another 2 hr period was used. If bicarbonate was added only at small concentrations or not at all, the buffer capacity of the medium was increased by adding Tris and phosphate buffer to yield final concentrations of 30 and 2 mM respectively.

The influence of pCO_2 and pO_2 on cell growth *in vitro* was studied in cultures loosely stoppered with gauze and placed in glass cylinders, ten in each, which were then closed, placed in the incubator and gassed continuously with the gas mixtures under investigation. The gas mixtures were purchased from "Dansk Ilt og Brintfabrik", Copenhagen, Denmark.

In a few experiments, the effects of various antiglycolytic compounds were tested. 2-deoxyglucose was purchased from Sigma and Koch-Light, Colnbrook, Bucks., England, while

iodoacetate and oxamic acid were purchased from British Drug Houses.

RESULTS

The glycolytic and respiratory metabolism of the ELT and the ELD cells were studied as described. From Table 1 it appears that both cell lines showed a higher rate of glycolysis in the culture medium than in Ringer–Locke's solution. Furthermore, it is seen that the ELTa cells showed a significantly higher rate of glucose consumption and lactate production in the culture medium than the ELDa cells. Cultivation *in vitro* for two years did not change the rate of glucose consumption significantly in either of the two lines, but both cell lines showed a significant increase in lactate accumulation at the end of the experimental period.

The respiratory metabolism of the two cell lines is shown in Table 2, from which it appears that both showed a higher rate of oxygen consumption in the culture medium than in Ringer–Locke's solution. No significant differences could be demonstrated between the respiration of the ELTa and the ELDa cells, and cultivation *in vitro* did not seem to have any effect on the respiratory activity.

The Warburg coefficient (Q lactate/Q oxygen) which is a measure of the balance between glycolysis and respiration varied considerably. When the Warburg coefficient of the *in vivo* propagated cells was measured in the culture medium, the ELTa cells showed significantly higher values than ELDa cells. This difference became even more pronounced during cultivation *in vitro*, under which conditions the Warburg coefficient of the ELT cells rose, while that of the ELD cells showed no significant changes.

The growth pattern of the ELT and the ELD cells was studied both *in vivo* and *in vitro*. Fig. 1 shows two characteristic growth curves in mice. The ELTa cells usually grew exponentially from the beginning of the experiments while the ELDa cells frequently showed an

Table 1. *Glycolytic metabolism of ELT and ELD cells propagated* in vivo *and* in vitro

Metabolic reaction medium	Method of propagation	Time of investigation	Aerobic glycolysis (μmoles per mg protein/hr)* Glucose ELT	Glucose ELD	Lactate ELT	Lactate ELD
R–L	*in vivo*	1964–66	0·56±0·09	0·43±0·07	0·78±0·08	0·60±0·10
R–L	*in vitro*	1964†	0·46±0·06	0·33±0·13	0·87±0·11	0·46±0·07
R–L	*in vitro*	1966‡	0·49±0·06	0·53±0·13	1·11±0·09	0·93±0·19
C–M	*in vivo*	1964–66	0·91±0·27	0·54±0·06	1·35±0·15	0·82±0·11
C–M	*in vitro*	1964†	0·82±0·05	0·56±0·13	1·61±0·16	1·07±0·19
C–M	*in vitro*	1966‡	0·96±0·11	0·68±0·13	2·15±0·18	1·23±0·16

R–L: Ringer–Locke's solution.
C–M: Culture medium.
*Mean with 95% confidence interval.
†Culture age: ELTc 3 months. ELDc 2 years.
‡Culture age: ELTc 2 yr. ELDc 4 years.

Table 2. *Respiratory metabolism and metabolic balance of ELT and ELD cells propagated* in vivo *and* in vitro

Metabolic reaction medium	Method of propagation	Time of investigation	Oxygen consumption (μmoles per mg protein/hr)* ELT	Oxygen consumption ELD	Metabolic balance Lactate: Oxygen* ELT	Metabolic balance ELD
R–L	*in vivo*	1964–66	0·25±0·03	0·26±0·03	3·24±0·57	2·41±0·80
R–L	*in vitro*	1964†	0·24±0·02	0·20±0·02	3·75±0·58	2·06±0·17
R–L	*in vitro*	1966‡	0·28±0·03	0·32±0·03	4·08±0·57	2·81±0·45
C–M	*in vivo*	1964–66	0·41±0·04	0·44±0·06	3·39±0·62	2·02±0·41
C–M	*in vitro*	1964†	0·37±0·06	0·48±0·06	4·67±0·75	2·33±0·58
C–M	*in vitro*	1966‡	0·40±0·04	0·50±0·05	5·79±1·10	2·67±0·65

R–L: Ringer–Locke's solution.
C–M: Culture medium with dialyzed serum.
*Mean with 95% confidence interval.
†Culture age: ELTc 3 months. ELDc 2 years.
‡Culture age: ELTc 2 years. ELDc 4 years.

initial lag phase. During the exponential growth phase no significant differences were observed between the doubling times of the ELTa and ELDa cells (Table 3), but as illustrated by Fig. 1, the growth curve of the ELTa cells usually started to fall off when the total population had reached a size of 50×10^6 cells per mouse, while a similar change of the growth rate did not occur in the ELDa tumors even at 150×10^6 cells.

Table 3. *Growth rate of ELT and ELD cells in mice and in cultures*

Cell strain	Cellular doubling time* (hr)	
	in vivo	*in vitro*
ELT	18·65±1·53	25·50±1·84
ELD	19·96±0·90	24·50±1·60

*Mean with 95% confidence interval.

In Fig. 2, the growth curves *in vitro* are shown. Due to the great loss of cells which often accompany the inoculation to culture flasks the initial values were not determined until after 24 hr of incubation. Thus, an initial lag phase might not be revealed by these studies. After the first day of incubation both cell lines showed exponential growth. It appears from Table 3 that the mean doubling time of the ELTc and the ELDc cells did not differ significantly, but it was somewhat longer than the corresponding values obtained for the ELTa and the ELDa cells.

The exponential part of the growth curves *in vitro* usually continued in the case of the ELDc cultures until the culture flasks were completely filled with cells, i.e. $6–8 \times 10^6$ cells per flask, while the growth curves of the ELTc cultures in all cases started to fall off, when a cell concentration of $3–5 \times 10^6$ cells per flask had been reached. During the experimental period, from 1964–66, no significant changes in the shape of the growth curves or in the doubling time during the exponential growth phase were observed.

The results of the present metabolic studies indicated that glycolysis plays an important role in the growth processes of the ELT and ELD cells. Therefore, it was not unexpected to find that lactate, lactate plus oxalacetate, and succinate were unable to substitute for glucose as substrates (Table 4) for the growth of the ELTc and ELDc cells in a medium with dialyzed serum.

In the hope to obtain more quantitative information about the role of glycolysis in growth, the effect of three antiglycolytic agents was studied. However, in most cases, these experiments were inconclusive, since the concentrations required for a depression of glycolysis was considerably higher than the doses

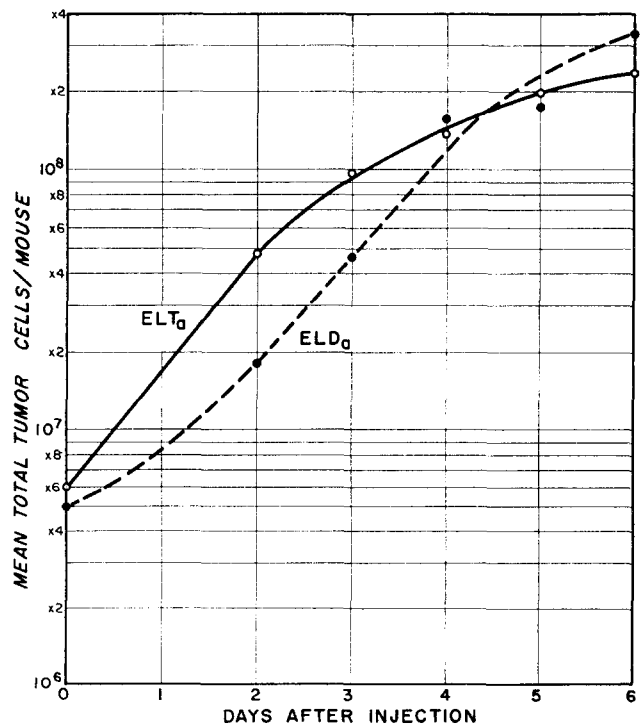


Fig. 1. *Growth of the ELTa and the ELDa cells* in vivo. *Each point represents mean total tumor-cell number per mouse, based on* 10 *mice.*

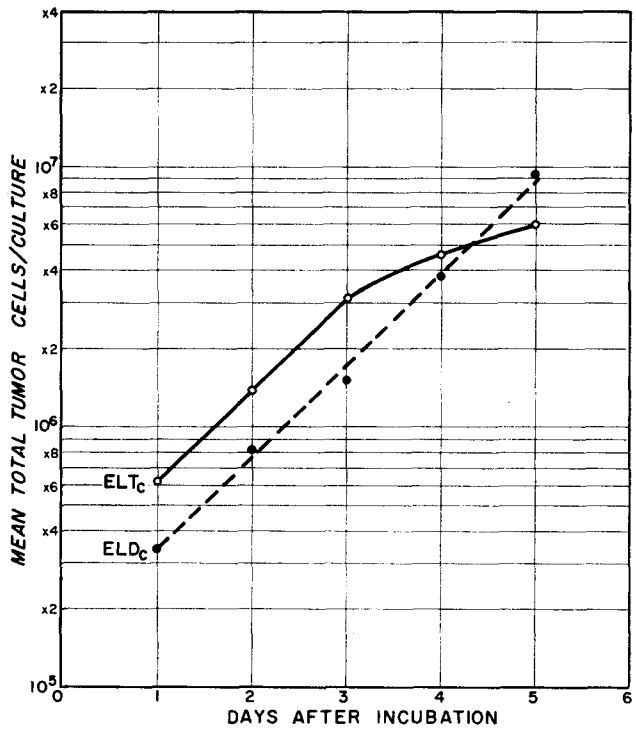


Fig. 2. Growth of the ELTc and the ELDc cells in vitro. *Each point represents mean total tumor-cell number per culture, based on* 10 *cultures.*

required for a significant cytostatic effect. Thus, 2-deoxyglucose caused a significant growth inhibition at a concentration of 0·5–1·0 mM which had no effect on glycolysis in a medium containing 7·5 mM glucose. Under the same conditions monoiodoacetate showed a significant cytostatic effect at a concentration of 0·01 mM, while a concentration of 0·05–0·1 mM was required to depress glycolysis. Only in the case of oxamic acid, a certain correlation between the anti-glycolytic and the cytostatic effect was indicated. As shown in Table 5 a significant cytostatic effect was obtained at 50–100 mM. At the same concentrations, glycosis, as measured by lactate accumulation, was inhibited by 46–87% (Table 6). At 25 mM, no significant growth inhibition was seen in spite of the fact that glycolysis was depressed by 35–46%. Thus, not all of the glycolytic energy production seemed required for maximum rate of growth.

The optimal glucose concentration for the growth of the ELDc cells has been studied in this laboratory in a perfusion system by Briand [19], who found a decrease of the growth rate, when the glucose concentration was reduced to

Table 4. The effect of substituting succinate, lactate, and lactate+oxalacetate for glucose on the growth of the ELTc and ELDc cells

Cell line	No. of cells per culture* ELTc	ELDc
Initial cell concn.	960,000±270,400	620,000±144,600
Final cell concn.		
Substrate:		
Control — 5 mM glucose	2,380,000±348,400	3,680,000±738,000
Substitute for glucose:		
None	14,600± 4600	50,000± 20,800
5 mM succinate	42,000± 41400	0
5 mM lactate	12,000± 9200	40,000± 20,700
5 mM lactate+ 1 mM oxalacetate	245,000±108,100	20,000± 8700

*Mean with 95% confidence interval.

Table 5. The influence of oxamic acid on the growth of the ELDc cells

Exp. no.	Duration of incubation (days)	Initial cell number × 10^6*	Finl cell number × 10^6 Oxamic acid concentration (mM) 0	10	25	50	100
1	4	1·840± 0·371	10·640± 1·426	10·680± 1·785	8·440± 1·812	6·800± 1·122	
2	4	1·020± 0·248	5·380± 0·934		5·720± 1·233	3·720± 0·773	1·500± 0·451

*Mean with 95% confidence interval.

2–3 mM. Under the standard conditions of the present experiments, a fall in glucose concentration below this critical value was to be expected several hours before the renewal of the medium in flasks with more than 2–3 × 10^6 cells. From Fig. 3 it appears that this was actually the case, and the continuous exponential course of the ELDc growth curve up to cell concentrations of 8–10 × 10^6 cells per flask was, therefore, perhaps more surprising than the falling off of the ELTc growth curve. Figure 4 shows that the decline of the ELTc growth curve might be delayed if the cultures are supplied with glucose at an initial concentration of 25 mM, but even in this case exponential growth could not be maintained until the flask was filled with cells. Thus, other limiting factors must be at work too.

Even if the glycolytic energy production exceeded the requirements for maximum growth under the present experimental conditions, neither of the two cell lines were able to grow under anaerobic conditions (Table 7). This effect might be due to lack of respiratory energy production or to lack of specific metabolites. No evidence in favour of the latter suggestion was obtained when the growth stimulating effect of succinate, ketoglutarate and oxalacetate was tested under anaerobic conditions. (Table 8). No growth occurred even in the presence of these metabolites.

Table 6. The influence of oxamic acid on the metabolism of the ELDc cells

Exp. no.	Oxamic acid concentration (mM)	µmoles × 10^{-6}/cell/hr 0	10	25	50	100
1	glucose consumption	0·23	0·25	0·17	0·17	0·13
	lactate production	0·39	0·30	0·21	0·21	0·08
2	glucose consumption	0·22	0·17	0·17	0·08	0·03
	lactate production	0·40	0·25	0·26	0·09	0·05
–	oxygen consumption	0·127	0·108	0·107	0·089	0·115

The influence of pCO_2 and pO_2 on cellular metabolism has previously been studied in this laboratory [20–28]. Table 9 shows that CO_2/HCO_3^- was essential for the growth of both the ELTc and the ELDc cells. Maximal rate of growth was usually reached at 5% CO_2 and maintained even at 15% CO_2 in spite of the strong anti-respiratory effect of such high CO_2 tensions [21, 22, 24, 26]. The effect of pCO_2 on glycolysis in cultures of the ELTc and ELDc cells was studied by measurements of

Table 7. Influence of anaerobiosis on the growth of the ELTc and the ELDc cells

Cell line	No. of cells per culture* ELTc	ELDc
Initial cell concn.	505,000±117,300	1,520,000±248,000
Final cell concn.		
0%oxygen	310,000± 50,600	460,000±110,400
20% –	2,000,000±436,000	3,580,000±772,800

*Mean with 95% confidence interval.

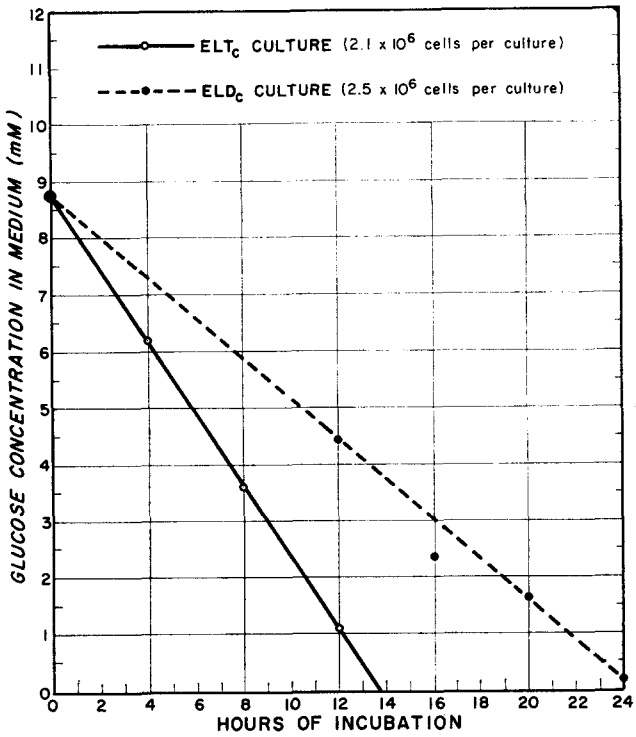


Fig. 3. *Changes in glucose concentration in ELTc and ELDc cultures during* 24 *hr of incubation. Each point represents mean values, based on* 10 *cultures.*

the glucose concentration in samples of the medium withdrawn directly from the growing cultures at 30 min interval for 4 hr. From Table 10 it appears that glycolysis in ELTc and ELDc cells was not influenced by variations in pCO_2 between 1 and 10% atm.

Our previous studies of the influence of pO_2 on growth and metabolism of Ehrlich's ascites tumor cells propagated *in vivo* showed [27, 28] that maximum rate of growth was obtained when the tumor carrying hosts were kept at 20% O_2. The present investigation included a

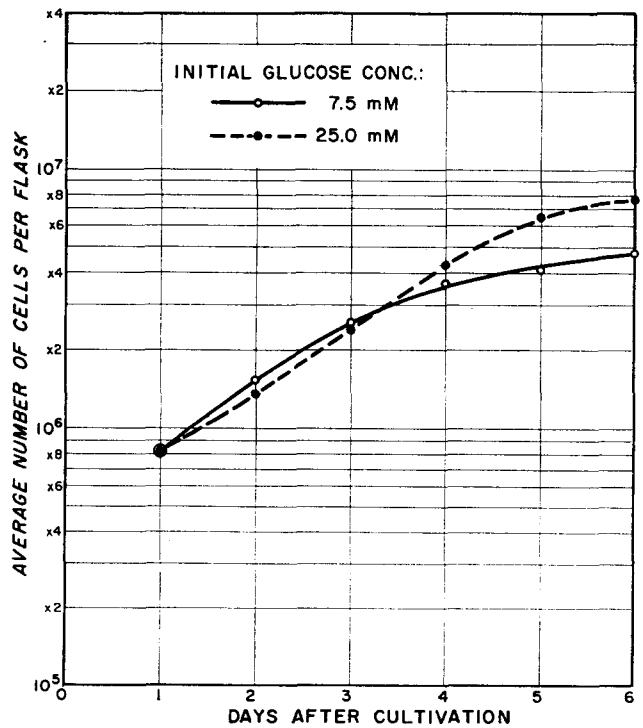


Fig. 4. *Influence of glucose concentration on the growth of ELTc cells in culture. Each point represents mean total tumor-cell number per culture, based on* 10 *cultures.*

Table 8. The influence of succinate, ketoglutarate and oxalacetate on anaerobic growth of the ELDc cells in vitro

pO_2	Medium	No. of cells per culture* Initial cell concentration	Final cell concentration
20%	Fib. 14 B	447,500±75,900	1,237,500±377,660
0%	Fib. 14 B	—	120,000± 7000
0%	Fib. 14 B fortified with: Ketoglutarate (1mM) Succinate (1mM) Oxalacetate (1mM)	—	255,000±104,650

*Mean with 95% confidence interval.

study of the effect of various oxygen tensions on the growth of the ELTc and the ELDc cells during a period of 3 and 4 days respectively. As seen from Fig. 5 maximum rate of growth *in vitro* occurred at 15% O_2.

DISCUSSION

Although a number of authors have succeeded in growing Ehrlich's ascites tumor cells *in vitro*, we still have little exact information about the conditions which support growth of these cells *in vitro*. According to Hull [1] Ehrlich's ascites tumor cells grow better directly on glass than in a clot. We have had the same experience during our attempts to adapt the ELT cells to *in vitro* conditions.

*Table 9. The influence of pCO_2 on the growth of the ELTc and the ELDc cells (number of cells per culture $\times 10^6$)**

Cell line		ELTc		ELDc	
Initial cell concn.		1·260±0·124	0·555±0·099	1·070±0·170	0·980±0·080
Final cell concn.					
pCO_2	0%	0·950±0·230	0·900±0·230	1·350±0·050	0·660±0·080
—	1%	2·980±0·309	2·560±0·633	2·060±0·370	—
—	5%	2·880±0·501	4·180±0·865	2·660±0·220	4·600±0·760
—	8%	—	—	2·710±0·330	3·170±0·900
—	10%	3·060±0·468	3·960±0·258	—	—
—	15%	—	—	—	3·770±0·400

*Mean with 95% interval.

Feeder cells were reported by Powell [3, 4] to stimulate growth of Ehrlich's ascites tumor cells, but with the improvement of the nutritional media feeder cells have become unnecessary. All media used so far have contained various biological fluids. Ely and Gray [10] found chicken embryo extract to be toxic. This is also our experience. Similarly we were able to confirm that fetal bovine serum is better than horse serum, ox serum and calf serum as originally reported by DiPaolo [12].

The biological fluids have been supplemented by a number of different synthetic media. According to DiPaolo [12] Eagle's medium produce damage to ELD cells *in vitro* within 24 hr. The same author found Hsu's medium to cause granulation, and medium 199 to cause the formation of clumps. Jackson *et al.* [7] on the other hand obtained good growth in static cultures with Eagle's medium supplemented with 10% calf serum, but for growth in suspension cultures they preferred Waymouth's medium. Postlethwaith and Macpherson [13] obtained good results with Eagle's medium modified to contain twice the recommended concentration of amino acids and vitamins supplemented with 10% calf serum and 10% tryptose phosphate broth in their cultivation of Ehrlich–Landschütz' near diploid cell line.

In our experiments the maximal rate of growth of ELTc as well as ELDc cells was obtained in Eagle's medium containing twice the recommended concentrations of amino acids, and four times the recommended concentrations of vitamins and of glutamine. This medium was supplemented with 20% fetal bovine serum. We have repeatedly tried to decrease the concentration of amino acids, vitamins, and glutamine, but each time we

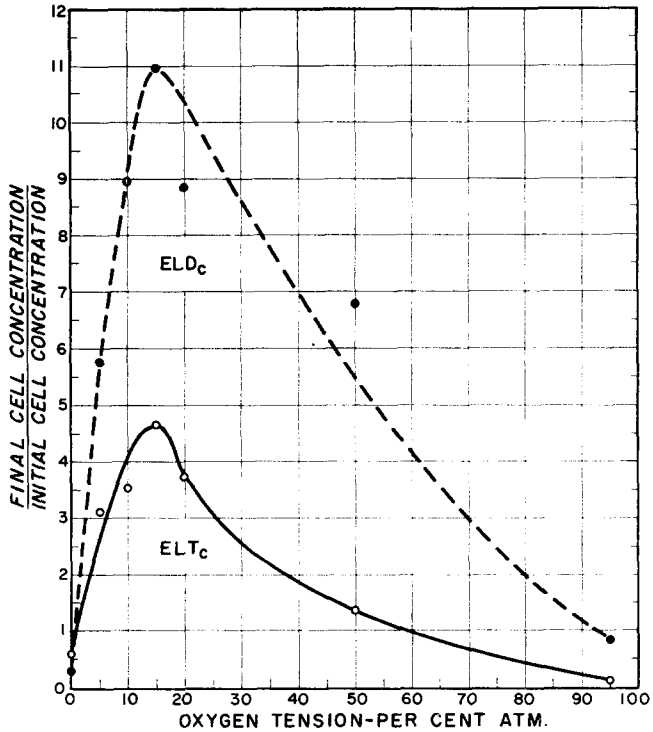


Fig. 5. *The influence of oxygen tension on the growth of the ELTc and the ELDc cells* in vitro. *Each point represents mean values based on* 10 *cultures counted after* 4 (*ELTc*) *and* 5 (*ELDc*) *days of cultivation.*

have observed an increase of granulation and a decrease of the growth rate in the course of a few days or weeks, but never in the course of 24 hr. Thus, the adverse effect of Eagle's medium reported by DiPaolo [12] was not confirmed by the present experiments.

During the two years of observation, the two cell lines of the present investigation underwent cytological dedifferentiation as will be described later. Most striking were the changes in cell size and chromosome number.

The present experiments show that these cytological changes were not accompanied by any major changes of the growth pattern or the metabolism of the ELT and the ELD cells. Apart from a slight prolongation of the doubling time the growth curves *in vitro* were basically the same as *in vivo*. Decelerating growth after an exponential growth phase was previously observed by Hauschka *et al.* [29] in ELT and ELD ascites tumors propagated in mice, and as in the present experiments deceleration was found to occur earlier in the ELT tumors than in the ELD tumors. The authors drew from their experiments the conclusion that total neoplastic mass, rather than cell surface or cell number as such, is the principle determinant of growth limits in the free-cell populations of Ehrlich's ascites tumor.

The present *in vitro* results do not support this conclusion. *In vitro* the ELTc cells continuously showed decelerating growth at a time when the ELDc cells still showed exponential growth. This was found not only at the beginning of the experimental period, when the mass of the ELTc cells was still larger than that of the ELDc cells, but also at the end of the experiments 2 years later, when the ELDc cells were slightly larger than the ELTc cells.

The differences between the growth curves of the two cell lines might indicate the presence of metabolic differences which lead to an

Table 10. *The influence of pCO_2 on glycolysis in ELTc and ELDc cultures*

pCO_2	Glucose consumption (μmoles $\times 10^{-6}$/cell/hr)* ELTc	ELDc
1% atm.	0·78±0·14	0·91±0·32
5% atm.	0·85±0·12	0·95±0·19
10% atm.	0·85±0·29	0·94±0·10

*Mean with 95% confidence interval.

earlier exhaustion of the nutritional resources in the host or in the culture in the case of the ELTc cells. Both cell lines retained their "malignant" type of metabolism with a high rate of glycolysis. The main difference between the two cell lines was the higher Warburg coefficient in the ELT cells, which increased during cultivation simultaneously with the disappearance of the cytological differences. The inability of other substrates to substitute for glucose indicates that glycolysis is essential for the growth of Ehrlich's ascites tumor cells. The effect of raising the initial glucose concentration to 25 mM on the growth curve of the ELTc cells shows that exhaustion of the glucose supply may only in part explain the early occurrence of growth deceleration in the ELTc cultures, which show a greater glycolytic activity than the ELDc cultures. However, other factors must be at work too, because the falling off of the growth curve was only delayed and not prevented by increased glucose concentrations.

Although glycolysis was found to be essential for the growth of Ehrlich's ascites tumor cells *in vitro*, no evidence was obtained in the present investigation indicating a regulatory function of glycolysis in the processes of cell growth. The exponential growth curve of the ELDc cells continued even if the supply of glucose was exhausted several hours before the daily renewal of the medium. Also the inability of oxamic acid to reduce the growth rate of the ELDc cells, before glycolysis is inhibited by about 50%, shows that there is no dependent correlation between growth and glycolysis.

In spite of the high rate of glycolysis, the ELTc and ELDc cells were unable to grow under anaerobic conditions. Evidently, respiration is necessary for the growth of these two cell lines, but as in the case of glycolysis the respiratory metabolism probably exceeds the needs of the cells.

Harris [30] and Geyer and Chang [31] were the first to demonstrate a growth supporting effect of CO_2/HCO_3^- on cells propagated *in vitro*. In the present study it was found that bicarbonate and carbon dioxide are essential for the growth of the ELTc and the ELDc cells, and that the optimal CO_2 tension is around 5% atm. In order to elucidate the mechanism of the growth supporting effect of CO_2/HCO_3^- we have previously studied the influence of pCO_2 on cellular respiration. A number of cells were found to show maximal rate of respiration when the gas phase contained 1–2% CO_2 and the medium a corresponding concentration of bicarbonate at pH 7·4 [20–23, 26–27], but a similar effect on the respiratory metabolism of the Ehrlich's ascites tumor cells could not be demonstrated. On the contrary, Nissen [24] found a depression of their oxygen consumption by 30–40 per cent already at 1 per cent CO_2. Thus, the growth promoting effect of CO_2 cannot be explained by its effect on cellular respiration. A more likely explanation is perhaps the participation of CO_2 in the non-mitochondrial fatty acid synthesis in the so-called malonyl reaction, for which we observed an optimal pCO_2 of about 5 per cent [32]. This effect was found to be accompanied by an increased oxidation of NADPH, which would make more NADP available for the pentose shunt and thereby for nucleic acid synthesis.

Loomis [33] has presented the theory that CO_2 may act as a physiological regulator of cell multiplication, and that cancer represents the de novo appearance of a cell that possesses metabolic mechanisms less susceptible to CO_2 inhibition than those of normal cells. In our previous studies we found that CO_2 tensions from 5 to 15% exert a strong antirespiratory effect, while aerobic glycolysis in tumor slices was greatly stimulated [26–27]. The optimal pCO_2 was found to be 8–15 per cent, which agrees well with Warburg's original studies of anaerobic glycolysis [34].

On the basis of these observations we drew the tentative conclusion [26, 27] that a high rate of glycolysis represents the mechanism which according to Loomis' theory would make cancer cells less susceptible to CO_2 inhibition than normal cells.

In support of this conclusion the present studies showed that the ELT and the ELD cells during propagation *in vitro* retain or even increase their high rate of glycolysis and at the same time show resistance to the cytostatic effect of high CO_2 tensions. However, in agreement with our previous studies of ascites tumor cells and cultured cells [26, 27], CO_2 had no stimulatory effect on glycolysis in ELTc and ELDc cultures. The explanation may be that CO_2 only exerts this metabolic effect in the presence of limiting factors, which are found in tumor slices but not in ascites tumor cells or cultured cells, but this does not exclude the possibility that the ELTc and the ELDc cells obtain compensation for the loss of respiratory energy caused by CO_2 through their excess glycolysis.

The influence of pO_2 on metabolism and growth *in vivo* of Ehrlich's ascites tumor cells has previously been described [27, 28]. The present findings show a similar effect on growth

in vitro. Maximal rate of growth occurred at 15% O_2, at which oxygen tension the ELTc and ELDc cells also show maximal endogenous respiration and fatty combustion, while glycolysis and carbohydrate combustion seem independent of variations in pO_2 [27, 28] as long as anaerobiosis does not exist. These observations indicate that other substrates than glucose are essential for the growth of Ehrlich's ascites tumor cells, and a more detailed study of their fatty acid metabolism seems desirable.

The growth rate of both the ELTa and the ELDa cells was found to be moderately, but significantly shorter than that of the ELTc and ELDc cells. This is somewhat surprising since it would seem that the cells are offered better conditions in the cultures than in the ascites fluid, where both the glucose concentration and the oxygen tension are very low [35, 36]. We have repeatedly observed that ascites tumor cells do not multiply *in vitro* in ascites fluid, and from the present experiments we know that they will not grow or only grow very slowly at the oxygen tensions which have been reported to exist in the ascites fluid. It seems, therefore, more likely that the biosynthetic processes which precede mitosis, *in vivo* take place on the surface of peritoneum rather than in the fluid.

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RESUME

Le métabolisme et la croissance de deux lignées, l'une hypertétraploïde (ELT), l'autre hyperdiploïde (ELD), de la tumeur ascitique d'Ehrlich, propagées in vivo *et* in vitro, *ont été étudiés. La culture* in vitro *n'avait qu'une faible influence sur l'allure du métabolisme et de la croissance de ces deux lignées cellulaires. La glycolyse et la respiration étaient toutes deux essentielles pour la multiplication de cellules ELD et ELT, mais aucune de ces deux sources d'énergie ne semblait être quantitativement liée à la vitesse de croissance. Les deux lignées de cellules nécessitaient le rapport CO_2/HCO^-_3, adéquat pour la croissance, mais ni l'une ni l'autre ne réagissaient à l'effet cytostatique d'une forte tension de CO_2. Les cellules ELD et ELT montraient un maximum de croissance à une pression partielle de O_2 de* 15%. *L'activité glycolitique des cellules ELT était plus élevée que celle des cellules ELD. Ceci peut expliquer, en partie, le fait que la courbe de croissance des cellules ELT s'abaisse* in vivo *comme* in vitro, *plus tôt que celle des cellules ELD. Cependant, il semble que le manque de glucose seul ne pourrait pas expliquer le ralentissement de croissance des cellules ELT.*

SUMMARY

Metabolism and growth of a hypertetraploid (ELT) and a hyperdiploid (ELD) subline of Ehrlich's asciles tumor cells propagated in vivo *and* in vitro *were studied. It was found that cultivation* in vitro *had only a slight influence on the metabolism and the growth pattern of the two cell lines. Both glycolysis and respiration were essential for the multiplication of the ELT and ELD cells, but neither of these two sources of energy seemed to be quantitatively correlated with the rate of growth. Both cell lines required CO_2/HCO_3^- for their growth, but neither of them responded to the cytostatic effect of high CO_2 tensions. Both cell lines showed maximal growth at* 15% O_2. *The glycolytic activity of the ELT cells was higher than that of the ELD cells. This may in part account for the fact, that the growth curve of the ELT cells falls off both* in vivo *and* in vitro *earlier than that of the ELD cells. However, lack of glucose could not alone explain the decelerating growth of the ELT cells.*

ZUSAMMENFASSUNG

Stoffwechsel und Wachstum einer hypertetraploiden (ELT) und einer hyperdiploiden (ELD) subline von Ehrlich-Aszitestumorzellen, wurden in vitro *und* in vivo *untersucht. Dabei stellte sich heraus, dass die* In-vitro-*Züchtung nur einen geringfügigen Einfluss auf Stoffwechsel und Wachstum der beiden Zellstämme ausübte. Sowohl Glycolyse als auch Atmung waren unerlässlich für die Vermehrung von ELT- wie ELD-Zellen, aber keine dieser beiden Energiequellen schien in einem quantitativen Verhältnis zur Wachstumsgeschwindigkeit zu stehen. Beide Zellarten bedurften für ihr Wachstum der CO_2/HCO_3^- Versorgung, aber keine sprach auf den zytostatischen Effekt hoher CO_2-*

Spannungen an. Bei beiden Zelltypen war maximales Wachstum bei einer O_2-Zufuhr von 15% festzustellen. Die glykolytische Aktivität der ELT-Zellen war höher als diejenige der ELD-Zellen. Dies mag teilweise dafür verantwortlich sein, dass die Wachstumskurve der ELT-Zellen sowohl in vivo *als auch* in vitro *früher abfällt als diejenige der ELD-Zellen. Allerdings vermöchte Glukosemangel allein das sich rascher verlangsamende Wachstum der ELT-Zellen nicht zu erklären.*

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Comparative Studies of a Near-Tetraploid and a Near-Diploid Line of Ehrlich's Ascites Tumor Propagated *in vivo* and *in vitro* II. Cytology and Transplantability

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IN A PREVIOUS WORK [1], the metabolism and growth of a hypertetraploid (ELT) and a hyperdiploid (ELD) subline of Ehrlich's ascites tumor propagated *in vivo* and *in vitro* were compared. It was found that the metabolism and the growth pattern shown by these cells during propagation *in vivo* only underwent minor changes, when the cells were grown *in vitro*. The purpose of the present investigation was to elucidate, whether the cytological characteristics of the same two lines and their tumor producing capacity in two strains of mice would maintain a similar stability during cultivation *in vitro*.

MATERIAL AND METHODS

The ELT and the ELD cells, which were originally obtained from the Department of Tumor Biology at the Karolinska Institutet in Stockholm, have frequently been described. Thus, Hauschka *et al.* [2] reported the modal chromosome number to be 90–92 and 45–46 in ELT and ELD tumors propagated *in vivo* in Buffalo, while the same tumor lines propagated in Stockholm showed modes of 92 and 46 respectively. These findings agree with earlier data [3–5]. The ELD cells seem more antigenic than the ELT cells, but both cell lines show a considerable lack of specificity in their histocompatibility. In our laboratory they have been carried as ascites tumors in inbred ST/a, C_3H and DBA/2 mice under the names ELTa and ELDa respectively. The ELT cells were explanted to cultures at the Fibiger laboratory in January 1964 and received the designation ELTc, while the ELD cells were adapted to *in vitro* conditions by Dr. Holmberg at the Research Division of Radiumhemmet in Stockholm in January 1962. They have been propagated *in vitro* in our laboratory since June 1962 under the name ELDc. The observations described in the present report were made during the period February 1964 to February 1967.

Cultivation

The methods of cultivation have previously been described [1]. The cells were grown on glass under a fluid medium (Fib. 14 B) composed of 20 per cent fetal bovine serum and 80 per cent Eagle's MEM [6] modified to contain

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twice the concentration of amino acids and four times the recommended concentration of glutamine and vitamins. Prior to 1964 the ELDc cells received calf serum instead of fetal serum.

Cytology

The average cell dimension was estimated by measurements of cell volume, dry weight and protein content during the logarithmic growth phase. For the determination of cell volume, the volume of packed cells was measured in MSE micro-haematocrit tubes after centrifugation for 15 min at $3500 \times \mathbf{g}$ in a MSE "Minor" centrifuge with a special micro-haematocrit head. Dry weight of the cells was determined at least three times at daily intervals after drying at 100°, until constant weight was obtained. Protein determinations were made as described by Oyama and Eagle [7], whose method is based on that of Lowry using a phenol reagent (Folin–Ciocalteau) for color development. Cell counts were made in a Bürker–Türk haemocytometer. For each experiment, cells were obtained from the different animals or from ten cultures.

Nuclear volume was measured under the phase contrast microscope (magnification $400\times$) using amicrometer with units representing 1/5 of a micron under the conditions of the present experiments. The longest and the shortest axis of ten nuclei in each of ten cultures or from each of ten animals were measured, and assuming that the nuclei form a prolate spheroid, the volume was calculated according to the formula $4/3\ \pi\ (a/2)\ (b/2)^2$, where a and b are the longest and the shortest axis respectively.

The number of nuclei and nucleoli per cell was counted under the phase contrast microscope (magnification $1000\times$) in one hundred cells from each of ten cultures or ten animals. Mitotic abnormalities were studied in haematoxylin–eosin stained preparations. One hundred mitotic figures were examined in each of ten cultures.

Chromosomes were counted after 4 hr of treatment with 0·04% colcemid in more than 100 well spread metaphases on aceto-orcein preparations, pretreated with 0·5% sodium citrate. At least 50 metaphases were counted on photomicrographs, while the remaining metaphases were counted directly under the phase contrast microscope (magnification $1000\times$).

Transplantation and implantation

For transplantation and implantation studies inbred mice of the C_3H and the ST/a strains were used. The H-2 type of these two mouse strains has been reported to be H-2k for the C_3H mice and H-2b for the ST/a mice [8]. The transplantability of the ELT and ELD cells propagated *in vivo* was studied by intraperitoneal and subcutaneous inoculation of 5×10^3–20×10^6 cells into 2–4 months old C_3H and ST/a mice. The animals were observed from 2 to 3 months. All i.p. inoculated animals which died within this period with ascites tumor were registered as takes. All subcutaneously inoculated animals, which showed palpable tumors 2 weeks after inoculation were registered as takes. Cases in which palpable tumors disappeared before the end of the second week after inoculation were considered negative, since it was felt that no safe distinction could be made between inflammatory reactions and true tumor formation at this early stage without histological examination. Animals, which died after the second week with tumor or which at the end of 3 months, showed growing tumors, were registered as takes with progressing tumor growth. Animals with palpable tumors after 2 weeks, which disappeared before the end of the observation period were registered as takes with regressing tumor growth. Animals, which died before the second week, were disregarded.

The tumor producing capacity on subcutaneous inoculation was also tested in newborn animals, i.e. animals less than 24 hr old. These animals were all inoculated in the interscapular region. Each animal received from 5×10^6 to 5×10^2 cells suspended in 30–50 µl of Tyrode's solution. The animals were observed for 2 months. During this period there was a high mortality which could not always be explained by the tumor. Insufficient nursing played an important role in many cases, and during the first 2–3 days after inoculation cannibalism was a dominating cause of death. Animals, which died before the second week, were disregarded in the present experiments. Animals, which showed no signs of tumor after 2 weeks or later were registered as negative cases. Animals with tumors after the second week were registered as positive cases. Due to the high mortality even after the second week no safe distinction could be made between progressing and regressing tumor growth.

The implantability of ELTc and ELDc cells were studied in a similar way. The cells were removed from the culture vessels by trypsinization, using Trypure trypsin (Novo, Copenhagen) dissolved in a balanced salt solution at a concentration of 0·25%. After trypsinization

for 10 min and centrifugation for 10 min at 200–450 × **g** the cells were resuspended in Tyrode's solution. After counting the cells in Bürker–Türk's haemocytometer the cell suspension was diluted with Tyrode's solution to contain the desired number of cells.

Tumor growth in young animals was studied by sacrificing animals selected at random at weekly intervals between the second and the sixth week of observation. The tumors were carefully removed and weighed. In addition, histological preparations were made from the tumor, the regional lymph nodes, the thoracic wall, the lung and the liver. Animals, which showed no palpable tumor at the end of 2 weeks, were not included in this study. On the other hand, animals, in which the tumor had completely regressed during the period of observation after the second week, were included as tumors weighing 0 g.

RESULTS

Cytology

The morphology of the ELTc and the ELDc cells did not differ appreciably. In dense cultures, most of the cells were round or polygonal (Figs. 1 and 2). In less dense cell populations, fibroblast like cells were frequently seen (Fig. 3). Pseudopodia were often observed in the form of fine needle shaped processes and signs of pinocytosis were not rare (Fig. 4). Even in dense cell populations one usually saw a clear separation between the individual cells (Fig. 5), which did not form true syncytia.

The dimensions of the cells varied greatly, and giant cells were regularly seen (Figs. 1–2). In Table 1 the mean dimension of the ELT and the ELD cells as measured by dry weight, protein content and cell volume is shown. *In vivo*, the dry weight and the protein content of the ELT cells were significantly greater than those of the ELD cells, while no significant differences were noticed between their cell volumes. During cultivation *in vitro* the dry weight, protein content and cell volume of both cell lines increased considerably. A great variability was observed, but eventually the two cell lines reached approximately the same values.

The nuclear volume was measured as described. It appears from Figs. 6 and 7 that there was a considerable spread without any definite indications of two or more distinct nuclear classes. The mean volume of the ELT nuclei did not change during cultivation *in vitro* for 2 years. The mean volume of the ELD nuclei was somewhat smaller, particularly after 2 years of cultivation *in vitro*. However, after 4 years of cultivation, the mean volume of the ELD nuclei did not differ significantly from that of the ELT nuclei.

Multinuclear cells were rarely seen in ELTa and ELDa cells (Table 2), and no significant differences could be demonstrated in this respect between the two cell lines. Cultivation *in vitro* led to a small but significant increase in the percentage of multinuclear cells. In 1964, this change was more pronounced in the ELTc than in the ELDc cells, but eventually both cell lines stabilized and showed the same percentage of multinuclear cells, still a little higher than in the ELTa and the ELDa cells. A similar development was observed as far as the number of nucleoli is concerned.

The main cytological difference between the ELTa and the ELDa cells is the difference between their numbers of chromosomes. As already pointed out, the ELT cells were

Table 1. *Dimensions* of ELT and ELD cells propagated* in vivo *and* in vitro

	Time of investigation	Dry weight (mg × 10^6/cell)		Protein content (mg × 10^{-6}/cell)		Cell volume (µl × 10^{-6}/cell)	
Cell line:		ELT	ELD	ELT	ELD	ELT	ELD
Method of propagation							
in vivo	1964–66	0·44± 0·06	0·31± 0·02	0·40± 0·04	0·27± 0·02	2·80± 0·31	2·54± 0·21
	1964†	0·98± 0·14	0·75± 0·14	0·82± 0·14	0·54± 0·13	4·56± 0·47	3·29± 0·40
in vitro	1966‡	0·57± 0·05	0·68± 0·04	0·53± 0·04	0·62± 0·03	4·00± 0·26	4·25± 0·53

*Mean values and 95% confidence interval.
†Culture age — ELT 2–3 months. ELD 2 years.
‡Culture age — ELT 2 years. ELD 4 years.

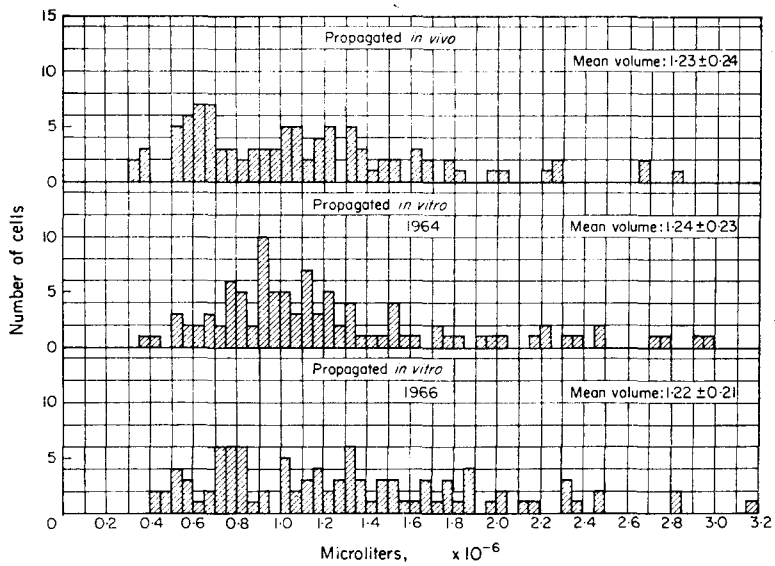


Fig. 6. *Nuclear volume of ELT cells propagated* in vivo *and* in vitro.

Table 2. *Number of nuclei and nucleoli in ELT and ELD cells propagated* in vivo *and* in vitro

	Time of investigation	Percentage of multinuclear cells*		Mean number of nucleoli per cell*	
Cell line		ELT	ELD	ELT	ELD
Method of propagation					
in vivo	1966	0·70± 0·16	0·90± 0·46	2·70± 0·32	2·34± 0·18
	1964†	4·80± 1·36	1·50± 0·69	4·15± 0·46	2·84± 0·19
in vitro	1966‡	2·70± 0·92	2·80± 1·38	3·69± 0·31	2·08± 0·18

*Mean values with 95% confidence interval.
†Culture age — ELT 2–3 months. ELD 2 years.
‡Culture age — ELT 2 years. ELD 4 years.

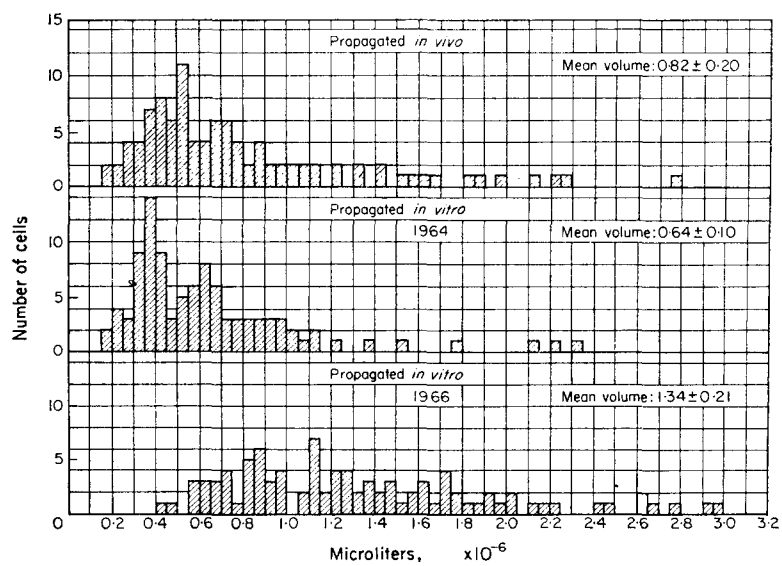


Fig. 7. *Nuclear volume of ELD cells propagated* in vivo *and* in vitro.

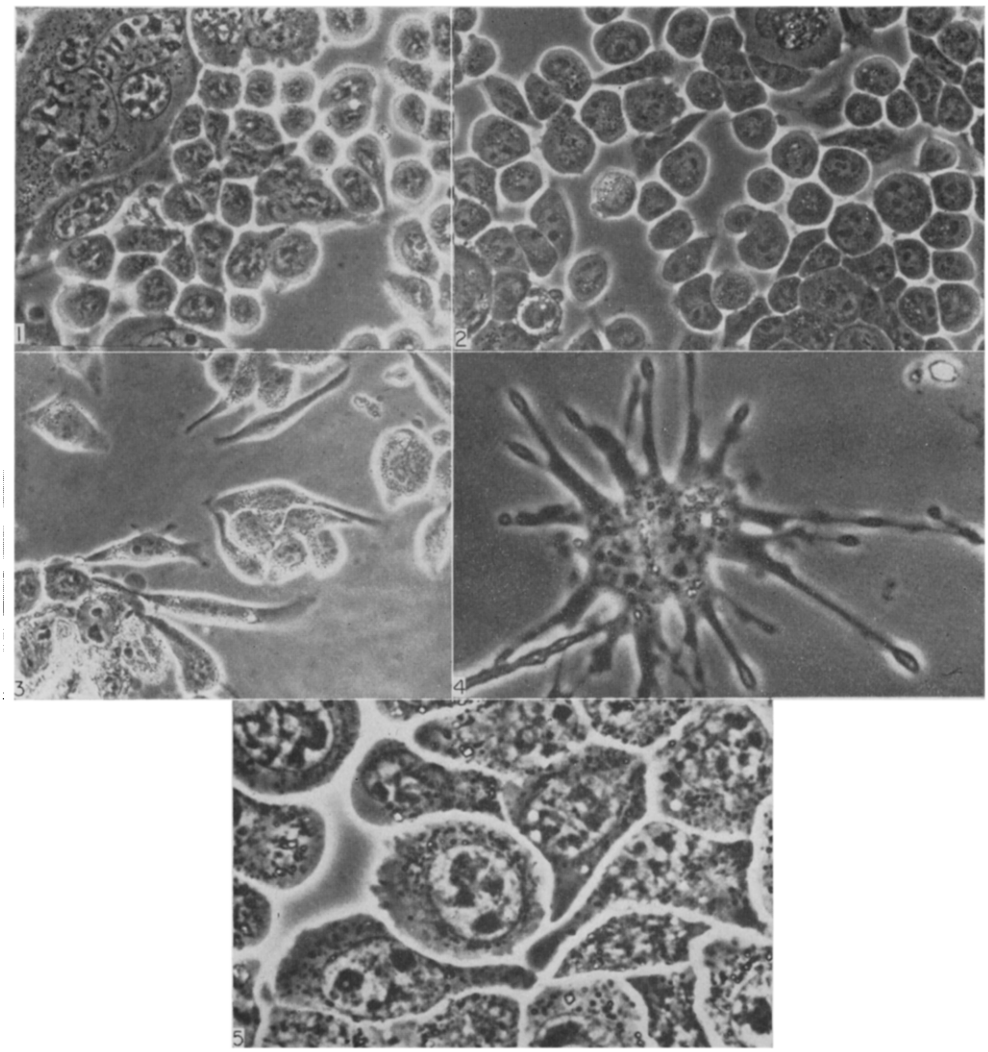


Fig. 1. *ELTc cells. Phase contrast microscopy.* (*Magnification* 400×).

Fig. 2. *ELDc cells. Phase contrast microscopy.* (*Magnification* 500×).

Fig. 3. *Fibroblast-like ELDc cells. Phase contrast microscopy.* (*Magnification* 400×).

Fig. 4. *ELTc cell showing pinocytosis. Phase contrast microscopy.* (*Magnification* 680×).

Fig. 5. *Epithelial-like ELTc cells without the formation of true syncytia. Phase contrast microscopy.* (*Magnification* 1000×).

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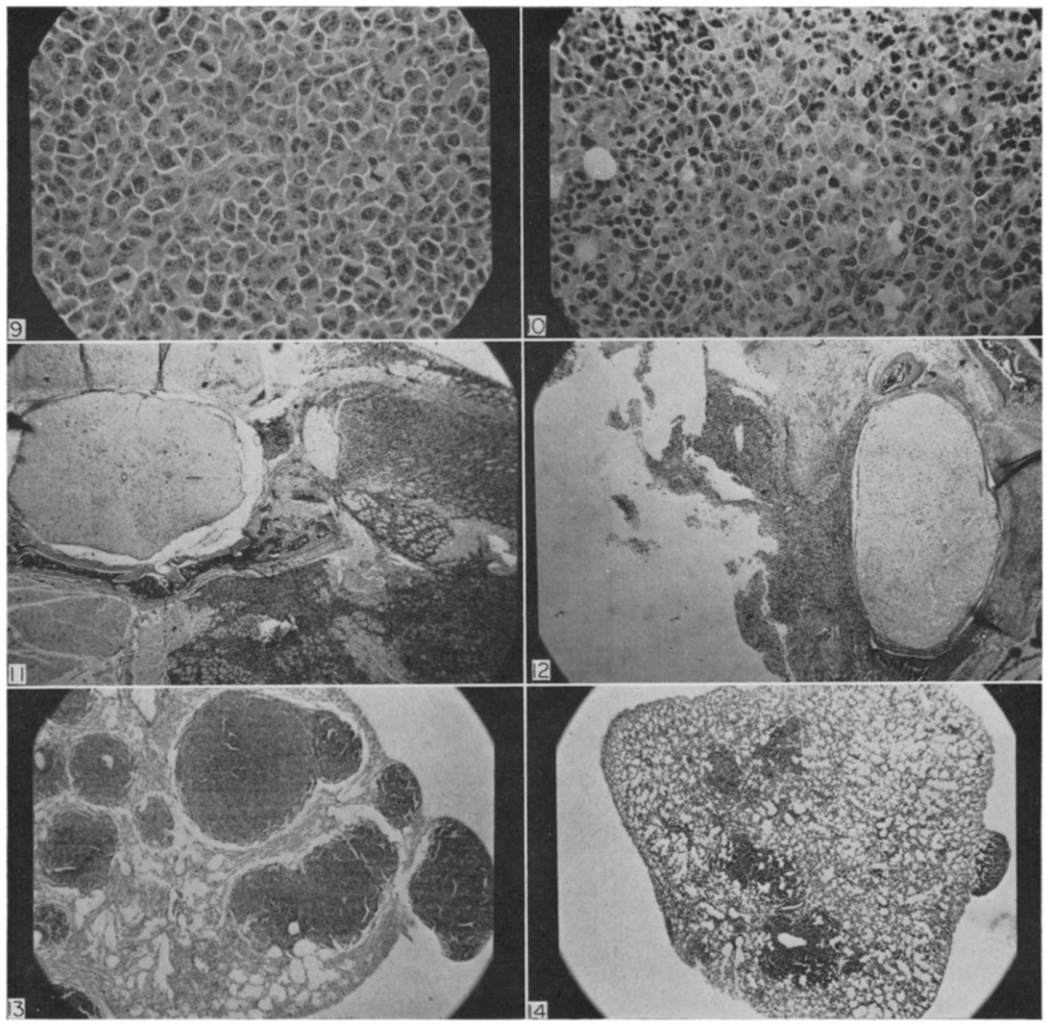


Fig. 9. *Anaplastic tumor produced in newborn ST/a mouse by subcutaneous inoculation of ELTc cells. Stain: Hematoxylin–eosin. Light microscopy.* (*Magnification* 400×).

Fig. 10. *Anaplastic tumor produced in newborn C_3H mouse by subcutaneous inoculation of ELDc cells. Stain: Hematoxylin–oesin. Light microscopy.* (*Magnification* 500×).

Fig. 11. *Invasive tumor growth in the spinal cord region after subcutaneous inoculation of ELTc cells into newborn C_3H mouse. Stain: Hematoxylin–eosin. Light microscopy.* (*Magnification* 40×).

Fig. 12. *Invasive tumor growth in the spinal cord region after subcutaneous inoculation of ELDc cells into newborn ST/a mouse. Stain: Hematoxylin–eosin. Light microscopy.* (*Magnification* 40×).

Fig. 13. *Pulmonary metastasis in newborn C_3H mouse after subcutaneous inoculation of ELTc cells. Stain: Hematoxylin–eosin. Light microscopy.* (*Magnification* 40×).

Fig. 14. *Pulmonary metastasis in newborn C_3H mouse after subcutaneous inoculation of ELDc cells. Stain: Hematoxylin–eosin. Light microscopy.* (*Magnification* 40×).

Table 3. Chromosome number of ELT and ELD cells propagated in vivo *and* in vitro

Cell line	Time of investigation	Culture age	Total no. of metaphases counted	Spread	Mode	Median
ELTc	1964	3 mths.	121	57–180	81	82
ELTc	1966	2 years	176	26–153	*	57
ELTc	1967	3 years	127	34–180	57	60
ELTa	1967		100	45–106	69	69
ELDc	1964	2 years	A. 90	42– 50	45	45
			B. 50	73–176	*	93
ELDc	1966	4 years	134	34–166	46	58
ELDc	1967	5 years	A. 68	19– 50	45, 37	40
			B. 54	54–157	*	80
ELDa	1967		100	29– 69	39	37

*Mode not statistically indicated.
A and B indicate the presence of two populations.

originally reported to be near-tetraploid and the ELD cells near-diploid. In this laboratory, a change in chromosome number was observed in both cell lines during propagation *in vivo* as well as *in vitro* (Table 3). Thus, in 1967, the chromosomal mode of the ELTa cells had fallen to 69, while that of the ELDa cells had fallen to 39. During propagation *in vitro* the mode of the ELT cells fell to a near-triploid value. The ELDc cultures contained two populations, A and B. A showed a mode of 45, while B showed higher chromosome numbers, but without any statistical indication of the mode. After 4 years of cultivation these two populations could no longer be distinguished, but a hyperdiploid mode was still indicated. One year later the ELDc cells seemed again separated into two populations, one with a mode of 45 and a secondary mode of 37, and one with higher chromosome numbers, but without any statistical indication of the mode.

Most of the chromosomes were acrocentric, but 1–2 metacentric or submetacentric chromosomes were also found in the majority of the cells (Fig. 8). During propagation *in vitro* the number of cells with more than two bi-armed chromosomes increased. The maximal number of bi-armed chromosomes was 6–8 in one cell.

Mitotic abnormalities were found in both cell lines as shown in Table 4. It appears from the table that there were no significant differences between the total number of abnormal mitoses in cultures of ELTc and ELDc cells.

Transplantability and implantability

The intraperitoneal transplantability of

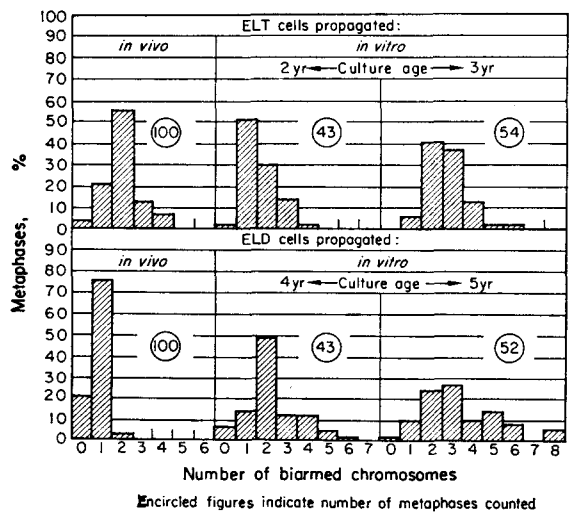


Fig. 8. Number of bi-armed chromosomes in ELT and ELD cells propagated in vivo *and* in vitro.

Table 4. Mitotic abnormalities in cultures of ELTc and ELDc cells

Cell line:	ELTc	ELDc
Mitotic abnormality	Percentage of mitotic figures*	
Binuclear cells with a single mitotic figure	0·3	0·1
Pyknotic chromosomes	1·0	0·1
Broken chromosomes	0	0
Chromosomal agglutination	0·1	0·6
Chromosomal bridge-formation	0·7	0·4
Unattached chromosomes	1·3	1·9
Incomplete spiralization	0·5	0·6
Multipolar mitoses	0·2	0·9
Incomplete spindle formation	3·9	3·5
Incomplete cleavage	0·1	0
Total percentage of abnormal mitoses†	7·5± 3·7	7·8± 1·4

*Mean values.
†Mean values with 95% confidence interval.

Table 5. Intraperitoneal transplantability of ELTa and ELDa cells into adult C3H and ST/a mice

Cell line:	ELTa		ELDa	
Recipient strain:	C3H	ST/a	C3H	ST/a
No. of cells inoculated:	Percentage of takes*			
20×10^6	100	100	95	95
5×10^6	100	100	100	100
1×10^6	100	100	45	80
5×10^5	100	100	5	85
5×10^4	80	100	–	0
5×10^3	35	95	–	0

*Each group contained 20 mice, 10 males and 10 females.

Table 6. Intraperitoneal implantability of ELTc and ELDc cells into adult C3H and ST/a mice

Cell line:	ELTc				ELDc				
Recipient strain:	C3H		ST/a		C3H		ST/a		
No. of Cells inoculated ($\times10^6$):	5	20	5	20	5	20	5	15	20
Culture age (years)	Percentage of takes (total No. of mice inoculated)								
1	—	95 (20)	—	86 (21)	—	—	0 (20)	0 (7)	—
2	60 (40)	97 (38)	68 (40)	86 (35)	—	—	—	—	—
2½	0 (25)	—	75 (12)	—	—	—	—	—	—
3	10 (20)	95 (20)	70 (20)	73 (30)	0 (20)	0 (20)	0 (20)	—	50 (24)
4	—	—	—	—	—	0 (20)	—	—	0 (20)
5	—	—	—	—	—	0 (20)	—	—	0 (20)

ELTa and ELDa cells into adult C3H and ST/a mice is shown in Table 5. As it is seen the ST/a mice were more susceptible to both cell lines than the C3H mice. It also appears from the table that a higher inoculum dosis of ELDa cells than of ELTa cells was required to obtain 100% of takes in both recipient strains, indicating a higher degree of virulence or of compatibility of the latter cell line.

During propagation *in vitro* the intraperitoneal implantability in adult C3H and ST/a mice decreased as shown in Table 6. This change of implantability seemed to occur earlier in the C3H mice than in the ST/a mice.

The table also shows that the ELTc cells retained their implantability in both strains of mice for a longer period than the ELDc cells.

The subcutaneous transplantability of ELTa cells differed from the intraperitoneal transplantability as regards the host strain preferred. As shown in Table 7 a higher percentage of progressively growing tumors was obtained in the C3H mice than in the ST/a mice, when the animals received 5000–50,000 cells. The subcutaneous transplantability of ELDa cells showed a pattern similar to that of the intraperitoneal transplantability (Tables 5 and 7). In both cases the ST/a mice were found to be more susceptible than the C3H mice, and in both cases the ELTa cells showed a higher rate of takes than the ELDa cells.

During propagation *in vitro* the ELTc cells lost their subcutaneous implantability into both

Table 7. Subcutaneous transplantability of ELTa and ELDa cells into adult C3H and StA mice

Cell line:	ELTa						ELDa					
Recipient strain:	C3H			ST/a			C3H			ST/a		
No. of cells inoculated	Percentage of takes*: (+)	+	Total no. inoc.	Percentage of takes: (+)	+	Total no. inoc.	Percentage of takes: (+)	+	Total no. inoc.	Percentega of takes: (+)	+	Total no. inoc.
10×10^6	0	100	10	0	100	10	80	20	20	50	50	20
5×10^6	0	100	10	0	100	10	65	15	20	65	35	20
1×10^6	0	100	10	0	100	10	70	5	20	50	50	20
5×10^5	10	80	10	20	80	10	35	5	20	50	45	20
5×10^4	2·5	70	40	32	24	50	—	—	—	—	—	—
5×10^3	0	20	20	0	5	20	—	—	—	—	—	—

*(+)=Regressive tumor growth.
\+ =Progressive tumor growth.

Table 8. Subcutaneous implantability of ELTc cells into adult C3H and ST/a mice*

Recipient strain:	C3H			ST/a		
Culture age (years)	Percentage of takes† (+)	+	Total no. of mice inocul	Percentage of takes† (+)	+	Total no. of mice inocul.
1	50	38	24	90	0	20
2	83	14	36	50	0	30
$2\frac{1}{2}$	36	0	42	63	0	38
3	12	0	50	9	0	23

*Inoculum dosis: 20×10^6 cells.
†(+)=Regressive tumor growth; +=progressive tumor growth.

Table 9. Subcutaneous implantability of ELDc cells into adult C3H and ST/a mice*

Recipient strain:	C3H			ST/a		
Culture age (years)	Percentage of takes† (+)	+	Total no. of mice inocul.	Percentage of takes† (+)	+	Total no. of mice inocul.
3	15	30	20	48	17	23
4	0	0	17	45	0	20
$4\frac{1}{2}$	–	–	–	46	0	92
5	0	0	20	26	0	43

*Inoculum dosis: 20×10^6 cells.
†(+)=Regressive tumor growth; +=progressive tumor growth.

Table 10. *Subcutaneous implantability* of ELTc cells into newborn C3H and ST/a mice*

Recipient strain:	C3H		ST/a	
Culture age:	Percentage of takes†	Total no. of mice inocul.	Percentage of takes†	Total no. of mice inocul.
1–4 mths.	97	39	100	29
7–11 mths.	97	35	62	102
2 years	94	31	56	25
3 years	94	49	24	51

*Inoculum dosis: 5×10^5 cells.
†Include both regressive and progressive growth.

strains of adult mice (Table 8). This change occurred sooner in the less susceptible ST/a strain than in the more susceptible C3H strain. Also the ELDc cells lost their subcutaneous implantability into adult mice (Table 9) after more than 3 years of cultivation. No differences between the two recipient strains of mice were observed in this respect, but regressive growth was still occasionally obtained in the ST/a mice after 5 years of cultivation, but not in the C3H mice.

Since this change of implantability might be due to immunological factors, the tumor producing capacity of ELT and ELD cells was also tested in newborn animals inoculated subcutaneously in the interscapular region. At a cell dosis of 5 million both cell lines produced tumors in about 100% of the inoculated mice, and this implantability did not decrease during the experimental period. At an inoculum dosis of half a million cells 100% of takes were obtained in both strains with *in vivo* propagated cells. The results obtained with *in vitro* propagated cells are shown in Tables 10 and 11. As it is seen, the implantability of both cell lines into newborn C3H mice was largely maintained during the first 3 years of cultivation. After this time, the ELDc cells, which have been grown in culture for more than 5 years, showed some decline in their implantability. In newborn ST/a mice, on the other hand, the ELTc cells showed a considerable decrease in their tumor producing capacity already during the second half-year period of cultivation, and this decrease continued until the end of the experimental period. The implantability of the ELDc cells into newborn ST/a mice did not differ appreciably from that observed in newborn C3H mice.

The tumors produced were anaplastic tumors composed of polygonal cells of varying size (Figs. 9–12). Invasion of the thoracic wall occurred, but not very frequently. Pulmonary metastases (Figs. 13–14) and metastases to the regional lymph nodes were regularly seen.

The growth of the tumors obtained by the subcutaneous inoculation of 5×10^6 ELTc and ELDc cells into newborn C3H and ST/a mice is shown in Fig. 15 and in Table 12. While there was no significant difference between the growth of the ELTc and the ELDc tumors in newborn C3H mice, the mean weights of the 3–6 weeks' old ELDc tumors in the ST/a mice were significantly higher than those of the ELTc tumors, which in this strain of mice grew more slowly than in newborn C3H mice.

The transplantability of the tumors produced in newborn animals into adult animals was

Table 11. *Subcutaneous implantability* of ELDc cells into newborn C3H and ST/a mice*

Recipient strain:	C3H		ST/a	
Culture age (years)	Percentage of takes†	Total no. of mice inocul.	Percentage of takes†	Total no. of mice inocul.
2	72	18	88	60
3	96	24	78	46
4	45	22	55	22
5	48	40	64	48

*Inoculum dosis: 5×10^5 cells.
†Include both regressive and progressive growth.

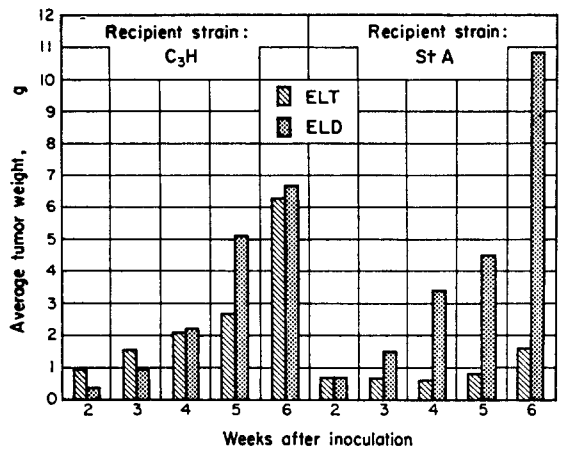


Fig. 15. *Weight of* 2–6 *weeks old tumors produced in newborn* C_3H *and StA mice by the subcutaneous inoculation of* 5×10^6 *ELTc and ELDc cells.*

Table 12. *Weight of tumors* produced in newborn mice after* 2–6 *weeks by* 5×10^6 *ELTc and ELDc cells*

Recipient strain of mice:	C_3H		ST/a	
Cell line inoculated:	ELTc	ELDc	ELTc	ELDc
Culture age (years)	1/2–1	2½–3	1/2–1	2½–3
Tumor age (weeks)	Tumor weight (grams)			
2	0·936±0·391	0·366±0·200	0·689±0·194	0·667±0·145
3	1·505±0·524	0·972±0·321	0·645±0·268	1·496±0·241
4	2·103±1·083	2·191±0·371	0·600±0·262	3·416±0·409
5	2·746±1·973	5·076±0·908	0·789±0·357	4·469±1·103
6	6·331±1·920	6·660±0·901	1·598±0·904	10·954±2·220

*Mean values and 95% confidence interval.

studied by the subcutaneous inoculation of 0·2 ml of tumor material from each of 21–23 tumors into each of two mice. From each tumor which developed further transplantations were made into two mice, until the fifth generation of transplantation had been reached. As it is seen from Table 13 the transplantability of the tumors from newborn mice rapidly decreased in adult mice.

Finally a study was made of the protective effect of primary inoculations into adult mice of ELTc and ELDc cells against the secondary inoculation of ELTa and ELDa cells. Two to four months old C_3H and StA mice were inoculated subcutaneously or intraperitoneally with ELTc and ELDc cells. The subcutaneous inoculum doses of both cell lines and the intraperitoneal doses of the ELDc cells were 20×10^6

Table 13. *Subcutaneous transplantability into adult mice of subcutaneous tumors produced in newborn mice by the inoculation of ELTc and ELDc cells*

Cell line	Strain of mice	No. of tumors originally transplanted	No. of tumors showing progressive growth in at least one adult mouse during passage no.				
			1	2	3	4	5
ELTc	C_3H	23	9	3	2	1	1
	ST/a	21	12	4	1	0	0
ELDc	C_3H	22	20	7	7	4	4
	ST/a	23	23	7	7	4	2

cells, which caused no progressive tumor growth. By the intraperitoneal inoculation of ELT cells the doses had to be reduced to 5×10^6 cells in order to avoid the development of lethal ascites tumors. Three weeks after this primary inoculation the same animals were charged intraperitoneally with ELTa and ELDa cells. Two doses of cells were used, one corresponding to the lowest doses which in preliminary experiments (see Table 5) caused 100% of takes in both strains of mice and one somewhat higher.

The results are presented in Tables 14–17, which show that intraperitoneal and subcutaneous preimmunization with both cell lines propagated *in vitro* protected the hosts against secondary i.p. inoculations of ELTa and ELDa ascites tumor cells. The protective effect was most pronounced in the C_3H mice, and it caused both a reduction in the percentage of takes and a prolongation of the life span of those mice, which developed ascites tumors. Partial cross protection was observed in the C_3H mice. In the ST/a mice pre-immunization with ELDc cells showed cross protection against the ELTa ascites tumor, but not vice versa.

Table 14. *The intraperitoneal transplantability of ELTa ascites tumor cells into C_3H mice immunized with ELTc and ELDc cells*

First inoculation (cells propagated *in vitro*)			Second inculation (cells propagated *in vivo*)				Controls (cells propagated *in vivo*)		
Cell line	No. of cells ($\times 10^6$)	Route of inocul.	No. of cells ($\times 10^5$)	No. of mice	No. of takes	Average survival time (days)	No. of mice	No. of takes	Average survival time (days)
ELTc	5	i.p.	5	19	0 (0%)	—			
ELTc	20	subcut.	5	15	0 (0%)	—	41	41(100%)	18·88±0·83
ELDc	20	subcut.	5	15	0 (0%)	—			
ELTc	5	i.p.	50	15	10(66·7%)	20·78±2·84			
ELTc	20	subcut.	50	15	4(26·7%)	17·25±6·24	45	44(97·8%)	13·86±1·68
ELDc	20	subcut.	50	15	7(46·7%)	20·00±4·03			

Table 15. *The intraperitoneal transplantability of ELTa Ascites tumor cells into StA mice immunized with ELTc and ELDc cells*

First inoculation (cells propagated *in vitro*)			Second inoculation (cells propagated *in vivo*)				Controls (cells propagated *in vivo*)		
Cell line	No. of cells ($\times 10^6$)	Route of inocul.	No. of cells ($\times 10^5$)	No. of mice	No. of takes	Average survival times (days)	No. of mice	No. of takes	Average survival time (days)
ELTc	5	i.p.	5	13	0(0%)				
ELTc	20	subcut.	5	15	1(6·7%)	19	48	48(100%)	13·40±0·69
ELDc	20	subcut.	5	15	0(0%)				
ELTc	5	i.p.	50	8	8(100%)	14·63±2·84			
ELTc	20	subcut.	50	20	16(80%)	13·81±1·85	40	39(97·5%)	11·67±0·99
ELDc	20	subcut.	50	15	14(93·3%)	14·14±1·84			

Table 16. *The intraperitoneal transplantability of ELDa Ascites tumor cells into C_3H mice immunized with ELDc and ELTc cells*

First inoculation (cells propagated *in vitro*)			Second inoculation (cells propagated *in vivo*)				Controls (cells propagated *in vivo*)		
Cell line	No. of cells ($\times 10^6$)	Route of inocul.	No. of cells ($\times 10^6$)	No. of mice	No. of takes	Average survival time (days)	No. of mice	No. of takes	Average survival time (days)
ELDc	20	i.p.	5	17	0(0%)	—			
ELDc	20	subcut.	5	9	0(0%)	—	37	34(91·8%)	14·88±1·84
ELTc	20	subcut.	5	17	5(29·4%)	15·20±9·11			
ELDc	20	subcut.	20	15	7(46·7%)	22·43±1·57	16	15(93·8%)	10·40±3·16
ELTc	20	subcut.	20	15	10(66·7%)	19·80±2·97			

Table 17. *The intraperitoneal transplantability of ELDa Ascites tumor cells into ST/a mice immunized with ELDc and ELTc cells*

First inoculation (cells propagated *in vitro*)			Second inoculation (cells propagated *in vivo*)				Controls (cells propagated *in vivo*)		
Cell line	No. of cells ($\times 10^6$)	Route of inocul.	No. of cells ($\times 10^6$)	No. of mice	No. of takes	Average survival time (days)	No. of mice	No. of takes	Average survival time (days)
ELDc	20	i.p.	5	15	10(66·7%)	15·60±1·58			
ELDc	20	subcut.	5	9	6(66·7%)	14·00±2·90	23	22(95·7%)	15·05±2·61
ELTc	20	subcut.	5	15	14(93·3%)	17·86±1·27			
ELDc	20	subcut.	20	14	8(57·1%)	11·38±2·51			
ELTc	20	subcut.	20	15	15(100%)	16·13±1·79	28	27(96·4%)	10·67±2·08

DISCUSSION

The cytology of Ehrlich's ascites tumor cells in culture has not received much attention in previous publications. Hull [9] found that these cells closely resembled those of Walker rat carcinoma except that they are slightly smaller and somewhat more elongated. The author concluded that it seems quite possible that they are epithelial cells. According to DiPaolo [10], the ELD cells appeared round, and lymphocyte-like with fibroblast-like and epithelial-like cells dispersed among them. Our observations agree in general with those of DiPaolo, but since no syncytia formation was seen, it seems doubtful whether the epithelial-like cells can be classified as true epithelial cells. On the other hand the cultures were far from showing the apothelial structure typical of connective tissue cultures. No characteristic morphological differences were seen between the ELTc and the ELDc cultures, which both contained the various cell types described. Since these cell types were also found in cloned sublines of ELTc and ELDc cultures, they show the great morphological variability of the ELT and ELD cells rather than the presence of various stem lines in mixed cell populations.

Multinuclear cells and mitotic abnormalities were reported by Deschner and Allen [11] to occur in cultures of Ehrlich's ascites tumor cells. We have made the same observations, and we also observed a moderate increase in the mean number of nucleoli. However, the incidence of these abnormalities was not great, and the difference between the present cell lines was small.

One of the most striking cytological changes in our experiments was the increase in cell size. Eventually the ELTc and the ELDc cells reached the same size, which was considerably larger than that of the ELDa cells and a little larger than that of the ELTa cells. The mean volume of the nuclei of the ELT cells remained unchanged, while that of the ELD cells increased after the substitution of fetal bovine serum for calf serum. At the end of the experimental period no significant differences could be demonstrated between the ELTc and the ELDc cells in this respect, which probably reflects the increased degree of aneuploidy which developed both in ELTc and in ELDc cultures.

The number of chromosomes in Ehrlich's ascites tumor cells cultivated *in vitro* has been studied by various authors. Deschner and Allen [11] observed polyploidy of Ehrlich's ascites tumor cells cultivated *in vitro* for 80 odd days. DiPaolo [10] found a change of the modal chromosome number of ELD cells from 44–46 to 67–75 during 12 months of cultivation, and at the same time increased aneuploidy was observed. In Postlethwaite and Macpherson's experiments [12] the ELD cultures seemed more stable. These authors reported a modal number 44–47 chromosomes after 4–16 months of cultivation. The present results agree with those reported by Postlethwaite and Macpherson. A hyperdiploid stemline was still seen in our ELDc cultures after 5 years of cultivation. It may be of some significance that our medium only differed slightly from that used by Postlethwaite and Macpherson. Eagle's medium with increased concentrations of amino acids and vitamins was used in both cases, but in addition our cultures received 20% fetal bovine serum, while Postlethwaite and Macpherson used 10% calf serum and 10% tryptose phosphate broth. DiPaolo on the other hand used 60% Earle's salt solution, containing 0·65% lactalbumin hydrolysate, 20% fetal bovine serum, 20% bovine amniotic fluid.

The modal number of the ELDc cells seemed more stable in culture under the present experimental conditions than in mice, where it decreased to 39 during the experimental period. On the other hand cultivation *in vitro*

led to an increased aneuploidy as also reported by DiPaolo.

The modal chromosome number of the ELT cells underwent greater changes both *in vivo* and *in vitro*. The change was most pronounced under *in vitro* conditions, where a hypotriploid mode developed in a population which originally showed a hypertetraploid mode. But also during propagation *in vivo* a considerable decrease in the modal number was seen.

Most of the chromosomes were acrocentric, but in both cell lines a few bi-armed chromosomes were seen in the majority of the cells. Most of the ELT cells contained two bi-armed chromosomes, while the ELDa cells usually contained only one. However, this difference disappeared during cultivation *in vitro*, when a moderate increase in the number of bi-armed chromosomes was observed, indicating that even in the ELDc cultures the original stem line could not be maintained.

Thus, the present studies have shown that the ELT and the ELD cells from a cytological point of view became almost indistinguishable during cultivation *in vitro*. Only their modal chromosome number differed, and even this difference tended to decrease during propagation in culture.

As previously reported [1] this cytological dedifferentiation, which lead to the gradual elimination of a number of differences between the two cell lines, was not accompanied by any major changes of their metabolism and growth pattern. Metabolic changes were perhaps not to be expected, since only very small differences were demonstrated between the metabolism of the two cell lines [1]. But it seems worth noticing that the previously described differences between the growth curves [1], the duration of the G_1 phase of the mitotic cycle [13], and the sensitivity to the toxic effects of colcemide [14] of the two cell lines were maintained in spite of cytological dedifferentiation.

On the other hand, the development of morphological similarity was accompanied by a decrease or complete loss of the implantability of both cell lines. At the present moment no satisfactory explanation can be offered for these changes, but they can hardly be regarded as a sign of dedifferentiation.

Ehrlich's ascites tumors are usually classified as "non-specific" tumors with regard to their histocompatibility in a wide range of mouse genotypes. However, the lack of specificity is not equally pronounced in the ELDa and ELTa cells. Thus, it has been possible to immunize animals with low doses of ELDa cells and thereby obtain immunoselection of ELTa cells [5]. The immunological advantage of the ELTa cells over the ELDa cells was also indicated by the present experiments. On the other hand the two tumor lines resembled one another in their greater compatibility with the ST/a mice than with the C_3H mice on intraperitoneal inoculation. The same difference between the susceptibility of the two recipient strains was seen on subcutaneous transplantation of the ELDa cells, but not of the ELTa cells which on this route of inoculation showed a greater compatibility with the C_3H mice than with the ST/a mice.

Although many authors have been able to grow Ehrlich's ascites tumor cells *in vitro*, they have not been equally successful in maintaining the ability of these cells to produce tumors in mice. Thus, Jackson *et al.* [15] and Deschner and Allen [11] found that 1000–4000 cells were unable to produce tumors in mice, while other authors have obtained tumors regularly by the re-inoculation of Ehrlich's ascites tumor cells [16, 17] and Ehrlich–Landschütz' ascites tumor cells [10, 12] into mice. Kolb and Siegel [18] speculated whether the loss of implantability is due to a reversion toward normal or to the selection of non-neoplastic incidental cells explanted with the tumor cells.

The present studies showed that the implantability of ELTc and ELDc cells depends greatly on the route of reimplantation, the number of reinoculated cells, the period of cultivation *in vitro*, and the strain and the age of the recipient mouse. On intraperitoneal implantation into adult mice a decrease or a complete loss of implantability was observed, but during this process the differences between the ELT and the ELD cells and between the compatibility of both cell lines with the C_3H and ST/a mice were maintained, suggesting that only quantitative changes in histocompatibility had occurred.

Also on subcutaneous implantation a decrease or a complete loss of implantability of both cell lines in both strains of mice was observed. In spite of the fact that ELTa cells on subcutaneous transplantation were found to be much more virulent than the ELDa cells, the opposite was found to be the case during propagation *in vitro*. Particularly in the ST/a mice the decrease or loss of implantability of the ELTc cells was more pronounced than that of the ELDc cells.

The greater susceptibility of newborn mice to *in vitro* propagated ELT and ELD cells points to an increased antigenicity as the

explanation of the change of implantability during cultivation *in vitro*. This explanation raises the question whether the changes are qualitative or quantitative, i.e. whether the cells have acquired new antigens during cultivation or whether the loss of implantability is due to an increased production of antigens or possibly to the removal of an inhibitor of the antigenic expression.

The chromosomal changes described indicate a change of the genotype which may be associated with changes of the antigenic properties of the two cell lines. However, as already pointed out, the changes in intraperitoneal implantability did not indicate any qualitative changes.

The present immunization and cross-immunization experiments showed that neither of the two cell lines propagated *in vivo* or *in vitro* were compatible with the C_3H and ST/a mice. The protection and cross-protection of C_3H mice against the secondary inoculation of the two ascites tumors by the primary immunization with the two *in vitro* cultured cell lines indicate the presence of at least one common antigen in ELT and ELD cells propagated *in vivo* and *in vitro*, an antigen which they do not share with the C_3H mice; but these observations do not explain the change of implantability occurring during cultivation *in vitro*.

In ST/a mice protection against both ELTa and ELDa ascites tumor cells was obtained by immunization with ELDc cells. A similar effect was not obtained with ELTc cells, which may indicate that ELDa, ELDc, and ELTa cells share an antigen, which is not present in ELTc cells. However, such a loss of an antigen does not explain the loss of implantability in the ST/a mice.

Finally, the protective effect of preimmunization of the ST/a mice with ELTc cells against ELTa ascites tumor cells indicate the presence of a third antigen shared by the *in vivo* and *in vitro* propagated ELT cells, but not by the ELD cells or the ST/a mice. However, also this observation fails to explain the change in implantability of cultured cells.

A qualitative change of the antigenic properties of the cells grown in culture may not be due to a change of the cellular genome, but rather to the absorption of bovine protein from the nutritional medium. However, the unsuccessful attempts to pass subcutaneous tumors produced in newborn animals in adult animals by serial transplantation do not support such an explanation of the loss of implantability.

Although the sera used in the present experiments were heat inactivated, they cannot entirely be excluded as a source of complement. However, considering the gradual decrease of implantability during cultivation and the lack of transplantability of tumors produced in newborn animals, it is highly unlikely that the rejection of ELTc and ELDc cells is due to complement obtained from the fetal bovine serum, which was present in the nutritional medium at a concentration of 20%.

Removal of an antigen barrier, which *in vivo* protects the tumor cells against the attack of host lymphocytes is another hypothetical explanation, which deserves consideration. Such a barrier may be humoral antibodies, which protect antigenic sites from immune lymphoid cells [19], or peri-cellular sialomucins which have been suggested [20] to present an important electrochemical barrier, which prevents host lymphocytes from making cell-to-cell contact with immunological competent cells. In support of the latter hypothesis it might be pointed out that Langley and Ambrose [21] have recovered a sialomucin from the surface of Ehrlich's ascites tumor cells.

Besides the immunological factors other causes of the change of implantability should be considered. The present experiments do not offer any explanation of either the rapid decline of the subcutaneous implantability of the ELTc cells in both adult and newborn ST/a mice, or of their reduced subcutaneous growth rate in ST/a mice as compared to the rate of growth in C_3H mice, which immunologically were less susceptible than the ST/a mice. Spärck [22] has found that X-irradiation may reduce the susceptibility of mice to subcutaneously inoculated isologous tumors. The author ascribes this observation to an inhibitory effect of irradiation on non-specific host responses, such as connective tissue proliferation and inflammation, which are required to form a growth promoting tumor-bed.

On the basis of her experiments with Ehrlich's ascites tumors, Hartveit [23 24] proposed that tumor growth is dependent on a balance between the sensitization of the tumor cells, obtained through the union of antigen with antibody, the amount of complement available, and an inhibitor of lysis which is present in the ascitic fluid. According to the author this inhibitor of lysis is called forth by the inflammatory response to the tumor tissue inoculated. Future studies will have to clarify, whether the inflammatory response to subcutaneously inoculated ELT cells is greater in the C_3H mice than in the ST/a mice, and whether this effect of the inoculated cells is reduced during propagation *in vitro*.

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RESUME

Durant la culture in vitro, *les lignées hypertétraploïde ELT et hyperdiploïde ELD de la tumeur ascitique d'Ehrlich font l'objet d'une dédifférentiation cytologique, ce qui a rendu difficile la distinction entre les deux lignées cellulaires. Même le nombre de chromosomes a montré une grande instabilité. En* 1967, *après* 3 *et* 5 *ans de culture respectivement, il était néanmoins toujours possible de reconnaître les deux lignées cellulaires par numération des chromosomes. Le nombre modal des cultures ELT était* 69, *et celui des cultures ELD* 45.

En même temps, la capacité des deux lignées cellulaires de produire des tumeurs chez des souris C3H et ST/a adultes a diminué, mais, au cours de ce processus, les différences de transplantabilité et de compatabilité envers les hôtes se sont maintenues pour l'essentiel. Chez des souris nouveau-nées, la transplantabilité s'est conservée dans une grande mesure. Ce fait, conjointement à l'effet protecteur obtenu par pré-immunisation de souris C3H et ST/a contre des cellules tumorales inoculées secondairement, montre l'activité antigénique des cellules cultivées in vitro. *La question de savoir si des altérations de cette antigénicité peuvent expliquer les modifications de la transplantabilité pendant la culture* in vitro *est discutée.*

SUMMARY

During propagation in vitro *the near-tetraploid ELT and the near-diploid ELD subline of Ehrlich's ascites tumor were found to undergo cytological dedifferentiation, which made the distinction between the two cell lines difficult. Even the number of chromosomes showed great instability. However, in* 1967 *— after cultivation for* 3 *and* 5 *years respectively — two different culture lines could still be recognized by chromosome counts, the ELT cultures showing a mode of* 69 *and the ELD cultures a mode of* 45.

At the same time, the tumor producing capacity of the two cell lines in adult C3H and ST/a mice decreased, but during this process the differences seen between their transplantability and their preference of host strain during propagation in vivo *were largely maintained. The persisting implantability of both cell lines in newborn mice, as well as the protective effect of preimmunization of C3H and ST/a mice with cultured cells against the secondary inoculation of ascites tumors carried* in vivo *showed the antigenicity of the two cell lines propagated* in vitro. *Changes in antigenicity are discussed as a possible explanation of the decrease or complete loss of the ability to produce tumors in adult mice.*

ZUSAMMENFASSUNG

Bei In-vitro-*Züchtung der nahezu tetraploiden ELT- und nahezu diploiden ELD-subline der Ehrlich-Aszitestumorzellen wurde eine Entdifferenzierung festgestellt, die die Unterscheidung der beiden Zelltypen schwierig machte. Selbst die Chromosomenzahl schwankte stark. Immerhin war es noch im Jahre* 1967, *nach drei- bzw. fünfjähriger Kultur möglich, durch Chromosomenzählung die beiden Zellstämme voneinander zu unterscheiden; bei den ELT-Kulturen war eine Modalzahl von* 69, *bei den ELD-Kulturen eine Modalzahl von* 45 *zu finden.*

Gleichzeitig nahm die tumorerzeugende Eigenschaft der beiden Zellarten bei erwachsenen C3H- und ST/a-Mäusen ab; aber die im Verlaufe dieses Vorgangs beobachteten Unterschiede in der Uebertragbarkeit und der Bevorzugung von Wirtsstämmen bei In-vivo-*Züchtung blieb weitgehend erhalten. Die weiterbestehende Implantierbarkeit beider Zellstämme in neugeborene Mäuse ebenso wie die Schutzwirkung einer Präimmunisierung der C3H- und ST/a-Mäuse mit gezüchteten Zellen gegen die sekundäre Inokulation von Aszitestumoren, demonstriert den Antigencharakter der beiden* in vitro *gezüchteten Zellformen. Veränderungen der Antigenität werden als mögliche Erklärung der verminderten oder völlig verlorenen Fähigkeit, bei erwachsenen Mäusen Tumoren zu erzeugen, in Betracht gezogen.*

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Chromosome Abnormalities in the Malignant Lymphomas

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INTRODUCTION

CHROMOSOME studies of lymph nodes in the malignant lymphomas are beset by certain difficulties. These are to obtain material from cases belonging to one histological category, the assessment of chromosome abnormalities in treated cases, and finally the hazards in interpreting results from a mixed population of cells, not all of which are neoplastic. Several case reports have been published [1–5]. The studies of Jacobs *et al.* [6] on Burkitt's lymphoma, and of Baker and Atkin [7], Speirs and Baikie [8] and Miles *et al.* [9] on various types of malignant lymphoma are more extensive. This paper reports observations of the chromosomes of lymph node cells in 14 cases of malignant lymphoma. It was hoped to discover if there are any specific chromosomal abnormalities in this group of disorders, and an attempt was made to classify the cases on a chromosomal basis.

MATERIALS AND METHODS

Altogether 17 lymph node biopsy specimens from patients with malignant lymphoma were examined. Satisfactory chromosome preparations were obtained in 13 cases. A lymph node aspirate was examined in one additional case (no. 5); the histological diagnosis was made from a previous biopsy.

Diagnosis

The histological diagnosis is given in Table 1; the classification was based on Gall and Mallory [10] and Rappaport [11]. As seen in Table 1, the cases are a heterogeneous group. Case 10 had long-standing chronic lymphocytic leukaemia (C.L.L.)*; the nature of the disease changed, and a lymph node biopsy showed a reticulum cell type of malignant lymphoma. Case 11 is unusual; from the histological sections the most likely diagnosis is malignant lymphoma of reticulum cell type. In view of the clinical features (particularly the long remission), it was suggested that Case 11 may have a granulomatous thymoma of the type described by Lowenhaupt and Brown [12]. Ackerman and del Regato [13] consider this to be a variant of Hodgkin's disease.

Technical procedures

The lymph node was cut up under sterile conditions in Tissue Culture (TC) Medium '199' (Glaxo) to make a fine cell suspension. This was divided into three parts which were examined:

(1) Directly, after 1½–2 hr incubation with 'Colcemid' (Ciba) at a concentration of 1 μg/ml, followed by hypotonic sodium citrate (0·95%) for 25 min, and finally fixed with 3:1 methyl alcohol: acetic acid.

(2) The cell suspension was added to a mixture of 3 parts AB serum and 7 parts TC 199, so that the final cell concentration was 1000–2000 cells/mm³. The culture was incubated at 37°C for 24 hr and was then treated with the same 'colcemid'-hypotonic-fixative sequence.

(3) A similar culture to (2) was set up with the addition of 0·05 ml Phytohaemagglutinin (PHA) (Burroughs-Wellcome). The culture was incubated at 37°C for 72 hr, and processed as in (2).

Slides were made by the air-drying method and were stained with 1% cresyl violet.

*Abbreviations to be used in this paper: HVT — high-voltage X-ray therapy; Ca. — carcinoma; LN — lymph node; CLL — chronic lymphocytic leukaemia; BM — bone marrow; PHA — Phytohaemagglutinin; RS cell — Reed-Sternberg cell; DNA — deoxyribonucleic acid; Letters A–G, Numbers '1', '2', etc. — Chromosomes corresponding to (or resembling) Patau and Denver classifications; fr. — acentric fragment; dic. — dicentric; del. — deletion; tr. — translocation; LA — long arm of a chromosome; SA — short arm of a chromosome; rl — random loss; Ch^1 — Christchurch chromosome.

Table 1. Clinical data

No.	Age & sex	Length of history at time of biopsy	Diagnosis (cell type)	Treated or untreated at time of biopsy	Other data
1	58 F	8 yr	Well-differentiated lymphocytic (C.L.L.)	T	8 yr ago — C.L.L. diagnosed 7 yr ago — Ca. Cervix, treated with radium
2	58 F	1 month	Well-differentiated lymphocytic	U	Mother and sister died of leukaemia, sister has C.L.L.
3	51 F	3 months	Well-differentiated lymphocytic (follicular)	U	
4	38 M	2 weeks	Moderately-differentiated lymphocytic	U	
5	50 M	4 yr	Moderately-differentiated lymphocytic (follicular) (LN biopsy 4 yr ago)	T	BM (2 yr after LN aspirate) 2/3 normal haemopoietic tissue, 1/3 small and medium atypical lymphocytes with cleft nucleus
6	81 F	1 month	Mixed cell type (lymphoblasts +reticulum cells)	U	
7	65 M	1 month	Mixed cell type (lymphoblasts +reticulum cells)	U	BM normal cytology
8	69 F	2 yr	Mixed cell type (lymphoblasts +reticulum cells) (follicular)	U	
9	73 F	1½ yr	Histiocytic (follicular)	U	
10	72 M	6 yr	C.L.L.→Reticulum cell	T	6 yr ago — C.L.L. diagnosed. Now fever, lymphadenopathy hepatosplenomegaly and anaemia
11	47 M	10 yr	? Reticulum cell	T	10 yr ago enlarged LN neck and mediastinum. HVT. LN biopsy? anaplastic Ca. 1 week ago — lump in axilla. LN biopsy same as 10 yr ago
12	46 F	2 yr	Reticulum cell (follicular)	U	
13	7 M	3½ yr	Hodgkin's disease	U	
14	44 M	2 yr	Hodgkin's disease	U	BM hyperplastic, normal cytology

Several specimens gave an adequate number of mitoses by the direct method (1); in other cases a greater number of dividing cells was obtained by short-term culture method (2). Both methods gave cells which are actively in division and which do not require stimulation; these cells include the malignant population. Method 3 yielded mitoses from a different population of cells (small lymphocytes) which responded to the stimulus of PHA.

The proliferative characteristics of the lymph node cells in Cases 1, 6, 7, 9, 11 and 12* were investigated by measuring (i) the nuclear labelling index following incubation with tritiated thymidine and (ii) the deoxyribonucleic acid (DNA) content of individual nuclei by microdensitometry. Full details of the results will be published elsewhere [14].

In three cases (nos. 5, 7 and 14) a sample of bone marrow was aspirated for diagnostic purposes, and a portion of this was processed for chromosome studies by the direct method [15].

*These cases correspond to Cases 6, 16, 15, 23, 27 and 25 respectively of Cooper *et al.* [14].

RESULTS

The clinical details are given in Table 1. Cases 1, 5, 10 and 11 had been treated with X-rays and/or chemotherapy previous to the biopsy.

The chromosomes of lymph node cells

In most cases, countable and analysable metaphases were obtained from either the direct method or 24 hr culture. No evidence of a difference in chromosome constitution was observed in some of these cases between direct and 24 hr culture and therefore for the purpose of analysis the results have been pooled. The chromosome counts are shown graphically in Figs. 1 and 2, the cases being grouped on a histological basis. The detailed chromosome analysis is presented in Table 2; the analysis of cells from Case 12 is given in Table 3.

Seventy-two hr cultures with PHA yielded

Table 2. Chromosome analysis

No.	Material	Analysis	Comment
1	LN 24 hr	2(43) small fr., 3(46) normal	fr.? due to radiation or chemotherapy
	LN 72 hr+PHA	1(46) normal	Very few mitoses
2	LN direct	3(46) normal	Family history of leukaemia
	LN 72 hr+PHA	11(46) normal	No evidence of Ch1 chromosome
3	LN 24 hr	1(46) normal, 1(46) one '18' has del. L.A.	Small extra chromosome
		7(47)+'18' del.	like no. '18' with del. L.A. ('18' del.)
		1(48)+'18' del.+extra C	(see Cases 4, 9)
	LN 72 hr+PHA	1(46) normal	
		1(47) '18' del.+'18' del.	Very few mitoses
4	LN direct	3(46) normal, 2(46) '18' del., 1(46)? '18' del.	
	LN 24 hr	5(46) normal, 10(46) '18' del., 2(46)? '18' del.	
		2(51) C+2, '16'+1, '18' del., F+1, G+1	'18' del. in most cells, rest normal
	LN 72 hr+PHA	8(46) normal, 1(46) '18' del.	Most cells have normal '18'. Very few mitoses
5	LN direct	All cells extra '1', missing '2', 1–2 extra C. Variable loss of '3', B, D, E or F chrom.	No normal cells
	BM direct	33(46) normal	No evidence of cells of abnormal clone
6	LN direct+LN 24 hr	Very long acrocentric 'marker' in most cells, extra '3', 3–4 extra C, missing D	No normal cells. 'Marker' in most cells from LN direct and in most cells from 24 hr culture
	LN 72 hr+PHA	9(46) normal, 1(46) large fr., 1(46)? extra C, missing F.	Mostly normal cells. No hyperdiploid cells with 'marker'. Very few mitoses
7	LN direct	Long acrocentric 'marker' in all cells; 1–2 extra D, 1–3 extra E, variable gain of B and C; 1–2 F missing, 1–3 G missing.	No normal cells. Stem lines at 46 and 50 inter-related (same 'marker').
		1(69), 1(98)+marker, 1(87)+2 markers+dic.	Cell with 100–110 chromosomes has elongated "beaded" chromosomes
	LN 96 hr+PHA	12(46) normal	No hyperdiploid cells with 'marker'
	BM direct	1(44), 3(45) rl, 5(46) normal	No hyperdiploid cells with 'marker'
8	LN direct +LN 24 hr	Long acrocentric 'marker' in all cells, missing '3', tr.→'2'; some cells extra '16', missing C. 3(44), 4(45), rl	No normal cells
	LN 72 hr+PHA	10(46) normal	No cells with 'marker'
9	LN direct	1(45) '18' del.	
		2(46) normal, 14(46) '18' del. 1(46)? '18' del.	'18' del. in most cells
	LN 72 hr+PHA	5(46) normal	All cells have normal '18'. Very few mitoses
10	LN 24 hr	1(46) normal, tetraploids regular (92)	Predominantly tetraploid. No 'marker'
	LN 72 hr+PHA	23(46) normal	Predominantly diploid
11	LN direct	1(43) dic., 2(46) normal, 1(46) '18' del.	'18' del. in 1 cell
	LN 24 hr	23(46) normal, 1(46) extra C, missing G	Hypotetraploid cells (78–92 chromosomes) have fuzzy, poorly-fixed chromosomes. No 'marker'
	LN 72 hr+PHA	11(46) normal, 3(46) dic.+fr., 2(46) fr., 1(46) extra C, missing E	Many mitoses. Gross radiation damage in 5 out of 20 cells. (HVT 10 yr ago)
12	LN 24 hr	See Table 3	
	LN 72 hr+PHA	13(46) normal, 1(46) 2 dic.+2 fr.	Many mitoses. Most cells have normal karyotype
13	LN direct +LN 24 hr	2(46) normal	
	LN 72 hr+PHA	10(46) normal	Many mitoses
14	LN 24 hr	4(46) normal, 2(46)? extra F, missing E 1(46)? extra C, missing B. Very long submetacentric 'marker'+many minute chromosomes in the aneuploid cells (64–80 chromosomes)	
	LN 72 hr+PHA	13(46) normal	No aneuploid cells with 'marker'
	BM direct	3(45) rl., 10(46) normal	No aneuploid cells with 'marker'

predominantly normal diploid cells, even when the result from direct or 24 hr culture was entirely abnormal (as in Cases 6, 7, 8). It appears that aneuploid cells with 'markers' (presumably the lymphoma cells) are not in division at 72 hr. The diploid metaphases in these cultures were probably derived from small lymphocytes in the lymph node which responded to PHA. In several cases, there were very few mitoses in the preparations,

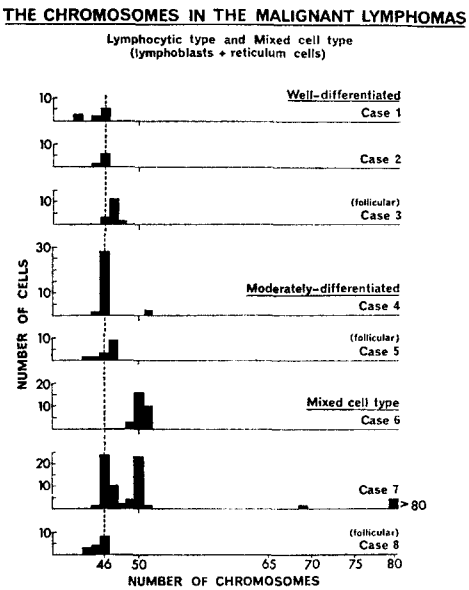


Fig. 1. *Chromosome counts, LN direct+24 hr culture. Cases* 1–8.

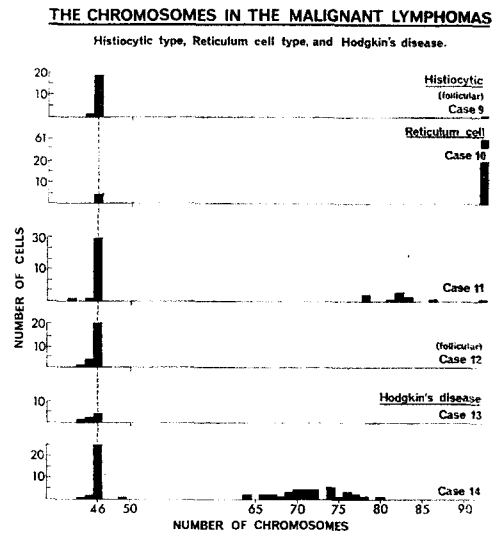


Fig. 2. *Chromosome counts, LN direct+24 hr culture. Cases* 9–14.

suggesting that the response of the cells to PHA was diminished.

The chromosome analysis from the direct method and 24 hr culture is as follows:

I. *Normal diploid* ... ... 3 cases (no. 1, 2, 13).

II. *Deletions and rearrangements*+ some normal diploid cells ... 4 cases (no. 3, 4, 9, 12).

III. *Gross abnormality* (pseudodiploid or hyperdiploid) ... ... 4 cases (no. 5, 6, 7, 8).

IV. *Tetraploid* ... ... 1 case (no. 10).

V. *Double population* (normal diploid cells+cells of higher chromosome number) ... 2 cases (no. 11, 14).

I. *Normal.* Case 13 had Hodgkin's disease; Cases 1 and 2 had well-differentiated lymphocytic lymphoma. The lymph nodes from these three cases yielded very few metaphases (see Figs. 1 and 2), nevertheless the cases are included in the series for the sake of completeness. Case 1 had chronic lymphocytic leukaemia (C.L.L.) and Case 2 had a family history of leukaemia. The Ch[1] chromosome was not observed. This is a small acrocentric chromosome with a deleted short arm; it is found infrequently in familial C.L.L. [16].

II. *Deletions and rearrangement.* The lymph nodes of Cases 3, 4, 9 and 12 differed histologically from each other (see Table 1). None of the patients has been treated. There was an apparently similar chromosome abnormality in two of the cases (no. 4 and no. 9). The abnor-

Table 3. *Case* 12. *Chromosome analysis of lymph node cells* (24 *hr culture*)

No. of cells	No. of chromosome	C	D	'18'	G	Comment
1	44					
1	45			−1	+1	One G large
1	45	−1	+1	−1	+1	
1	45	−1	+1	−2	+2	Two G large, one like '18' with del. S.A.
4	46 normal					
1	46			−1	+1	'18' has del. S.A.
3	46			−2	+2	One G large in 2 cells Two G large in 1 cell
8	46	−1	+1	−1	+1	
2	46	−1	+1	−2	+2	

Cells with 44 and 45 chromosomes have random loss.

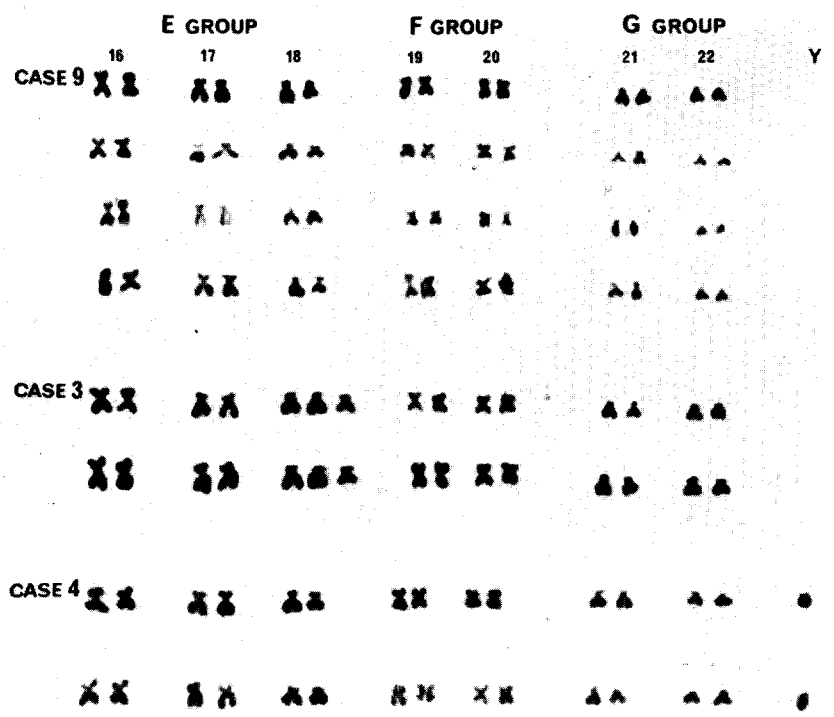


Fig. 3. *The* '18 *deletion' abnormality.*

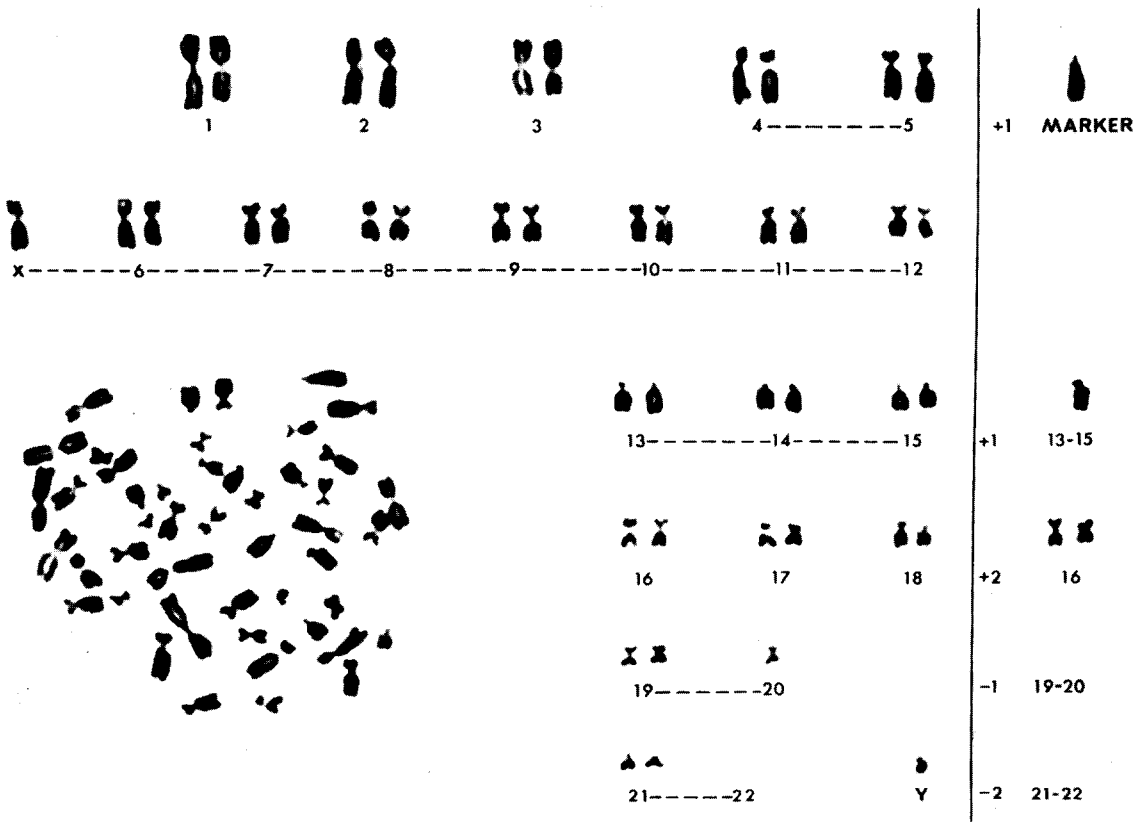


Fig. 4. *Cell from Case* 7 (*LN direct*) *with* 47 *chromosomes.*

(to face p. 100)

mality most often resembled a deletion of the long arm of one no. 18 chromosome: it is called the '18 deletion' in this paper. In some cells the '18 deletion' chromosome was only slightly larger than the chromosomes of the 21–22 (G) group, but invariably it had longer short arms. Satellites were not observed. Figure 3 illustrates the abnormality. In the bottom row the second no. 18 chromosome is condensed; the abnormality is borderline in this cell. A similar '18 deletion' was observed in a single diploid cell from Case 11.

In Case 3 (stem line 47 chromosomes) there was an additional chromosome which resembled closely the '18 deletion' found in Cases 4 and 9 (see Fig. 3). This interpretation requires two separate events, deletion and non-disjunction. In some cells, the extra chromosome could possibly be one of the G group with long short arms, the chromosome being acquired by a single non-disjunctional event. However, on morphological grounds the extra chromosome is considered to be a deleted no. 18. One cell from Case 3 and two cells from Case 4 had additional chromosomes of other groups (see Table 2).

The chromosome abnormalities in Case 12 are more complex (see Table 3). In some cells there was a small rearrangement, one or two no. 18 chromosomes missing and one or two extra chromosomes indistinguishable from the G group. The simplest explanation is a large deletion of a no. 18 chromosome giving an apparent extra G; alternatively there could be a loss of no. 18 and a gain of G by non-disjunction. In 12 cells there were rearrangements involving chromosomes of the C and D groups and in two cells there was a deletion of the *short* arm of a no. 18 chromosome.

In each of these four cases there were a few cells with normal karyotypes in direct preparations and 24 hr cultures. Seventy-two hr cultures with PHA yielded predominantly normal diploid cells (see Table 2). Thus in the cases with the '18 deletion' a form of constitutional mosaicism is unlikely; and the abnormality is probably related to the disease.

III. *Gross abnormality.* Of the four cases with the most extensive chromosome abnormalities, one was moderately differentiated lymphocytic type and three were mixed cell type (see Table 1). On case (no. 5) had been treated. All the cells from these cases had an abnormal karyotype, with extra and missing chromosomes; in three of the cases there was a similar long acrocentric 'marker' chromosome. This 'marker' was probably formed differently in each case, as each karyotype was quite different from the other (see Table 2). This type of gross karyotypic abnormality with variable gains and losses of chromosomes has been observed in some cases of acute leukaemia. The karyotype of a cell from Case 7 is shown in Fig. 4. In Case 7 the three cells with 69, 87 and 98 chromosomes all had the same 'marker' as the hyperdiploid cells. By another method, Cooper *et al.* [14] have shown that the hyperdiploid cells and also the cells of higher ploidy are lymphoma cells (Case 7=their Case 15).

IV. *Tetraploid.* Case 10 (C.L.L. reticulum cell type) had a tetraploid stem line in 24 hr culture; these were probably the malignant reticulum cells. The polyploidy could be a radiation-induced change. The cells in 72 hr culture with PHA were predominantly diploid (small lymphocytes).

V. *Double Population.* In Case 11, the cells of higher chromosome number were hypotetraploid and in Case 14 the counts spanned a wide range of chromosome number (triploid–hypertriploid). In Case 11, (? reticulum cell type, ? Hodgkin's disease), the chromosomes of the hypotetraploid cells were fuzzy and poorly fixed; this feature has been observed in bone marrow cells in some cases of acute leukaemia [17, 18]. Cooper *et al.* [14] found that in Case 11 (their Case 27) the lymphoma cells have a hypotetraploid amount of DNA. Seventy-two hr culture with PHA yielded normal diploid cells (small lymphocytes) with a 25% incidence of radiation-induced damage.

The chromosomes of bone marrow cells

The bone marrow cells of Cases 5, 7 and 14 had normal chromosomes (see Table 2): there were very few metaphases in the aspirate from Case 7. In Cases 7 and 14 the bone marrow was morphologically normal. In Case 5 it was infiltrated with many lymphocyte-like cells which were probably lymphoma cells. However, the chromosomes of the bone marrow cells were entirely normal, in contrast to the abnormal chromosomes of the lymphoma cells from the lymph node aspirate.

DISCUSSION

The relationship of the karyotype to the histology and to diagnosis and prognosis

In spite of a lack of close correlation between the histology and the karyotype in this heterogeneous group of cases, a few generalisations can be made. Lymph nodes showing gross chromosomal changes and complete absence of normal cells are all less well-differentiated types. Conversely, the better differentiated types tend to have normal chromosomes or

minor abnormalities. In this group of disorders a similar chromosome abnormality (such as the '18 deletion') does not indicate a particular histological diagnosis. In chronic granulocytic leukaemia the Philadelphia (Ph^1) chromosome is a considerable help in diagnosis; with this exception there is no known malignant condition in which a chromosome abnormality is of positive diagnostic value.

Because of the diversity of the chromosome findings in relation to the histology, the response to treatment and the prognosis cannot be predicted by the type of chromosome derangement. Of the four cases with the most abnormal karyotypes, Case 6 died shortly after the biopsy, Case 7 did not respond to any form of treatment, Case 8 responded well to chlorambucil, and Case 5 has survived for several years. The three cases with the '18 deletion' have responded well to treatment, but in such a very small series the prognostic value of this abnormality is of no reliance.

Chromosome no. 18 *in the malignant lymphomas*

An abnormal chromosome resembling a no. 18 with a deletion in the long arm ('18 deletion') was observed in three out of the 14 analysed cases, and in a single cell in a fourth case. In another recent series of 15 cases, a similar '18 deletion' was seen in two cases — Hodgkin's paragranuloma and reticulum cell sarcoma (G. Seif, personal communication). Further evidence for abnormalities of chromosomes no. 18 in the malignant lymphomas comes from Speirs and Baikie [8] who analysed the chromosomes in ten cases. They found a deletion of the *short* arm of one chromosome no. 18 in three of their cases (two Hodgkin's disease and one follicular lymphoma); the defect is similar to the abnormality found in two cells in Case 12. No deletion of the long arm of chromosome no. 18 was observed by Speir and Baikie, although the extra chromosome (?G) in their Case 1 does resemble the extra chromosome ('18 deletion') in Case 3 of the present study (Baikie, personal communication). In certain cases of malignant lymphoma, therefore, there may be mutational events (spontaneous, or induced by viral, chemical or physical agents) affecting chromosome no. 18 either side of the centromere. The most obvious explanation of these structural changes is a deletion of either the long or the short arm, with an actual loss of chromosomal substance. However, it is possible that some other abnormality of chromosome structure is involved, such as an alteration in the degree of chromosomal coiling. In some cases additional chromosomal changes occurred in cells containing the same marker chromosome. This would suggest that they came from the same ancestral cell. For example, in Case 12, 11 cells had the same basic change (18−1, G+1) and nine of these cells showed an additional chromosomal change (C−1, D+1). This is evidence of clonal evolution within a cell population.

In the present study, no chromosome other than no. 18 appears to be specifically affected. Others have reported abnormalities of the small acrocentrics [two cases, 8]; and of a no. 2 chromosome in four cases of Burkitt's Lymphome [6]. Miles *et al.* [9] who examined the lymph nodes of 12 cases of malignant lymphoma by the direct method, found in most cases a pronounced secondary constriction in chromosome no. 9. This feature was not observed in the present series.

The cases showing the '18 deletion' are histologically distinct from each other. However, there appears to be a histological interrelationship in the malignant lymphomas. According to Rappaport [11] certain types of malignant lymphoma can progress to another type with time, so possibly the histological types may not be rigidly separate categories. Evidence of interrelationship on a cellular level has hitherto been lacking.

The significance of the normal diploid cells

Except in the four most abnormal cases, some normal diploid metaphases were observed in direct and 24 hr cultures of the lymph nodes, and in Cases 11 and 14 they form an appreciable proportion of the dividing cell population. In normal lymph nodes, cells in metaphase are rarely seen except after incubation with PHA. Others [7, 8, 9] have also observed normal as well as abnormal karyotypes in the lymph nodes of some cases of malignant lymphoma. These cells with an apparently normal karyotype may be lymphoma cells; alternatively they may be non-malignant cells (such as reticulum cells, plasma cells, large and medium lymphocytes) which are stimulated to divide by the presence of malignant cells. Thus in Case 11, the small lymphocytes, reticulum cells and histiocytes have a diploid DNA content, whereas the lymphoma cells have an aneuploid DNA content [14]. A disadvantage of current cytogenetic techniques is that following 'colcemid'-hypotonic pre-treatment, it is not possible to identify a metaphase (diploid or aneuploid) as a specific cell type. The same disadvantage applies to the study of other tissues, such as the bone marrow.

The normal diploid cells in the 72 hr cultures

with PHA are probably derived from small lymphocytes. These cells may originate either from a normal stem-line in the node or from unaffected lymphoid tissue elsewhere in the body. In several cases the response of the cells to PHA was poor as shown by the scarcity of metaphases. Several workers have shown that the response of peripheral blood lymphocytes to PHA is depressed in the malignant lymphomas [19, 20, 21]. The reason for this is unknown.

The Reed-Sternberg cell

As there were only two cases of Hodgkin's disease in this series, few comments can be made concerning the chromosomes of lymph node cells in this disease, in particular, the chromosomes of the Reed-Sternberg (RS) cell. Cases 13 and 14 both showed these cells in sections; yet diploid metaphases were found in one, and in the other there was a double population of diploid and hypertriploid cells. Several workers have found a predominantly or entirely diploid mode in the lymph nodes of some cases of Hodgkin's disease [2, 8, 9]; in one case the mode was hyperdiploid [7]. In none of these cases was it conclusively proved that the metaphases were RS cells. However, a case of Hodgkin's disease with many RS cells in the blood was studied using the blood leucocyte culture method, and a hyperdiploid mode was found [22]. It might be expected that the RS cell which is frequently multi-lobed or multinucleate would have a high chromosome number, and it has been suggested that in some cases the RS cell is hypotetraploid or tetraploid [1, 3, 9]. Here, again there was no conclusive proof. Further observations on the DNA content of these cells together with autoradiographic and chromosome studies may clarify this problem.

The cytogenetics of the bone marrow

In malignant lymphoma whether or not the chromosomes of the bone marrow are abnormal usually depends upon the degree of infiltration of the bone marrow with lymphoma cells. Abnormal karyotypes in infiltrated bone marrow have been reported [5, 23, 24]. An interesting case of lymphosarcoma with a leukaemoid reaction showed the same abnormal pseudodiploid cell line in lymph node and in the bone marrow [4]. However, the latter was not infiltrated with lymphoma cells, but showed granulocytic hyperplasia and eosinophilia. It was suggested that there was a common stem cell for the haemopoietic tissue and the lymphoma cells. Case 5 of the present study demonstrates the reverse situation; the bone marrow was infiltrated with lymphoma cells but the chromosomes were normal. It is concluded that the lymphoma cells in the bone marrow were not in division.

Suggestions for future work

More lymph nodes from cases of malignant lymphoma (especially Hodgkin's disease) should be studied, using various culture times (direct, 24 hr, 74 hr) to obtain cells in division. Serial lymph node biopsies or aspirates might throw light on the mechanism of clonal evolution and also on the process by which abnormal cell lines originate. Biopsies of nodes at different sites could determine whether an abnormal stem line was localised or generalised. There is still some uncertainty about the multifocal or unifocal origin of the malignant lymphomas, particularly Hodgkin's disease. The combination of chromosome studies with measurement of the labelling index and the DNA content of the cells could yield further information about the proliferation patterns in these disorders [14]. The chromosomes of the bone marrow cells should be analysed in all cases. It is not known if the case of Tjio *et al.* [4] is an isolated instance; possibly a subgroup of the malignant lymphomas is characterised by a common stem-cell in both tissues.

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RESUME

Les chromosomes des cellules de ganglion provenant de 14 *cas de lymphomes malins furent examinés. Trois cas avaient un nombre diploïde normal;* 3 *cas présentaient la même anomalie qui semblait être une délétion du bras long d'un chromosome* 18*;* 1 *cas avait des réarrangements complexes et une délétion du bras court d'un chromosome* 18*, dans deux cellules;* 4 *cas avaient un caryotype profondément altéré, avec un chromosome 'marqueur'*

dans 3 cas; 1 cas était tétraploïde, et 2 cas avaient une double population de cellules, l'une diploïde normale, l'autre avec un plus grand nombre de chromosomes. Il n'y avait pas de relation étroite entre l'histologie et le caryotype; cependant, les ganglions avec les anomalies chromosomiques les plus accentuées étaient du type le plus indifférencié, tandis que les ganglions du type bien différencié avaient des chromosomes normaux ou seulement des anomalies minimes.

Sauf dans les 4 cas avec caryotypes très anormaux, un certain nombre de métaphases diploïdes normales furent observées à l'examen direct, et après une culture de 24 heures. Il s'agissait probablement de cellules normales, non malignes. Dans les cultures de 72 heures avec PHA, la plupart des métaphases étaient normales (petits lymphocytes transformés). Les chromosomes des cellules de la moelle osseuse furent examinés dans 3 cas; le caryotype était normal dans chaque cas, bien que l'un des spécimens fût infiltré par des cellules lymphomateuses. On en conclut que ces cellules ne se divisaient pas.

SUMMARY

The chromosomes of lymph node cells from 14 cases of malignant lymphoma were analysed. Three cases were normal diploid; three cases showed a similar abnormality resembling a no. 18 chromosome with a deletion in the long arm; 1 case had more complex rearrangements and a deletion in the short arm of a no. 18 chromosome in 2 cells; 4 cases had a grossly abnormal karyotype with a 'marker' chromosome in 3 cases; 1 case was tetraploid, and 2 cases had a double population of normal diploid cells and cells of higher chromosome number. There was no close correlation between the histology and the karyotype; however, lymph nodes with the most extensive chromosomes abnormalities were less well-differentiated, and the better differentiated nodes had normal chromosomes or minor abnormalities.

Except in the 4 cases with very abnormal karyotypes, some normal diploid metaphases were found in direct and 24 hr cultures; these were probably non-malignant cells. Seventy-two hr cultures with PHA yielded predominantly normal diploid metaphases (transformed small lymphocytes). The chromosomes of bone marrow cells were analysed in 3 cases; the karyotype was normal in each case, although one of the samples was infiltrated with lymphoma cells. It was concluded that these cells were not in division.

ZUSAMMENFASSUNG

In 14 Fällen von malignem Lymphom wurden die Chromosomen der Lymphknotenzellen untersucht. In 3 Fällen waren die Zellen normal, d.h. diploid; 3 Zellproben wiesen eine Anomalie auf, die aussah, wie wenn ein Stück des langen Arms des 18. Chromosoms fehlte ("Deletion"). In 1 Zellprobe waren komplexere Translokationen festzustellen und bei 2 Zellen wies der kurze Arm von Chromosom 18 eine "Deletion" auf. In 4 Fällen war der Karyotyp stark abnorm, in 3 davon mit einem "Markierchromosom". Das Zellmaterial eines Patienten war tetraploid und dasjenige von 2 weiteren Kranken bestand aus zwei verschiedenen Populationen, die eine diploid, die andere mit einer höheren Anzahl von Chromosomen. Es bestand kein enger Zusammenhang zwischen histologischen Befund und Karyotyp. Immerhin: diejenigen Lymphknoten aus denen das Probematerial mit den höchstgradigen Chromosomenanomalien stammte, waren weniger gut ausdifferenziert und die gut ausdifferenzierten Knoten hatten normale Chromosomen oder solche mit nur geringfügigen Anomalien.

Ausser in den 4 Proben mit den starken Karyotypanomalien waren in allen Direktpräparaten und 24-Stunden-Kulturen einige diploide Metaphasen zu finden; vermutlich handelte es sich dabei um nicht-maligne Zellen. In 72-Stunden-Kulturen mit PHA waren überwiegend normale diploide Metaphasen (umgewandelte kleine Lymphozyten) zu sehen. In 3 Fällen wurden auch die Chromosomen der Knochenmarkzellen untersucht. Der Karyotyp aller 3 Proben war normal, wenn auch eine davon mit Lymphomzellen durchsetzt war. Man gelangte zum Schluss, diese Zellen seien nicht in Teilung begriffen.

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Lactate Dehydrogenase Isoenzyme Patterns in Bronchogenic Carcinoma

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THE HIGH glycolytic capacity of malignant tissue remains the most prominent biochemical feature of neoplasia so far recognized.

Lactic acid dehydrogenase (LDH) is considered a key enzyme in glycolysis. Five LDH-isoenzymes have been found. Each isoenzyme has been shown to be composed of four subunits which may be one of two polypeptides called subunit *H* and subunit *M*. The synthesis of the polypeptides is controlled by separate genes. The five isoenzymes represent the five possible variants of tetrameric combination of the two polypeptides. Thus the five tetramers range from $LDH_1(H_4)$ to $LDH_5(M_4)$ the three hybrid enzymes being $LDH_2(H_3M_1)$, $LDH_3(H_2M_2)$, and $LDH_4(H_1M_3)$. The physiological significance of the isoenzymes was better understood when it was found that each subunit bestowed metabolic characteristics upon the individual isoenzymes according to its relative representation in the tetramer [1–9].

It is generally accepted that in malignant tissue the macromolecular synthesis is changed in favour of subunit *M*, resulting in a dominance of the cathodic isoenzyme. This is thought partly to explain the high capacity for anaerobic glycolysis in malignant tissue.

Changes in the LDH of body fluids in advanced malignancies have been studied for a number of years [10–14]. More recently a number of comparative studies of the LDH-isoenzyme patterns of malignant and non-malignant tissue homogenates have been published [15–24]. The results reported all indicate that the relative isoenzyme activity in malignant tissues is shifted towards the cathodic isoenzymes LDH_5 and LDH_4. The change of the isoenzyme synthesis has been found to occur in regions of tumour bearing organs exhibiting morphological changes accepted as being pre-malignant [17–18, 25]. The author has shown that the isoenzymatic changes may be found in fields of the tumour bearing colon in the absence of morphological changes [26].

The present report deals with the LDH-isoenzymatic changes found in the bronchogenic carcinoma and in the apparently non-affected lung structures.

MATERIALS AND METHODS

Three hundred and fifty-five specimens from 34 cases of bronchogenic carcinoma were assayed for LDH-isoenzyme pattern and histolgoical structure. Similar investigations were carried out on 25 specimens from 3 patients with lung lesions clinically considered to be metastases. The two groups of tissue are hereafter referred to as group 1 and group 2.

As soon as possible following the removal of the tissue at operation the bronchial tree was cut open and sketched along with the tumour. Tissue samples were taken from the tumour, from the lung parenchyma, and from different sites of the bronchial tree. Care was taken to avoid necrotic areas or obvious pneumonic parenchyma.

The bronchial wall was dissected from the surrounding parenchyma. The samples were divided along the longitudinal axis of the bronchi. One half was fixed in 5% neutral

*Under the Danish Cancer Society.

formalin. Paraffin sections were stained with haematoxylin eosin. The remaining half of the sample was washed meticulously for contaminating blood. Homogenization was carried out with an Ultra-Turrax for two 5-sec periods, temperature being kept below 5°C. After centrifugation for 15 min at 1700 x **g**, 30 µl-samples of the supernatant were used for disc-electrophoresis.

Samples which could not be electrophoresed immediately were kept at tissue fragments at −70°C. The stability of the LDH-isoenzymes was not affected by this method of storage.

Human erythrocytes were washed three times with Tyrode's solution. After centrifugation haemolysis was effected by freezing and thawing. The supernatant was used for disc-electrophoretic assay of the LDH isoenzymes.

Disc-electrophoresis was carried out essentially as described by Davis and Ornstein [27, 28]. The electrophoresis was conducted in an electrophoresis cell constructed in this laboratory (See Fig. 1). Forty samples may be run in parallel under identical conditions. Cooling was effected by the immersion of the gel-tubes in the large lower buffer reservoir at 4°C.

Spacer and separation gel were 3 and 7·5% acrylamide at pH 6·7 and 8·8 respectively. The buffer was glycine-Tris(hydroxymethyl) amino-methane (Sigma, St. Louis, MO, U.S.A.) pH 8·3. The running time was 55 min at a constant current of 3 mA (source 'Vitratron', Viatron Scientific Instruments, Dieren-Holland).

The LDH-activities were visualized by the conversion of Nitro Blue Tetrazolium (Sigma) into formazan. The gels were incubated at 37°C for 4 min in the dark. The incubation medium is shown in Table 1. Following the development of the stained bands the gels were fixed and stored in 7·5% acetic acid.

The bands were quantitated by densitometry in a Canalco Model F microdensitometer (Canal Industrial Corporation, Rockville, MD, U.S.A.). The relative isoenzyme activity was expressed by the ratio LDH_4/LDH_2 $[(M_3H_1)/(M_1H_3)]$ which in the following is referred to as the "isoenzyme ratio". The choice of this ratio instead of the total M/H ratio was necessary for technical reasons. The blood content of lung tissue may vary considerably. The hemoglobin thus contaminating the sample may represent a source of error in the quantitation of the enzyme activities. One of the electrophoretic hemoglobin fractions migrates so close to the LDH_1 that overlapping due to diffusion of hemoglobin may easily occur. This interferes with the accurate densitometric reading of the LDH_1. Therefore, the calculation of the total amount of M and H subunits was not possible.

In the disc-electrophoretic separation of LDH in human tissues, one or more subfractions are frequently seen trailing the LDH_2 and LDH_3 while LDH_4 may exhibit splitting into several fractions. The subfractions are situated close to the corresponding main fractions and their activities have been included into the latter.

RESULTS

Of the total 353 tissue samples derived from lungs with bronchogenic carcinoma 77 proved to contain tumour tissue on histological examination.

In one of the patients where the clinically recognized tumour was localized to the sixth bronchial segment of the right lung, a second invasive primary carcinoma was found on microscopic examination of the second bronchial segment. The epithelium adjacent to the tumour showed intra-epthelial carcinoma and pronounced atypical metaplasia. In all the other cases only solitary tumours were found.

Hyperplasia as well as metaplastic areas alternating with normal ciliated epithelium were common findings in this group of patients.

Table 1. *Incubation medium*

Tris–HCL buffer pH 7·5	(0·05 M)	15·00 ml
Sodium d, l-lactate pH 7·5	(0·50 M)	6·00 ml
Nitro-blue tetrazolium	(2 mg/ml)	7·00 ml
Phenazine methosulphate	(2 mg/ml)	0·30 ml
Nicotinamide adenine dinucleotide	(NAD)	20·00 mg
$MgSO_2 \cdot 6\ H_2O$		0·82 mg

Varying degrees of lymphocyte and reticulum cell infiltration were seen in the tumour area as well as in the parenchyma and the submucous structure of the non tumourous bronchi. Erythrocytes were present in varying amounts. Polymorphonuclear leucocytes were present

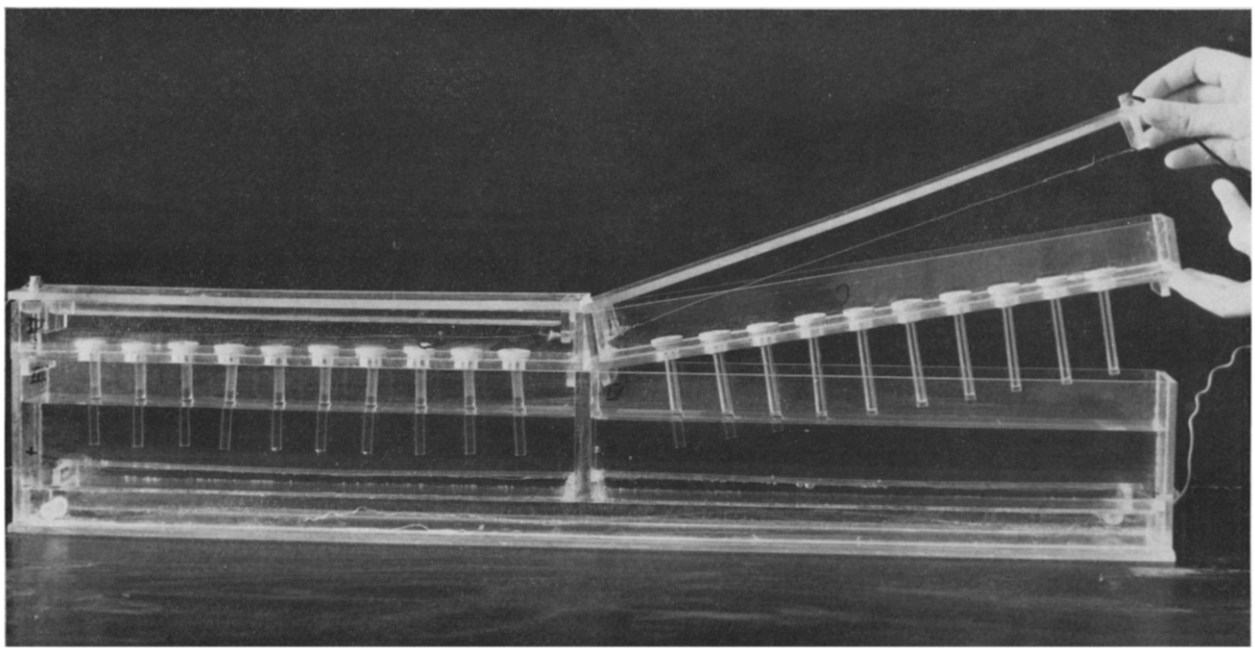


Fig. 1. *Electrophoresis cell. Note upper compartment with tubes and the lid carrying the cathode.*

(to face p. 108)

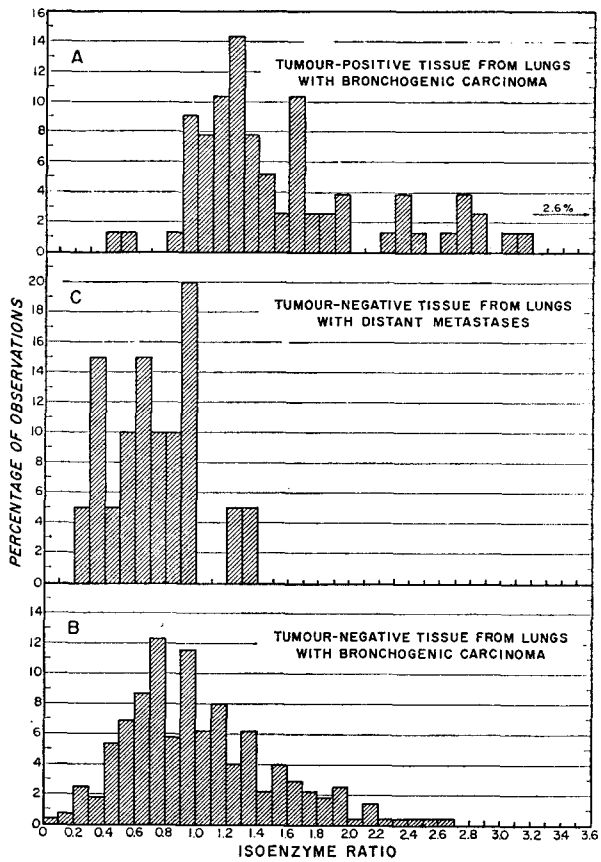


Fig. 2. Distribution of isoenzyme ratios of tumour-positive (A) *and tumour-negative* (B) *samples of group* 1 *and of tumour-negative samples* (C) *of group* 2.

although in amounts not comparable to those of lymphocytes and reticulum cells.

In the lung parenchyma areas with pronounced fibrosis were common findings especially in the near pleural regions. The arteriolar walls often were strikingly hyperplastic in such areas. Minute areas of necrotic tissue were seen in some of the tumour areas.

The distribution of the isoenzyme ratios of the tumour-negative and tumour-positive material is shown in Figs. 2A–B. Both distribution curves rose steeply towards a maximum, subsequently showing a considerably slower fall. The modal values of tumour-negative and tumour-positive samples were 0·7–1·0 and 1·2–1·3 respectively. The two materials showed some overlapping. Of the tumour-positive material 13·3% showed ratios of 1 or less, but only 3·9% showed values below 0·9. In 30% of the tumour-negative samples a ratio of 1·2 or more was found.

The total material of group 1 is presented in Table 2. It appears from this table that the individual patient in the majority of cases showed pronounced variations of isoenzyme ratio in the different non-affected regions varying from values much below those found in the tumours to values which in a few cases even exceeded those of the tumour-positive regions. In a minority of cases, a more constant isoenzyme ratio was found in the tumour-negative control material. These ratios were, in some cases, much below those of the tumour-positive samples, but in other cases they were found very close to the tumour values.

The results of the isoenzyme analysis of the tumour-negative samples in group 2 are shown in Fig. 2C and Table 3. The modal value was 0·9–1·0 and the maximal value 1·3–1·4. Only 10% of the samples showed values of 1·2 or more as compared to 30% of the tumour-negative samples in group 1. The comparison between the tumour-negative samples of group 1 and 2 also shows that ratios exceeding the upper limit of the latter group, i.e. 1·4 were found in 19·8% of the former. Furthermore, it is seen that only 44·5% of the tumour-negative samples in group 1 showed ratios below 0·9, while the corresponding percentage in group 2 was 70.

The mean ratio of the different cell and tissue

Table 2. LDH_4/LDH_2 *isoenzyme ratios of samples from* 32 *patients with bronchogenic carcinoma (Group* 2)

Patient no.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Tumour-positive samples	1·15	1·68	2·33	0·91	1·24	1·64	4·00	1·03	2·80	1·87	1·92	1·41	1·14	1·20	1·70	1·64	5·61
		1·86	1·39	2·76	2·00	1·35	1·28	0·98	1·54			1·25	1·38	1·26	1·21	1·57	
				2·76	1·07	1·18	2·61		1·50				1·06				
						1·61	3·08										
						1·33											
Tumour-negative samples	3·98	1·00	0·57	1·33	1·53	1·80	0·76	1·28	0·67	1·67	0·11	1·81	0·64	1·20	0·27	0·89	0·95
	1·29	1·81	0·44	1·95	1·38	0·98	0·89	1·09	1·25	1·00	0·44	2·22	1·00	1·67	0·28	1·28	0·74
	1·24	1·41	0·47	0·65	0·97	1·50	0·83	0·91	1·17	1·50	0·82	2·50	1·78	0·75	0·56	1·33	0·31
	1·94	1·99	1·14	0·68	1·19	0·97	1·08	1·87	1·33	0·90	1·37	1·63	1·51	1·43	0·79	1·59	0·45
	1·86	0·61	0·48	0·95	1·00	0·80	0·81	1·38	1·00	0·93	1·05	1·20	0·99	2·25	0·65	1·39	
	1·00	2·00	0·48	0·85	1·14	0·66	1·41	1·25	1·00	1·40	2·00	1·61	0·75	1·80	0·49	0·76	
	1·31	2·35	0·41	0·96	0·85	0·60	2·00	0·67	0·63	1·14	1·37	1·40	0·77	1·11	0·39		
	1·55	1·07	0·63	0·84	1·12	0·50	0·70	1·03	0·56	1·19	0·83	1·60	0·80	1·80			
	0·79	1·09	1·31	2·52	0·50	1·33	0·72	1·16	0·56	1·15	1·00	1·56		1·00			
	0·71	1·18	0·85	0·35	0·80	1·00	0·76	1·09	0·50	0·89	0·77	1·75					
	1·40	0·74	0·60		1·18	0·67	0·64	0·88	0·60	1·00	1·33	1·80					
			0·54		1·00	0·75	0·86	1·64		1·00		1·60					
			0·62		1·09	0·57	0·80			0·75		1·00					
			0·71		1·06	0·61	0·80					2·73					
			0·80		1·44	0·60	0·66					2·30					
							0·64										

Patient no.	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
Tumour-positive sample	2·38	1·29	1·40	1·72	1·70	2·42	2·00	1·27	0·95	1·23	0·99	2·84	1·11	0·43	0·71	0·97	1·46
	3·15			2·29		3·36	2·90	0·60	1·23	1·08	1·09		0·84	1·08		1·19	1·26
	1·17						1·50			1·67				0·96		1·18	
										1·69				1·23			
										1·74							
										1·40							
Tumour-negative samples	2·69	0·44	0·80	1·13	1·74	0·85	1·54	1·53	0·02	1·10	0·13	1·16	0·53	0·21	0·92	0·35	0·69
	0·78	0·80	1·32	1·20	1·29	1·17	2·04	0·61	0·25	1·25	0·22		0·60	0·51		0·54	0·96
	1·36	0·92	1·07	1·60	0·93	0·74	1·63	0·53	0·52		0·65			0·56		0·57	0·59
	1·02	0·97	1·09	1·14	1·23	1·13	0·74	0·42			0·24						0·58
	2·00	0·26	1·57	0·78	1·05	1·07		0·44									
	0·59	0·76	1·25	1·24	1·07	1·67		0·64									
	0·97	0·48	0·72	0·89	0·75	1·11		1·00									
	1·18	0·62	0·67		0·68	1·04		1·00									
		0·78	0·37		0·98			1·00									
			0·73														

Table 3. LDH_4/LDH_2 *isoenzyme ratios of samples from* 3 *patients with lung metastases (Group* 2)*

Patient no.	1	2	3
Tumour-positive samples	0·90	1·25	8·70
	2·63		
	1·15		
	1·26		
Tumour-negative samples	1·33	0·25	0·74
	1·23	0·35	0·68
	0·76		0·69
	0·83		0·40
	0·72		0·49
	0·94		0·40
	1·00		
	0·99		
	0·63		
	0·52		
	1·00		
	0·58		

*Primary tumours: 1. Larynx; 2. Mamma; 3. Adenocarcinoma of unknown origin.

types represented in the homogenates is shown in Table 4. It appears that lymphocytes and erythrocytes, which by far are the dominating cellular contaminants of the tissues investigated, had very low isoenzyme ratios while polymorphonuclear leucocytes showed a mean ratio intermediate to tumour-negative and tumour-positive lung tissue. The tumour-negative specimens from lungs with distant metastases showed mean values considerably below those of the corresponding tissue in bronchogenic carcinoma.

DISCUSSION

The literature on lactate dehydrogenase isoenzymes in bronchogenic carcinoma is scarce and the material presented is very limited as compared to the quantitatively much more impressive material of gastro-intestinal malignancies. However, the findings of Goldman and co-workers [15], by driect determination of *M* and *H* LDH, indicated the same trend for bronchogenic carcinoma as shown in tumours of the gastro-intestinal tract. However, Goldman and co-workers [15] in their studies of 7 lung carcinomas found the same trend as found in tumours of the gastro-intestinal tract, i.e. a rise in the *M/H* ratio. An attempt to elucidate the physiological significance of this finding was made by McBeth and Bekesi [29] who found an increased rate of anaerobic glycolysis in lung tumours and many other human tumours.

The main difficulty in evaluating the present results as well as those of other authors is the unobtainability of a fresh control material from healthy persons. In studies of the LDH-isoenzymes in the tumour bearing colon the author observed that high isoenzyme ratios may be found in regions remote to the tumour without any morphological changes. These observations could, to some extent, be correlated with the anamnestic and clinical course of the malignant disease. It was concluded that the mean isoenzyme ratio of the non affected tissue in a tumour bearing organ may be expected to be well above the physiological level [26].

In the present study, the mean isoenzyme ratio of tumour-negative tissue from lungs with

Table 4. *Mean ratio of tissues and cells represented in the tissue homogenates*

Tumour-negative bronchial tissue in lung with distant metastasis	0·73
Tumour-negative lung parenchyma in bronchogenic carcinoma	0·82
Tumour-negative bronchial tissue in bronchogenic carcinoma	1·15
Tumour-positive tissue in bronchogenic carcinoma	1·62
Erythrocytes	0·10
*Lymphocytes	0·26
*Polymorphonuclear leucocytes	1·28

*The ratio has been calculated on the basis of the results published by Rabinowitz and Dietz [30].

bronchogenic carcinoma was shown to be appreciably higher than the mean ratio found in control-tissue derived from lungs containing distant metastases. This difference of the isoenzyme ratios indicated the existence of a basic dissimilarity in an organ with a primary malignancy as opposed to a metastatic tumour.

Goldman and co-workers pointed out [15] that metastases from the same primary tumour showed a uniform isoenzyme structure irrespective of their localisation and they arrived at the conclusion that no intermingling of metastatic tissue and that of the locality had taken place.

Thus, the pulmonary metastasis might be considered a circumscript tumour graft in an otherwise normal environment, while the bronchogenic carcinoma may be considered a local manifestation of a pathological process in which a large section of the lung or perhaps the entire lung is involved. A comparison of the tumour-negative samples from lungs with primary and secondary tumours is, therefore, of great interest.

It appears from Fig. 2 that neither the tumour-negative nor the tumour-positive material of group 1 was normally distributed. Both curves showed a rise much steeper than the subsequent fall.

The tumour-negative specimens from the individual patients with bronchogenic carcinoma showed a pronounced variance of the isoenzyme ratio in different parts of the bronchial tree. Comparing the variance of the individual cases statistically significant differences were found by Bartletts test ($P<0\cdot001$) which indicates that this group of patients is inhomogenous. A trend towards high individual mean isoenzyme ratios accompanied by high variance and low mean ratios accompanied by a low variance was found.

No statistically significant difference of the individual variances of the isoenzyme ratios in tumour-negative tissue was found among the patients with lung metastases ($P>0\cdot05$). Therefore in contrast to the patients with bronchogenic carcinoma, these patients may belong to one group.

These conclusions might have been expected on the basis of the histopathological findings in patients with bronchogenic carcinomas.

In recent years several reports on pulmonary pathology in bronchogenic carcinoma have shown that widespread pre-neoplastic changes, intra-epithelial carcinomas and micro-invasive carcinomas are frequent findings along with the tumour clinically recognized (34–39). The changes are found with equal frequency in both lungs as an indication of the generalized tumour proneness of the organ.

The lung which is the seat of distant metastases does not exhibit the changes otherwise accompanying primary pulmonar malignancy. Consequently a low variance of the isoenzyme ratio in the individual patient and among the patients as well as a low mean ratio would be expected in this group. Although the present material of patients with pulmonary metastases does show this trend, the limited number of patients does not permit statistically safe conclusions to be drawn.

As an explanation of the great variability of the isoenzyme ratio observed the various non malignant cellular elements infiltrating the tissue samples have to be considered.

The dominant cellular contaminants are erythrocytes and lymphocytes. Both of these have isoenzyme ratios below that of normal lung tissue [30] and may, therefore, tend to lower the resultant ratio of the homogenate. This may partly explain why the isoenzyme ratio of lung parenchyma being rich in erythrocyte content, is lower than that of bronchial tissue.

The presence of polymorphonuclear leucocytes might influence the isoenzyme ratio in a different way. On the basis of the figures reported by Rabinowitz and Dietz [30] the LDH_4/LDH_2 ratio in human leucocytes was calculated to be 1·28. This value is below the ratios found in 54·6% of the present tumour-positive material shown in Fig. 2A, and above the ratios found in 74·3% of the tumour-negative material shown in Fig. 2B. Thus, assuming that the cellular contaminants are randomly and equally represented in the tumour-positive and the tumour-negative material the polymorphonuclear leucocytes would tend to decrease the difference which was found to exist between the mean isoenzyme ratios of the two materials, while lymphocytes and erythrocytes would have no such influence. Furthermore, it is clear that leucocyte infiltration cannot explain the differences observed between the ratios in tumour-negative samples from lungs with bronchogenic carcinoma and the ratios in lungs with distant metastases.

In spite of the limitations of the control material the present observations have shown that the presence of tumour tissue can be excluded with great confidence at LDH isoenzyme ratios below 0·9. Furthermore, they indicate that not only the malignant tumour, but also large parts of the histologically non-malignant lung tissue have undergone isoenzymatic and metabolic changes in cases of

bronchogenic carcinoma. This may be interpreted as a pre-neoplastic environmental change contributing to the formation of a tumour bed, where cells may subsequently manifest themselves as malignant tumour cells. The formation of a malignant environment at the subsequent site of a primary tumour might offer some explanation of the different growth patterns of primary and secondary neoplasms. While the primary neoplasm is characterized by the direct continuous infiltrative growth, merging into the surrounding structures, the secondary tumours tend to grow expansively as if depending on an internal optimal environment which is not present in the tissue surrounding the metastatic graft.

This would be in accordance with the concept of the broad field of origin of tumours [31]. The observations on the LDH-isoenzymatic changes in the tumour bearing colon are also in agreement with such a concept which, furthermore, is sustained by the clinically experienced tendency towards multiplicity of colonic tumours [32–33]. The reason why the bronchogenic carcinoma does not present itself clinically as multicentric may be found in the bad prognosis following the stage of the clinical recognition of the initial tumour.

The present findings of isoenzymatic changes in morphologically normal lung tissue indicate that a widespread metabolic change is an early step in advancing carcinogenesis, the development of tumour being one possible end point.

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RESUME

Les isozymes de la déshydrogénase lactique sont étudiés, par la méthode d'électrophorèse à disques, dans les homogénats de tumeurs pulmonaires.

L'augmentation des isozymes migrant vers la cathode, caractéristique du tissu malin, est confirmée.

Il est démontré que la synthèse des isozymes, dans le tissu pulmonaire tumoral, peut être modifiée sans accompagnement de modifications morphologiques. On en conclut que le changement métabolique ne s'accompagne pas nécessairement d'un changement morphologique de la tumeur.

Il est suggéré que la tumeur se développe au sein d'un tissu où une modification métabolique s'est déjà établie. Il apparaît donc que cette modification de synthèse enzymatique pourrait être le signe précurseur d'une transformation prénéoplasique, l'apparition de la tumeur en étant le signe ultime.

SUMMARY

The lactic acid dehydrogenase isoenzyme pattern of the tumour-bearing lung has been studied in homogenates by disc-electrophoretic separation.

The cathodic shift of the isoenzyme pattern characteristic for malignant tumour tissue has been confirmed.

It has been shown that the enzyme synthesis may be altered in the absence of morphological changes. Thus, the metabolic change is not specifically tied to the parenchymatous structure, i.e. the morphologically recognizable structures of the tumour.

It is suggested that the tumour develops within a field of tissue where a metabolic modification has already been established. It appears, therefore, that the alteration of the enzyme synthesis might be symptomatic of an established pre-neoplastic condition, the appearance of the tumour being the ultimate symptom.

ZUSAMMENFASSUNG

Das Milchsäuredehydrogenase-Isoenzym-Muster von tumorbefallenem Lungengewebe wurde mittels Elektrophorese von Homogenaten untersucht.

Die für malignes Tumorgewebe charakteristische Verschiebung des Isoenzymmusters in Richtung Kathode wurde bestätigt.

Es erwies sich, dass die Enzymsynthese gestört sein kann, auch wenn keine morphologischen Veränderungen vorliegen. Die Stoffwechselstörung ist also nicht spezifisch von

der Parenchymstruktur, d.h. von der morphologisch erkennbaren Struktur der Tumoren abhängig.

Es wird die Annahme zur Diskussion gestellt, dass Tumoren in einem Gewebebereich mit bereits abnormem Stoffwechsel entstehen. Trifft dies zu, so kann die gestörte Enzymsynthese als Hinweis auf einen präneoplastischen Zustand gelten, dessen letzte Manifestation das Auftreten des Tumors wäre.

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Mobilization of Plasma Free Fatty Acids During the Growth of Various Experimental Tumors

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THE DEVELOPMENT of a tumor is generally accompanied by a debilitation of the general condition of the host. Several reasons may be responsible for this effect including: (a) increased requirement of energy induced by the growth of the tumor itself, (b) elimination of toxic factors by the tumor or, (c) establishment of metastases in vital organs.

Mueller and Watkins [1] observed that some patients with neoplastic diseases show a concentration of plasma free fatty acids (FFA) higher than healthy subjects. Also in experimental animals the growth of certain tumors induces depletion of fat stores and debilitation.

It is well known that plasma FFA play an important role as a source of energy [2]. Therefore it may be possible that a continuous mobilization of FFA may lead to an exhaustion of the adipose tissue with the consequent observed debilitation.

The aim of the present work was to study how the growth of transplantable tumors affects the concentration of plasma FFA in their hosts.

MATERIAL AND METHODS

Male Sprague-Dawley rats weighing 140 ± 10 g and female Swiss mice, weighing 20 ± 2 g, both from ALAL breeding were used throughout all the experiments.

Rats were kept singly in makrolon plastic cages, while mice were housed in the number of 6 per cage.

Room temperature was 22°C and relative humidity 60%. Food and water were available *ad libitum*.

The following strains of solid tumors were transplanted in rats: Walker carcinosarcoma 256, Flexner–Jobling carcinoma, uterine epithelioma T_8 Guérin. Yoshida H 130 in rats, Ehrlich carcinoma and sarcoma 180 in mice were transplanted in the ascite form.

Solid tumors were transplanted by inoculating subcutaneously about 100 mg of a 15-day-old tumor. Ascites tumors were injected intraperitoneally in the volume of 0·2 ml/rat of an ascites fluid diluted to contain 5×10^6 tumor cells/ml. Plasma FFA concentration was determined according to Trout *et al.* [3] with minor modifications.

RESULTS

Table 1 reports the values of plasma FFA in rats bearing the solid tumors, namely Walker, Flexner–Hobling and Guérin T_8.

It is evident that responses were different according to the various strains of tumor: the plasma FFA concentrations in rats bearing Walker carcinosarcoma or T_8 epithelioma increased as the tumor grew while no changes

Table 1. Plasma concentration of FFA in rats with solid tumors

Tumor strain	Experimental condition	Plasma FFA μeq/l ± S.E. after tumor transplantation (days)				
		7	14	21	29	36
Controls	fed	284±13		269±39		239±14
	fasted*	650±17				
Walker	fed	297±30	477±57†	414±34†		
	fasted*	714±19	471±54†	313±46†		
T_8 Guerin	fed		296±62	349±27†		
	fasted*		631±38	464±41†		
Flexner–Jobling	fed		259±24	320±22	245±30	245±12
	fasted*		595±24	705±30	610±12	615±31

The weight of Walker carcinosarcoma at the 21st day after the transplantation was 43±3 g that of T_8 Guérin carcinoma was 42±9 g that of Flexner–Jobling carcinoma was 42·4±9·0 at the 36th day after transplantation.
*Overnight fasting. All animals were sacrified between 9·30 and 10·30 a.m.
Each figure is the average of at least 5 rats.
†$P<0·01$ in respect to controls.

were induced by the growth of Flexner–Jobling carcinoma.

Another difference is present when animals were submitted to overnight fasting, a condition which increased the level of plasma FFA. Twenty-one days after the transplantation of Walker tumor the plasma FFA level was constantly lower in fasted animals compared with those of fed animals. A similar picture was observed in mice with Ehrlich or S 180 ascites and in rats with Yoshida H 130 ascites (see Table 2). The levels of plasma FFA in fed resting animals increased with the enlargement of the ascites, and as the ascites became severe the capacity to mobilize lipids during fasting was impaired.

Rats bearing Flexner–Jobling carcinoma maintained, on the contrary, the capacity to mobilize plasma FFA during fasting. Further experiments were carried out in order to establish the food intake in normal and tumor bearing rats. Figure 1 shows that the food intake was strongly decreased in rats bearing Walker tumor, the extent of the decrease was less in rats with T_8 tumor, while the presence of Flexner–Jobling tumor did not affect the consumption of food.

In order to establish whether the decreased food intake was responsible for the increase of plasma FFA and the subsequent failure to mobilize FFA during fasting, in one experiment controls and other tumor bearing animals received the food intake of the animals bearing Walker carcinosarcoma. The results obtained 21 days after tumor transplantation are shown in Table 3.

The increase in body weight was similar in all groups with the exception of the Walker

Table 2. Plasma FFA concentration in rats and mice bearing ascites tumors

Tumor strain	Experimental condition	Animal species	Plasma FFA μeq/l ± S.E. after tumor transplantation (days)		
			7	10	14
Controls	fed	mice	238±18		
	fasted*	mice	1019±78		
Ehrlich	fed	mice			749±81†
	fasted*	mice			326±5†
S 180	fed	mice	405±63†	753±64†	
	fasted*	mice	926±53	786±47†	
Controls	fed	rat	142±44		
	fasted*	rat	581±62		
Yoshida H 130	fed	rat	289±60†	542±22†	
	fasted*	rat		523±45	

Each figure is the average of at least 5 animals.
*Overnight fasting
†$P<0·01$ in respect to controls.

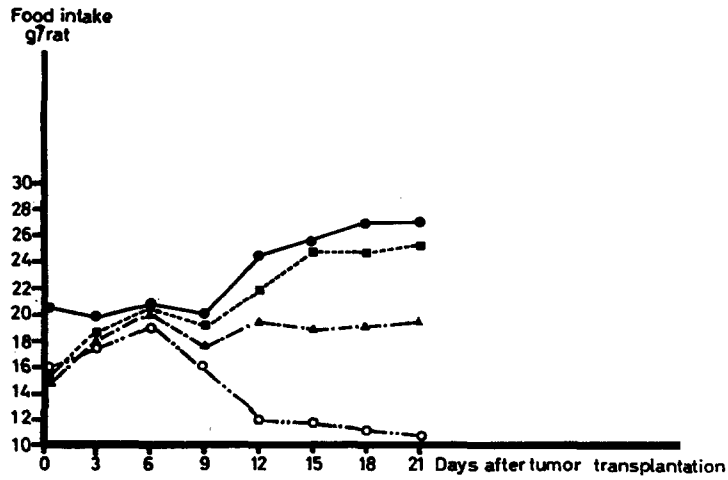


Fig. 1. *Daily food intake of rats bearing Walker tumor.*

●———— *Controls*
○—..— *Walker*
▲·—·—·— *T_8 Guérin*
■ - - - - *Flexner–Jobling*

rats. The tumor weight was higher for the Guérin followed by Walker and Flexner–Jobling. The latter tumor was apparently sensitive to the restriction of food intake.

The mobilization of FFA during fasting was present only in controls and rats with Flexner–Jobling tumor. In the case of Walker tumor, there was a decrease in FFA during fasting and a high death rate.

The data on the mobilization were explained by the decrease in weight of the epididymal adipose tissue. In fact, in the case of Walker bearing rats, the weight of the adipose tissue was less than 10% compared to controls. Also the content of triglycerides was reduced in these animals on a weight basis.

The levels of FFA in the adipose tissue of Walker-bearing rats before fasting was always very high and comparable to the level found for the other groups in the fasting conditions.

DISCUSSION

A number of experimental tumors have been transplanted in rats and in mice, and the level of plasma FFA has been followed during the growth of the tumor. All the solid and ascitic tumors tested induced an increase in the basal concentration of plasma FFA except for the

Table 3. *Effect of food restriction on plasma FFA concentration and adipose tissue weight of normal and tumor bearing rats*

Tumor strain	Experimental condition	% body weight change	Tumor weight (g±S.E.	Plasma FFA (μeq/l)	Epididymal adipose tissue		
					(g/rat)	FFA μeq/g	Triglycerides (g/100 g)
Controls	fed	+109	—	174±11	1·24	2·0	52
	fasted	+97	—	629±42	1·12	7·4	53
Walker	fed	+31	42±4	331±27‡	0·09	11·7	37
	fasted*	+18	35±4	121±34‡	0·06	11·8	24
T_8 Guerin	fed	+108	65±12	246±22‡	0·54	2·6	53
	fasted†	+86	60±5	334±48‡	0·38	12·9	45
Flexner Jobling	fed	+110	12±5	276±41‡	0·98	2·2	54
	fasted	+93	9±3	578±52	0·87	7·7	49

Control rats and rats bearing T_8 Guérin epithelioma and Flexner–Jobling carcinoma had daily the food intake of the Walker carcinosarcoma bearing rats.
Each figure is the average of at least 5 rats. Tumors were transplanted 21 days before the experiment.
4/10* and 1/10† of the animals died during the overnight fasting.
‡$P<0·01$ in respect to controls.

Flexner–Jobling carcinoma, which reached a considerable weight, without affecting the plasma FFA concentration.

The increase of plasma FFA induced by overnight fasting decreased progressively with the growth of all tumors tested, with the exception of the Flexner–Jobling carcinoma.

In the case of Walker carcinosarcoma and Ehrlich carcinoma there was a decrease of the plasma FFA level as a consequence of the fasting condition. In relation with this effect there was a high percentage of mortality. Since it is well known that the growth of Walker carcinosarcoma is accompanied by a decrease in food intake [4], the Flexner–Jobling and T_8 tumors were implanted in animals maintained on the same food intake as the Walker-bearing animals.

In this condition, the growth of tumor was accompanied by a complete loss of epididymal adipose tissue only in the Walker tumor. T_8 responded in the same manner but to a much lower degree, particularly considering that the weight of the T_8 tumor was higher than that of the Walker. It may still be possible that the absorption of food could be impaired in Walker bearing rats in respect to the other experimental groups. Therefore, the simple reduction of food intake does not seem essential for the interpretation of this effect. These data can be better explained assuming that the Walker tumor requires more FFA for its growth, and because of the concomitant hypoglycemia [5].

Concerning this matter, it should be recalled that cancer cells can utilize fatty acids [6].

It may also be possible that the Walker tumor releases in the plasma a factor able to stimulate mobilization of FFA from adipose tissue.

This suggestion remains to be proved, but it is interesting to recall that the capacity to produce ACTH [7], a polypeptide with strong lipomobilizing activity, had been showed for several human tumors of nonendocrine origin [8].

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RESUME

Plusieurs tumeurs de la souris et du rat, et notamment le carcinome d'Ehrlich, le sarcome 180, *la tumeur H*130 *de Yoshida, le carcinome de Walker, l'épithélioma utérin* T_8 *de Guérin et le carcinome de Flexner–Jobling, produisent une augmentation du niveau de FFA dans le plasma des animaux qui en sont porteurs.*

La mobilisation de FFA, provoquée par le carcino-sarcome de Walker, réduit considérablement les réserves de triglycérides dans le tissu adipeux.

Cet effet n'est pas dû à la réduction d'alimentation observée chez les animaux porteurs du carcino-sarcome de Walker. On pense que le carcinosarcome de Walker produit des facteurs capables de mobiliser les lipides du tissu adipeux.

SUMMARY

Several tumors of mice and rats including Ehrlich-carcinoma, Sarcoma 180, *Yoshida H* 130, *Walker carcinosarcoma,* T_8 *uterine epithelioma, and Flexner–Jobling carcinoma, induced an increase in plasma FFA.*

Walker carcinosarcoma induced a strong mobilization of FFA which depleted the stores of triglycerides in the adipose tissue.

This effect was not due to the reduction of food intake shown by Walker-bearing animals. It is suggested that Walker tumor was producing factors able to mobilize lipids from adipose tissue.

ZUSAMMENFASSUNG

Die Implantation von verschiedenen Mäuse- und Rattentumoren (z.B. Ehrlich-Karzinom, Sarkom 180, *Yoshida-Tumor H* 130, *Walker-Karzinosarkom, Uterus-Epitheliom* T_8 *und Flexner–Jobling Karzinom) führte zu einer Erhöhung der frein Fettsäuren (FFA) im Plasma.*

Das Walker Karzinosarkom erzeugt eine starke Mobilisierung von FFA, wobei eine Entleerung der Triglyzeridlager im Fettgwebe erfolgt.

Diese Wirkung hängt außerdem mit der verminderten Nahrungszufuhr zusammen, die bei Ratten mit Walkertumor beobachtet werden kann.

Man nimmt an, daß Walkertumoren einige Faktoren ausscheiden, die Lipoide aus dem Fettgewebe mobilisieren können.

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Absence de Propriété Sarcomogène chez le Dibenzo [*a*, *c*] Anthracène; Nette Activité de son Dérivé 10-Méthylé

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INTRODUCTION

Le rôle capital joué par le dibenzo [*a*, *c*] anthracène (I), dans l'élaboration des idées actuelles sur les processus de la cancérisation par les hydrocarbures, contraste quelque peu avec l'incertitude qu'on éprouve encore à l'endroit de deux points essentiels:

(1) le dibenzo [*a*, *c*] anthracène est-il totalement dépourvu de propriétés cancérogènes, ou faudrait-il plutôt le classer parmi les cancérogènes à très faible activité?

(2) existe-t-il, parmi les nombreux homologues possibles de ce corps, des substances qui seraient, elles, nettement cancérogènes, malgré l'absence d'une zone K dans leur molécule?

Le présent travail, destiné à fournir les éléments d'une réponse à ces deux questions, a consisté dans la recherche d'une activité sarcomogène éventuelle chez le dibenzo [*a*, *c*] anthracène d'une part, et chez un de ses homologues connus, le 10-méthyldibenzo [*a*, *c*] anthracène (II).

MATERIEL ET METHODES

Les *animaux* utilisés étaient des souris de notre lignée habituelle XVII *nc*/$\mathcal{Z}$ de l'Institut du Radium, dans laquelle nous n'avons jamais constaté l'apparition spontanée de sarcome sous-cutané, au sein d'une population de plus de 70.000 animaux autopsiés.

Ces animaux, des deux sexes, étaient âgés de 4 ou 5 mois au début de l'expérience. Ils reçurent, selon notre technique de routine, dans la région du flanc droit, trois injections sous-cutanées de 0,6 mg du produit à étudier, dissous dans 0,2 ml d'huile d'olive stérile et neutralisée, à intervalles d'un mois entre chacune des injections.

Les deux *hydrocarbures* avaient été synthétisés dans notre laboratoire de chimie, par des méthodes univoques, afin d'éliminer tout doute sur leur pureté; les produits obtenus furent purifiés par chromatographie [1].

(a) avec le dibenzo [*a*, *c*] anthracène lui-même, deux expériences indépendantes, faites à 18 mois d'intervalle, portèrent: la première sur 32 mâles et 26 femelles, la seconde sur 47 mâles et 32 femelles.

(b) avec le 10-méthyldibenzo [*a*, *c*] anthracène, deux expériences indépendantes furent également effectuées; la première intéressait 28 mâles et 28 femelles, la seconde 27 mâles et 26 femelles.

RESULTATS

(a) Cinquante pour cent des souris appartenant à la première expérience effectuée avec le dibenzo [*a*, *c*] anthracène moururent entre les 320e et 592e jours d'expérience; chez aucune d'elles ne s'était manifesté le développement d'une tumeur. Les survivants (2 mâles et 2 femelles) furent sacrifiés au 602e jour de l'expérience; aucun cas de tumeur n'a non plus été décelé parmi eux.

Même résultat totalement négatif avec la seconde expérience. La survie des animaux s'était échelonnée de 292 à 566 jours; les survivants (32 mâles et 22 femelles) furent sacrifiés, indemnes de tumeur, au 572e jour de l'expérience.

(b) En ce qui concerne les deux expériences avec le 10-méthyl-dibenzo [*a*, *c*] anthracène, tableau 1 en donne les résultats.

De ces résultats, il ressort que, si le dibenzo [*a*, *c*] anthracène (I) peut être considéré comme totalement dépourvu de propriétés sarcomogènes, son homologue méthylé (II) possède, à ce point de vue, une activité certaine et reproductible. Les mâles se sont montrés nettement plus susceptibles que les souris femelles à cette action cancérogène (7 cas sur 55 d'une part contre 1 sur 54 de l'autre), avec un temps moyen de latence égal à 250 jours. Si l'on considère l'ensemble des animaux des deux expériences, l'index de Iball atteint environ 5. Cette même différence importante entre les sexes avait été signalée à propos d'autres hydrocarbures polycycliques [2–5].

Tableau 1

Expérience I

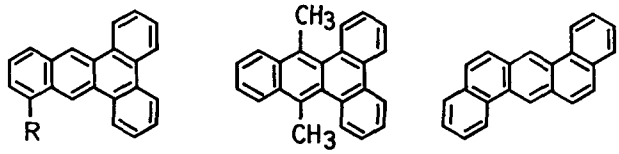
Souris mâles		*Souris femelles*	
Survie en jours	Latence en jours à l'apparition des sarcomes	Survie en jours	Latence en jours à l'apparition des sarcomes
143		167	
185			
192		192	
199		199	
217	190	213	
230		232	
245	228	246	210
275	256	310	
346	316	340	
359			
370			
522		502	
540		555	
570		572	
676	sacrifice des 14 animaux survivants et indemnes de cancer	676	sacrifice des 17 animaux survivants et indemnes de cancer

Expérience II

Souris mâles		*Souris femelles*	
Survie en jours	Latence en jours à l'apparition des sarcomes	Survie en jours	
176			
196		192	
196			
213		202	
245	195	230	
249		236	
262	240	246	
270			
289			
299			
360	326	390	
422			
462		462	
502		465	
515		515	
606	Sacrifice des 11 animaux survivants et indemnes de cancer	606	Sacrifice des 16 animaux survivants et indemnes de cancer

DISCUSSION

(1) Kennaway, après qu'il eut démontré que la fraction cancérogène des goudrons est constituée par un ou plusieurs composés du groupe des hydrocarbures cycliques, essaya, dès 1930, avec Hieger, un certain nombre d'hydrocarbures dont ils disposaient alors, dont le dibenzo [*a*, *c*] anthracène. Appliqué sur la peau de souris, ce corps dissous dans le benzène fut considéré comme inactif, [6] mais la courte durée de cette expérience (133 jours) ne permettait pas qu'on en tire des conclusions certaines. Deux ans plus tard, Cook publia des résultats d'une expérience de longue durée (plus de 700 jours), au cours de laquelle quelques épithéliomas de la peau furent observés [7]. Finalement, à la suite d'une troisième expérience, les auteurs précités et leurs collaborateurs conclurent à l'absence de pouvoir cancérogène du dibenzo [*a*, *c*] anthracène, [8] ce qui fut confirmé par ORR [9]. Mais aucun des expérimentateurs précédents n'avait recherché l'activité du corps administré en injection sous-cutanée. Cette dernière technique fut utilisée par Berenblum [10] pour l'examen d'un homologue supérieur, le 9, 14-diméthyldibenzo [*a*, *c*] anthracène (III). Bien que cet hydrocarbure soit de structure très voisine de celle du 7, 12-diméthyldibenzo [*a*] anthracène (un des plus puissants cancérogènes existants), sa totale inactivité, tant par badigeonnage que par injection, fut avérée.



I ; R=H
II ; R=CH_3

III

IV

A la suite des expériences de ces divers auteurs, et bien que des cancers eussent été obtenus par Cook, il avait été généralement admis que le dibenzo [*a*, *c*] anthracène et ses dérivés sont inactifs. Cela cadrait parfaitement avec la théorie électronique de la cancérisation chimique, puisqu'il n'existe pas, chez ces composés, de zone méso-phénanthrénique, dite zone K de Pullman [11], an niveau de laquelle se réaliserait la réaction déterminant entre l'hydrocarbure et le substrat cellulaire mis en cause dans la cancérisation.

Cependant, Heidelberger et Moldenhauer [12], utilisant la méthode des molécules marquées au C14, constatèrent que le dibenzo [*a*, *c*] anthracène se fixe, sur les protéines de la peau, dans les mêmes proportions que son isomère fortement cancérogène, le dibenzo [*a*, *h*] anthracène. Ils expliquaient le fait par une différence dans les sites de fixation, tant au niveau des protéines qu'à celui de la molécule d'hydrocarbure: la fixation se ferait par la zone K dans le cas du dibenzo [*a*, *h*] anthracène cancérogène (III), par la zone L (méso-anthracénique) dans le cas du dibenzo (*a*, *c*) anthracène non-cancérogène. (D'après ces auteurs, seule la fixation par la zone K conduirait à la cancérisation. Plus récemment, Heidelberger et Davenport ont signalé l'existence d'une "interaction" entre les acides nucléiques de la peau de Souris et le dibenzo [*a*, *h*] anthracène; mais, ici encore, le dibenzo [*a*, *c*] anthracène serait, lui aussi, capable de se fixer sur l'acide désoxyribonucléique.

(2) Nos expériences, dont les résultats ont été rapportés dans ce travail, répondent aux deux questions posées dans l'introduction.

D'une part, en utilisant la méthode par injections, l'absence de propriété cancérogène du dibenzo [*a*, *c*] anthracène a été vérifiée.

D'autre part, on a fait apparaître, à partir de ce composé de base inactif, des propriétés cancérogènes par l'introduction d'un groupement méthyle sur une position appropriée de la molécule comme cela avait déjà été observé dans beaucoup d'autres séries, entre autres dans les séries benzo [a] acridine et benzo [c] acridine. [14]

Il en résulte que, s'il est légitime de considérer que la cancérisation par le 10-méthyldibenzo [*a*, *c*] anthracène est liée à une possibilité qu'a cet hydrocarbure de se fixer au préalable sur des récepteurs cellulaires déterminés, cette fixation doit pouvoir se faire par des sites moléculaires autres que la zone K, comme le laissaient entrevoir nos recherches antérieures avec des hydrocarbures dépourvus de zone K, par exemple certains dérivés du fluoranthène [3].

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RESUME

Par injection sous-cutanées de dibenzo [a, c] *anthracène à des souris, on a confirmé l'absence d'action cancérogène de cet hydrocarbure.*

En revanche, on a obtenu des sarcomes, au lieu d'injection, avec son dérivé le 10-*méthyldibenzo* [a, c] *anthracène. Les souris mâles sont plus sensibles que les femelles. La latence moyenne est de* 250 *jours.*

Pour exercer cette action cancérogène, le 10-*méthyldibenzo* [a, c] *anthracène, qui ne possède pas de région K, doit se fixer sur des récepteurs cellulaires appropriés, par des sites de sa molécule autres que la zone mésophénanthrénique.*

SUMMARY

Subcutaneous injections in mice of dibenz [a, c] *anthracene have confirmed the absence (hitherto determined only by skin-painting) of carcinogenicity in this hydrocarbon.*

On the other hand, sarcomas at the site of injection were obtained with its derivative, 10-*methyldibenz* [a, c] *anthracene. The male mice were more sensitive than the females; the mean latency period was* 250 *days.*

In order to exert this carcinogenic effect, 10-*methyldibenz* [a, c] *anthracene must bind to the appropriate cell receptors by sites of its molecule other than the K-region, which does not exist here.*

ZUSAMMENFASSUNG

Die nichtkanzerogene Wirkung des Dibenz [a, c] *anthrazens für das subkutane Gewebe der Mäuse wird bestätig.*

Sein höherer Homologe, des 10-*Methyldibenz* [a, c] *anthrazen, im Gegenteil, ist sarkomogen. Die Mänchen sind sensibler als die Weibchen. Die mittlere Latenzzeit ist* 250 *Tage.*

Das 10-*Methyldibenz* [a, c] *anthrazen besitzt keine K-Zone in seinem Molekül; es wird angenommen, dass die Bindung des Karzinogens mit den Zellreceptoren von einer anderen Molekülzone vermittelt ist.*

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Résultats d'un Essai d'une Méthylhydrazine dans le Traitement des Cancers Epidermoïdes et Anaplastiques des Bronches

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Organisation Européenne de Recherche sur le Traitement du Cancer (G.E.C.A.)

LE BUT de cet essai était de déterminer si un dérivé de la méthylhydrazine‡ avait une efficacité dans la traitement des cancers épidermoïdes et anaplasiques des bronches.

MATERIEL ET METHODE

(*I*) *Technique de l'essai*

Il s'agit d'un essai simple, de type "screening", sans série témoin. Il n'en répond pas moins à des règles statistiques précises [1].

Les malades, entrant dans l'essai, doivent être dans un état tel que l'évolution ne puisse comporter aucune amélioration spontanée. Pour cela, une "période-témoin" d'observation est nécessaire; elle doit permettre de ne retenir que les affections dont le mode évolutif, en l'absence de traitement spécifique, est soit stationnaire, soit en aggravation. Cette "période-témoin" est de durée variable; elle peut être très réduite si l'on dispose de données cliniques et radiologiques suffisantes pour conclure à une extension évidente du processus tumoral. Elle dépend aussi du traitement antérieur, dans la mesure où il faut être sûr que ce dernier a épuisé ses effets.

Les conditions précédentes étant remplies, on administre la méthylhydrazine; si une amélioration se produit, on peut alors compter un succès à l'actif du produit.

Dans une première étape, on a d'abord cherché à savoir si la méthylhydrazine était efficace ou non, sans préjuger le degré d'efficacité. Il suffit d'une réponse positive pour prouver l'efficacité du produit. On peut donc arrêter l'essai au premier succès. Cependant, si l'efficacité du produit est faible, ce succès peut demander un nombre élevé de malades; il est donc nécessaire de fixer une limite. Le calcul permet de déterminer le nombre de

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Roche d'avoir mis ce prodiut a notre disposition.
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‡L'Ibenzmethysine: 1 méthyl-p.-isopropyl-carbomoyl-benzyl-hydrazine. Nous remercions les Laboratoires Roche d'avoir mis ce produit à notre disposition.

malades nécessaire en fonction du risque de se tromper que l'on choisit. On a retenu le nombre n=15 malades, qui correspond à une probabilité inférieure ou égale à 10 chances sur 100 de laisser échapper un produit donnant au moins 15 succès sur 100 malades.

Dans une seconde étape, nous avons continué l'essai pour préciser le degré d'efficacité de la méthylhydrazine. Finalement, l'essai a porté sur 50 malades, 25 cancers épidermoïdes et 25 cancers anaplasiques.

(II) Choix des malades

(1) *En ce qui concerne la maladie.* (a) Le diagnostic devait être établi par un examen histologique de la tumuer bronchique ou d'éventuelles métastases. L'essai portait sur deux groupes distincts:

— les cancers épidermoïdes, quelle que soit la précision de différenciation,

— les cancers anaplasiques à petites ou à grandes cellules.

(b) On éliminait les cas relevant de la chirurgie ou de la radiothérapie.

(2) *En ce qui concerne les malades.* Les malades inclus dans l'essai devaient pouvoir, a priori, supporter un traitement par la méthylhydrazine durant au minimum 4 à 6 semaines. Etaient donc exclus les malades en très mauvais état général, à un stade terminal ou dont l'examen hématocytologique était anormal du fait d'une anaplasie médullaire.

(III) Définition du traitement

(1) *Méthylhydrazine.* (a) la dose quotidienne fixe de 300 mg/jour était atteinte progressivement en donnant 100 mg, soit 2 gélules, le premier jour, puis en augmentant d'une gélule par jour, jusqu'à atteindre 6 gélules par jour.

(b) la dose totale a varié de 2,1g à 28,4g; elle dépendait des résultats objectifs observés et de la tolérance hématologique de la chimiothérapie.

Si une régression objective était observée, on pouvait conclure à l'efficacité de la chimiothérapie.

S'il n'y avait aucune régression, la chimiothérapie était poursuivie jusqu'à la dose maximum tolérée, soit jusqu'à ce que l'examen hématologique montre un nombre de leucocytes inférieur à 3.000/mm^3 ou un nombre de plaquettes inférieur à 120.000/mm^3.

S'il n'y avait aucune amélioration objective alors que la chimiothérapie restait bien tolérée au point de vue hématologique, il semblait inutile de poursuivre ce traitement plus de 6 semaines; le produit était alors jugé inefficace après ce temps d'observation.

(2) *Traitements associés.* On ne prescrivait aucun autre traitement spécifique que la méthylhydrazine. La corticothérapie était évitée autant que possible; si sa prescription s'imposait, la raison en était notée dans l'observation.

(IV) Critères de surveillance

(1) *En ce qui concerne l'efficacité du traitement.* On se basait sur les critères les plus objectifs possible, jugés à la fin de la "période-témoin" et à la fin de la chimiothérapie. Ce sont: (a) l'évolution de la lésion bronchique appréciée par un bilan radiologique et bronchoscopique, (b) la recherche des métastases.

(2) *En ce qui concerne la tolérance.* Elle était étudiée, sur le double plan clinique et hématologique, par des examens hebdomadaires. Dans chaque service, un médecin était responsable de la conduite de l'essai. C'est lui qui pratiquait les différents bilans prévus. Il reportait les données recueillies sur une observation codifiée. A la fin de l'essai, tous les dossiers ont été discutés par un groupe de médecins comportant un représentant de chaque service.

RESULTATS

(I) Cancers epidermoides

(A) Dans une première étape, sur une série de 15 malades ayant reçu un traitement à la méthylhydrazine pendant 4–6 semaines, un seul cas fut considéré comme présentant une amélioration objective certaine (voir le résumé de l'observation en annexe n° 1). Selon les règles mêmes de l'essai, énoncées plus haut, on pouvait donc conclure que la méthylhydrazine avait une certaine efficacité, sans toutefois pouvoir fixer le degré de cette efficacité.

(B) Dans une seconde étape, on continua l'essai. Au total, 25 cas de cancers épidermoïdes furent traités par la méthylhydrazine. Aucune autre amélioration ne fut observée. La proportion de succès est donc finalement de 1 sur 25.

(II) Cancers anaplasiques

(A) Dans une première étape, sur une série de 16 malades traités, 1 seule rémission objective fut observée (Voir le résumé de l'observation en annexe n° 2). Là encore, on pouvait conclure que la méthylhydrazine avait une certaine efficacité, sans pouvoir préciser davantage.

(B) L'essai fut donc continué jusqu'à un total de 25 malades. Une nouvelle rémission objective fut observée (Voir le résumé de l'observation en annexe n° 3). La proportion de succès est finalement de 2 sur 25.

CONCLUSION

On peut conclure que, dans les conditions précises d'expérimentation de cet essai, le dérivé de la méthylhydrazine étudié n'est pas totalement dépourvu d'efficacité dans le traitement des cancers épidermoïdes et anaplasiques des bronches, mais que cette efficacité est faible.

Certes, on peut penser que les résultats seraient différents avec une autre posologie ou un autre mode d'administration (voie intraveineuse). Toutefois il faut noter que la technique retenue pour l'essai est las plus couramment utilisée, en particulier en hématologie ou le produit s'avère efficace.

Ces résultats fixent, de façon assez nette, les limites d'utilisation du produit. Il n'est pas à rejeter dans le traitement des cancers bronchiques primitifs, mais son efficacité est insuffisante pour l'utiliser seul. En revanche, peut envisager de l'associer à d'autres produits.

Enfin, il faut noter que c'est une chimiothérapie relativement bien tolérée puisque certains malades ont pu en prendre pendant 4 mois, sans incidents.

ANNEXE

Observation N°. 1

"Monsieur A... Gustave, 76 ans, est vu pour hémoptysie en septembre 1964. La radiographie montre une opacité para et sus hilaire droite et la bronchoscopie révèle s'agit d'un carcinome épidermoïde nettement différencié et moyennement mature de la bronche intermédiaire. En raison de contre-indications opératoires, on institue un traitement par la méthylhydrazine, du 16 octobre au 16 novembre, à raison de 300 mg par jour. Sur les radiographies faites à l'issue du traitement, il apparaît une régression nettement appréciable de la tumeur, de plus de la moitié de ses dimensions. En octobre 1965, soit un an après le début du traitement, il persiste sur les radiographies une image de condensation para-cardiaque droite avec état stationnaire. On apprend que le malade est décédé le 8 décembre 1965".

Observation N°. 2

"Monsieur G ... Armand est un homme de 72 ans, vu pour une dyspnée progressive et une altération de l'état général. La radiographie thoracique montre une opacité hilaire droite, avec opacité systématisée des lobes supérieur et moyen et attraction de la trachée. La bronchoscopie montre une infiltration sténosante de la bronche lobaire supérieure droite. La biopsie permet une confirmation histologique: "carcinome ayant la structure d'un carcinome anaplasique à petites cellules". Il y avait de plus une métastase cutanée du cuir chevelu, confirmée histologiquement. Le traitement par la méthylhydrazine a été administré du 15.10.64 au 10.02.65, soit pendant 119 jours. La dose totale reçue est de 21,450g. Les résultats sont bons: diminution importante de l'opacité hilaire et reperméabilisation des bronches lobaires supérieure et moyenne. La bronchoscopie montre un orifice lobaire supérieur normal et une infiltration de l'orifice lobaire moyen. L'amélioration se maintient jusqu'en mai 1965. Il apparaît alors une métastase cérébrale et le malade meurt le 25.09.65. Il y a donc eu une régression partielle ayant duré 5 mois".

Observation N°. 3

"Monsieur de N ... Victor, âgé de 65 ans, est vu en mai 1965 pour une opacité hilaire gauche. La bronchoscopie montre une végétation de la bronche lobaire supérieure. Il y a également une adénopathie sus-claviculaire droite dont la biopsie confirme le diagnostic de cancer indifférencié à petites cellules. Le traitement par la méthylhydrazine est donné pendant 120 jours à une dose totale de 28.400g. En juillet 1965, on note: disparition complète de l'adénopathie sus-claviculaire droite et régression de l'opacité hilaire à la radiographie. Cette rémission partielle dure 6 mois. En décembre 1965, il apparaît une nouvelle adénopathie sus-claviculaire droite et une aggravation des images radiologiques; le malade décède le 17 juillet 1966".

RESUME

Un essai simple d'efficacité d'un dérivé d'une méthylhydrazine a été entrepris dans le traitement des cancers épidermoïdes et anaplasiques des bronches ne relevant ni de la chirurgie, ni de la radiothérapie.

On a obtenu une amélioration objective sur 25 cas de cancers épidermoïdes, et deux améliorations objectives sur 25 cas de cancers anaplasiques.

On peut conclure que, dans les conditions précises d'expérimentation de cet essai, le produit étudié n'est pas totalement dépourvu d'efficacité dans le traitement des cancers épidermoïdes et anaplasiques des bronches, mais que son efficacité est faible et apparaît insuffisante pour l'utiliser seul.

SUMMARY

A screening trial of a methylhydrazine derivative was established in a group of patients with epidermoid or anaplastic carcinoma of the lung. The patients were considered not suitable for surgery and radiation therapy.

One objective response among 25 *cases of epidermoid carcinoma and* 2 *objective improvements among* 25 *cases of anaplastic carcinoma were observed.*

It is concluded that, in the strict experimental conditions of this trial, the drug is not completely devoided of therapeutic activity in far-advanced lung cancer, but that its activity is poor and seems unsufficient to recommend its use alone.

ZUSAMMENFASSUNG

In einer einfachen Untersuchung wurde die Wirksamkeit eines Methylhydrazin- Derivats zur Behandlung von epidermoiden und anaplastichen Bronchialkarzinomen geprüft, die weder chirurgisch noch radiotherapeutisch beeinflussbar waren.

Es wurde eine objektive Besserung unter 25 *Fällen von epidermoiden Karzinomen und zwei objektive Besserungen unter* 25 *Fällen von anaplastischen Karzinomen erreicht.*

Aus den Ergebnissen zieht der Autor den Schluss, das Präparat habe sich unter den gegebenen Untersuchungsbedingungen als nicht völlig unwirksam erwiesen, sein Effekt sei iedoch gering und erschiene als unzureichend für die alleinige Anwendung des Medikaments.

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Letter to the Editor

Prolonged Effect of Pentobarbital in Tumor Bearing Rats

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AN INCREASING body of evidence indicates that drug disposal and metabolism are influenced by many physiological or pathological conditions [1, 2]. Very little information is known about the possible influence of tumor growth on drugs metabolism. It is known, however, that neoplastic cells, originating from the liver (hepatomas), have little, if any, capacity to metabolize drugs through microsomal enzymes [3, 4]. It is possible that the presence of a growing tumor may alter the microsomal enzymes of the host liver.

The experiments reported here have been designed to determine whether there is a difference in the metabolism and the pharmacological effects of pentobarbital between tumor bearing rats and normal rats. Sprague-Dawley male rats, weighing about 120 g at the beginning of the experiment, were used. Two tumors transplanted subcutaneously were studied: Walker 256 carcino-sarcoma which, after producing a considerable weight loss, kills the rat host in 20–25 days, and Flexner-Jobling carcinoma, which kills the host in a longer period, 35–40 days [5].

Pentobarbital metabolism was investigated by determining its level in the brain according to a spectrophotometric method, described by Brodie [6]. Pentobarbital activity was estimated, *in vivo*, by measuring the sleeping time from the time of loss of the righting reflex until the reflex was regained. The dose of sodium pentobarbital administered was always 25 mg/kg ip (kindly supplied by Abbott).

Table 1 shows that, after administration of pentobarbital, rats bearing Walker carcino-sarcoma or Flexner-Jobling carcinoma sleep longer than normal rats. This difference was also present when pentobarbital was given i.v. This effect is already present 4 days after transplantation of the tumor, when no visible growth is present.

In normal rats, subcutaneous transplantation of fragments of normal tissues, like liver and lung, did not produce any change in the sleeping time or in the brain levels of pentobarbital.

Biochemical determinations, reported in Table 2, indicate that the decreased effect of pentobarbital in tumor bearing animals may be due to its increased metabolism. In fact, the levels of pentobarbital in the plasma and brain of the tumor bearing animals are significantly higher than are the levels in the plasma and brain of the control rats. The effect of phenobarbital, an inducer of microsomal enzymes [7], or of SKF 525A, an inhibitor of microsomal enzymes [8], responsible for the metabolism of pentobarbital, was still present in tumor bearing animals. Phenobarbital pretreatment (40 mg/kg twice a day for 4 days until 24 hr before the experiment) increased the sleeping time induced by pentobarbital to a similar extent in both normal and tumor bearing rats, SKF 525A (100 mg/kg 1 hr before) decreased this sleeping time.

These data show that the sensitivity to pentobarbital, and possibly to other drugs, is

changed in tumor bearing animals. A change in the rate at which pentobarbital is metabolized by the liver may explain part of this effect, although further studies are required to elucidate the problem.

Other studies from this laboratory suggest that amphetamine hydroxylation may also be reduced in rats bearing an advanced Walker tumor [9]. These results open the possibility of extending similar investigations to other drugs with particular reference to possible extrapolations to cancer patients.

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Table 1. *Values of sleeping time in normal and tumor bearing rats after intraperitoneal administration of sodium pentobarbital* (25 mg/kg ip)

No. rats	Body wt. without tumor (g±S.E.)	Tumor	Days after transplantation	Tumor weight (g±S.E.)	Sleeping rats (%)	Sleeping time (min)	Increase (%)
24	161±6	—	—	—	58	29±5	—
24	165±3	Walker	4	—	87*	29±7	92
8	158±2	Flexner	4	—	87*	68±6	141
8	192±2	—	—	—	75	34±5	—
8	184±2	Walker	8	0·4±0·1	100	57±6	65
8	188±4	Flexner	8	0·6±0·1	87	55±5	60
24	237±9	—	—	—	75	33±5	—
24	194±6	Walker	12	5·9±1·0	83	56±6	68
16	171±9	Flexner	12	4·4±1·2	93	70±5	115
37	238±8	—	—	—	54	31±4	—
38	152±5	Walker	16	18·3±2·1	92	65±8	107
16	170±10	Flexner	16	22·5±3·7	87†	110±8	250
8	286±6	—	—	—	62	36±4	—
8	151±12	Walker	21	37·6±3·0	100*	83±10	129
8	192±5	Flexner	21	40·4±4·6	100	97±5	169
8	241±5	—	—	—	87	16±3	—
6	183±5	Walker	16‡	15·6±2·7	100	80±8	385

*A animal dead during narcosis
†2 animals dead during narcosis.
‡Pentobarbital was given intravenously (25 mg/kg).

Table 2. Plasma and brain levels of pentobarbital in normal and Walker bearing rats

No. rats	Body wt. without tumor (g±S.E.)	Group	Tumor weight	Time after pentobarbital (min)	Pentobarbital	
					plasma (μg/ml+S.E.)	brain (μg/g±S.E.)
5	234±8	normal	—	20	13·5±1·0	16·1±1·1
5	226±4		—	40	9·1±0·6	12·0±1·6
5	248±10		—	60	7·1±0·4	6·6±1·1
5	146±16	Walker 16 day old	15·9±3·8	20	16·0±0·4	16·0±0·8
5	134±8		14·8±2·8	40	*15·6±0·4	*18·8±0·9
5	137±15		17·0±3·5	60	*14·1±0·3	*18·2±2·8

Na pentobarbital: 25 mg/kg ip.
*$P < 0·01$.

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Announcements

Tenth International Cancer Congress

Houston, 22–29 *May* 1970

THE TENTH International Cancer Congress, sponsored by the International Union against Cancer, will be held at Houston, Texas, U.S.A.

The Congress will feature the following events:

Preliminary Special Sessions of the Congress
Congress Lectures
Panel Discussions
Sectional Meetings with proferred papers on a variety of topics or subjects
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Fifth International Congress on Photobiology

The *Fifth International Congress on Photobiology* will be held in 1968 [August 26–31] at Dartmouth College, Hanover, New Hampshire, U.S.A. The Second Announcement of the Congress is now available. It contains detailed information on the Congress and application forms for registration and presentation of papers. The Announcement or further information may be obtained by writing to the Secretariat, Vth International Photobiology Congress, Argonne National Laboratory-202, Argonne, Illinois, U.S.A. 60439.

The present quadrennial meeting, sponsored by the Comité International de Photobiologie, will be under the auspices of the United States National Academy of Sciences, and organized by its Committee on Photobiology. Sections of the Congress will emphasize topics under the categories:

Photochemistry

Photodynamic action

Photosynthesis

Bioluminescence

Photochemistry of macromolecules

Photodermatology including carcinogenesis

Photomorphogenesis

Phototropism and phototaxis

Vision

Rhythms

Instrumentation and action spectra

Repair mechanisms

Interactions of ionizing and nonionizing radiations

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Les 17 Cétostéroïdes Plasmatiques Libres et Totaux dans les Cancers Génitaux

Etude chez les Femmes Atteintes de Cancer Mammaire ou Génital et chez les Hommes Atteints de Cancer Prostatique ou Testiculaire

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L'ETUDE des 17 cétostéroïdes libres dans le plasma ou le sang total de sujets bien portants a été entreprise par de nombreux chercheurs. Migeon et Plager [1], Clayton *et al.* [2], Migeon [3], Ceresa et Cravetto [4], Cohn *et al.* [5], Puche et Nes [6], Oertel [7], Baulieu [8], Deshpande et Bulbrook [9], Gandy [10], Wieland [11] ont relevé, chez les sujets bien portants, des taux indosables, ou de l'ordre de 1 à 3 μg/100 ml. Pour Jayle [12], il n'existe que des traces de 17 cétostéroïdes libres dans le sang humain.

A l'encontre des auteurs précédents, Dubrulle et Clayes [13], Tamm *et al.* [14], Shida *et al.* [15], ont trouvé jusqu'à 75 μg de 17 cétostéroïdes libres pour 100 ml de sang ou de plasma, chez quelques sujets normaux.

Rares sont les cas dans lesquels on a mis en évidence la présence des 17 cétostéroïdes libres dans le sang périphérique de malades. Dans la fièvre périodique, pendant les crises, le sang renferme de l'étiocholanolone libre, comme l'ont montré Kappas *et al.* [16], Bondy *et al.* [17, 18]. Cohn *et al.* [5], et Jacobs [19] trouvent, pendant les accès de fièvre périodique, des quantités importantes d'androstérone, d'étiocholanolone et de déhydroépiandrostérone libres. Tisdale [20] a mis en évidence la présence d'étiocholanolone libre au cours des accès de fièvre dans la cirrhose de Laennec, Cara [21] au cours de l'hyperplasie surrénalienne congénitale et Schenker [22] dans la maladie granulomateuse du foie. Dans le syndrome de féminisation testiculaire, Morris et Mahesh [23], et Kase et Morris [24], ont décelé un taux élevé de déhydroépiandrostérone libre.

Chez 10 femmes, dont 5 atteintes d'un épithélioma mammaire, 4 d'un épithélioma de l'utérus et 1 d'un épithélioma bilatéral de l'ovaire, nous avons trouvé des 17 cétostéroïdes libres dans le sang ovarien prélevé au cours d'interventions de Wertheim [25]. Mahesh *et al.* [26] ont étudié la présence des 17

cétostéroïdes dans le syndrome de Stein-Leventhal. Au cours de ce syndrome, ils ont mis en évidence, dans le sang de la veine ovarienne, l'existence de déhydroépiantrostérone libre (9,4 et 15,4 µg/100 ml) et de la Δ4 androstènedione libre (20,9 et 13,1 µg/100 ml).

Smith *et al.* [27] ont montré, dans le plasma sanguin de la veine spermatique d'un enfant atteint d'une tumeur du testicule, la présence d'androst-4-ène-3, 17-dione (105 µg/100 ml) et de la 11β hydroxy-androst-4-ène-3, 17-dione (29,5 µg/100 ml) libres, tandis qu'ils ne trouvent pas ces stéroïdes libres dans le sang périphérique du même malade.

Nous nous sommes attachés nous-mêmes au problème des 17 cétostéroïdes libres et conjugués dans le plasma, ou parfois dans le sang total périphérique.

Chez des femmes bien portantes, nous n'avons trouvé que rarement des 17 cétostéroïdes libres.

Au contraire, chez des femmes souffrant d'un cancer mammaire ou génital, nous avons observé, avec une fréquence importante, la présence de 17 cétostéroïdes libres plasmatiques: 9 fois sur 9 [25], 21 fois sur 21 [28], 66 fois sur 107 [29].

Nous avons complété ces recherches, et ce sont les résultats globaux obtenus qui font l'objet de la présente note.

Notre étude a porté sur 60 femmes bien portantes et sur 298 malades dont:

Tableau 1. 17 *Cétostéroïdes libres et conjugués chez des femmes témoins âgées de* 15 *à* 60 *ans*

Dosages dans le plasma sanguin			Dosages dans le sang total		
Sujets	17 Cétostéroïdes (µg/100 ml) Libres	Conjugués	Sujets	17 Cétostéroïdes (µg/100 ml) Libres	Conjugués
Du · ·	16	31	CNTS*	14	15
Vi · ·	18	64	,,	34	29
Bi · ·	31	46	,,	Indosables	12
Bo · ·	16	62	,,	,,	38
Cr · ·	18	39	,,	,,	101
Ca · ·	Indosables	57	,,	,,	43
Ta · ·	,,	31	,,	,,	—
Bo · 2	,,	44	,,	,,	—
Do · ·	,,	30	,,	,,	16
Ba · ·	,,	39	,,	,,	53
Re · ·	,,	37	,,	,,	—
Le · ·	,,	98	,,	,,	16
De · 2	,,	51	,,	,,	36
La · ·	,,	97	,,	,,	23
Fr · ·	,,	62	,,	,,	51
Tr · ·	,,	79	Ro · ·	,,	40
CNTS†	,,	106	Re · ·	,,	46
,,	,,	31	Co · ·	,,	46
,,	,,	92	Fa · ·	,,	52
,,	,,	140	An · ·	,,	61
,,	,,	83	Pe · ·	,,	6
,,	,,	93	Yu · ·	,,	59
,,	,,	45		,,	60
,,	,,	56			
,,	,,	38			
,,	,,	59			
,,	,,	42			
La · 2	,,	61			
Ma · ·	,,	53			
Ve · ·	,,	50			
Ha · ·	,,	22			
De · ·	,,	43			
Ch · ·	,,	54			
Ga · ·	,,	84			
Ba · ·	,,	54			
Se · ·	,,	8			
L'H ·	,,	31			
Le · ·	,,	53			

*CNTS = Centre National de Transfusion Sanguine.

155 atteintes de cancer du sein,
48 „ „ „ du col utérin,
4 „ „ „ du vagin,
7 „ „ „ de l'ovaire,
et 84 malades castrées pour l'une ou l'autre de ces affections.

Nous avons examiné, en outre, 32 hommes bien portants, 18 sujets porteurs de cancer de la prostate et 10 castrés pour cancer du testicule.

Le diagnostic de néoplasme a été confirmé par l'examen histologique (Melle Hébert).

La technique que nous avons suivie est celle que nous utilisons de façon habituelle [30]. Elle est inspirée de la méthode de Gardner [31] et Migeon [32], et a été soumise au contrôle du rendement par le procédé de la dilution isotopique [33].

Le plasma est extrait par l'éther, une heure environ après le prélèvement sanguin, pour obtenir les 17 cétostéroïdes libres. Après cette extraction, la phase aqueuse résiduelle est soumise à une hydrolyse acide, pendant dix minutes, à la température d'ébullition. Après refroidissement, une nouvelle extraction par l'éther, permet de doser les 17 cétostéroïdes qui étaient primitivement conjugués. Après l'élimination des oestrogènes par la soude et lavage à l'eau, l'extrait éthéré est évaporé à sec. Le résidu est repris par l'alcool à 70%; la solution alcoolique est lavée avec l'éther de pétrole. La majorité des lipides passe dans l'éther de pétrole alors que les stéroïdes restent dans l'éthanol. La phase éthanolique est additionnée d'un demi-volume d'eau distillée, puis extraite par le chloroforme. L'extrait chloroformique est purifié par un passage sur une colonne de florisil, de façon à éliminer des substances interférantes polaires. L'éluat de la chromatographie, évaporé à sec, est soumis à la réaction de Zimmermann. Pour améliorer la spécificité de cette réaction, on extrait, par le chloroforme, le milieu coloré terminal. Le chloroforme laisse, dans la phase aqueuse, des

Tableau 2. 17 Cétostéroïdes plasmatiques libres et conjugués chez des femmes jeunes atteintes d'un cancer du sein

Sujets	17 Cétostéroïdes (µg/100 ml) Libres	Conjugués	Sujets	17 Cétostéroïdes (µg/100 ml) Libres	Conjugués
Av · ·	24	63	On · ·	Ind.	58
La · ·	6	45	Pa · ·	„	54
Ba · ·	18	72	Na · ·	„	47
Ma · ·	30	52	Kl · ·	„	74
Ma · 2	37	45	La · ·	„	77
Si · ·	42	75	Pa · 2	„	54
Sa · ·	14	27		„	58
Ju · ·	18	77	So · ·	„	98
Mi · ·	11	86	Hé · ·	„	45
Fa · ·	37	48	Be · ·	„	36
Mu · ·	21	50	Gr · ·	„	80
An · ·	49	57	Ba · ·	„	43
La · ·	25	22	Ch · ·	„	78
Bi · ·	43	17	Gr · 2	„	80
He · ·	12	61	Ma · ·	„	42
	16	69	Co · ·	„	52
Le · ·	23	44	Be · 2	„	88
Mo · ·	17	39	Ba · 2	„	52
Le · ·	23	44	Ma · 2	„	43
Bo · ·	22	29	Ma · 3	„	57
Bu · ·	14	70	Ca · ·	„	68
Co · ·	23	72	Ma · 4	„	54
Ma · 3	31	45	Ma · 5	„	63
Co · ·	11	52	Ro · ·	„	45
Ba · 2	34	39	Ma · 6	„	63
Se · ·	22	34		„	52
Ta · ·	26	62	Hi · ·	„	45
			Ga · ·	„	9
			Th · ·	„	38
			St · ·	„	110
			Ob · ·	„	25
			Sa · ·	„	54

impuretés qui, malgré les séparations antérieures, ont pu rester dans l'extrait.

Comme le montre le Tableau 1, sur 60 femmes bien portantes, d'un âge compris entre 15 et 60 ans, nous n'avons mis que 7 fois la présence de 17 cétostéroïdes libres en évidence dans le sang périphérique (5 fois sur 32 dans le plasma) (2 fois sur 22 dans le sang total).

En ce qui concerne les malades atteintes de cancer du sein, nous avons séparé les femmes jeunes (en période d'activité génitale) des femmes âgées (ou ménopausées).

Sur 56 malades jeunes (Tableau 2) les 17 cétostéroïdes plasmatiques libres ont été présents 26 fois, soit dans 46,4% des cas.

Chez 99 malades âgées, atteintes également de cancer du sein (Tableau 3 a et b), les 17 cétostéroïdes libres ont été trouvés présents dans 54 cas, soit dans une proportion de 54,5%.

Chez les malades atteintes de cancer du col utérin (Tableau 4), quelques dosages ont été faits dans le sang total, mais la plupart dans le plasma. Sur 48 malades étudiées, des 17 cétostéroïdes libres ont été observés 33 fois, soit dans 68,7% des cas.

Les résultats, obtenus chez quelques malades souffrant d'un épithélioma de l'ovaire ou du vagin, sont présentés dans les Tableaux 5 et 6.

Des 17 cétostéroïdes libres sont présents dans 6 cas sur 7 (85,7%) dans l'épithélioma de l'ovaire, et dans 3 cas sur 4 (75%) dans l'épithélioma du vagin.

La castration étant fréquemment pratiquée comme traitement du cancer du sein et des cancers génitaux, il nous a été possible de doser les 17 cétostéroïdes plasmatiques libres et conjugués chez des malades privées de leurs ovaires. Les malades castrées chirurgicalement pour cancer du sein (Tableau 7) ont montré des 17 cétostéroïdes plasmatiques libres dans 23 cas sur 35 (65,7%). Sur 7 malades, atteintes de la même affection, et castrées par radiothérapie, des 17 cétostéroïdes plasmatiques libres ont été trouvés dans tous les cas.

En ce qui concerne le cancer du col utérin (Tableau 8), on a pu déceler des 17 cétostéroïdes plasmatiques libres, chez 8 malades sur 20, soit dans 40% des cas.

Chez 6 femmes castrées pour épithélioma du vagin, l'existence de 17 cétostéroïdes plas-

Tableau 3a. 17 Cétostéroïdes plasmatiques libres et conjugués chez des femmes atteintes de cancer du sein, âgées de 47 à 78 ans

	17 Cétostéroïdes libres présents				
Sujets	17 Cétostéroïdes (µg/100 ml)		Sujets	17 Cétostéroïdes (µg/100 ml)	
	Libres	Conjugués		Libres	Conjugués
Bl ··	31	36	Pr ··	14	58
Br ··	29	50	Ja ··	20	29
Av ··	28	54	Ma··	12	40
Co ··	29	46	Po ··	34	48
	27	48	Be ··	28	39
Ch ··	23	52	Do ··	14	45
Ch ·2	38	63	Da ··	14	51
Pi ··	27	43	Mi ··	16	59
As ··	7	45	Pl ··	51	35
Tu ··	30	40	Fa ··	10	68
Du ··	12	35	Pe ··	18	41
Le ··	32	53	Ch ·3	27	61
Al ··	8	40	Ja ··	25	65
La ··	17	40	Ba ··	17	70
Sa ··	20	70	Ta ··	25	52
Co ·2	17	44	Gi ··	18	52
Sc ··	11	61	Ta ·2	10	68
Re ··	11	68	Sa ··	45	66
Da ··	28	47		17	68
Ca ··	28	74	Vo ··	40	45
Au ··	18	27	Bi ··	21	66
Co ·3	19	63	Mo··	17	39
Re ·2	11	61	Re ··	17	23
Du ··	29	45	St ··	65	32
Ca ··	52	70	Di ··	26	51
Ma·2	13	69	Ur ··	30	58
Fa ··	34	36	Av ··	28	45
Ni ··	17	63	De ··	47	56

Tableau 3b. *17 Cétostéroïdes plasmatiques libres et conjugués chez des femmes atteintes de cancer du sein, âgées de 47 à 83 ans*

Sujets	17 Cétostéroïdes libres indosables (µg/100 ml)		Sujets	17 Cétostéroïdes libres indosables (µg/100 ml)	
	Libres	Conjugués		Libres	Conjugués
Ch ·4	Ind.	64	Be ·5	Ind.	65
Pa ··	,,	89	Co ·5	,,	67
		78	Pl ··	,,	48
Be ·2	,,	34	Au ··	,,	45
Ge ··	,,	79	Pi ·2	,,	56
Ma·2	,,	25	Ro ··	,,	54
Be ·3	,,	57	Ca ·3	,,	65
Fi ··	,,	31	La ·2	,,	52
Ba ·3	,,	60	La ·3	,,	60
Ca ·2	,,	36	Ma·3	,,	63
Co ·3	,,	72	La ·4	,,	50
Be ·3	,,	48	Go ··	,,	54
		45	Me··	,,	50
Le ·2	,,	39	Ma·4	,,	34
Co ·4	,,	46	Le ·3	,,	36
Bi ··	,,	35	Gr ··	,,	89
So ··	,,	45	Bo ··	,,	57
Ma·5	,,	45	Ga ··	,,	9
Ma·6	,,	43	D'A ·	,,	40
Pe ··	,,	52	Mi ··	,,	54
La ·3	,,	74	At ··	,,	50
Bo ·2	,,	58	Du ··	,,	32
Be ·6	,,	63	Pr ··	,,	25
Ja ··	,,	52			

matiques libres a été notée 5 fois, soit dans 83,3% des cas (Tableau 9).

Enfin, nous avons trouvé des 17 cétostéroïdes plasmatiques libres 4 fois sur 7 (57%) chez des malades castrées pour épithélioma du corps utérin (Tableau 10), et 6 fois sur 9 (66,6%) chez des malades castrées pour épithélioma de l'ovaire (Tableau 11).

Le Tableau 12 récapitule les résultats obtenus dans les tableaux précédents. Chez les sujets témoins, le pourcentage de la présence des 17 cétostéroïdes libres est de 11,8, tandis que chez les malades atteintes de cancer mammaire ou génital, il varie de 46,4 à 85,7.

Chez les malades castrées pour cancer du sein (castration chirurgicale ou radiothérapique), pour cancer du col de l'utérus, de l'ovaire et du corps utérin, le pourcentage de la présence des 17 cétostéroïdes libres oscille entre 40 et 100.

Dans le Tableau 13, nous avons étudié le taux des 17 cétostéroïdes plasmatiques totaux, suivant qu'il existât ou non des 17 cétostéroïdes libres.

Dans tous les groupes examinés, le taux moyen des 17 cétostéroïdes totaux s'est montré plus élevé lorsqu'il existait dans le sang des 17 cétostéroïdes libres, que lorsque ceux-ci y faisaient défaut. La différence est significative avec t respectivement égal à 3,2 — 10 — 3,21 — 3,5 et 2,4, correspondant à une probabilité pour l'hypothèse hasard comprise entre—1‰ et 1% ou entre 2 et 5%.

Dans 299 dosages sur 308, effectués chez des femmes atteintes de cancer mammaire ou génital, le taux des 17 cétostéroïdes conjugués dépasse, dans le plasma, celui des 17 cétostéroïdes libres.

Depuis que le problème des perturbations du métabolisme hormonal dans le cancer a été soulevé, nombreuses sont les études des hormones stéroïdes chez des malades atteints de cancer de la prostate. Parmi les plus importantes, se placent celles de Frame et Jewett [34], Huggins et Scott [35], Robinson et Goulden [36], Gemzell *et al.* [37], Birke *et al.* [38, 39], Burt *et al.* [40], Hellström *et al.* [41], Chwalla [42], Bulbrook *et al.* [43,44], O'Connor *et al.* [45], Brendler et Winkler [46], Zimmermann [47], Nakano [48], Gallagher *et al.* [49], Hohlweg et Dorner [50], Gallagher *et al.* [51], Stern *et al.* [52], Zimmerman *et al.* [53], Szendroi *et al.* [54], Marmostan *et al.* [55], Tamm *et al.* [56], Klosterhalfen *et al.* [57], Tamm *et al.* [58], Wiener *et al.* [59].

Nous nous sommes proposé de voir si, chez les hommes souffrant d'un cancer de la prostate

Tableau 4. 17 *Cétostéroïdes libres conjugués chez des femmes atteintes d'épithélioma du col utérin*

Dosages dans le sang total

Sujets	17 Cétostéroïdes (µg/100 ml) Libres	Conjugués
Mo··	53	66
Bu ··	30	52
Ri ··	28	70
	32	52
Si ··	45	63
Co ··	45	68
Hu··	35	28
Ma··	79	29
De ··	12	49
Ra ··	29	56
Se ··	13	116
Fo ··	28	40

Dosage dans le plasma sanguin

17 Cétostéroïdes libres présents			17 Cétostéroïdes libres indosables		
Sujets	17 Cétostéroïdes (µg/100 ml) Libres	Conjugués	Sujets	17 Cétostéroïdes (µg/100 ml) Libres	Conjugués
Le ··	25	86	Se ··	Ind.	45
Si ··	15	31	So ··	,,	45
De ··	34	60	Mi ··	,,	79
Da ··	19	40	Go ··	,,	49
Bo ··	29	45	Vi ··	,,	61
Mo··	7	34	Pe ··	,,	39
Gi ··	27	47	Ch ··	,,	65
Ja ··	18	76	Ro ··	,,	54
Do ··	14	16	An ··	,,	47
De ·2	12	49	Sc ··	,,	63
Hu··	10	—	Ba ··	,,	56
Pi ··	27	64	Ro ·2	,,	52
Ga ··	56	62	Bl ··	,,	61
Gu ··	17	84	Ch ··	,,	25
Bo ·2	13	73	Pi ··	,,	38
Gr ··	35	46			
Ja ·2	20	39			
Si ··	15	31			
Pi ··	32	56			
Ne ··	31	63			
De ·3	29	35			
La ··	22	46			

Tableau 5. 17 *Cétostéroïdes plasmatiques libres et conjugués chez des malades atteintes d'épithélioma de l'ovaire*

Sujets	17 Cétostéroïdes plasmatiques (µg/100 ml) Libres	Conjugués
Cu ··	Ind.	45
Ja ··	37	53
Ge ··	18	25
Le ··	50	71
Ro ··	72	Ind.
Ca ··	14	70
Ja ·2	22	79

Tableau 6. 17 Cétostéroïdes plasmatiques libres et conjugués chez des femmes atteintes d'épithélioma du vagin

Sujets	17 Cétostéroïdes (µg/100 ml)	
	Libres	Conjugués
Le · ·	Ind.	62
Bu · ·	10	—
Da · ·	20	45
Be · ·	12	43

Tableau 7. 17 Cétostéroïdes plasmatiques libres et conjugués chez des femmes castrées pour cancer du sein

Castration chirurgicale

17 Cétostéroïdes libres présents

Sujets	17 Cétostéroïdes (µg/100 ml)	
	Libres	Conjugués
Gu · ·	40	56
Bo · ·	11	29
	30	25
Ba · ·	29	24
Sa · ·	14	27
Mu ·	33	58
Ma · ·	10	29
Fa · ·	48	58
Gu · 2	20	59
De · ·	17	37
Ti · ·	34	36
Fe · ·	35	24
Sa · ·	17	68
Li · ·	51	68
Bo · 2	29	55
Ba · ·	17	70
Ge · ·	8	26
De · 2	18	24
He · ·	23	52
	22	34
Ge · 2	40	16
Ig · ·	38	45
Co · ·	28	60
Hi · ·	20	96
Gu · 3	40	56

17 Cétostéroïdes libres indosables

Sujets	17 Cétostéroïdes (µg/100 ml)	
	Libres	Conjugués
Bi · ·	Ind.	35
Ar · ·	,,	37
Br · ·	,,	60
Mu ·	,,	48
Pe · ·	,,	39
Be · ·	,,	26
Ma · 2	,,	50
Ay · ·	,,	48
Be · ·	,,	59
Se · ·	,,	62
Sa · ·	,,	52
Ma · 3	,,	68

Castration radiothérapique

Sujets	17 Cétostéroïdes (µg/100 ml)	
	Libres	Conjugués
Vi · ·	20	30
Ar · ·	20	58
Ar · 2	16	60
Al · ·	18	44
Na · ·	20	38
Fa · ·	22	45
Bo · ·	11	47

Tableau 8. 17 *Cétostéroïdes plasmatiques libres et conjugués chez des femmes castrées pour épithélioma du col utérin*

17 Cétostéroïdes libres présents			17 Cétostéroïdes libres indosables		
Sujets	17 Cétostéroïdes (µg/100 ml) Libres	Conjugués	Sujets	17 Cétostéroïdes (µg/100 ml) Libres	Conjugués
Pr ··	45	56	Ll ··	Ind.	52
Ro ··	38	48	Di ··	,,	43
Le ··	28	74	Ta ··	,,	87
La ··	32	20	Bo ··	,,	56
Be ··	18	25	Ja ··	,,	43
Ad ··	11	56	Mo··	,,	42
Fa ··	33	68	Cu ··	,,	31
La ·2	16	58	Th ··	,,	48
			Pr ··	,,	52
			He ··	,,	68
			Fo ··	,,	92
			Br ··	,,	22

Tableau 9. 17 *Cétostéroïdes plasmatiques libres et conjugués chez des femmes castrées pour épithélioma du vagin*

Sujets	17 Cétostéroïdes (µg/100 ml) Libres	Conjugués
Le ··	Ind.	62
La ··	16	58
Be ··	12	43
Da ··	20	45
Hu ·	10	—
Be ·2	12	43

Tableau 10. 17 *Cétostéroïdes plasmatiques libres et conjugués chez des femmes castrées pour épithélioma du corps utérin*

Sujets	17 Cétostéroïdes (µg/100 ml) Libres	Conjugués
Pe ··	Ind.	58
Gr ··	,,	79
Al ··	,,	60
Se ··	20	45
Ga ··	19	35
Bu ··	12	29
Le ··	18	47

Tableau 11. *17 Cétostéroïdes libres et conjugués chez des malades castrées pour épithélioma de l'ovaire*

17 Cétostéroïdes libres présents			17 Cétostéroïdes libres indosables		
Sujets	17 Cétostéroïdes (µg/100 ml) Libres	Conjugués	Sujets	17 Cétostéroïdes (µg/100 ml) Libres	Conjugués
De · ·	15	65	Ca · ·	Ind.	34
Cr · ·	27	58	Qu ·	,,	40
Me · ·	23	25	Ro · ·	,,	59
Br · ·	34	64			
Cl · ·	14	49			
Cl · 2	13	68			

Tableau 12. *Tableau récapitulatif: pourcentage de cas avec 17 cétostéroïdes libres chez les normaux et les malades atteintes de cancer mammaire et genital*

Sujets		17 Cétostéroïdes	
		Nombre de cas	Proportion
Femmes témoins	(60 cas)	7	11,8%
Cancer du sein;	(155 cas)		
Malades jeunes	(56 cas)	26	46,4%
Malades âgées	(99 cas)	54	54,5%
Cancer du col de l'utérus	(48 cas)	33	68,7%
Cancer de l'ovaire	(7 cas)	6	85,7%
Malades castrées pour:			
Cancer du sein (castration chirurgicale)	(35 cas)	23	65,7%
(castration radiothérapique)	(7 cas)	7	100,0%
Cancer du col de l'utérus	(20 cas)	8	40,0%
Cancer de l'ovaire	(9 cas)	6	66,6%
Epithélioma du corps utérin	(7 cas)	4	57,1%
Epithélioma du vagin	(6 cas)	5	83,0%

Tableau 13. *Taux moyen des 17 cétostéroïdes plasmatiques totaux en µg/100 ml*

Sujets	17 Cétostéroïdes plasmatiques libres présents	17 Cétostéroïdes plasmatiques libres indosables
	Malades atteintes d'un cancer du sein	
Malades jeunes	(26 cas) 75,6	(30 cas) 57,6
Malades âgées	(54 cas) 75,0	(45 cas) 50,0
Malades castrées chirurgicalement	(23 cas) 73,5	(12 cas) 48,6
Malades castrées par radiothérapie	(7 cas) 64,0	(0 cas)
	Malades atteintes de cancer du col	
Malades	(21 cas) 75,2	(15 cas) 52,0
Malades castrées	(8 cas) 78,2	(12 cas) 53,0
	Malades atteintes de cancer de l'ovaire	
Malades	(6 cas) 85,1	(1 cas)
Malades castrées	(6 cas) 75,8	(3 cas) 44,3

Tableau 14. 17 *Cétostéroïdes libres et conjugués chez des hommes témoins âgées de* 18 *à* 55 *ans*

Sujets	17 Cétostéroïdes (µg/100 ml)	
	Libres	Conjugués
Bo ··	16	62
Me··	20	40
CNTS	24	120
,,	15	110
CNTS	Ind.	22
,,	,,	56
,,	,,	125
,,	,,	170
,,	,,	100
,,	,,	71
,,	,,	70
,,	,,	52
,,	,,	88
,,	,,	156
,,	,,	36
,,	,,	145
,,	,,	72
,,	,,	25
,,	,,	62
,,	,,	39
,,	,,	61
Pi ··	,,	64
Ro ··	,,	45
Wi ··	,,	61
Vi	,,	66
Ho ··	,,	53
Ga ··	,,	54
Le ··	,,	67
Ma··	,,	51
Po ··	,,	51
Ca ··	,,	74
La ··	,,	84

ou du testicule, la quantité des 17 cétostéroïdes plasmatiques libres était autre que chez les hommes normaux.

Chez 32 hommes normaux, la présence des 17 cétostéroïdes libres n'a été relevée que 4 fois dans le plasma (Tableau 14) (12,5%).

Le Tableau 15 réunit les résultats du dosage des 17 cétostéroïdes libres, ou conjugués, chez des hommes atteints d'un cancer de la prostate.

Seize malades ont été étudiés. Chez certains, plusieurs dosages ont été effectués. Sur 31 dosages, des 17 cétosteroïdes plasmatiques libres ont été décelés 23 fois, soit dans 74,2% des cas. Chez Du., Be., He., Mu., Po., des examens répétés confirment la présence des 17 cétostéroïdes plasmatiques libres. Chez 2 des 7 malades (He. et Be.), où ces stéroïdes libres étaient indosables au début, des examens ultérieurs ont montré leur présence à un stade plus avancé de la maladie, mais c'est l'inverse qui s'est produit chez le malade Ne. Sur 23 dosages, les 17 cétostéroïdes plasmatiques libres ne dépassent qu'une seule fois le taux des conjugués.

En ce qui concerne le cancer du testicule, on trouve, dans la littérature, des études portant sur l'excrétion urinaire des hormones stéroïdes, ou sur leur modification au sein d'homogénats: Venning *et al.* [60], Cook *et al.* [61], Schmidt et Tonutti [62], Savard *et al.* [63, 64], Camin *et al.* [65], Dominguez *et al.* [66], Schoen *et al.* [67], Dominguez [68], Gual *et al.* [69], Martin *et al.* [70], Engel *et al.* [71], Bayer *et al.* [72].

Le Tableau 16 a trait à 10 malades castrés pour cancer du testicule. Contrairement à ce qui se passe chez les malades atteints de cancer de la prostate, les 17 cétostéroïdes plasmatiques libres n'ont été trouvés que rarement (dans 2 cas sur 11 dosages); mais, dans 1 de ces cas négatifs (De.), ils sont apparus plus tard avec la dissémination des métastases. Il faut noter la différence d'âge entre les deux groupes de malades (52 à 77 ans pour nos sujets atteints de cancer de la prostate, contre 16 à 41 ans pour ceux castrés pour cancer du testicule).

Nous ne disposons pas de dosages avant la castration chez des sujets atteints de cancer du testicule.

Tableau 15. *17 Cétostéroïdes plasmatiques libres et conjugués chez des hommes atteints d'un cancer de la prostate* (52 *à* 77 *ans*)

17 Cétostéroïdes libres présents				17 Cétostéroïdes libres indosables			
Sujets	Date	17 Cétostéroïdes (µg/100 ml) Libres	Conjugués	Sujets	Date	17 Cétostéroïdes (µg/100 ml) Libres	Conjugués
Ch · ·		34	56	Qu · ·		Ind.	37
Be · ·		27	46	Sc · ·		,,	63
Fo · ·		40	54	Gr · ·		,,	70
Jo · ·		16	61	Pr · ·		,,	74
Ne · ·	14.6.66:	13	67	Ne · ·	30.6.66:	,,	57
Pa · ·		51	56				
Du · ·	10.3.66:	34	88				
	17.3.66:	27	64				
Be · ·	25.9.66:	Pulpectomie		Be · ·	19.8.66:	,,	46
	1.10.66:	51	68		24.9.66:	,,	108
	7.10.66:	34	85				
He · ·	4.6.66:	45	54				
	29.7.66:	25	56				
	9.8.66:	47	52				
Ha · ·	27.7.66:	20	56	Ha · ·	9.12.65:	,,	71
	17.8.66:	35	38				
	7.9.66:	17	62				
Mu · ·	16.9.66:	45	67				
	26.9.66:	31	45				
	26.9.66:	Pulpectomie					
	29.9.66:	20	63				
	4.10.66:	23	68				
Po · ·	11.10.66:	45	56				
	12.10.66:	Pulpectomie					
	18.10.66:	34	24				
	24.10.66:	17	51				

Tableau 16. *17 Cétostéroïdes plasmatiques libres et conjugués chez des hommes castrés pour cancer du testicule* (16 *à* 41 *ans*)

Sujets	Date	17 Cétostéroïdes plasmatiques (µg/100 ml) Libres	Conjugués
Re · ·		32	78
Bi · ·		38	79
Bo · ·		Ind.	51
Go · ·		,,	84
De · ·		,,	67
Tr · ·		,,	84
De ·2	8.3.66:	,,	54
	17.1.67:	22	65
Ra · ·		Ind.	65
Ba · ·		,,	116
Du · ·		,,	39

CONCLUSIONS

Les résultats que nous présentons montrent qu'au cours du cancer du sein et des cancers génitaux chez la femme, et du cancer de la prostate chez l'homme, il existe fréquemment, dans le plasma sanguin (dans plus de 50% des cas), des 17 cétostéroïdes libres, alors que ces mêmes stéroïdes libres font pratiquement défaut dans le sang des sujets normaux. La raison de ce phénomène est difficile à donner car cette présence se maintient chez les malades largement opérés. Nos observations sont à rapprocher des constatations de Sandberg et Slaunwhite qui ont montré que des stéroïdes radioactifs libres, administrés à des malades atteints d'un cancer du sein, disparaissaient du plasma sanguin plus lentement que chez les femmes bien portantes.

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RESUME

Chez 298 *femmes atteintes de cancer du sein, de l'utérus (col ou corps), ou de l'ovaire, castrées ou non, nous avons trouvé* 175 *fois des* 17 *cétostéroïdes libres dans le sang périphérique, soit dans* 58,7% *des cas.*

Chez des hommes souffrant de cancer de la prostate, les 17 *cétostéroïdes plasmatiques libres sont plus souvent présents* (12 *fois sur* 19) *que chez les hommes castrés pour cancer du testicule* (3 fois sur 11).

Les 17 *cétostéroïdes libres font pratiquement défaut dans le sang des sujets bien portants, tant chez les hommes que chez les femmes.*

SUMMARY

In 298 *women with cancer of the breast, of the uterus (cervix or body), or of the ovary, castrated or not, we found, in* 175 *cases, free* 17 *ketosteroids in the peripheral blood, which represents* 58·7%.

In men with cancer of the prostate, the free plasmatic 17 *ketosteroids were found more often (in* 12 *men out of* 19) *than in men castrated for cancer of the testicle* (3 *out of* 11).

Free 17 *ketosteroids are practically never found in the blood of subject in good health, either men or women.*

ZUSAMMENFASSUNG

Bei 298 *Frauen, die an einem Krebs der Brust, des Uterus oder des Eierstockes leiden, haben wir* 175 *mal freie* 17 *Ketosteroide im Blutplasma gefunden* (58,7% *der Fälle). Diese Beobachtung gilt sowohl für die nicht kastrierten wie die kastrierten Patientinnen.*

Bei Männern, die an einem Krebs der Prostata leiden, sind freie 17 *Ketosteroide im Blutplasma viel öfter vorhanden,* (12 *mal bei den* 19 *untersuchten Kranken) als bei wegen Hodenkrebsen kastrierten Männern* (3 *mal in* 11 *Untersuchungen).*

Freie 17 *Ketosteroide fehlen normalerweise im Blutplasma gesunder Männer und Frauen.*

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Inhibitory Effect of Polycations on the Transplantability of Mouse Leukaemia Reversed by Heparin

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INTRODUCTION

CORRELATION of the behaviour and function of tumour cells with their surface charge has gained much interest in the recent time. Properties like the invasiveness of tumour cells, lack of contact inhibition, and decreased mutual adhesion compared to most other types of cells may all be parameters of the cell surface conditions. Thus Ambrose *et al.* [1] demonstrated that carcinoma cells carry a higher negative electric charge in the surfaces than normal fibroblasts, and Purdom *et al.* [2] have advocated the idea that the invasiveness of tumour cells may be due to the increased negative surface charge. The inhibitory effect on the growth of mouse mammary tumours, which has been obtained by incubation of the tumour cells before inoculation with polylysine and lysine-rich histone fractions by Shah and Reilly [3], falls very well in line with the supposition that the cell surface charge is of importance for the function and behaviour of tumour cells. Similar results have been obtained by Richardson *et al.* [4] and by Vorobyer and Bresler [5].

The purpose of the present investigation has been to study in further details the mechanism of polycationic inhibition of tumour cell transplantability by reversing the probably positive surface charge resulting from adsorption of polycations with the polyanion heparin.

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MATERIALS AND METHODS

Biological testing

C3H/A mice weighing 20–25g were used in the experiments. The C3H/A mouse strain has been maintained in the Cancer Research Institute, Aarhus. The leukaemic tumor, leukaemia NJA, has been carried by subcutaneous transplantations by weekly serial transfers of approximately 10^6 tumour cells/C3H/A mouse, since it occurred as a spontaneous leukaemia in C3H mice in Aarhus, 1960. The tumor has been thoroughly described by Olsen [6] and used as a screening tumour for cancer chemotherapeutics [7].

Solid tumors were clipped with scissors and strained through one layer of gauze in suspension in one part saline and one part N/10 phosphate buffer pH 7·4. Suspensions containing 2×10^6/ml NJA tumour cells were incubated with equal volumes containing the appropriate concentrations of polycations in the same saline–phosphate buffer. In order to make sure that each animal received the same amount of cells, the incubation took place at room temperature in 1 ml syringes containing 10^6 NJA cells per ml. If heparin was used after the incubation with polycation, this was effected by subsequent suction of a concentrated heparin solution into the syringe. One ml tumour cell suspension was given subcutaneously to each C3H/A mouse in groups of 10 animals.

The evaluation of the effect on the tumour cells was based on the observation of the average survival time of groups of inoculated C3H/A mice. An increase in life span (ILS) of less than 40% over untreated controls was taken as insignificant [8]. Organs and tumours were removed from each animal for histology and routine

examinations were done after staining with ordinary hematoxylin/eosin stain.

Oxygen uptake was measured in standard Warburg flasks on cells in the same buffer as used for incubation. The metabolic bath was maintained within $\pm 0 \cdot 1°$ of the stated temperature.

Materials

Polyornithine HBr was purchased from Yeda Research & Development Co. Ltd., The Weisman Institute, Israel. Protamine sulphate with a molecular weight of about 5000 and 60% arginin prepared from salmon sperm was kindly supplied by the late Dr. Poul Stroier Rasmussen. DEAE-Dextran, a soluble ion exchanger with a molecular weight of about 2×10^6, was from Pharmacia, Uppsala, and Heparin containing 140 i.u./mg was a product from Leo Pharmaceuticals, Copenhagen.

RESULTS

Polyornithine HBr is rather toxic to C3H/A mice. Injections of 30 mg/kg i.p. are tolerated but the animals exhibit signs on paralysis in the hind part of the body, lasting for about one day. The dosages used in the experiments of 4 mg polyornithine/kg (6·8 mg polyornithine, HBr/kg) are well tolerated and animals do not exhibit signs of toxic manifestations on inspection after autopsy. Protamine and DEAE-Dextran are less toxic than polyornithine as the animals tolerated well five daily injections of each 40 mg/kg. Polyornithine HBr, Protamine, and DEAE-Dextran failed to show any significant increase of the life span in mice with well established leukaemic NJA tumour. Incubation of NJA tumour cells has been carried out with different concentrations of polyornithine, DEAE-Dextran, and protamine for different lengths of time. Polyornithine in concentrations of 2 μg/ml containing 10^6 NJA tumour cells and 10 μg/ml for 30 minutes gave less than 40% increase of life span. 50 μg/ml polyornithine significantly increased the survival time. Experiments on incubation of 10^6 NJA tumour cells with DEAE-Dextran in concentrations of 50 μg/ml, 100 μg/ml, and 200 μg/ml all resulted in less than 40% increase of life span and attempts to demonstrate an effect of protamine in concentrations from 50 μg/ml to 1000 μg/ml for 30 and 60 minutes of incubation gave just about 40% increase of life span and no survivors. A definite effect was obtained by incubations with 100 μg polyornithine/ml or 1000 μg DEAE-Dextran/ml. The results from experiments with these concentrations used for varying lengths of incubation time are depicted in Figs. 1 and 2. It is seen in Fig. 1 that incubation of 10^6 NJA tumour cells with 100 μg polyornithine for 60 minutes in three experiments with 10 and 20 C3H/A mice in each group resulted in the survival of 70% of the animals and significantly increased life span for the rest of the animals. If the 60-minutes incubation period is followed by incubation for 5 minutes with 430 μg heparin, subsequent inoculations resulted in the death of all animals without any significant increase of life span. Figure 2 shows the results after incubation with DEAE-Dextran. Heparin again reverses the inhibitory action of the polybase after incubation for one hour.

Histological examination of organs and tumour tissue verified that the development of NJA leukaemia in untreated controls and in mice who have received polycation inhibited

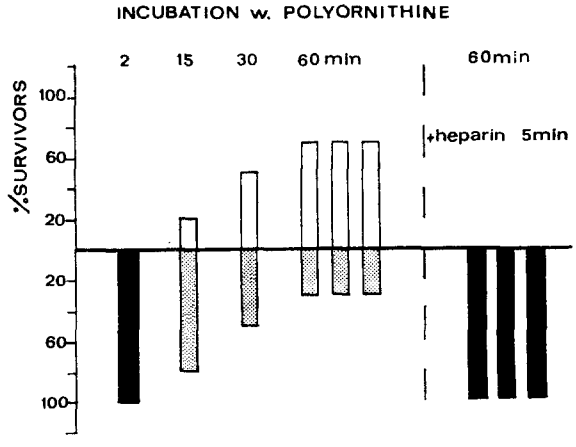


Fig. 1. Effect of incubation of NJA tumour cells with polyornithine for 2, 15, 30, and 60 minutes at room temperature before inoculation into C3H/A mice. Each mouse received 10^6 tumour cells in one ml buffered saline (equal parts of saline and N/10 PO_4, pH 7 4) containing 0·1 mg polyornithine. Heparin treated cells were incubated for 5 minutes after 60 minutes of incubation with polyornithine. The bars represent groups of 10 or 20 mice. The parts of the bars above the vertical line represent the fraction of the groups who survive, i.e. observed for 45–50 days, which are 3–4 times the life span of control animals. Below the vertical line is depicted the fraction of the groups which developed tumour. Black bars illustrate that the increase in life span compared to untreated controls was less than 40%, grey bars more than 40%.

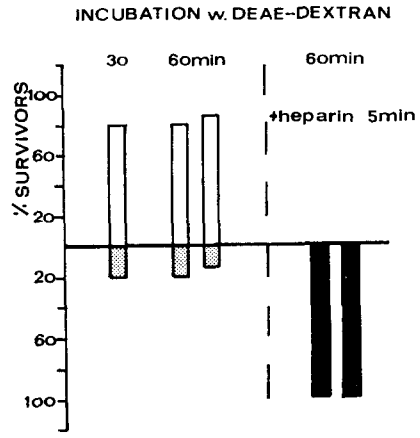


Fig. 2. Effect of incubation of NJA tumour cells with DEAE-Dextran for 30 and 60 minutes at room temperature before inoculation into C3H/A mice. Each mouse received 10^6 tumour cells in one ml buffered saline (equal parts of saline and N/10 PO_4, pH 7·4) containing 1·0 mg DEAE-Dextran. Heparin treated cells were incubated for 5 minutes after 60 minutes of incubation with DEAE-dextran. Signatures as in Fig. 1.

tumour cells subsequently incubated for 5 minutes with heparin was similar in appearance and distribution. Surviving animals examined from 45th to 50th day after inoculation exhibited no signs of tumour.

Two series of experiments were made to study whether the effect of heparin could be demonstrated *in vivo* without *in vitro* incubation. After 30 minutes of incubation with 100 µg polyornithine the NJA tumour cells were subcutaneously inoculated. Immediately hereafter each mouse received an intraperitoneal injection of 1 mg heparin. In this case no effect of heparin was noted. A similar experiment with 1 mg DEAE-Dextran showed a slight effect of heparin, with two surviving animals out of ten compared to eight survivors out of ten in the group which had no heparin.

Upon incubation with the polycations used in this study a heavy aggregation of the tumour cells was observed even after a short time exposure to low concentrations. This phenomenon was supposed to cause severe disturbances of the cell respiration which could explain the low transplantability. Therefore, the cellular uptake of oxygen was measured with conventional Warburg technique. Measurements were performed on cell suspensions incubated with the polycations in the same buffer as used for inoculations. The cell concentration was kept about 10^7 NJA tumour cells/ml. Addition of heparin to a final concentration of 1 mg heparin per ml tumour cell suspension preincubated with polyornithine or DEAE-Dextran had no demonstrable effect neither on the respiratory rate nor on the aggregation effect.

Oxygen consumption of the tumour cells was increased from 10 to 50% over the controls if polyornithine or DEAE-Dextran in concentrations of 100 µg/ml was added to the cell suspensions both at 24° and at 37°. With higher concentrations of polyornithine and also of DEAE-Dextran, an inhibition of the oxygen uptake down to about 50% of the controls was found at 37° for 1·0 mg/ml of polyornithine HBr whereas concentrations of DEAE-Dextran of 1 and 2 mg/ml resulted in values of about 65% of the control cell respiration.

DISCUSSION

Polycations and basic proteins have been shown to be able to influence the behaviour and metabolism of cells in many ways. Besides being able to agglutinate cells, polycations have also been shown to affect the cellular uptake of proteins as reported by Ryser and Hancock [9] who were able to produce a marked increase in albumin uptake into monolayers of Sarcoma 180 cells by very low concentrations of histones and polyornithine. A similar effect of polyornithine and protamine on the permeability for albumin has been produced on artificial protein membranes [10]. The polycations and basic proteins, among which mainly histones and histone fractions have been studied, permeate readily into the interior of the cells, where other functions have been demonstrated. Effects of histones on mitochondrial membranes causing marked changes in mitochondrial metabolism and in mitochondrial adenosine triphosphatase activity have been reported by Schwartz [11], and Allfrey *et al.* [12] have demonstrated a

decreased RNA biosynthesis when histones, protamines, or polylysine are added to isolated nuclei.

The inhibitory effect of polycations on transplantable tumour cells reported by Shah and Reilly [3] and found in our experiments could be due to one or more of the functions mentioned above. In an attempt to discuss the mechanism of the inhibitory effect, it occurs to us, however, that the observed effect of heparin may exclude some of those functions of polycations as probably causes of the tumour cell inhibition. The oxygen consumption of the cells is influenced by polycations. Our results resemble the observations of Schwartz [11] regarding the effect of histones and other polycations on the oxygen uptake by isolated mitochondria. Addition of heparin to cells inhibited with polycations did not alter the oxygen consumption, and even in experiments where heparin and polyornithine were added simultaneously, the oxygen uptake was the same as in experiments where no heparin was added. According to studies by Sherman [13], heparin does not enter ascites tumour cells, and as indicated by the inability of heparin to influence the oxygen consumption, this may also be true for the NJA tumour cells. The target of the polybase action may, therefore, not be via the inhibition of the DNA dependent RNA polymerase or any action on mitochondria.

Apparently, the inhibitory effect of polycations on the transplantability of tumour cells points to the rôle of the cell surface charge. Generally the cell membrane is considered to carry a net negative charge. The agglutination which takes place when cells are exposed to positive polyelectrolytes is due to interaction between the negatively charged cell membranes and the positively charge compounds. The mechanism of this type of agglutination has been studied by Katchalsky *et al.* [14, 15] who have shown that the agglutination of red blood cells is dependent on the chemical nature and the molecular weight of the polycations. Incubation of the leukaemic tumour cells in our experiments resulted in agglutinations which followed within two minutes even after addition of very small concentrations of polyornithine (2 μg polyornithine/ml with 10^6 NJA tumour cells). Addition of heparin did not disperse the aggregates. Therefore, still the greater part of the cell bound polycation is present in the aggregate, possibly because of the inability of the relatively high molecular weight heparin molecules to penetrate into the agglutination seam [14].

If polyornithine is injected intraperitoneally to mice with NJA leukaemia cells in peritoneum, cell aggregates can be found 24 hr after injection. This supports the view that the aggregates are not dispersed during the first hours after inoculation. Heparin treatment of the aggregates presumably reverses the effect of polycations on the peripheral cell surfaces. Originally the cells may have been negatively charged. Polycations which are considered to bind very firmly to surfaces, will change the surface charge to more positive, and finally heparin will form salt bonds to the free, positive groups of the polycations, whereby cleavage of cell surfaces for polycations may take place. Although only a few of the original tumour cells are affected in this way, there should be enough tumour cells to establish a tumour. Experiments with 10^5 NJA tumour cells, i.e. ten per cent of the inoculated number does not result in significant increases of life span (I.L.S.$<40\%$) [6].

When the tumour cells are inoculated after being incubated with 100 μg polyornithine for two minutes, the amount of polycation injected together with the cells is the same as when inoculated after 60 minutes of incubation. In the former experiment the cells 'take' and in the latter a pronounced inhibition is found. This may indicate that something reminiscent of polyanion action takes place *in vivo* if the cells have been short-time exposed to polycation *in vitro*. Is the *in vitro* incubation period longer than about 15 minutes, this *in vivo* effect does not appear.

Whereas there should be no doubt that cell surface charges vary due to interaction with the strong polyions used in these experiments, there is considerable uncertainty whether the changes in tumour cell behaviour are entirely due to a charge effect. According to Abercrombie *et al.* [16], many functions which are exerted by tumour cells are due to their negative surface charges. However, the stimulatory effect of basic polyaminoacids and basic proteins on the cellular uptake of protein [9] which is supposed to be due to conformational rearrangements in the membrane proteins comparable to the reversible changes in the fine structure which have been demonstrated in the membranes of red blood cell ghosts treated with polylysine [14] may be another effect of great importance.

RESUME

Les auteurs ont étudié l'effet de l'incubation des cellules leucémiques NJA, avec diverses substances, sur leur transplantabilité chez la souris. L'incubation avec la polyornithine et le Dextran-DEAE, pendant une heure, provoquait une inhibition complète de la transplantabilité, chez 70 *à* 85% *des animaux, et augmentait, de façon significative, la survie des animaux restants. La protamine n'avait que peu d'effet.*

Cet effet inhibiteur était réversible. Les cellules tumorales, qui avaient été incubées pendant une heure avec la polyornithine ou le Dextran-DEAE, pouvaient, à la suite d'une incubation de 5 *minutes avec l'héparine, se comporter comme les cellules non traitées, et étaient capables de produire une leucémie NJA chez les souris.*

On peut supposer que la capacité des polycations d'inhiber les cellules tumorales serait due à des altérations des structures de la membrane cellulaire.

SUMMARY

The effect on the transplantability of NJA leukaemic tumour cells to mice after in vitro *incubation with polyornithine, DEAE-Dextran and protamine has been studied. Incubation with polyornithine and DEAE-Dextran for one hour resulted in complete inhibition of the transplantability in* 70 *to* 85% *of the animals and in a significantly increased life span for the remaining animals. Protamine had only little effect.*

This inhibitory effect was found to be reversible. Tumour cells which were incubated for one hour with polyornithine or with DEAE-Dextran would, after subsequently 5 *minutes of incubation with heparin, behave as untreated controls with regard to their ability to produce NJA leukaemia in mice.*

It is assumed that the capability of polycations to induce inhibition of tumour cells is due to alterations produced in cell membrane structures.

ZUSAMMENFASSUNG

NJA-Leukämiezellen wurden in vitro *mit Polyornithin, DEAE-Dextran und Protamin inkubiert. Die Wirkung dieser Inkubation auf die Transplantabilität der Zellen in Mäuse wurde untersucht. Polyornithin und DEAE-Dextran (Inkubation von einer Stunde) ergaben eine Hemmung der Uebertragungsfähigkeit in* 70 *bis* 85% *der Tiere und eine signifikante Lebensverlängerung der restlichen Tiere. Protamin hatte nur eine geringe Wirkung.*

Diese Hemmwirkung war reversibel. Tumorzellen, die während einer Stunde mit Polyornithin oder DEAE-Dextran inkubiert worden waren, verhielten sich nach anschliessender 5*-minütiger Heparin-Inkubation bezügich ihrer Fähigkeit, NJA-Leukämie in Mäusen zu erzeugen, wie unbehandelte Zellen.*

Es wird angenommen, dass die Fähigkeit von Polykationen, Tumorzellen zu hemmen von Einwirkungen auf die Zellmembranstruktur abhängt.

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The Effects of X-Rays on a Human Tumour Growing in Organo-Typic Culture

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INTRODUCTION

DURING the past 5 years, a technique has been developed by Wolff and Wolff [1–3] which permits human tumours to be maintained continuously in organo-typic culture. The latest advances have recently been published by Wolff, Wolff and Croisille [4], Wolff and Wolff [5] and Wolff, Croisille, Mason and Wolff [6].

In this system, small pieces of biological material, approximately 4 mm^2 in area and 10–20 cells thick show active growth which results in an increase in the overall size of each piece of tumour but there is no cell migration to form a monolayer sheet as in the more usual explant cultures. A system of this type has two advantages. First it is essentially a continuous culture and thus the conditions of growth in the controls can be expected to remain approximately constant for the duration of any experiment. This is a condition which can usually be satisfied in monolayer tissue culture work by using established cell lines but only rarely in embryological studies. Secondly it is possible to study the responses of cells which are in close contact in all directions with similarly treated material. It is probably impossible to satisfy this condition in any conventional tissue culture work, and the facility takes us a step nearer, in many respects, to the situation which obtains with solid tumours *in vivo*.

The disadvantages of organo-typic cultures are the heterogeneous nature of the cell population and the limitation placed on many quantitative assay methods by the necessity for preparing sections.

A study of this type of culture ought to form another important link between *in vitro* and *in vivo* conditions and the work is therefore concerned with tumour cell metabolism in unirradiated and X-ray irradiated cultures.

MATERIALS AND METHODS

The biological material was originally an hepatic metastasis from a human tumour, Z 200, which almost certainly originated in the gastro-intestinal tract. It was cultured by Wolff and Wolff and has now been maintained in organo-typic culture for over 5 years. Full details of the culture techniques are given in papers by Wolff and co-workers [2–4]. For the present work it was grown on a medium which had the following composition: 11 drops (each approximately 0·05 ml) agar-agar, 4 drops horse serum, 4 drops chick embryo extract and 1 drop of undialysed yeast extract. The nutritive substances were limited to those which could pass through the vitelline membrane of an unincubated egg, since each piece of tumour tissue was completely enfolded in this

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membrane. Two pieces of tumour, each about 4 mm^2 in area, were placed in each chamber, then the chamber was sealed. Observation suggested that the quality of the cultures could be improved if the chambers were opened every 4 days to replace the air in contact with the medium. The results on cellular respiration to be presented later confirm that appreciable O_2 consumption and CO_2 release does occur in this time interval. Every 10 12 days the control cultures were removed from their membrane and two new pieces of tumour, each approximately 4 mm^2 in area, were cut from the available material, enfolded in a new membrane and placed on fresh culture medium. After irradiation, owing to the fragility of the residual tumour, results were most consistent if there was only one subculture at 5 days post-irradiation and all the available material was placed on the fresh medium.

All irradiations were performed using a Picker X-ray machine working at 60 kV and 8 mA, giving a dose rate at the level of the biological material of 500 R/min in air. Doses ranging from 100 R to 800 R could be delivered reasonably quickly and a correction could be made for the error in dose during the transient period when the current was rising from zero to the required value. X-ray dose rates recorded in air have frequently had to be modified in biological studies to allow for the back-scatter of secondary electrons [7, 8], but since these cultures are growing on a gel which has an aqueous base, any correcting factor will be very small.

To perform an irradiation, the glass top of the culture chamber was replaced by a piece of cellophane 100μ thick and the X-ray tube was brought down to the predetermined height of 14 cm above the pieces of tumour. During irradiations the cultures were maintained at 37° in a water bath and immediately afterwards they were returned to the incubator.

For studies on the mitotic index and rate of DNA synthesis, ½ ml of ^3H thymidine at 25μc/ml and 4·8 C/mM in a medium comprising 50% Tyrode solution, 25% chick embryo extract and 25% horse serum was added to the culture medium at various times up to 150 hr after irradiation. Assuming that the thymidine mixed with the culture medium completely, it was at a final concentration of 12·5μc/ml. One hour later the cultures were washed well in Tyrode solution and immediately fixed in 25% HAc in EtOH overnight followed by 70% EtOH for 1 hr. The usual procedures were adopted for preparing paraffin sections 5μ thick cut vertically through the tumour at right angles to the surface which *in vivo* had been in contact with the medium gel. After taking the slides down to water, those which were to be used for autoradiography were hydrolysed in 5% $HClO_4$ at 4° for 30 min to remove low molecular weight precursors of DNA and autoradiographs were prepared using either Ilford K2 liquid emulsion or Kodak AR 10 stripping film. Twelve days later the autoradiographs were developed in 50% Kodak D19 b in water for 4 min, followed by rapid fixation and thorough washing in running tap water. All the sections were stained with haematoxylin and counter-stained with eosin.

Measurements on tumour respiration were made using a modified Warburg apparatus following closely the procedure of Le Douarin and Kirrmann [9]. The change in volume in a closed air space in contact with the tumour was measured by observing the movement of a coloured-water meniscus at constant pressure in a capillary tube of 1 mm dia. To facilitate the measurement of respiration each culture in this experiment contained 8 pieces of tumour each between 1 and 2 mm dia. Under normal conditions, the volume measured was the difference between the oxygen consumed and the CO_2 liberated but for most experiments two small pieces of filter paper soaked in caustic soda were introduced into the air space. It was then possible to measure simply the oxygen consumed. After the respiration measurements, the size of each piece of tumour in mm^2 was measured under the microscope and the total nitrogen content was estimated as follows.

The culture was removed from its feeding medium and washed well in Tyrode solution followed by distilled water. After drying, complete mineralization was effected by a mixture of one part selenium, five parts $HgSO_4$ and 30 parts K_2SO_4 in 5N sulphuric acid at 170° for at least 12 hr. A diluted solution was then treated with Nessler's reagent and spectro-photometric measurements were made at 490 mμ. Solutions of ammonium sulphate of known concentration were used to establish a calibration scale.

RESULTS

Measurement of mitotic index during the first 150 *hr after irradiation*

An accurate absolute measure of mitotic index is not possible for sections of material 5μ thick since only a small portion of a nucleus has to be seen for it to be recorded as a cell, but far more has to be seen if it is to be scored as a mitotic cell. To offset this fact a cell was only

registered if approximately $\frac{1}{3}$ or more of its nucleus, estimated visually fell within the 5μ section.

The whole of a section was searched at 800× magnification for mitoses but for each section a detailed cell count was made on only one field of view which frequently contained between 150 and 200 cells in the controls. This figure was then multiplied by the size, in fields of view, of the section to obtain the total number of cells, probably accurate to about 10%. Sections from all parts of the piece of tumour were examined and the error involved in taking a sample of this type was estimated as shown below.

Slide BA 4 was from a control culture fixed 24 hr after other cultures of this series had been irradiated.

Seven sections were scanned and gave mitotic indices ranging from 1·3% to 2·4%. In all there were 99 mitoses in 5250 cells giving an index of 1·8% and the standard error of the mean, calculated from the percentages for the seven individual sections was 0·13%.

Experience showed that this error was considerably less than the variation between cultures given the same treatment and a sample of approximately 5000 cells chosen in this manner was considered satisfactory.

Figures 1 and 2 show how the mitotic index varies after irradiation—comparison of mitotic indices between cultures which received different doses of radiation is not seriously affected by the factors which prevent an accurate absolute measure. Where errors are shown the figures have been compounded from results for two or more cultures. The most marked effect seems to occur at about 24 hr and Fig. 1 is a simple picture of the variation of mitotic index with dose at that time. A dose of 600 R is sufficient to reduce this index almost to zero but some mitoses (0·05%) were still recorded.

Figure 2 is a more composite presentation of changes in mitotic index with time after various doses of X-rays. The earliest measurements were made 6 hr after irradiation but since the mitotic indices did not differ from those obtained at 12 hr, the two groups have been combined. The curves show a well-known pattern of mitotic delay followed by recovery and a wave of mitoses particularly at lower doses. Little information was obtained beyond 72 hr and the curve is somewhat speculative but its form is not inconsistent with results which have been well established in tissue culture. The very large error in the (200 R, 50 hr) point which was compounded from five cultures confirms that it is a time when the mitotic index is changing rapidly and corresponds to the development of the wave of mitosis.

The mitotic index 6 hr after a dose of 400 R for one culture was 2·95%. This single observation is not recorded in Fig. 2 but since practically all of the mitotic cells were in metaphase it gives some indication that one of the earliest effects of radiation may be to produce a block at metaphase.

Autoradiographic study of DNA synthesis during the same period

Figure 3 shows the autoradiograph over a section from a control culture. The localization of label over the peripheral nuclei is most striking and will be considered again in the discussion.

Examination of autoradiographs over sections prepared from irradiated cultures showed that at any time during the first 24 hr after irradiation the reduction in ^{3}H-thymidine uptake, and

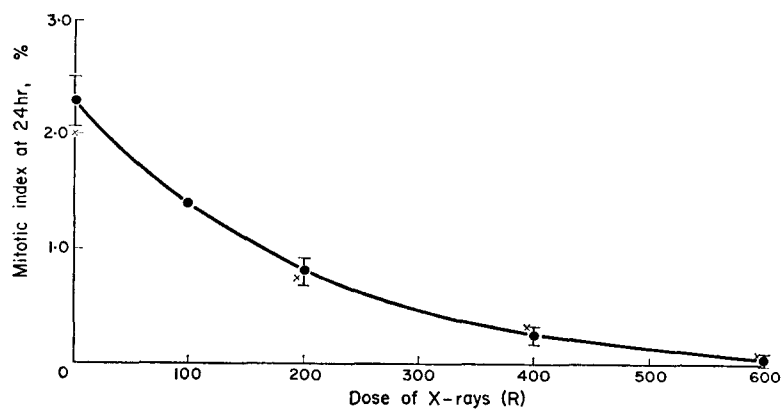


Fig. 1. *The solid circles show values of the mitotic index measured* 24 *hr after various doses of X-rays. Where errors are included, results from several cultures which all received the same radiation treatment have been averaged and the standard error of the mean is recorded.*
The crosses show the results of a similar experiment performed on cultures which had already received, and recovered from, a dose of 400 *R X-rays* 42 *days previously.*

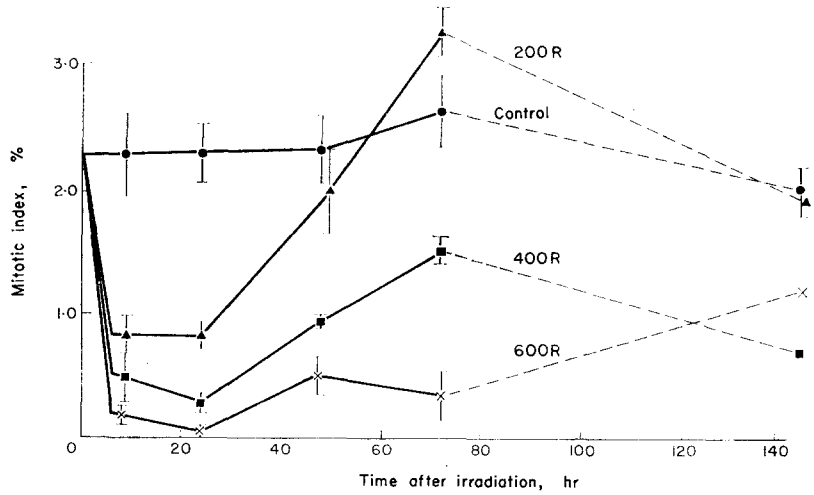


Fig. 2. *Changes in the mitotic index during the first* 140 *hr after X-ray irradiation. Results for several cultures have been compounded as in Fig.* 1.

presumably in the rate of DNA synthesis was not appreciable. This point is illustrated in Fig. 4. Peripheral cells were well labelled by ^3H thymidine administered 24 hr after 600 R but the mitotic index in this culture was only 4 per cent of that in the controls. Owing to the inhomogeneity of label and the difficulties in interpretation when short range ^3H β-particles emerging from a tissue section are examined, no attempt was made to measure any slight reduction in the rate of DNA synthesis, but it is clear that this could not be a primary event leading to the severe reduction in mitotic index.

Tumour respiration

Within a series of experiments, great care was taken to prepare each culture in exactly the same way and with approximately the same volume of tumour. Figure 5 shows respiration measurements for several unirradiated cultures which were all established on the same day and studied either in the presence or absence of caustic soda paper. Each measurement is characterised by a lag period during which time respiration appears to be nil. This is simply because a certain pressure difference must be established in order

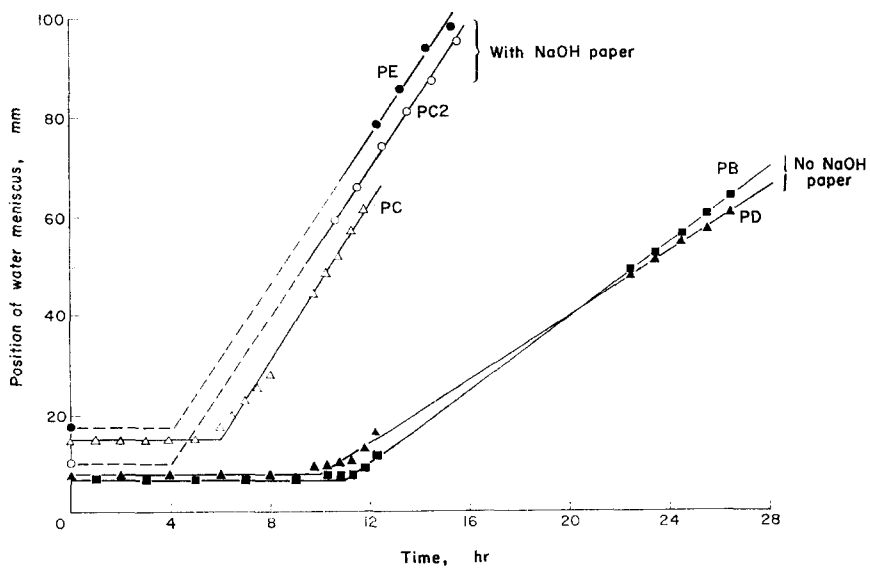


Fig. 5. *Measurements of respiration on a series of cultures (series P). After a lag period (see text), the change in index position in a capillary of constant bore (*1*mm dia.) is proportional to the change in gaseous volume at constant pressure. For cultures PB and PD, this was the difference between the* O_2 *consumed and the* CO_2 *released. For cultures PC and PE, the* CO_2 *released was absorbed in caustic soda.*

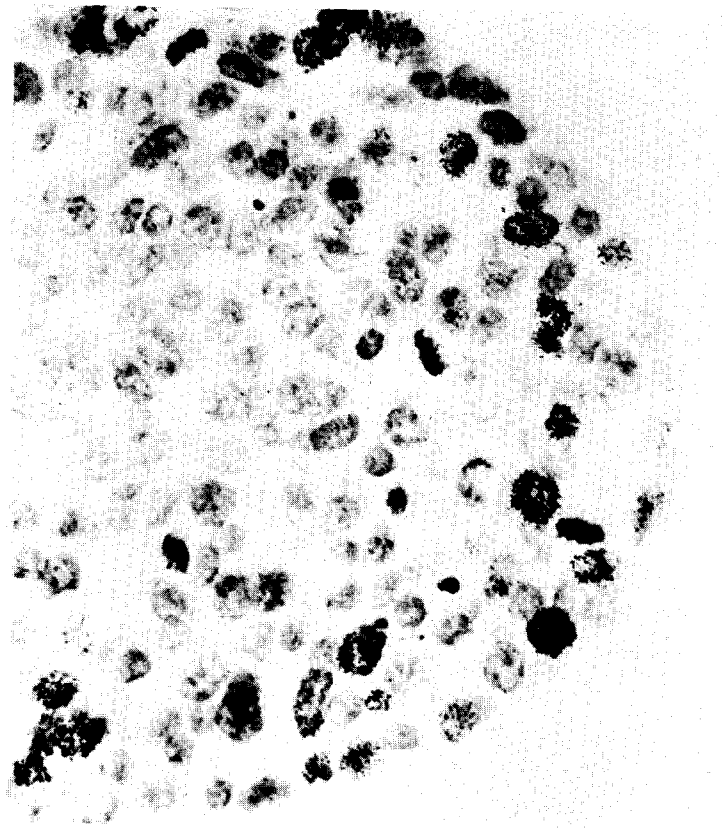


Fig. 3. *The autoradiograph over part of a section through a control culture which had been treated with* ^{3}H*-thymidine for* 1 *hr immediately prior to fixation.*
Three cells are in metaphase. Only nuclei at or near the periphery are labelled. Magnification × 540.



Fig. 4. *The autoradiograph over part of a section through a culture which had received* 600*R X-rays* 24 *hr prior to fixation and* ^{3}H*-thymidine for 1 hr immediately prior to fixation. There are no mitotic figures but many of the peripheral cells are heavily labelled. Magnification* × 540.

(to face p. 166)

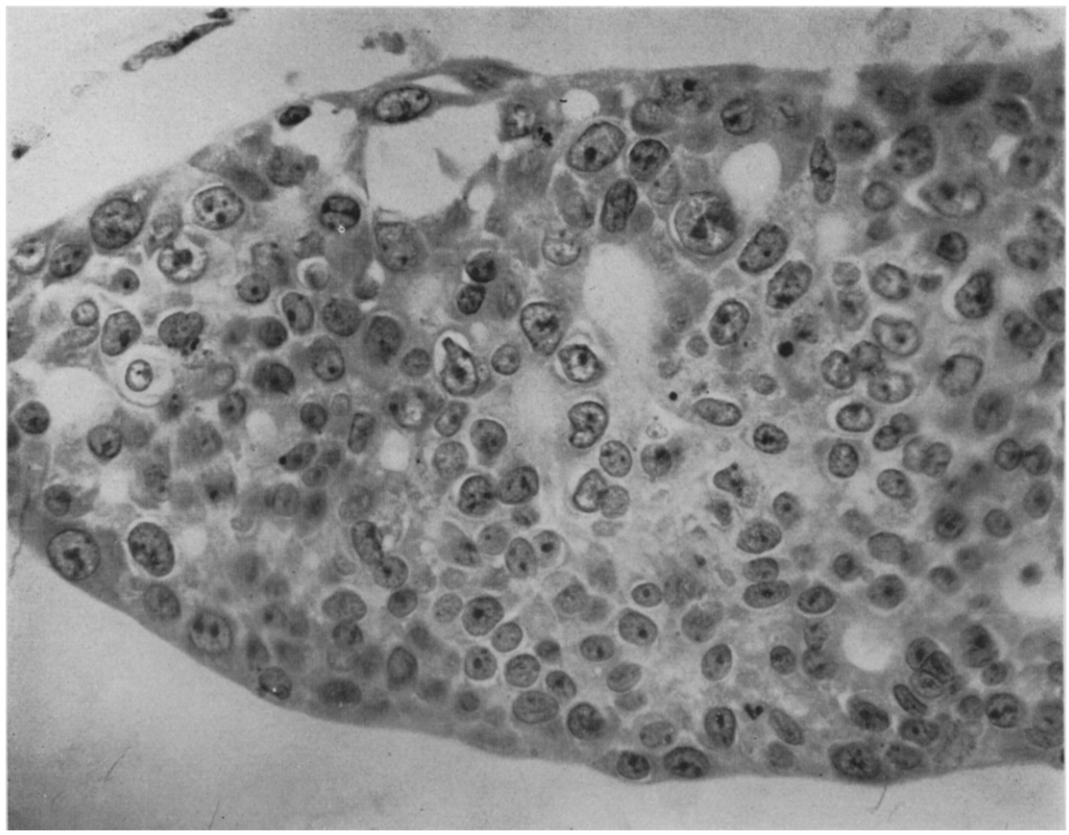


Fig. 7. Part of a section prepared 5 days after 400R X-rays. Nuclei are enlarged and well separated. Magnification × 350.



Fig. 8. *Regenerating nodules photographed* 52 *days after a dose of* 800*R. Episcopal lighting was used, thus the healthy nodules appear nearly black in a white necrotic mass. Magnification* × 42.

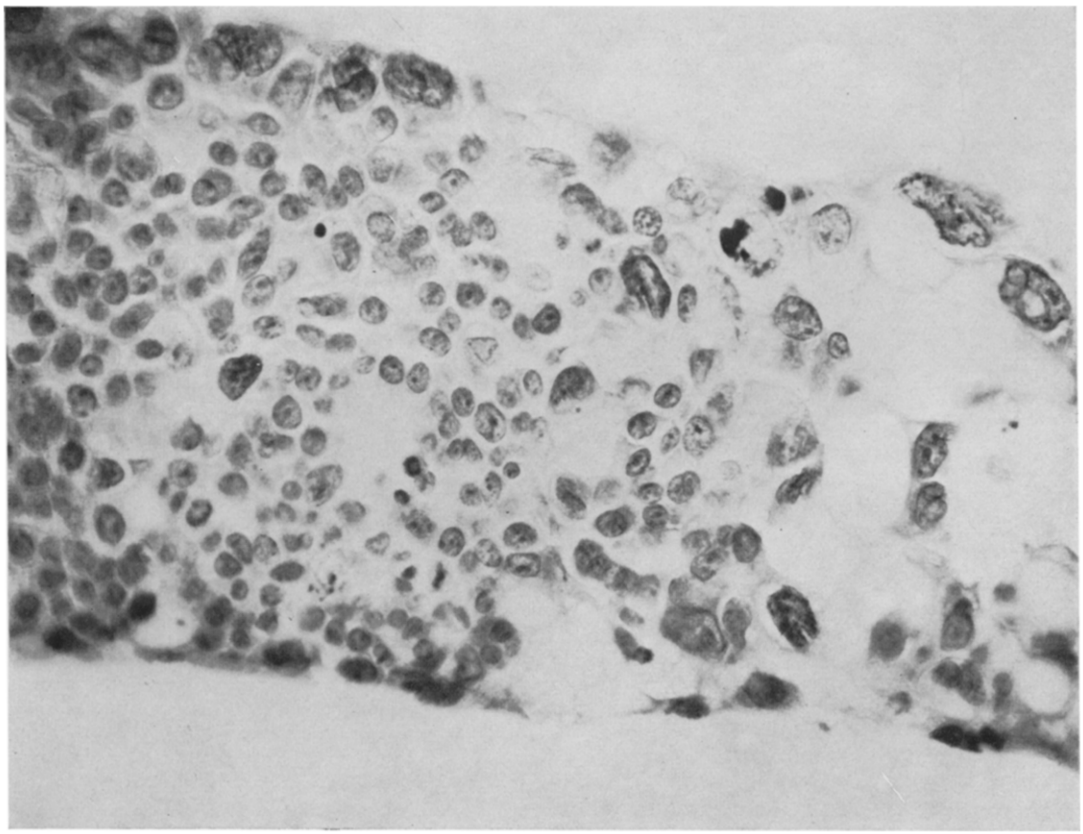


Fig. 10. *Section through a culture fixed* 44 *days after* 400*R. On left-hand side of the photograph there are now many normal cells. Magnification* × 350.

Table 1. *Measurements of respiration for a number of control cultures*

Culture	Total area (mm^2)	Total N_2 content (μg)	Index movement (mm/hr)	Respiration ($\mu l O_2$ /μg N_2.hr)
PC 1	12·7	120	7·9	0·052
PC 2	12·7	120	7·5	0·049
PE	17·9	175	7·5	0·034
WA	8·0		7·5	
XA 1	13·0		3·5	
XD 1	13·0		3·6	
XA 2	13·0		5·0	
XD 2	13·0		3·2	
ZD	18·8	132	7·0	0·042
ZA	18·6	135	5·8	0·034

to move the index. Once the movement has started, the index position changes linearly over a period of hours.

The variations of respiration rate within a series of cultures were much less than those between cultures prepared on different days and attempts to correlate the respiration with some physical or chemical parameter of the tumour were only partially successful. For example a measure of the total area of the tumours took no account of differences in the third dimension, and these could be appreciable particularly after irradiation. When the total nitrogen content was estimated, it was difficult to wash off completely all the 'jellylike' culture medium and thus an unknown and variable error was introduced.

The variations in these parameters are readily seen from Table 1 which gives measurements of the respiration rate for a number of control cultures in the presence of caustic soda paper.

For those cultures for which the nitrogen content was estimated we have a respiration value of $(0·042 \pm 0·004)$ μl $O_2/\mu g$ N_2.hr.

In the absence of caustic soda paper, the figure was $(0·015 \pm 0.002)$ $\mu l/\mu g$ N_2.hr. Hence the CO_2 released is $(0·027 \pm 0·004)$ μl $CO_2/\mu g$ N_2.hr and the Q value for the tumour under these conditions is approximately 0·64.

Respiration has also been measured at various times up to 100 hr after the cultures had received doses of 800 R or 8000 R of radiation. When the respiration of a culture

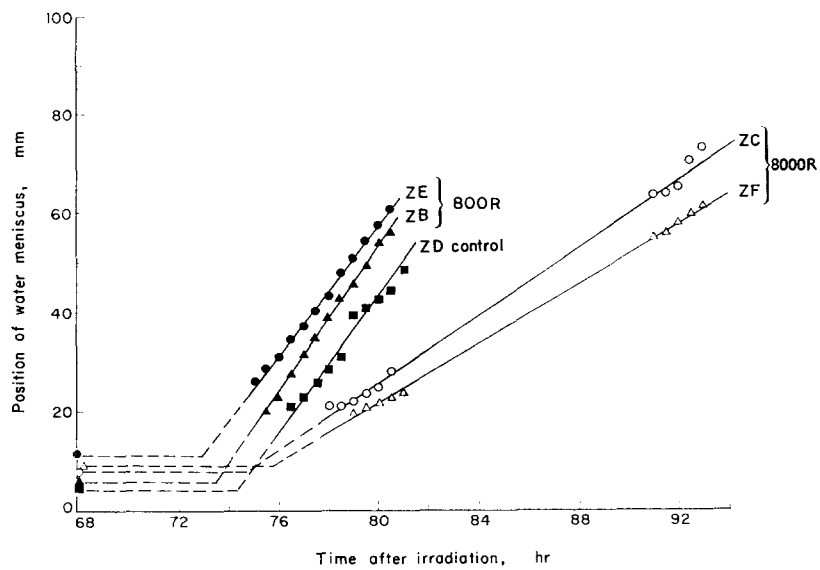


Fig. 6. *Measurements of respiration on a series (series* Z*) in which some of the cultures had been irradiated. In these experiments, the water meniscus was not introduced until* 68 *hr after irradiation.*

which has received 800 R is compared with the respiration of a control from the same series, this dose can be shown to have no effect on the oxygen consumption at any time. About 70 hr after a dose of 8000 R the respiration rate is apparently beginning to fall (see Fig. 6) but this is partly offset by a reduction in the total amount of tissue measured on a total nitrogen basis and the calculated value of 0·029 μl O_2/μg N_2.hr for cultures ZC and ZF, while lower than the control figure, indicates that there is still appreciable oxygen metabolism.

Morphological and cytological changes

A preliminary study by Dumas [10] on the same tumour material (Z 200) had shown that a single dose of 200 R caused necrosis and reduced the growth rate but did not prevent complete recovery of the tumour after several transfers. Doses of 550 R, however, caused complete degeneration of the cultures if they were subcultured weekly as for the controls.

In general the observations recorded below, some of which were noted in 5μ sections prepared after fixation at various times after irradiation, while the remainder were made on the living culture using episcopal lighting at 16× magnification on a dissecting microscope, confirm Dumas' findings. (a) 5 days—a section stained with haematoxylin and eosin from a culture which had received 400 R shows a typical radiation response. The density of nuclei has been drastically reduced and there is a marked increase in the amount of eosinophilic material (Fig. 7).

(b) 6 days—first signs of small necrotic regions in the living tumour.

(c) 10–15 days—stage of most severe necrosis usually characterized by the following features:

200 R–large number of necrotic centres

400 R–necrotic centres are so extensive that there is no visible evidence for healthy tissue.

800–a large number of dead cells are eliminated, holes develop in the tumour and it becomes very fragile. Any attempt to transfer the culture to a fresh medium, as in the experiments of Dumas, would seriously impair the chance of survival if a few viable cells were present. Irradiated cultures were therefore left on the same medium and this factor probably made possible the ensuing tumour behaviour which has not previously been reported.

(d) Any time after 15 days—tiny regions of healthy regenerating tissue, mostly round the periphery, may be seen. These nodules of regeneration contain very few unhealthy cells and are clearly seen in Fig. 8 which was of course taken several days after the first detection. A notable feature is the large variation in the sizes of the regenerating nodules.

Regeneration in a culture was scored as positive when at least one healthy nodule 0·2 mm in diameter was present and this criterion provided a basis for the evaluation of the long term effects of radiation as shown in Fig. 9. A somewhat arbitrary limit of 50 days, largely governed by practical considerations, was set for the appearance of regeneration.

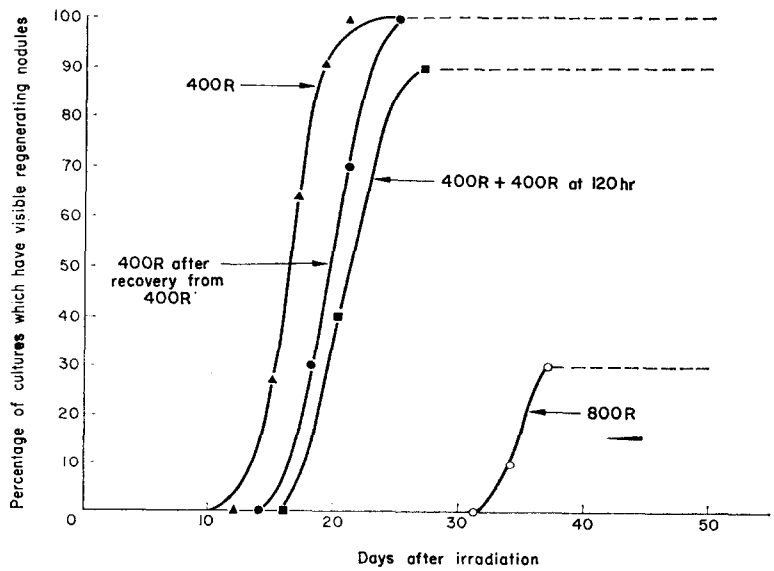


Fig. 9. Results of various experiments in which cultures were examined for the appearance of regenerating nodules following X-ray irradiation. The curves from left to right show the appearance of regeneration following:

a single dose of 400 *R*

400 *R to cultures which had recovered from* 400 *R given* 42 *days previously*

two doses of 400 *R separated by* 120 *hr and*

a single dose of 800 *R.*

In each instance the time scale commences with the last irradiation given.

After the appearance of the nodules, regeneration continued steadily and Fig. 10 shows a section prepared from a culture which was fixed 44 days after 400 R. Clusters of normal healthy nuclei are found between the small areas of necrosis which still persist and are characterized by giant, frequently bizarre nuclei.

Observations after repeat irradiation

A total dose of 800 R X-rays was delivered to the culture in two equal amounts of 400 R. In one set of studies the interval between the doses was 120 hr (5 days) and the time interval between the second dose of radiation and the appearance of regenerating nodules was recorded and is shown in Fig. 9. The delay corresponds much closer to that observed after 400 R than after 800 R and indicates that already at 5 days appreciable recovery has occurred.

For the other studies the second dose of 400 R was given 42 days after the first and constitutes an examination of the radiosensivity of the cells in the regenerated tumour. Once again the appearance of nodules of regeneration was recorded and the values are incorporated into Fig. 9. Some cultures in this radiation category were fixed 24 hr after the second dose and measurements of mitotic index are shown on Fig. 1 as crosses. At 200 R, 400 R, and 600 R these have been displaced slightly to the left for clarity. Both experiments show that the radiosensitivity of the regenerated cells is not greatly different from the original cultures.

DISCUSSION

Many of the results reported here are qualitatively similar to those obtained by workers using cells growing in tissue culture and lend further encouragement to the hope that most of the observations made in artificial systems will be valid *in vivo*.

A review of work on radiation induced mitotic delay for example has been made by Dewey *et al.* [11]. The reappearance of cellular multiplication was their criterion for the end of division delay which was expressed in min/rad, and a correlation was claimed between this figure and the generation time of the culture. The results in Fig. 2 at 200 R and 400 R indicate that the value for these cultures might be approximately 5 min/rad. This is rather higher than any of the figures of Dewey *et al.*, indicating a longer generation time.

Studies on both mitotic delay and modifications in DNA synthesis have been made on mouse L strain cells [12, 13] on U 12 fibroblasts [14] and on mouse ascites tumour cells [15]. Mitotic index measurements agree qualitatively with Fig. 2 but show quantitative differences which suggest that tumour Z 200 is much more radiosensitive. All these workers find that the rate of DNA synthesis is relatively insensitive to radiation and doses in excess of 1500 R are required to produce appreciable changes. We must conclude that changes in the DNA synthetic mechanism are unlikely to provide an explanation for the severe changes in mitotic index and viability.

The respiration characteristics of normal cells usually fall into one of two categories [16]. For example, most resting cells except brain and retina have a low respiratory quotient (Q value) and a low ability to form lactate anaerobically, whereas growing tissues have both a high Q value and a high glycolytic rate. Tumour cells on the other hand are characterised by a low Q value but a high rate of glycolysis. The Q value of approximately 0·64 reported here is very low indeed and suggests that a study of lactic acid production in the culture medium would be most important and might confirm the persistence of this tumour-like characteristic in organo-typic culture.

It has already been shown for both normal and tumour tissues [17, 18] that respiration is a very radioresistant process and the results obtained here after doses of 800 R and 8000 R are quite consistent with previous findings. Modifications of respiration after X-ray irradiation thus seem to be almost negligible by comparison with the changes in mitotic index and survival.

Clear evidence for cell recovery in this tumour was obtained when the X-ray doses were fractionated. This phenomenon has been studied in great detail by Elkind and Sutton since their first paper on the subject in 1959 [19] and the experiment reported here in which the X-ray doses were separated by 5 days is in good agreement with the ideas that recovery is practically complete at the time of the wave of mitosis which follows the radiation induced mitotic delay. Experiments performed on regenerated tumour 42 days after the first irradiation show that it exhibits the same radiosensitivy as a stock culture.

The wide variation in the sizes of the regenerating nodules (Fig. 7) is of interest in view of the report of Nias *et al.* [20] that there was a marked spreading in the number of cells per clone which developed when isolated HeLa cells were subjected either to a single dose of X-rays or continous irradiation with tritium β-particles.

The nature of the regeneration indicates that relatively few cells survive to develop as three dimensional clones, and two possible explanations may be relevant. The surviving cells may be privileged by their position in the cell cycle at the time of irradiation [21, 22] and have a high resistance which is purely transient. This idea alone is unlikely to be adequate to explain the effects following high doses of radiation when only a small percentage of cells survive and the viable cells are then probably chosen also on a statistical basis. In the latter case we must suppose that there must be a number of critical events within each cell since a large number of contributing events and appreciable interaction between degenerating cells would rapidly average out statistical variations. The most obvious choice for these events is a direct action on the DNA molecule, but it is difficult to reconcile this idea with the relative insensitivity of the rate of DNA synthesis unless much of the ^{3}H-thymidine is incorporated after irradiation into non-functional DNA.

There are many similarities between this phenomenon of regenerating nodules and the results of experiments in which survival curves are plotted for cells in tissue culture. Over a limited range of radiation doses, the method reported here can perhaps be developed to supplement the normal procedure. However, since there is no technique by which the initial cell concentration can be modified it is unlikely that survival over several orders of magnitude can be studied. In the present series of experiments, for doses lower than 400 R regeneration was so extensive that it did not appear in the form of nodules while Fig. 9 shows that already after 800 R, the most probable number of surviving nodules per culture is zero. If an approach based on survival curves is developed successfully, the average number of regenerating nodules per culture at each radiation dose will have to be recorded, but to learn anything about the type of dose-survival curve, it will certainly be necessary to extend the range of doses beyond that which is at present possible.

Most of the nodules of regeneration which appear between 20–30 days after radiation are at the periphery of the tumour. A comparison of Fig. 3 with recently published work by Rajewsky [23] and supporting evidence from our own respiration measurements would suggest however, that this is the oxygenated part of the tumour at the time of irradiation and would be expected to be more sensitive. Thus it is possible that the post-irradiative conditions, particularly nutritional factors, for the suppression of recovery are even more important than the conditions at the time of irradiation and also completely different. No comment can be made concerning the time after irradiation at which the conditions should be changed.

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RESUME

La tumeur humaine, utilisée dans ces expériences, est maintenue en culture organotypique par Wolff et Wolff depuis quelques années. Quand ce matériel est soumis à une seule dose de rayons X, les résultats indiquent que des changements de l'index mitotique, une nécrose intense et la mort de la tumeur sont des conséquences de l'irradiation, tandis que le métabolisme de l'ADN et la respiration sont peu altérés. Si l'on administre une deuxième dose de rayons X, égale à la première, 5 jours plus tard, on constate que, au niveau cellulaire, la récupération est déjà presque terminée. La régénération morphologique n'apparaît que 15 jours au moins après irradiation, et, aux doses les plus élevées, elle se manifeste sous forme d'un petit nombre de nodules de tissu sain. Après une deuxième dose de rayons X, appliquée 42 jours après la première, la tumeur irradiée a la même radiosensibilité que les cellules primitives, ce qui prouve qu'après ce delai la récupération est complète.

SUMMARY

The human tumour used in these experiments has been maintained by Wolff and Wolff in organo-typic culture for a number of years. When the material was subjected to a single

dose of X-rays, results showed that changes in mitotic index and the production of severe necrosis and tumour death are radiosensitive processes while DNA metabolism and respiration are relatively insensitive.

By delivering a second equal dose of X-rays 5 days after the first, it could be shown that recovery at the cellular level was already practically complete. Morphological recovery was not visible until at least 15 days after irradiation and at higher doses took the form of regenerating nodules of healthy tissue. A second dose of X-rays at 42 days indicated that this new material exhibited the same radiosensitivity as the original.

ZUSAMMENFASSUNG

Der in diesen Versuchen verwendete menschliche Tumor wurde von Wolff und Wolff seit mehreren Jahren in organotypischer Kultur weitergezüchtet. Wurde dieses Material einer Röntgenbestrahlung ausgesetzt, so treten als Folge Veränderungen des Mitoseindexes, starke Nekrotisierung und Tod des Tumors ein, während DNS-Stoffwechsel und Atmungsvorgänge relativ strahlenunempfindlich sind. Durch eine zweite, gleich dosierte Bestrahlung, fünf Tagen ach der ersten, konnte nachgewiesen werden, dass auf Zellebene eine schon nahezu vollständige Erholung stattgefunden hatte. Eine morphologische Restitution war erst nach wenigstens 15 Tagen sichtbar; bei Verabreichung höherer Dosen nahm sie die Form von Knötchen regenerierenden gesunden Gewebes an. Eine weitere Strahlendosis nach 42 Tagen wies darauf hin, dass dieses neue Gewebe in der gleichen Art strahlenempfindlich ist wie das ursprüngliche.

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Tumour Reoxygenation During Fractionated Radiotherapy; Studies with a Transplantable Mouse Osteosarcoma

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INTRODUCTION

THE PRESENCE of radioresistant poorly oxygenated or anoxic cells in tumours has in the last decades been recognized as a major obstacle to successful radiotherapy [1, 2]. According to this concept, which has been heavily supported by many experimental studies, small numbers of anoxic cells may have a decisive importance. Even if only about 1% of the cells are initially anoxic, these cells will determine the radio-curability because a moderate dose of radiation will eliminate the well-oxygenated cells, leaving a radioresistant anoxic cell population which requires very high radiation doses for complete elimination [3].

Recently evidence has been accumulated showing that in a number of experimental tumours the preferential elimination of the more radio-sensitive well-oxygenated cells by radiotherapy is followed by an improved oxygenation of the surviving anoxic cells [4, 5] and in one experimental tumour this reoxygenation mechanism completely prevented the expected increase in the fraction of anoxic cells among the surviving tumour cell population during fractionated irradiation [6]. The existence of such a reoxygenation mechanism during radiotherapy of tumours is most important for our understanding of the consequences of the oxygen effect in radiotherapy and further studies about its occurrence in different tumours were thought desirable.

The present report concerns experiments with a transplantable osteosarcoma, in which the occurrence of reoxygenation in the course of fractionated radiotherapy was studied in a similar way as has been done earlier in a subcutaneous sarcoma [6]. The results show that the osteosarcoma has a much lower capacity for reoxygenation of its anoxic cells than was observed in the subcutaneous sarcoma, and the possibility cannot be excluded that under the pretreatment regimes tested no reoxygenation occurred at all.

MATERIALS AND METHODS

Tumours

Osteosarcoma C22LR developed in 1957 in a female (C57BL/Rij × CBA/Rij)F_1 hybrid mouse that had been injected with strontium-90 [7]. It was serially transplanted by subcutaneous injection of a coarse tumour brei until 1959 and then stored as a cell suspension with 10% glycerol at low temperature (initially at −80°C, later at −196°C) until 1964. Since then it has been serially transplanted by subcutaneous injection of cell suspensions. For the present experiments use was made of passages 70–91, and since a large number of cells of passage 75 was stored in liquid nitrogen, the majority of the studies could be performed with passages 76–81.

Tumour cell suspensions were prepared by the trypsinization method described by Rein-

hold [8]. Endpoint dilution titration was carried out by a modification [9] of the method of Hewitt [10]. One further modification was introduced; the four injections per mouse were administered subcutaneously on the back of the animal, in the neck, the flanks and over the sacrum, rather than in the axillary and inguinal areas. This was done in order to facilitate differentiation between tumour growth at the inoculation sites and possible lymph node metastases.

Before and after the irradiation studies, tests were made to ascertain whether the tumour was antigenic in its host of origin.

Irradiations

All exposures of tumours *in situ* were given as whole body irradiation of unanaesthetized mice. The animals contained in a perforated lucite box, were exposed to X-rays from a Maxitron, operated under the following conditions: 250 kVP, 30 mA, half-value layer of the beam 2·1 mm Cu, distance from target to centre of the mouse 34 cm, dose rate about 170 rad/minute. In order to obtain a homogeneous dose distribution, full back-scatter was employed, using a 10 mm thick stack of masonite plates under the lucite box. Dose variations over a mouse phantom of tissue-equivalent materials was measured to be less than 5%. Each irradiation was monitored by exposure of a Baldwin "Ionex" dosimeter in a position on top of the box.

In vitro irradiation of tumour cell suspensions was performed under intensive aeration in a rotating suspension bottle as described by Reinhold [8]. *In vitro* irradiation under anoxic conditions was performed by blowing nitrogen containing less than 5 ppm oxygen over the suspension before and during irradiation. A Hersch cell [11] was used to monitor the oxygen content of the gas after passing over the suspension.

Oxygenation studies in situ

For each experiment in the oxygenation study, mice were inoculated subcutaneously in the flank. Four to six animals were randomly allocated to each of the following treatment groups:

(a) to serve as unirradiated controls,
(b) to be irradiated while breathing air,
(c) to be irradiated after having been killed with nitrogen.

After irradiation, tumours were collected, pooled for each group and cell suspensions were prepared which were inoculated in suitable dilutions into groups of recipients. These were observed for 100 days and the number of inocula that gave rise to the growth of a palpable tumour was registered. The method of Spearman and Karber was used to estimate for each suspension the number of cells required to produce a tumour in 50% of inocula (TD_{50}) and the fraction of surviving cells was calculated by comparing the TD_{50}'s of irradiated and control suspensions. The accuracy of these titrations is usually such that the 95% confidence limits are between 70 and 140% of the TD_{50}.

As has been described in detail [6] it is possible to derive the fraction of anoxic tumour cells in the living mouse by comparing dose-effect curves for irradiation of the tumour in living and dead animals. At high radiation doses, where the number of surviving well-oxygenated cells is negligible compared to the number of surviving anoxic cells, the ratio of the fraction of surviving cells after equal radiation doses given to the tumour *in vivo* and to the completely anoxic tumour is identical to the fraction of anoxic cells in the tumour. In the present studies the minimal radiation dose used in such studies was 1000 rad.

Pretreatment

In the course of fractionated irradiation the well-oxygenated cells are preferentially eliminated. In order to study the degree of reoxygenation of previously anoxic cells in the course of fractionated treatment, oxygenation studies were carried out on tumours that had been exposed to 250 rad on each of the four days preceding the oxygenation tests.

RESULTS

(a) *Tests of the suitability of the tumour for use in the endpoint titration system*

It is well-known that the presence of antigenic differences between tumour and host may interfere with tumour growth and tumour cell survival [9, 12]. Similarly, a growth-stimulating effect of radiation sterilized cells may affect the titration results [13, 14]. In order to test the suitability of the osteosarcoma, tests were performed of the effect of adding heavily irradiated cells to the titration suspension, and of the effect of prior immunization of the recipients. For the latter purpose, two subcutaneous injections of 10^6 tumour cells that had been irradiated *in vitro* with 10,000 rad were given at two-week-intervals before titration of a viable tumour cell suspension. Titrations were then performed simultaneously in immunized and non-immunized control mice with and without addition of heavily irradiated cells. The results are presented in Table 1.

Table 1. *Effect of immunization of the recipients and of addition of heavily irradiated cells on the* TD_{50}

Controls	Immunized	Controls +H.R. cells	Immunized +H.R. cells
390	112	6·6	
458	161	5·5	
2826	3821	3·7	11·2

The results are expressed as the TD_{50}, that is the number of cells calculated to produce a take of a tumour in 50% of inoculation sites. The differences between simultaneous titrations in immunized and control mice are not significant. The effect of addition of heavily irradiated cells is highly significant ($P<0·01$).
+H.R. cells=to each inoculum 250,000 heavily irradiated (10,000 rad) cells had been added.

Although there is a wide variation in titration results a systematic difference between titrations in control and immunized recipients is not evident. The marked influence of the addition of heavily irradiated cells to the tumour inocula stresses the importance of precautions to prevent distortion of dose-survival curves [14]. For this reason, 250,000 irradiated cells (10,000 rad) were added to each inoculum in all tumour cell titrations.

(b) In vitro *irradiations*

The results of *in vitro* irradiation are presented in Fig. 1. The parameters of the dose-survival curve for irradiation of tumour cells in equilibrium with air are:

$D_0=127$ rad, $n=5·3$

and for cells in equilibrium with nitrogen:

$D_0=273$ rad, $n=10·1$

the ratio of the slopes of the curves in air and nitrogen is 2·2.

(c) In situ *irradiation*

Under anoxic conditions as obtained by killing the mice with nitrogen 1 minute before the irradiation, the survival curve is characterized by:

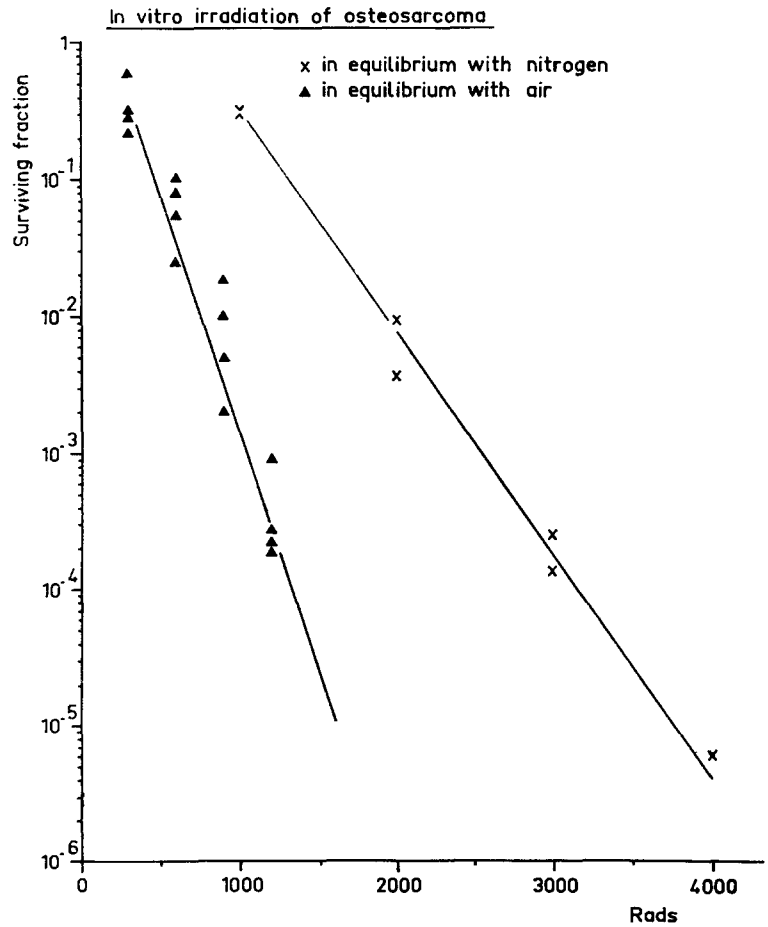


Fig. 1. *Semilogarithmic representation of the dose survival curves obtained by irradiation of tumour cells* in vitro. (*Straight lines were fitted by the method of least squares.*)

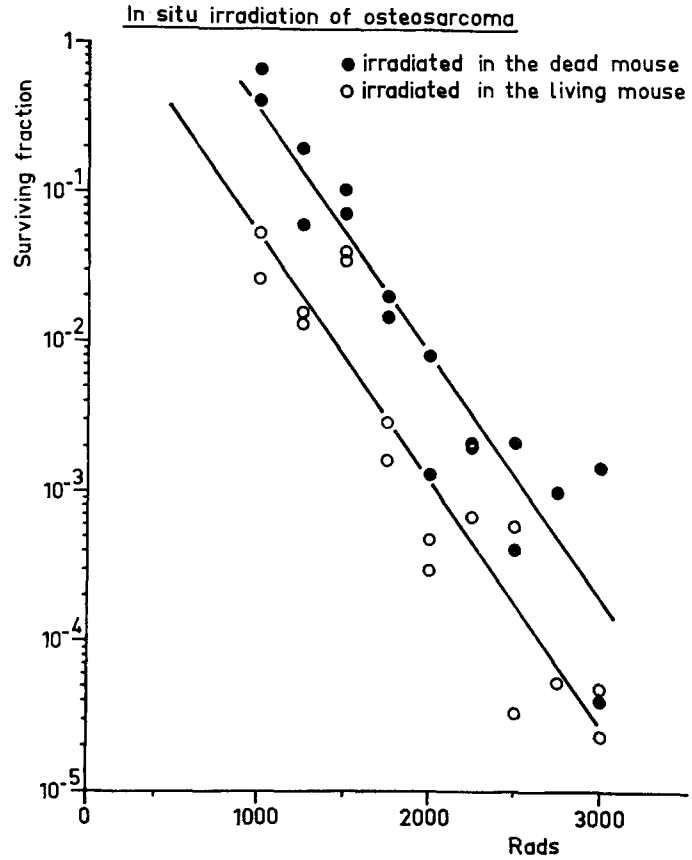


Fig. 2. Semilogarithmic representation of the dose survival curves for tumour cells irradiated in situ.

D_0=263 rad, n=15·3 (Fig. 2).
If the animals are breathing air during irradiation the parameters are:
D_0=262 rad, n=2·3.
Thus the two *in situ* curves are almost parallel and since the slope is typical for the anoxic cell population it seems logical to assume that in the living mouse the survival of well-oxygenated cells after irradiation with doses in excess of 1000 rad is negligibly small compared to the

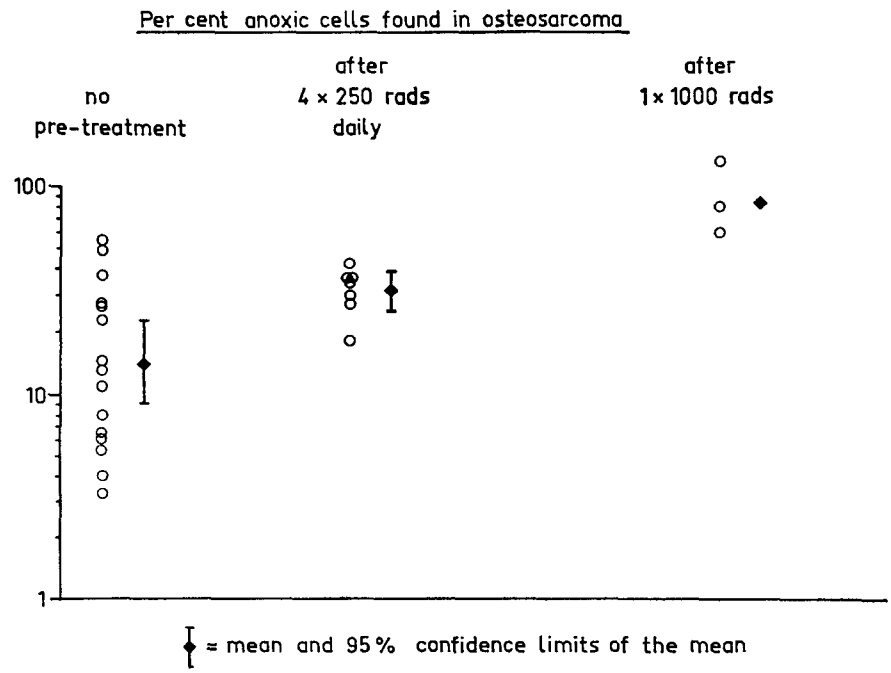


Fig. 3. Graph of the observed anoxic cell fractions in each simultaneous test performed under various experimental conditions in the osteosarcoma.

survival of the anoxic cells which are present. The surviving fractions after irradiation in the living mouse are lower than in the dead mouse since the anoxic population is smaller in the living mouse. It will be evident that the ratio of the surviving fractions observed under these two types of irradiation is identical to the anoxic cell fraction [6]. Figure 3 shows the anoxic cell fractions, as observed at each separate experiment; the geometric mean is 14·3% anoxic cells.

(d) *Effect of preceding fractionated irradiation*

The studies in mice that had been pretreated with four daily doses of 250 rad are presented in Fig. 4. For irradiation in the dead mouse the D_0 was 278 rad and n was 6·5; the survival curve of living mice gave a D_0 of 282 rad and an extrapolation number of 1·8. The anoxic cell fractions observed in these studies are also presented in Fig. 3. The mean anoxic cell fraction, 31·8%, differs significantly from that observed in tumours that had not been pretreated ($P<0·001$).

(e) *Effect of pretreatment with a single dose*

The anoxic cell fraction was determined after a pretreatment dose of 1000 rad. The survival ratios for the 1000 rad test dose given 48 hours later to the tumour in the living and in the dead mice were: 0·818, 1·367 and 0·605; and the mean content of anoxic cells, calculated from these figures was 88%.

DISCUSSION

Reinhold's rhabdomyosarcoma in the rat contained 15% anoxic cells [15]; the C3H sarcoma in which oxygenation studies were performed earlier [6] contained 14% anoxic cells and the osteosarcoma on which this paper reports also contains 14% anoxic cells. This similarity might be coincidental; the figure of 14% is the average of a number of widely varying observations as is shown in Fig. 3. On the other hand it is not impossible that some inherent parameter of transplantable tumour growth around capillaries with anoxic cells at the margin of the viable cells leads to a condition where the mean fraction of anoxic tumour cells may show little variation from one tumour to another. Hewitt [16] described a sarcoma with >50% anoxic cells and a differentiated squamous carcinoma with 18% anoxic cells. Clifton *et al.* [17] found 21% anoxic cells in an adenocarcinoma.

The radiation survival curves after pretreatment are different from those in the non-pretreated mice. A comparison of the

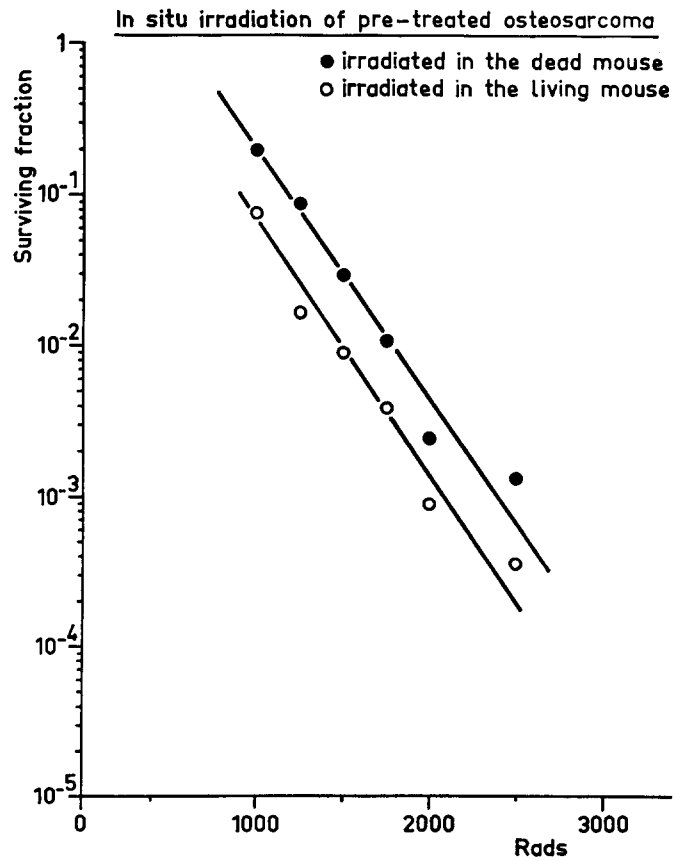


Fig. 4. *Semilogarithmic representation of the dose survival curves for cells from tumours irradiated* in situ, *which had been subjected to pretreatment with* 4×250 *rad.*

parameters of the curves shows that — contrary to expectation — the increase of the anoxic cell fraction seems mainly due to a displacement of the curve describing the survival of tumour cells irradiated in dead mice.

The survival curve of tumour cells irradiated in living mice has been displaced to a much smaller extent. This phenomenon seems to cast some doubts on the validity of our conclusion that the technique used is valid for the measurement of anoxic cell fractions, and for this reason it seems indicated to formulate here an unambiguous interpretation of our observed survival ratio. If the high-dose-regions of cell survival curves are represented by two parallel lines for two conditions of irradiation, the interpretation is that both curves represent the survival of the most radioresistant cells common to both populations and that the relative fractions of these cells in the populations determine the distance of these parallel lines. Since the only difference between the two conditions in these studies is the life or death of the mouse bearing the tumour, it seems logical to consider anoxia as the only relevant parameter. On theoretical grounds a case could be made for an effect of other nutritional influences of an intact circulation on the radiosensitivity, but relevant factors other than oxygen have so far not been identified. Thus we have to assume that the difference between the curves in living and dead mice after pretreatment is caused by a difference in the size of the fraction of anoxic cells. The fact that the lower curve seems hardly displaced, whereas the upper curve was lowered in comparison to the non-pretreated controls might be interpreted to show that perhaps in the pretreated tumour killing of the mouse does not cause complete anoxia. Such a possibility seems absurd; no change in oxygen uptake of tumours is noted after the radiation doses involved and in the mouse a rapid decrease in blood and tissue oxygen occurs after breathing nitrogen [18].

Thus our conclusion must be that the different survival ratio represents a true difference in the anoxic tumour cell fraction in the living mouse and that the similarity of the survival curves in living mice with and without pretreatment is a coincidence. This implies that another explanation must be found for the displacement of the survival curve for tumour cells under complete anoxia. Perhaps this could be found in the occurrence of incomplete recovery from the pretreatment irradiation damage in the survivors or by a partial synchronization due to the preceding treatment. However this may be, it seems likely that such factors will affect the survival curves both in living and dead mice and so the results may confidently be interpreted as showing that after four doses of 250 rad the mean anoxic cell fraction in this osteosarcoma rises to 32%. Table 2 compares these results in the osteosarcoma with some data obtained in the C3H sarcoma mentioned earlier.

In order to evaluate to what degree reoxygenation of previously anoxic cells occurred under these conditions, it is necessary to make an estimate of the minimal value of the anoxic cell fraction if no reoxygenation were present. For this estimate we must know the fraction of the cells surviving the four pretreatment doses; this can be approximated by comparing the control tumour cell titrations with those of the tumour after four times 250 rad. From the ratio of the TD_{50} results of these titrations a survival of 15% of the tumour cells can be estimated. In addition some suppositions have to be made of the relative radiosensitivity of the oxygenated and anoxic cells*.

If we assume a minimum oxygen enhancement ratio (O.E.R.) of 2·0 we can conclude that a similar surviving fraction can be reached by a dose of 125 rad to the oxygenated cells and by 250 rad to the anoxic cells. Next we assume that the effect of 250 rad to oxygenated cells is approximately similar to that of two separate doses of 125 rad. These suppositions lead to the conclusion that the surviving fraction after 250 rad to oxygenated cells will be identical to the square of the surviving fraction after 125 rad to oxygenated cells. The latter is identical to the survival after 250 rad in anoxic cells. (Both this approximation and the supposition of an O.E.R. of 2 tend to underestimate the difference between anoxic and oxygenated cell sensitivity, and the anoxic cell fraction calculated on the basis of these assumptions will thus be a minimal value). If the survival after 250 rad in anoxic cells is S_a and in oxygenated cells S_o, $S_o=S_a^2$. Now we can write out the effect of four pretreatment irradiation doses of 250 rad each to a tumour with 14% anoxic cells:

$$\text{The overall survival } S=0\cdot15 \text{ (observed)}$$
$$=0\cdot86(S_o)^4+0\cdot14(S_a)^4=$$
$$=0\cdot86(S_a)^8+0\cdot14(S_a)^4$$

Solving this equation leads to an anoxic surviving fraction after a single dose of 250 rad, $S_a=0\cdot766$ and an oxygenated surviving frac-

*The cell survival at these low doses cannot be taken from the survival curve (Fig. 1) with enough accuracy for these calculations.

Table 2. Anoxic cell fractions in two transplantable tumours

	Not previously irradiated	After 4×190 resp. 4×250 rad	After 950 resp. 1000 rad
KHT sarcoma (C3H)	14%[6]	14%[6]	34% [19]
Osteosarcoma C22LR (CBA×C57BL)F_1	14%	32%	88%

tion, S_o=0·587 and the final survivors of the original total population are 4·8% anoxic cells and 10·2% oxygenated cells; total survival 15%. The 4·8% anoxic cells represent 32% of the total surviving population which is almost identical to the mean measured value and this suggests that it is possible that no reoxygenation occurred in this tumour at all.

It is of interest to compare this estimate for the osteosarcoma with earlier studies in another tumour [6], a transplantable sarcoma in the C3H mouse. Similar calculations for this tumour in which the cell survival after 4 doses of 190 rad was 9%, yields after each dose a surviving fraction of 71% of the anoxic and of 50% of the oxygenated cells resulting after 4 doses in a minimum anoxic fraction of 40%. This is significantly different from the observed value of 14%; thus in this C3H tumour a marked degree of reoxygenation of the previously anoxic cells must have occurred. The difference in capacity for reoxygenation is further emphasized by the results after 1000 rad resp. 950 rad. In the osteosarcoma this resulted in a mean anoxic cell fraction of 88%, whereas in the C3H sarcoma Kallman [19] found 34%; in both cases the value expected without reoxygenation is about 90%. Although the former data were collected 48 hr and the latter 72 hr after irradiation it seems unlikely that this can explain the different results. The osteosarcoma is evidently much slower in reoxygenating its anoxic cells after irradiation than the C3H sarcoma. It should be stressed that our observations do not cover all possible pretreatment schedules or time intervals; it is very likely that other conditions may indeed show that some reoxygenation does indeed occur, although too late to be of importance for fractionated radiotherapy. Thus the findings do not argue against the validity of Thomlinson's general reoxygenation model [5]; they merely indicate a pronounced difference in degree or in time-course.

The observation of such a difference in reoxygenation between different tumours is very important since it implies another addition to the list of parameters which determine the curability of a tumour by fractionated radiotherapy. In Table 3 these parameters have been listed for a hypothetical — highly radiosensitive — tumour and the total dose of

Table 3. Fifty per cent cure doses for an assumed standard tumour after treatment in 20 fractions as influenced by various parameters

Parameters		Standard	Modifications					
N, cells/tumour		10^8	**10^{10}**	10^8	10^8	10^8	10^8	10^8
D_0 (rad)*		100	100	**175**	100	100	100	100
n†		3	3	3	**10**	3	3	3
O.E.R. of the slope‡		2·3	2·3	2·3	2·3	**3·0**	2·3	2·3
Fraction of anoxic cells		1%	1%	1%	1%	1%	**15%**	1%
Repopulation§		—	—	—	—	—	—	**×64**
50% cure doses (rad) when reoxygenation between fractions is:	complete	4200	4750	7350	7600	4200	5200	4700
	absent	8060	9400	14,105	17,460	10,513	8900	9300

*D_0 is the dose increment required to reduce survival of well-oxygenated cells to e^{-1} in the exponential portion of the dose-effect curve.

†The extrapolation number, obtained on a semilogarithmic plot by extrapolating the exponential portion of the survival curve to zero dose.

‡O.E.R.=oxygen enhancement ratio. Here it is the ratio relating D_0 for anoxic cells to D_0 for well-oxygenated cells.

§Multiplication of cells during the fractionated treatment.

Table 4. Fifty per cent cure dose for a hypothetical tumour with a survival curve similar to that of T_1 cells [20] *and HeLa cells* [21]

Parameters		Standard	Modifications		
N (cells/tumour)		10^8	**10^{10}**	10^8	10^8
Fraction of anoxic cells		15%	15%	**1%**	15%
Repopulation		—	—	—	**×64**
50% cure doses (rad) when reoxygenation between fractions is:	complete	6300	7600	5700	7500
	absent	15,300	17,500	13,400	17,200

radiation needed to cure 50% of tumours in 20 fractions has been calculated. Similar calculations have been presented for tumours in which each of the parameters in turn is changed. This permits an evaluation of the importance of each parameter in determining the dose necessary for cure of the tumour. It is evident that the degree of reoxygenation capacity of the tumour is the variable which causes the most marked change in the cure dose. Of course the parameters for this tumour have been selected arbitrarily and the dose-survival curve is perhaps not representative for any experimental cell population. In Table 4 data for another hypothetical tumour have been presented. In this case the dose survival curve was made identical to that observed experimentally for Barendsen's T_1 cells [20], which is very similar to the radiosensitivity of HeLa cells as observed by Hall *et al.* [21]. The calculated cure doses are in this case also very markedly affected by the phenomenon of reoxygenation. It seems reasonable to assume the differences in reoxygenation occur between spontaneous tumours subjected to radiotherapy and these differences appear to be important in determining the cure dose. Conversely it may be supposed that the wide variation in curability between different tumour types may be mainly due to the variation that exists between these tumours in their capacity to reoxygenate the surviving tumour cells continuously in the course of fractionated radiotherapy. The phenomenon of reoxygenation may not only explain the empirical superiority of fractionation in radiotherapy, but it may also be of major importance in determining the curability of a tumour.

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RESUME

Nous avons étudié l'importance de l'oxygénation des cellules d'un ostéosarcome transplantable de souris C22LR. Des suspensions de cellules ont été faites, afin de déterminer la survie cellulaire selon la méthode de Hewitt, après irradiation de la tumeur in vivo, *ainsi qu'après irradiation des cellules tumorales en suspension.*

L'étude des courbes de survie a montré que le taux de cellules en anoxie s'élève à 14%. *A la suite de quatre irradiations de* 250 *rad chacune, ce pourcentage peut atteindre* 32%, *et, deux jours après une irradiation de* 1000 *rad, il s'élève à* 88%. *Les calculs de la survie des cellules tumorales montrent que la teneur en oxygène n'augmente pas (ou très rarement) dans les cellules en anoxie qui survivent. Ces résultats vont à l'encontre des observations précédentes, faites sur une autre tumeur transplantable, et il faut conclure que la capacité de réoxygénation peut varier d'une tumeur à l'autre.*

On a démontré que la capacité de réoxygénation des cellules en anoxie, au sein des tumeurs irradiées, était un facteur prédominant dans la détermination de la dose fractionnée curative. En clinique, en revanche, les différences de la radiocurabilité des tumeurs pourraient dépendre de cette capacité de réoxygénation.

SUMMARY

The oxygenation status of transplantable mouse osteosarcoma C22LR was studied from the radiation dose survival curves. Cell suspensions were prepared from this tumour and the radiosensitivity of both the suspensions in vitro *and the tumour* in situ *were determined by the endpoint dilution assay of Hewitt.*

From these survival curves, the mean anoxic cell fraction in the tumour in situ *could be determined at* 14%. *After four daily doses of* 250 *rad to the tumour, the anoxic cell fraction rose to* 32% *and two days after a single dose of* 1000 *rad* 88% *anoxic cells were found. A calculation of the fractions of surviving cells shows that little or no reoxygenation of previously anoxic cells had occurred in this tumour after the preferential elimination of oxygenated cells by the radiation. This contrasts markedly with earlier observations for another tumour and it seems likely that different tumours may differ greatly in their capacity to reoxygenate anoxic cells during fractionated therapy. Since this capacity was calculated to be most important in determining the radiation dose needed to cure a tumour, a difference in reoxygenation may well be responsible for a large part of the variation in curability by radiotherapy between different tumours.*

ZUSAMMENFASSUNG

Der Sauerstoffsättigungszustand von transplantierbarem Mausosteosarkom C22LR wurde an Hand seiner Strahlenempfindlichkeit studiert. Zellsuspensionen des Tumors wurden hergestellt, und die Strahlenempfindlichkeit sowohl der Suspension in vitro *wie des Tumors* in situ *wurde nach der Methode von Hewitt (d.h. durch den Endpunktverdünnungsversuch) bestimmt. Mittels dieser Empfindlichkeitskurven konnte die mittlere anoxische Zellfraktion des Tumors* in situ *bestimmt werden. Sie beträgt* 14%. *Bestrahlt man den Tumor täglich während* 4 *Tagen mit je* 250 *rad, so steigt diese Fraktion auf* 32% *an* 2 *Tage nach einer Einzeldosis von* 1000 *rad beträgt die Fraktion sogar* 88%. *Durch Berechnung der Fraktion der überlebenden Zellen konnte festgestellt werden, dass nur wenig oder keine Reoxygenation von vorher anoxischen Zellen stattfand, nachdem die sauerstoffgesättigten Zellen durch Bestrahlung eliminiert wurden. Diese Feststellung differiert von früheren Beobachtungen mit anderen Tumoren. Es scheint, dass verschiedene Tumoren sich ganz verschieden verhalten in Bezug auf ihre Fähigkeit, anoxische Zellen während fraktionierter Therapie zu reoxygenieren. Da diese Fähigkeit als sehr wichtig angesehen wird zur Feststellung der zur Heilung eines Tumors benötigten Bestrahlungsdosis, scheint die Differenz der Oxygenationsfähigkeit zum grossen Teil verantwortlich zu sein für die unterschiedliche Heilungsmöglichkeit verschiedener Tumoren durch Bestrahlung.*

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Epidermal Cell Population Kinetics after Repeated Applications of some Hyperplasia-producing Substances

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INTRODUCTION

IN A PREVIOUS paper [1], some alterations in the population kinetics of the hairless mouse epidermis, following a single application of benzene, cantharidin, and 20-methylcholanthrene, were reported. It was found that the reactions provoked by benzene and cantharidin could well be interpreted as simple regenerative phenomena. The late effects observed after a single application of methylcholanthrene could not, however, easily be explained in this way.

The aim of the present study is to follow some growth parameters of the epidermal cell population for a prolonged period after repeated applications of the same three hyperplasia-inducing agents. Three different growth parameters have been followed; the mitotic count, the mitotic rate, and the mitotic duration (for precise definition of these parameters, see reference 1).

MATERIAL AND METHOD

Hairless mice (hr/hr) were used in all experiments. The histology of their skin and the growth of the epidermal cell population have been well examined [2–6].

Three to four drops of benzene, 0·063% cantharidin in benzene, and 0·6% methylcholanthrene in benzene, respectively, were applied 3 times weekly to the interscapular area by means of a Pasteur pipette. Groups of 8 mice were killed at intervals after the first application. For further details about the experimental procedures, see [1] and [7].

The Colcemid method was used to estimate the mitotic rate and the mitotic duration. The mitotic rate was calculated by means of the equation: $R=C/t$, where R is the average mitotic rate, and C the number of arrested mitoses t hours after the injection. In the experiments $t=4$ hr. The mitotic duration was calculated by means of the equation: $D=$ M/R, where D is the mitotic duration, M the mitotic count, and R the mitotic rate. These equations should be used with precaution when working with tissues where changes in the mitotic rate occur rapidly. This has been thoroughly discussed elsewhere [7, 8].

The normal values for the mitotic count, the mitotic rate, and the mitotic duration in the epidermis of this particular strain of hairless mice were evaluated in a previous study [7], comprising 100 mice not injected with Colcemid, and 100 mice injected with Colcemid. The results were as follows:

Mitotic count (per 6 mm interfollicular epidermis): $6·74 \pm 1·29$;

Mitotic rate (per 6 mm interfollicular epidermis per hour): $6·36 \pm 1·19$;

Mitotic duration (hours): $1·07 \pm 0·016$.

EXPERIMENTS AND RESULTS

A. Benzene. The animals were treated 3 times weekly with 3–4 drops of benzene on the interscapular area of the back. Groups of 8 mice were killed 1, 3, 5, 7, 9, and 11 days, and 2, 3, 4 and 5 weeks after the first application. The animals killed on the 1st and on the 3rd day were sacrificed 1 day after the preceding application. All other groups were killed 2 days after the last application.

Table 1. *Repeated applications of* 3–4 *drops of benzene*

Time after first application	Mean mitotic count without Colcemid	SD of the mean	Mean mitotic count after Colcemid	SD of the mean	Mitotic rate	Mitotic duration
1 day	8·75	6·99	25·50	7·55	6·38	1·37
3 days	9·25	2·99	27·75	5·68	6·94	1·38
5 days	10·25	4·03	34·00	10·42	8·50	1·21
7 days	10·00	0·82	20·50	3·32	5·13	1·95
9 days	7·50	2·24	25·00	12·68	6·25	1·20
11 days	8·75	2·36	30·25	4·51	7·56	1·15
14 days	9·50	2·89	27·25	5·68	6·81	1·40
3 weeks	9·75	3·56	44·25	11·95	11·06	0·88
4 weeks	16·75	3·95	66·35	6·19	16·56	1·01
5 weeks	17·75	6·50	66·00	18·46	16·50	1·08

The results are shown in Table 1 and in Figs. 1 and 2. The mitotic rate remained within normal limits during the first 2 weeks, but from the 2nd to the 4th week it increased gradually to values about 3 times the normal. This increase was maintained for the rest of the experimental period. The mitotic duration was increased during the first 2 weeks, with a peak on the 7th day after the first application. During the period of increasing mitotic rate the mitotic duration decreased and was significantly lower than normal 3 weeks after the first application. For the rest of the period the mitotic duration was within normal limits. The mitotic count was increased by the 5th and by the 7th day after the first application, and during the period of increasing mitotic rate and nearly constant mitotic duration it reflected the alterations of the mitotic rate.

B. Cantharidin. The animals were treated 3 times weekly with 3–4 drops of 0·063% cantharidin in benzene on the interscapular area of the back. Groups of 8 mice were killed 1, 3, 5, 7, 9, and 11 days, and 2, 3, 4, and 5

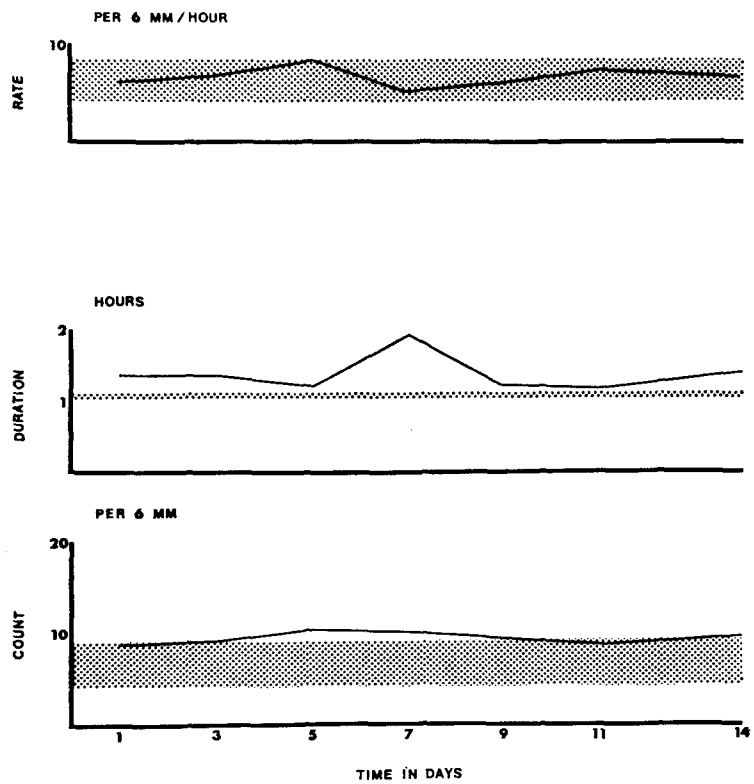


Fig. 1. *The mitotic rate, duration, and count during the first* 14 *days of repeated application of benzene. The dotted area represents* ±2 *standard errors of the method.*

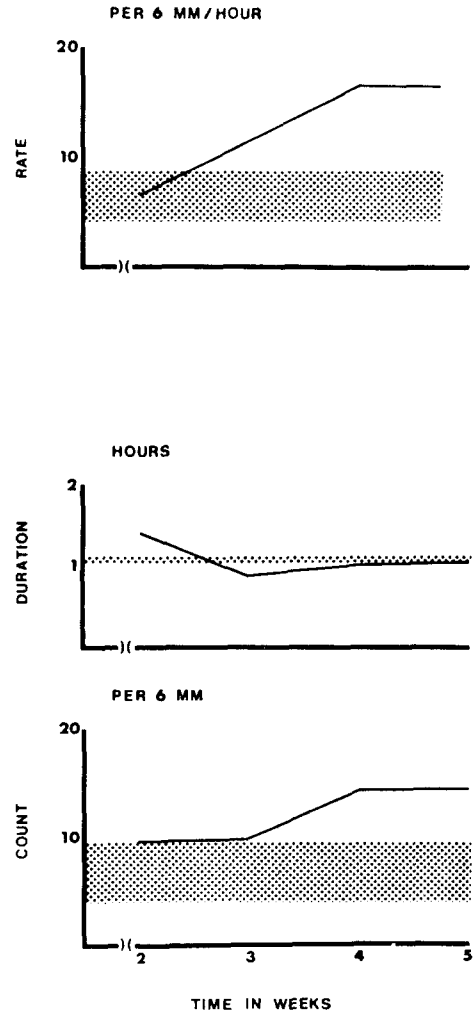


Fig. 2. *The mitotic rate, duration, and count from the* 3*rd to the* 5*th week inclusive, of repeated applications of benzene. The dotted area represents* ± 2 *standard errors of the method.*

weeks after the first application. Apart from the first 2 groups all groups were killed 2 days after the last preceding application.

The results are shown in Table 2 and in Figs. 3 and 4. The mitotic rate was greatly increased from the first day on, and continued to increase in a stepwise manner during the whole period. The increase was most pronounced during the last week and attained values about 8 times the normal by the end of the experimental period. The mitotic duration was slightly increased by the 5th and by the

Table 2. *Repeated applications of* 3–4 *drops of* 0·063% *cantharidin in benzene*

Time after first application	Mean mitotic count without Colcemid	SD of the mean	Mean mitotic count after Colcemid	SD of the mean	Mitotic rate	Mitotic duration
1 day	26·50	7·34	87·50	5·80	21·87	1·21
3 days	24·25	9·40	120·50	10·28	30·13	0·81
5 days	33·00	4·97	96·00	12·13	24·00	1·38
7 days	28·75	15·41	90·75	19·04	22·69	1·27
9 days	19·75	5·06	87·50	14·01	21·88	0·90
11 days	23·50	2·38	137·50	46·28	34·38	0·68
14 days	19·75	4·57	122·25	29·47	30·56	0·65
3 weeks	11·00	2·83	57·25	10·43	14·31	0·77
4 weeks	16·75	4·19	85·75	16·58	21·44	0·78
5 weeks	35·50	6·35	189·75	94·77	47·44	0·75

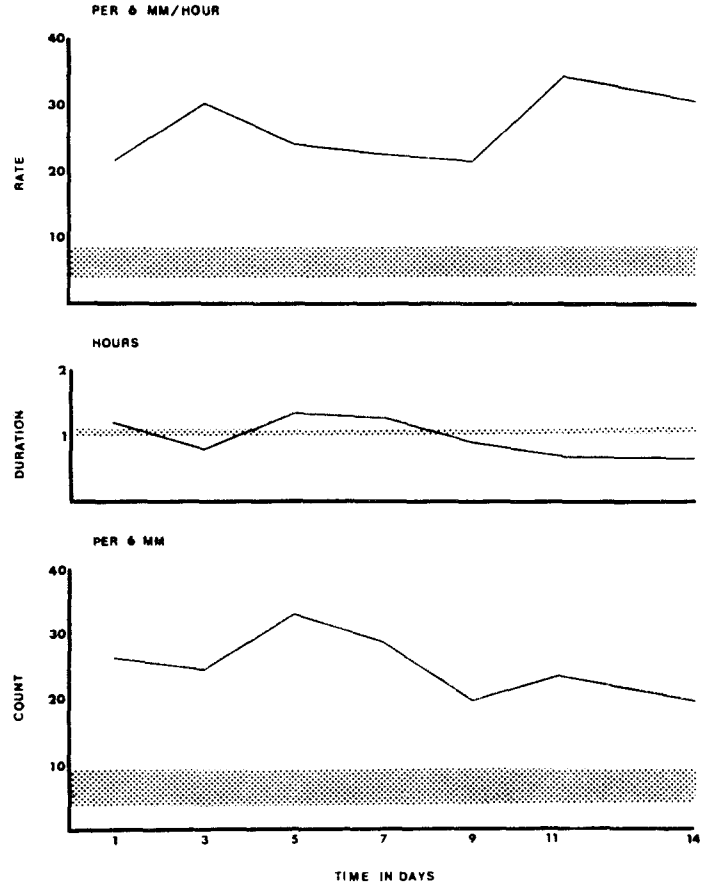


Fig. 3. The mitotic rate, duration, and count during the first 14 days of repeated applications of cantharidin in benzene. The dotted area represents ±standard errors of the method.

7th day. For the rest of the period, it was significantly shorter than normal, and, in spite of the wide fluctuations of the mitotic rate, the mitotic duration was relatively stable at values between 0·65 hr and 0·75 hr. The mitotic count was greatly increased during the whole period, mainly caused by the high mitotic rate. And as the mitotic duration was nearly constant during the last part of the experiment the mitotic count roughly reflected the fluctuations of the mitotic rate.

C. Methylcholanthrene. The animals were treated 3 times weekly with 3–4 drops of 0·6% methylcholanthrene in benzene on the interscapular area. Groups of mice were killed 1, 5, 7, 9, 12, 14, and 17 days, and 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, and 13 weeks after the first application. Apart from the first group all groups were killed 2 days after the last preceding application. The results are shown in Table 3 and in Figs. 5 and 6. The mitotic rate was slightly elevated on the first day. Further applications brought about a moderate decline in the rate which, however, was within the normal limits during the first week. After the 7th day, there was a sharp rise in the mitotic rate culminating on the 9th day. Two weeks after the first application, the rate returned to the normal range, only to exhibit a new increase by the 17th day. Between the 4th and the 12th week, the rate of cell proliferation was consistently elevated to values about twice the normal, but during the whole of this period it showed fluctuation which gave an impression of periodicity with an interval of 2 weeks between each peak of the curve. During the last week, i.e. from the 12th to the 13th week, the mitotic rate decreased to normal values.

The longest mitotic duration was found on the 5th day, when the cells spent about 3·4 hr in mitosis. During the first 3 weeks the mitotic duration tended to decrease relatively when the mitotic rate increased, and vice versa. From the 3rd week to the end of the experimental period, it increased gradually, and fluctuations in the mitotic rate did not affect the steady increase of the mitotic duration. The mitotic count was highly increased during the whole experiment but fluctuated widely. The high mitotic count was due partly to the long mitotic duration, partly to the increased mitotic rate.

Table 3. Repeated applications of 3–4 drops of 0·6% methylcholanthrene in benzene

Time after first application	Mean mitotic count without Colcemid	SD of the mean	Mean mitotic count after Colcemid	SD of the mean	Mitotic rate rate	Mitotic duration
1 day	19·25	5·44	39·25	5·44	9·81	1·96
5 days	23·25	6·70	27·50	2·38	6·88	3·38
7 days	15·75	4·57	20·75	5·56	5·19	3·03
9 days	29·50	7·55	65·50	17·52	16·38	1·80
12 days	22·75	5·56	45·00	8·79	11·25	2·02
14 days	19·00	2·16	30·50	11·03	7·63	2·49
17 days	26·50	6·61	46·75	6·51	11·68	2·27
3 weeks	15·50	1·73	33·25	4·34	8·31	1·87
4 weeks	16·50	1·73	42·00	1·41	10·50	1·57
5 weeks	18·25	0·96	40·25	10·40	10·06	1·81
6 weeks	26·00	3·46	54·75	10·26	13·69	1·90
7 weeks	22·25	4·11	38·50	6·58	9·63	2·31
8 weeks	36·00	2·16	59·50	17·12	14·88	2·42
9 weeks	28·50	2·65	43·25	7·81	10·81	2·64
10 weeks	40·25	7·14	56·25	8·99	14·06	2·86
11 weeks	30·25	7·14	42·75	3·95	10·69	2·83
12 weeks	29·00	7·07	52·50	8·81	13·13	2·21
13 weeks	22·25	4·19	30·25	11·41	7·56	3·01

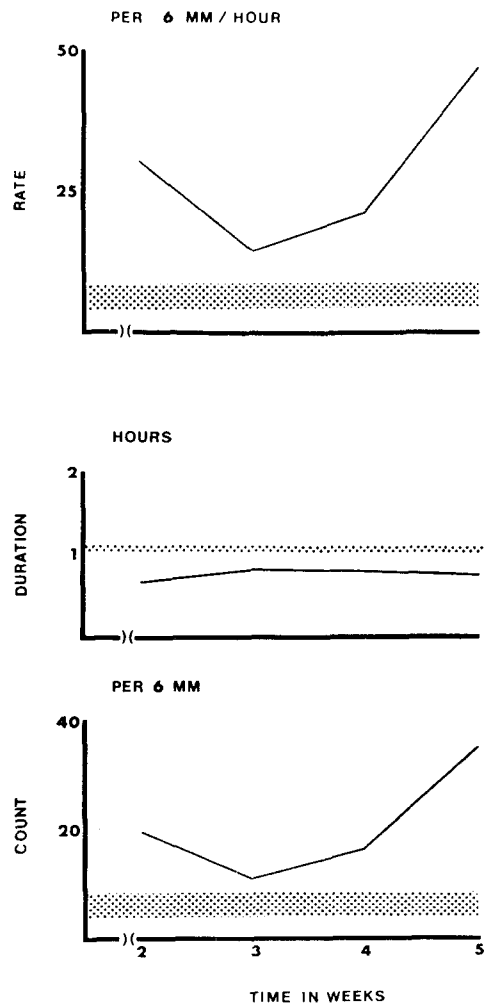


Fig. 4. The mitotic rate, duration, and count, from the 3rd to the 5th week inclusive, of repeated applications of cantharidin in benzene. The dotted area represents ±2 standard errors of the method.

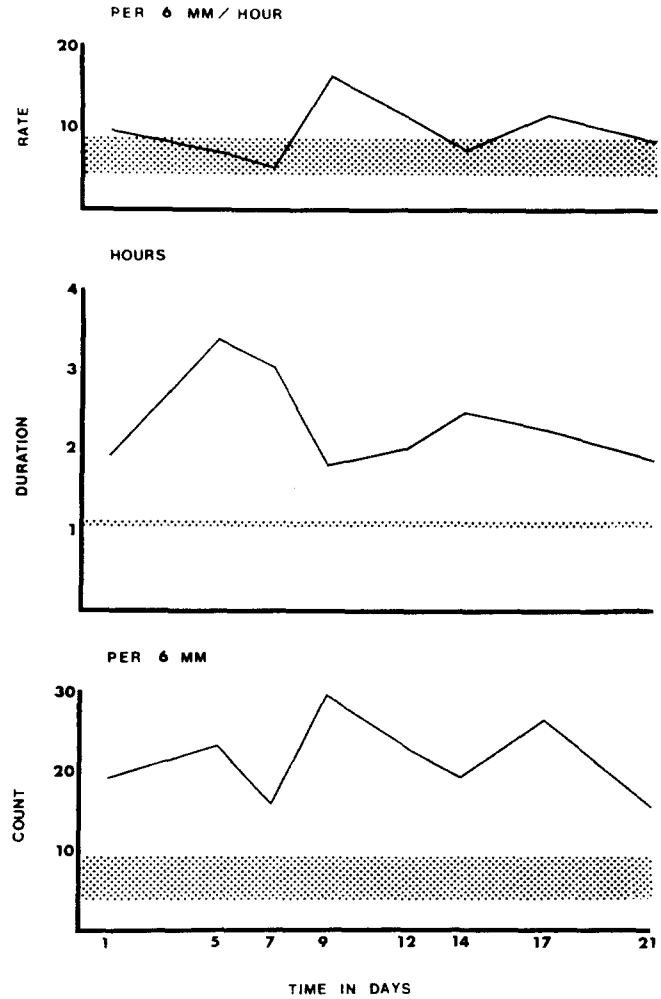


Fig. 5. The mitotic rate, duration, and count during the first 21 days of repeated applications of 0·6% methylcholanthrene in benzene. The dotted area represents ±2 standard errors of the method.

DISCUSSION

All the three agents tested in this series of experiments induced profound alterations in the kinetics of the epidermal cell population. The cell renewal of this population is most probably regulated by some negative feedback mechanism [6–14]. The feedback signal from the differentiating cells to the dividing basal cells is probably represented by the "chalone" described first by Bullough in 1962 [15]. Within the framework of this hypothesis the results presented in this study can be divided into 2 categories. The first category comprises the alterations brought about by benzene and cantharidin, as well as those found during the first 2 weeks of treatment with methylcholanthrene. These alterations are well compatible with regenerative phenomena in a cybernetic system, and they can even to a great extent be predicted by using a mathematical model based on the principles mentioned above [12]. From a strictly cybernetic and somewhat theoretical point of view, the alterations of the mitotic rate induced by benzene and cantharidin seem to ressemble oscillations with some tendency to self-excitation (for further discussion of this, see reference 7).

Another feature which characterizes this first category is that some inverse relationship between the mitotic rate and the mitotic duration can be discerned. An increasing mitotic rate is usually accompanied by a decreasing mitotic duration, and vice versa. But there is apparently some lower limit below which the mitotic duration cannot come, even if the mitotic rate is further increased. This is well illustrated by Figs. 3 and 4, and by Table 2.

The second category is represented by the alterations of the growth parameters observed after more than 4 weeks of repeated applications of methylcholanthrene. The outstanding feature during this part of the experiment was the continuous prolongation of the mitotic duration. By the 13th week, the mitotic duration thus lasted for about 3 hours. This prolonged

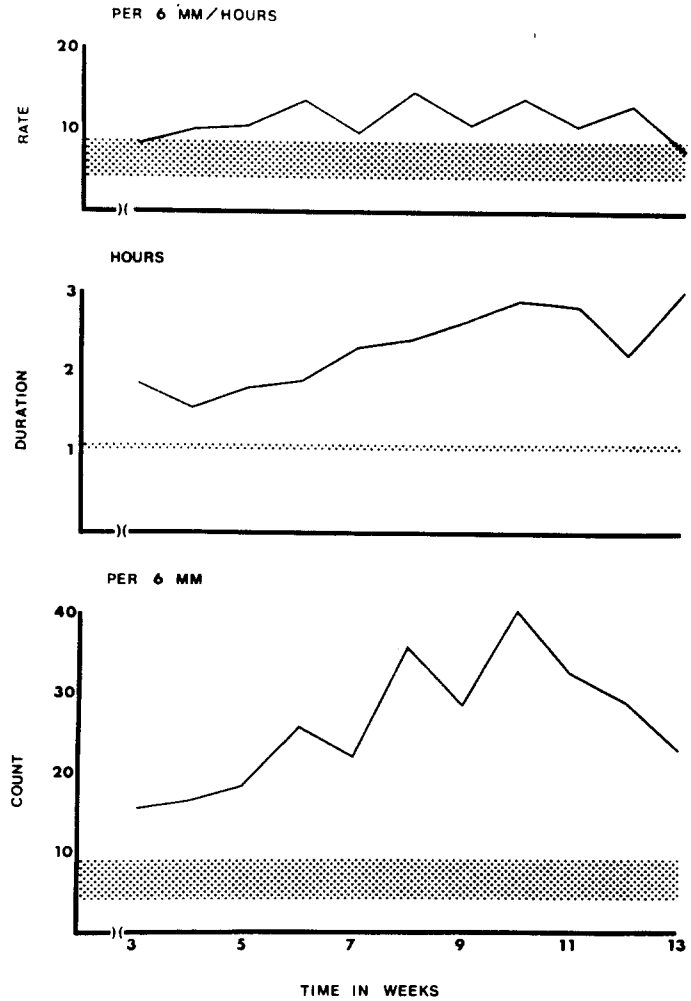


Fig. 6. *The mitotic rate, duration, and count, from the 4th to the 13th week inclusive, of repeated applications of* 0·6 % *methylcholanthrene in benzene. The dotted area represents* ± 2 *standard errors of the method.*

mitotic duration was accompanied by a slightly elevated mitotic rate, which oscillated around values twice the normal. The normal inverse relationship between these two parameters thus seemed to have disappeared, and the reaction cannot well be explained or predicted only on the basis that the epidermal cell population reacts as a cybernetic system. In this respect, the reaction to repeated applications of methylcholanthrene is similar to that found after a single topical application of this compound, which is also followed by a very long-lasting prolongation of the mitotic duration, without any corresponding alteration in the mitotic rate [1, 7]. Neither the experiments with a single application of methylcholanthrene nor the findings presented in this paper (Table 3 and Fig. 6) indicate why the mitotic duration is altered in this way.

The possibility exists that the epidermal cells treated with methylcholanthrene do not react to Colcemid in the same manner as do normal epidermal cells. Setälä [16] has shown that the topical pharmacological effect of Colchicine can be different in a normal epidermal cell population and in one treated with a carcinogen. To examine the effect of Colcemid on epidermal cells treated with methylcholanthrene groups of mice were killed at short intervals after the injection of Colcemid (for further details, see reference 7). It was found that the Colcemid-arrested mitoses accumulated proportionally to the time elapsed from the injection. Thus, the use of the Colcemid method seems to be justified even in these experimental circumstances and it is not probable that the prolongation of the mitotic duration can be accounted for by such methodological errors.

Application of a carcinogen to the mouse skin does not affect the epidermis only. Profound changes are regularly seen both in the dermis with its blood vessels [17–19] and at the dermo-epidermal junction [20]. As the dermis is of capital importance for the differentiation of the normal skin [19] the changes brought about in the dermis by methylcholan-

threne might well influence the growth of the epidermal cell population. It is still not known, however, how the interaction between the dermis and the epidermis takes place. At present, it is very difficult to see how the dermal changes could be related to the alteration of the various growth parameters.

When methylcholanthrene is applied 3 times weekly to the mouse skin the hydrocarbon is constantly present in the epidermis as well as in the dermis. The presence of methylcholanthrene can therefore consistently interfere with the metabolism of the epidermal cells. Several investigators have demonstrated that hydrocarbon carcinogens combine with the proteins of the skin and that this binding takes place at specific sites in the cells [21–27]. There is still controversy, however, as to which parts of the cells are most affected by the carcinogens. Some of the alterations found in the epidermal cell population kinetics have been related to an affection of the mitochondria [6], and atypical mitochondria have been observed in the electron microscope in methylcholanthrene-induced fibrosarcomas [28]. Other workers have emphasized the accumulation of hydrocarbon in the lysosomes [29, 30]. The progressive accumulation of carcinogens in the lysosomes may well interfere with their elimination of various wastes, and it may even damage the lipid membrane of the lysosomes resulting in a release of enzymes [30]. Amlacher and Graffi [31] have demonstrated that when cultures of fibroblasts are treated with 9,10-dimethyl-1,2-benzanthracene this hydrocarbon accumulates in the nuclei too. So, even though it may be difficult to evaluate at the moment what role these various findings can play it is evident that profound changes can well be brought about both in the metabolism of the single epidermal cell and in the kinetics of the epidermal cell population by repeated application of methylcholanthrene. On the other hand, the experiments with a single application of methylcholanthrene [1] revealed that the alterations of the growth parameters, in particular the alterations of the mitotic duration, can persist long after the methylcholanthrene had disappeared.

As mentioned above it is probable that the epidermal cells produce a "chalone" that act on the dividing basal cells, thus adjusting the production of new cells to the cell loss from the epidermal surface. Bullough and Laurence [32] have shown that high concentrations of "chalone" prolong the mitotic duration in *in vitro* experiments. It is therefore possible that the prolonged mitotic duration can be related to an increased amount of "chalone" produced by an increasing number of differentiating cells. But under these circumstances the mitotic rate is increased too, and that would imply that the "recognition" mechanism of the dividing cells were altered in such a way that they were only partially sensitive to an increased concentration of the inhibitor substance. At present, however, no experiments have been performed that can affirm or reject this hypothesis.

It would have been very interesting to know if the mean generation time and the other phases of the life cycle of the basal cells were prolonged too. This was not examined in the experiments presented in this paper, but some other investigations indicate that at least in malignant tumours there is no consistent correlation between the mean generation time and the malignancy of the cells [33]. Reiskin and Mendelsohn [34] have even shown that the various phases of the life cycle of the cells were shorter than normal in carcinomas induced by DMBA. The existing evidence thus indicates that the prolongation of the mitotic duration may be a unique phenomenon and that it is not accompanied by prolongation of other phases of the life cycle of the epidermal cells. Work is in progress at our institute to elucidate this problem.

CONCLUSION

The concept of the epidermal cell population reacting as a cybernetic system may well be applied to the alterations found after repeated applications of benzene or cantharidin, and it is even applicable to the alterations found during the first 3 weeks of repeated applications of methycholanthrene. The later changes observed after treatment with the latter agent are, however, not compatible with this point of view. Some more fundamental changes must be discovered.

RESUME

On a examiné les effets initiaux et les effets à long terme d'applications répétées, au niveau de la surface cutanée des souris "hairless" (hr/hr), de benzène, de cantharidine et de 20-méthylcholanthrène. Les paramètres de croissance de l'épiderme ont été évalués au moyen de la méthode à la Colcémide. Les réactions des cellules épidermiques ont été interprétées en tant que phénomène biologique et au point de vue de la cybernétique. Le benzène,

comme la cantharidine, provoque des altérations dans le dynamisme de la population épidermique, qui se prêtent à une explication en termes de cybernétique. Après des applications de méthylcolanthrène, la réaction initiale se trouve également en conformité avec ce concept. Mais plus tard, une prolongation considérable de la durée mitotique a été observée, en dépit d'un taux mitotique augmenté. Il était difficile de concilier ces résultats avec le système cybernétique simple. D'autres mécanismes semblent devoir être impliqués pour expliquer ces dernières réactions. Quelques explications plausibles sont discutées.

SUMMARY

The initial and the long term effects of repeated surface applications to the skin of hairless mice (hr/hr) of benzene, cantharidin in benzene, and 20-methylcholanthrene, respectively, have been examined. The growth parameters have been evaluated by means of the Colcemid method. The reactions of the epidermal cell population have been interpreted from cybernetic point of view and as biological phenomena. Both benzene and cantharidin provoked alterations in the dynamics of the epidermal cell population that can readily be explained in terms of cybernetics. The initial reaction after application of methylcholanthrene was also in conformity with this concept, but later on after treatment with this agent a striking prolongation of the mitotic duration was observed in spite of the fact that the mitotic rate was increased too. These findings were difficult to reconcile with the simple cybernetic system. Other mechanisms must be discovered. Some possible explanations are discussed.

ZUSAMMENFASSUNG

Die anfänglichen und langandauernden Wirkungen bei oberflächlichen Applikationen von Benzen, Cantharidin in Benzen und 20-Methylcholanthren in Benzen auf die Haut haarloser Mäuse (hr/hr) wurden untersucht. Die Wachstumsparameter wurden mit Hilfe der Colcemid-Methode bewertet. Die Reaktion der Epidermiszellen wurden von kybernetischen und biologischen Standpunkt gedeutet. Beide Substanzen, Benzen und Cantharidin, lösen Veränderungen in der Dynamik der Epidermiszellen, die man vom kybernetischen Standpunkt erklären kann, aus. Die anfängliche Reaktion auf die Applikation von Methylcholanthren stand ebenfalls mit dieser Auffassung im Einklang. Später, aber, wurde trotz einer schnellen Zellproliferation eine erhebliche Verlängerung der Dauer des mitotischen Prozesses gefunden. Diese Veränderungen konnten nicht mit einem einfachen kybernetischen System in Übereinstimmung gebracht werden. Es müssen also einige andere Mekanismen angenommen werden, um die Veränderungen der Wachstumsparameter während der letzten Wochen der Behandlung mit Methylcholanthren zu erklären.

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Particules Subcellulaires dans les Tumeurs

I. Distribution intracellulaire de la cytochrome oxydase, la glucose-6-phosphatase, la catalase et plusieurs hydrolases acides dans un hépatome chimique transplantable (hépatome HW)

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INTRODUCTION

L'ETUDE de la distribution intracellulaire d'enzymes, par les méthodes de centrifugation, permet non seulement d'identifier des organites subcellulaires dans un tissu donné, mais aussi de caractériser certaines propriétés physico-chimiques, et même fonctionnelles, de ces entités; les travaux de de Duve et son groupe, sur le foie de rat, en sont une illustration [1–4]. Le but de nos travaux est précisément d'analyser la distribution intracellulaire de différentes enzymes dans des tumeurs expérimentales, en mettant à profit les techniques de centrifugation différentielle et de centrifugation en gradient de densité utilisées par ces auteurs. De nombreux auteurs ont déjà étudié la distribution intracellulaire d'enzymes dans les tissus tumoraux, mais la seule méthode pratiquement utilisée est la centrifugation différentielle suivant la technique de Schneider et Hogeboom [5, 6].

Les tumeurs auxquelles nous nous sommes intéressés d'abord sont les hépatomes chez le rat. Ces tumeurs sont facilement induites par des moyens chimiques et leur transplantation est d'habitude aisée. De plus, au cours de ces dernières années, il a été possible d'obtenir des hépatomes transplantables de degrés divers de différenciation [7], aussi ces tumeurs représentent-elles un matériel biologique intéressant, non seulement d'un point de vue cancérologique mais aussi dans l'étude de la différenciation cellulaire.

Les premiers résultats que nous présentons ont été obtenus sur un hépatome chimique transplantable chez le rat, l'hépatome HW. Dans cet article, nous décrivons comment la cytochrome oxydase, la glucose-6-phosphatase, la catalase et plusieurs hydrolases acides se distribuent, après centrifugation différentielle d'homogénats de cette tumeur, suivant le schéma de de Duve *et al.* [1]. Les distributions sont comparées avec celles établies pour ces enzymes dans le foie de rat.

MATERIEL ET METHODES

Tumeur

L'hépatome HW est un hépatome induit à la nitrosomorphiline chez le rat; il est maintenu en transplantation sur des rats R, depuis de nombreuses générations, au laboratoire du Dr. C. Deckers (Institut du Cancer de l'Université de Louvain). Il se développe en 5 à 6 semaines et peut atteindre un poids de 40 à 50 g;

toutefois, nous avons utilisé généralement des tumeurs ne dépassant pas 20 g, à un stade où les phénomènes de nécrose sont peu marqués.

Centrifugation différentielle

Le rat est sacrifié par décapitation, et la tumeur rapidement disséquée et découpée en gros fragments qui sont placés dans du saccharose 0,25 M glacé. Une quantité pesée est utilisée pour l'expérience, elle est d'habitude de l'ordre de 10 g. Le tissu est découpé en petits morceaux, à l'aide de ciseaux, puis homogénéisé dans le saccharose 0,25 M à l'aide de l'homogénéiseur du type de Potter et Elvehjem [8], constitué d'un tube en pyrex et d'un piston en Teflon actionné par un moteur tournant à 3.000 tours/min. La suspension est centrifugée pendant 10 min à 1·200 tours/min dans une centrifugeuse réfrigérée International PR 2 (rotor no 253); le sédiment est passé à l'homogénéiseur, puis dilué et recentrifugé dans les mêmes conditions; l'opération est encore répétée une fois, après cette seconde centrifugation. Finalement, le culot est resuspendu dans un volume de saccharose 0,25 M correspondant, en ml, à un poids qui représente 4 à 5 fois le poids en g du tissu. Cette supension constitue la fraction *N*. Les liquides surnageants de chaque centrifugation sont mélangés, portés à un volume correspondant à un poid qui représente 5 fois le poids en g du tissu, pour constituer l'extrait cytoplasmique *E*. Le fractionnement de cet extrait se fait suivant la technique de de Duve *et al.* [1, 3], à l'aide d'une ultracentrifugeuse préparative Spinco L-HV (rotor 40). Une fraction mitochondriale 'lourde' *M*, une fraction mitochondriale 'légère' *L*, une fraction microsomiale *P*, et une fraction soluble *S*, sont isolées suivant le schéma de fractionnement décrit par ces auteurs.

Mesures enzymatiques

La cytochrome oxydase (E C 1.9.3.1.) a été mesurée suivant la méthode d'Appelmans, Wattiaux et de Duve [9]; la glucose-6-phosphatase (E C 3.1.3.9.), par la méthode de de Duve *et al.* [1]; la phosphatase acide (E C 3.1.3.2.), la ribonucléase acide (E C 2.7.7.16.), et la cathepsine, suivant les techniques décrites par Wattiaux et de Duve [10]. Les procédés préconisés par Vaes [11] ont été suivis pour la mesure de la β-galactosidase (E C 3.2.1.23.) et de la β-N-acétyl-glucosaminidase (E C 3.2.1. 30.) avec, respectivement, de l'*o*-nitrophényl-β-D-galactoside et du *p*-nitrophényl-N-acétyl-β-D-glucosaminide comme substrats. Comme nous le décrirons, les hydrolases acides de l'hépatome HW existent, sous forme latente, dans l'homogénat; aussi, les mesures de ces enzymes ont-elles été faites en présence de Triton X-100 à 0,1%, comme il est recommandé par Wattiaux et de Duve [10]. La mesure de la catalase (E C 1.11.1.6.) a été réalisée suivant la technique de Baudhuin *et al.* [3]; quant aux protéines, nous les avons déterminées par la méthode de Lowry *et al.* [12], en nous référant à la sérum albumine bovine, comme étalon.

Les unités d'activité enzymatique sont définies, pour les hydrolases, comme étant la quantité d'enzyme capable de décomposer une µmole de substrat par min, dans les conditions de l'essai. Une unité de cytochrome oxydase correspond à la quantité d'enzyme qui catalyse en une minute l'oxydation de 90 pour cent du cytochrome c réduit présent dans 100 ml [9]. Pour la catalase, une unité d'activité se définit également comme la quantité d'enzyme qui provoque la destruction de 90 pour cent du substrat en une minute mais dans un volume de 50 ml [3].

L'hydrolyse de l'ARN est évaluée en mononucléotides sur la base d'un coefficient d'extinction à 260 mµ de $8{,}5 \times 10^6 cm^2 mole^{-1}$ [13].

RESULTATS

Activités enzymatiques

Le Tableau 1 rapporte les valeurs moyennes d'activités enzymatiques que nous avons mesurées dans les homogénats d'hépatome. Il permet aussi de comparer les activités spécifiques de ces enzymes à celles que l'on trouve dans les homogénats de foie de rat. La cytochrome oxydase, la glucose-6-phosphatase et la catalase sont nettement moins actives dans la tumeur; au contraire, les hydrolases acides manifestent une activité plus élevée dans l'hépatome que dans le foie.

Distribution des enzymes après centrifugation différentielle

Les distributions observées sont signalées au Tableau 2, et représentées graphiquement à la Fig. 1, suivant la méthode proposée par de Duve *et al.* [1].

Avant d'examiner chaque distribution en particulier, remarquons que la fraction *N* contient un pourcentage élevé des protéines totales de l'homogénat et de toutes les enzymes mesurées; sauf pour la glucose-6-phosphatase, le taux d'enzyme présent dans cette fraction est semblable, quel que soit l'enzyme considérée.

La cytochrome oxydase est récupérée principalement dans la fraction mitochondriale 'lourde' *M;* néanmoins une proportion très significative de l'enzyme se retrouve dans la fraction mitochondriale 'légère' *L*, fraction

Tableau 1. *Activités enzymatiques de L'hepatome HW3*

	Activités spécifiques		Activités spéc hepatome Activités spéc. foie
	Hépatome	Foie	
Ribonucléase acide	(15) 1,14 ±0,13	(10) 0,46 ±0,05	2,46
Phosphatase acide	(16) 3,09 ±0,43	(10) 2,69 ±0,23	1,15
β-Galactosidase	(13) 0,364±0,034	(10) 0,176±0,033	2,06
Cathepsine	(12) 0,739±0,062	(10) 0,689±0,052	1,07
β-N-Acétylglucosaminidase	(10) 2,16 ±0,25	(10) 1,68 ±0,30	1,28
Glucose-6-phosphatase	(9) 1,04 ±0,12	(10) 7,85 ±1,63	0,13
Cytochrome oxydase	(15) 3,80 ±0,78	(9) 13,6 ±2,1	0,27
Catalase	(12) 0,561±0,049	(10) 18,7 ±2,3	0,03

*Les activités sont exprimées en unités/100 mg de protéines. Les résultats sont présentés sous forme de moyennes ±écart type (SD). Entre parenthèses, le nombre de déterminations individuelles. La quantité de protéines, trouvée dans un g de tissu frais, est de 91,9±7,0 mg pour l'hépatome, et de 227±9 mg pour le foie.

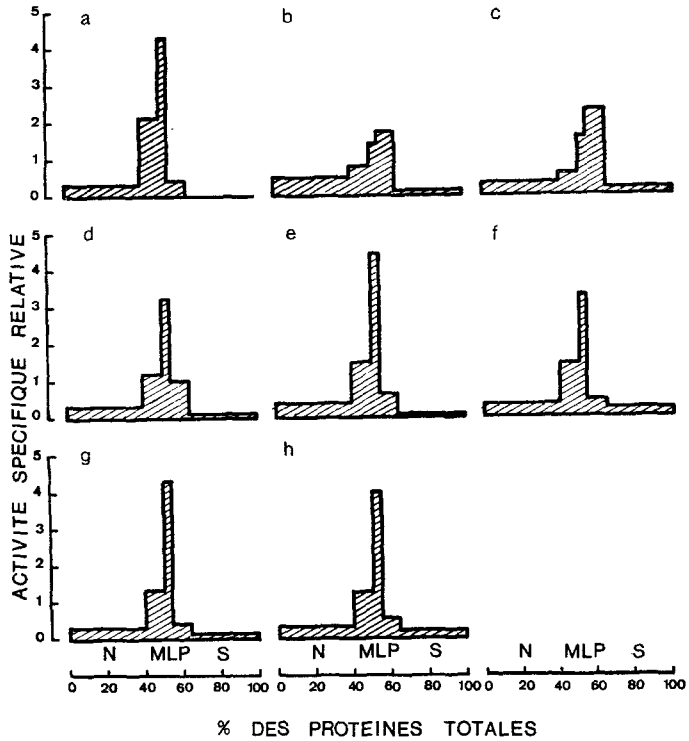


Fig. 1. *Profil de distribution des enzymes après centrifugation différentielle:* (a) *cytochrome oxydase;* (b) *glucose-6-phosphatase;* (c) *catalase;* (d) *phosphatase acide;* (e) β-*N-acétylglucosaminidase;* (f) *ribonuclease acide;* (g) β-*galactosidase;* (h) *cathepsine.* En ordonnée: *l'activité spécifique relative moyenne des fractions* (*pourcentage de l'activité totale récupérée/pourcentage des protéines totales récupérées*). En abscisse: *pourcentage des protéines totales récupérées* (*cumulativement de gauche à droite*).

dans laquelle, d'ailleurs, l'oxydase est la plus purifiée. Le taux de glucose-6-phosphatase associé à la fraction *N* excède nettement celui que l'on trouve pour les autres ferments; dans l'extrait cytoplasmique, l'enzyme est localisée surtout dans la fraction *P*; aussi son profil général de distribution est-il du type microsomial. Les hydrolases acides se caractérisent par des distributions très comparables; c'est dans les fractions mitochondriales *M* et *L* qu'on les retrouve principalement, leur activité spécifique étant de loin la plus élevée dans la fraction légère *L*. On observe toutefois quelques différences appréciables d'une enzyme à l'autre, surtout quant au taux d'enzyme trouvé dans la fraction soluble *S*. Quant à la catalase, elle sédimente tout particulièrement dans la fraction microsomiale *P*, et il est à noter que 10% seulement de l'enzyme sont récupérés dans la fraction soluble.

*Tableau 2. Distribution intracellulaire des enzymes**

	No. d'exp.	Valeurs absolues $E+N$	Pourcentages						
			$E+N$	N	M	L	P	S	Récupération
Cytochrome oxydase	4	3,34 ±0,71	100	28,8±7,6	40,5±3,9	21,3±4,8	7,5±1,3	—	98,1±5,0
Glucose-6-phosphatase	3	0,897±0,063	100	42,5±3,7	14,6±1,2	7,0±1,4	34,0±4,9	9,6±4,2	107,7±5,7
Catalase	4	0,499±0,070	100	26,3±4,9	9,9±0,9	7,4±0,8	42,6±6,9	9,8±1,0	96,0±6,0
Phosphatase acide	4	2,58 ±0,41	100	28,3±4,0	22,8±3,0	18,7±4,1	19,7±1,3	9,8±1,4	99,3±0,5
Cathepsine	4	0,645±0,070	100	29,5±3,6	24,2±4,1	20,4±2,9	11,5±0,5	16,4±2,2	102,0±3,0
Ribonucléase acide	4	1,041±0,200	100	27,4±3,1	27,4±3,1	16,7±2,0	8,4±0,8	21,1±2,9	101,0±2,1
β-galactosidase	4	0,316±0,050	100	30,4±4,5	25,8±2,8	21,9±2,8	8,1±0,9	14,5±2,7	100,7±1,4
β-N-acétylglucosaminidase	4	1,92 ±0,10	100	33,2±4,9	29,1±3,1	23,0±3,5	12,4±1,2	4,7±1,3	102,4±2,1
Protéines	4	90,9 ±6,8	100	41,5±2,1	9,8±2,4	2,6±0,4	10,0±1,0	38,6±2,1	102,5±1,8

*Les valeurs absolues sont données en mg/g pour les protéines et en unités/g de poids frais pour les enzymes; les résultats sont présentés sous forme de moyennes ± écart type. *E*, extrait cytoplasmique; *N*, fraction des noyaux; *M*, fraction mitochondriale lourde; *L*, fraction mitochondriale légère; *P*, fraction microsomiale; *S*, fraction soluble.

Latence des enzymes

Les hydrolases acides sont présentes, sous forme lantente, dans l'extrait cytoplasmique; c'est ce qu'illustre la Fig. 2. Leur activité peut être démasquée par des congélations e décongélations répétées, par la mise en milieu hypotonique, par l'addition de Triton X-100, par un traitement dans un Waring Blendor. La catalase aussi existe sous forme latente (Fig. 2); on remarque toutefois que seuls le Triton X-100 et le traitement au Blendor sont efficaces; ni les congélations et décongélations, ni la mise en milieu hypotonique, ne peuvent démasquer l'enzyme.

DISCUSSION

Les distributions enzymatiques que nous avons observées après centrifugation différentielle d'homogénats d'hépatome HW, et celles établies par de Duve et ses collaborateurs sur le foie de rat [1, 3], diffèrent sous plusieurs aspects. Nous les discuterons ici.

Tout d'abord, la fraction N de l'hépatome contient une proportion nettement plus élevée des protéines et des enzymes de l'homogénat. La similitude entre les taux des différentes enzymes (sauf celui de la glucose-6-phosphatase), présents dans cette fraction, suggère fortement une contamination élevée, non spécifique de cette fraction, par du matériel cytoplasmique. Celui-ci peut provenir de la présence, dans la fraction N de la tumeur, d'un nombre appréciable de cellules intactes: le broyage de la tumeur est en effet plus difficile que celui du foie de rat. Remarquons aussi que d'après Dounce [14], lors du broyage ménagé d'une tumeur en milieu aqueux, du matériel cytoplasmique reste toujours 'collé' aux noyaux, phénomène qui ne se produit pas lors du broyage du foie. De plus, une tumeur contient une trame conjonctive appréciable, dont les fragments sédimentent certainement avec la fraction N et peuvent entraîner, dans les agglomérats qu'ils forment, des particules cytoplasmiques.

La distribution de la cytochrome oxydase se signale par la présence d'une proportion notable de l'enzyme dans la fraction mitochondriale 'légère' L. Cela suggère que les mitochondries de la tumeur ont un coefficient de sédimentation moyen dans le saccharose 0,25 M, plus faible que celui des mitochondries du foie et que, par conséquent, leur taille ou (et) leur densité sont en moyenne plus petites que celles des mitochondries hépatiques. Nos résultats ne permettent toutefois pas d'exclure la possibilité qu'un certain nombre de mitochondries de l'hépatome soient lésées au cours de la préparation de l'homogénat et que l'activité cytochrome oxydasique de la fraction L soit due principalement à des fragments mitochondriaux, auxquels l'enzyme reste fermement attachée. D'autre part, l'activité spécifique de la cytochrome oxydase dans la fraction M est nettement inférieure à celle de l'enzyme dans la fraction L, soit que les mitochondries, sédimentant dans M, aient une teneur en protéines supérieure à celle que l'on trouve dans L, soit qu'il existe une proportion importante d'éléments protéiques non mitochondriaux dans M. La seconde hypothèse est la plus vraisemblable, l'examen de la fraction M au microscope de phase révèle la présence de noyaux; de plus, comme nous le montrerons [15], il est possible de séparer les mitochondries d'une fraction $M+L$ de certains contaminants protéiques, non mitochondriaux, par centrifugation isopycnique dans un gradient de saccharose.

L'activité glucose-6-phosphatasique, trouvée dans la fraction N, laisse supposer une contamination sélective de cette fraction par l'enzyme. Rappelons toutefois que, dans le foie comme dans le rein, la fraction N contient un taux nettement plus élevé de glucose-6-phosphatase que des autres enzymes cytoplasmiques [1, 16]. Ce phénomène est vraisemblablement da à la présence, dans cette fraction, de gros fragments du reticulum endoplasmique. L'estimation de l'activité glucose-6-phosphatasique de l'hépatome pose, d'autre part, un certain problème En effet, elle est nettement plus faible que celle du foie, et même que celle de la phosphatase acide non spécifique de la tumeur. Or, cette dernière enzyme est capable d'hydrolyser le glucose-6-phosphate et son activité peut donc fausser la mesure de l'activité glucose-6-phosphatasique vraie; ce problème est à l'étude actuellement dans notre laboratoire.

Dans l'hépatome HW, la distribution de la catalase est nettement différente de celle trouvée dans le foie de rat. Dans cet organe, la catalase est localisée dans les peroxysomes (microbodies) qui sédimentent surtout dans les fractions mitochondriales et sont le plus purifiés dans la fraction 'légère' L [3]; de plus, dans les homogénats de foie de rat, 30% de l'activité catalasique sont récupérés dans la fraction soluble [3]. Dans l'hépatome HW, la catalase est avant tout microsomiale et le taux d'activité soluble ne dépasse pas 10%. Du point de vue de sa latence dans l'homogénat, la catalase de l'hépatome ressemble à celle du foie, en ce sens qu'elle peut être démasquée par le Triton X-100 et par un traitement au Blendor

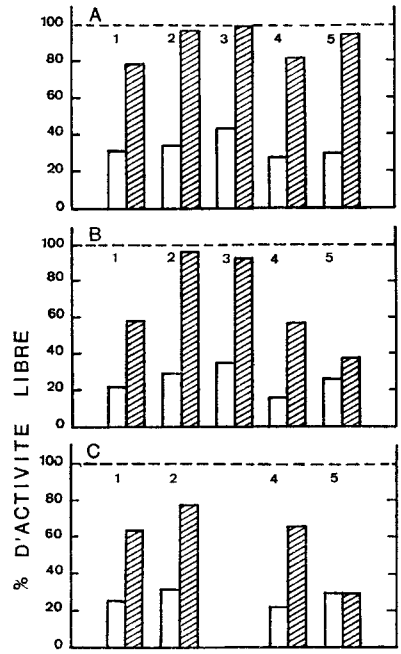


Fig. 2. Activation des hydrolases acides et de la catalase de l'extrait cytoplasmique d'un hépatome HW.A. La préparation est traitée pendant 16 *min dans un Waring Blendor MSE tournant à vitesse maximale. B. La préparation subit 5 congélations et décongélations successives. C. L'extrait est préparé dans l'eau distillée au lieu de saccharose* 0,25 M. *Les activités libres et totales sont déterminées sur un échantillon traité et sur un échantillon témoin provenant d'un même extrait. L'incubation pour la mesure de l'activité libre est de* 10 *min et le milieu contient du saccharose* 0,25 M; *l'activité totale correspond à l'activité mesurée en présence de Triton X-*100 *à* 0,1%. *Elle est assumée comme valant* 100% *de l'activité enzymatique (traits pointillés).* (1) *phosphatase acide;* (2) β-*Nacétylglucosaminidase;* (3) β-*galactosidase; ribonucléase acide;* (5) *catalase. Bloc ouvert avant et bloc bhachure après traitement.*

mais non par la mise en milieu hypotonique; néanmoins les congélations et décongélations ne peuvent activer la catalase de la tumeur, alors qu'elles démasquent la catalase des peroxysomes hépatiques [17]. Les relations éventuelles entre les structures porteuses de catalase dans l'hépatome HW et les peroxysomes seront discutées plus loin, en fonction des résultats obtenus après centrifugation en gradient de densité. Signalons que nous n'avons pu mettre en évidence, jusqu'à présent, dans l'hépatome HW, ni urate oxydase, ni D-amino acide oxydase, deux enzymes localisées dans les peroxysomes de foie de rat [3, 4, 17].

Le critère biochimique fondamental de l'existence de lysosomes, dans un tissu, est la présence, dans un homogénat de ce tissu, d'hydrolases acides de spécificités variées, sédimentables, et dont l'activité est latente. Ce critère est bien vérifié dans l'hépatome HW; de plus, les distributions des hydrolases acides, observées après centrifugation différentielle, sont comparables à celles que ces enzymes ont dans le foie; dans ces deux tissus, les enzymes sont associées principalement aux fractions mitochondriales et présentent leur activité spécifique la plus élevée dans la fraction *L*. Ces considérations, jointes au fait que les activités totales de ces enzymes sont élevées, laissent déjà présumer que le système lysosomial est bien développé dans l'hépatome HW.

RESUME

1. *Les activités de la cytochrome oxydase, glucose-*6*-phosphatase, catalase, et plusieurs hydrolases acides, ont été mesurées dans les homogénats d'un hépatome transplantable chez le rat (hépatome HW) et comparées avec celles trouvées dans le foie de rat. Les trois premières enzymes sont beaucoup moins actives dans la tumeur, tandis que les hydrolases acides sont plus actives.*

2. *Les distributions intracellulaires de ces enzymes ont été étudiées suivant un schéma de centrifugation semblable à celui décrit par de Duve et ses collaborateurs* [1]. *Les homogénats ont été fractionnés en une fraction nucléaire* N, *des fractions mitochondriales 'lourde' et 'légère',* M *et* L, *une fraction microsomiale* P, *et une fraction soluble* S.

3. *La cytochrome oxydase et les hydrolases acides sont retrouvées, en majeure partie, dans les fractions mitochondriales; elles sont le plus purifiées dans la fraction 'légère'* L. *La glucose-6-phosphatase et la catalase présentent un profil de distribution microsomial.*
4. *Les hydrolases acides présentent le phénomène de latence; elles peuvent être activées dans l'extrait cytoplasmique par congélations et décongélations, traitement au Waring Blendor, hypotonicité, et par le Triton X-100, un détergent non ionique. La catalase aussi est trouvée sous forme latente dans l'extrait cytoplasmique, mais ne peut être démasquée ni par hypotonicité, ni par congélations et décongélations.*
5. *Une comparaison est faite entre les distributions enzymatiques dans l'hépatome HW et dans le foie de rat. Des différences significatives se sont avérées, sauf pour les distributions des hydrolases acides.*

SUMMARY

1. *The activity of cytochrome oxidase, glucose-6-phosphatase, catalase and several acid hydrolases has been measured in homogenates of a rat transplantable hepatoma (hepatoma HW) and compared with that found in rat liver. The first three enzymes are much less active in the tumour; on the contrary, acid hydrolases are more active in the hepatoma.*
2. *The intracellular distribution of these enzymes has been investigated according to a centrifugation scheme similar to that described by de Duve and his co-workers* [1]. *The homogenates were fractionnated in a nuclear fraction* N, *a 'heavy' and a 'light' mitochondrial fraction,* M *and* L, *a microsomal fraction* P *and a soluble fraction* S.
3. *Cytochrome oxidase and acid hydrolases are chiefly recovered in the mitochondrial fractions; they are the most purified in the 'light' one* L. *Glucose-6-phosphatase and catalase exhibit a microsomal distribution pattern.*
4. *Acid hydrolases display the phenomenon of structure-linked latency; they can be activated in the cytoplasmic extract by freezing and thawing, Blendor treatment, hypotonicity and the non-ionic detergent Triton X-100. Catalase is also found in a latent form in the cytoplasmic extract but cannot be unmasked by hypotonicity nor by freezing and thawing.*
5. *A comparison has been made between the distributions of the enzymes in the hepatoma HW and in the rat liver. Significant differences have been shown to exist except for the acid hydrolases distribution.*

ZUSAMMENFASSUNG

1. *Die Aktivität der Zytochromoxydase, der Glukose-6-Phosphatase, der Katalase und mehrerer saurer Hydrolasen wurde in Homogenaten eines bei Ratten transplantablen Hepatoms (HW Hepatom) gemessen und mit derjenigen in der Leber der Ratte verglichen. Während die drei erstgenannten Enzyme im Tumor viel weniger aktiv sind, entfalteten die sauren Hydrolasen im Hepatom eine grössere Aktivität.*
2. *Die intrazelluläre Verteilung dieser Enzyme wurde—gemäss einem Zentrifugierungsschema, wie es in ähnlicher Weise de Duve und seine Mitarbeiter beschrieben haben* [1]*—festgestellt. Die Homogenate wurden nach diesem Verfahren in folgende Fraktionen ausgeteilt: Kernfraktion* (N), *schwere und leichte Mitochondrialfraktion* (M *und* L), *Mikrosomalfraktion* (P) *und lösliche Fraktion* (S).
3. *Man findet die Zytochromoxydase und die sauren Hydrolasen vorwiegend in den mitochondrialen Fraktionen; in der reinsten Form erscheinen sie in der leichten Fraktion* (L). *Die Glukose-6-Phosphatase und die Katalase hingegen sind in der Mikrosomalfraktion zu finden.*
4. *Die sauren Hydrolasen sind durch eine strukturbedingte Latenz gekennzeichnet; sie können im Zytoplasmaextrakt durch folgende Massnahmen aktiviert werden: Gefrieren und Auftauen, Behandlung im Waring Blendor, Herabsetzen der osmotischen Spannung und Zugabe von Triton X-100 einem nichtionisierten Detergens. Die Katalase ist ebenfalls latent im Zytoplasmaextrakt enthalten; sie kann aber weder durch verminderte osmotische Spannung noch durch Gefrieren oder Auftauen aktiviert werden.*
5. *Man vergleicht die Enzymverteilung im HW-Hepatom und in der Leber der Ratte. Dabei ergeben sich mit Ausnahme der Verteilung der sauren Hydrolasen signifikante Unterschiede.*

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Particules Subcellulaires dans les Tumeurs

II Analyse de fractions mitochondriales et microsomiales de l'hépatome HW par centrifugation isopycnique

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INTRODUCTION

LA CENTRIFUGATION isopycnique, appliquée à l'étude des particles subcellulaires permet de sous-fractionner des préparations de granules obtenues par centrifugation différentielle, mais aussi de préciser certaines propriétés physiques des granules; des positions d'équilibre que ceux-ci atteignent dans des gradients de densité, il est en effet possible de déduire des renseignements sur la perméabilité des granules, leur densité en milieu isotonique, etc. [1–3].

Nous avons appliqué cette méthode à l'étude des structures dans lesquelles sont localisées les enzymes de l'hépatome HW, dont nous avons décrit précédemment la distribution après centrifugation différentielle [4]. Nos résultats sont exposés dans cet article et comparés aux résultats obtenus par des techniques semblables sur le foie de rat, par de Duve et ses collaborateurs [2, 5, 6].

MATERIEL ET METHODES

Centrifugation en gradient de densité

Nous avons utilisé deux types de gradients de densité. Dans l'un, les variations de densité sont assurées par des variations de concentration en saccharose dans l'eau, dans l'autre par des variations de concentration en glycogène, cette substance étant dissoute dans une solution de saccharose [2, 5, 6]. Dans le premier type de gradient, les granules sont soumis, au cours de leur sédimentation, à des concentrations croissantes en saccharose, et donc à des pressions osmotiques croissantes; dans le second, la concentration en saccharose est constante dans toute la colonne liquide centrifugée, les granules sont donc toujours dans un milieu d'égale tonicité, le glycogène n'ayant pratiquement pas d'effet osmotique.

Nos expériences ont été réalisées soit sur des fractions mitochondriales totales correspondant à la somme des fractions *M* et *L* [4, 7] soit sur des fractions microsomiales *P*.

La méthode de centrifugation en gradient que nous avons appliquée est celle décrite par Beaufay *et al.* [5, 6]. Les gradients sont préparés à l'aide d'un appareil semblable à celui qu'utilisent ces auteurs; il est constitué de deux seringues jumelées, de même section, débitant l'une une solution lourde, l'autre une solution légère, dans une petite chambre de mélange

d'où la mixture s'écoule dans le tube de centrifugation. Le débit de la seringue contenant le liquide lourd augmente linéairement au cours de temps, tandis que celui de la seringue contenant le liquide léger décroît linéairement, le débit total restant constant; on obtient ainsi un gradient linéaire [1, 8]. Toutefois, dans les gradients de glycogéne, il se produit, au cours de la centrifugation, une certaine distorsion du gradient due à la sédimentation du glycogène [6]. Dans les gradients de saccharose, un échantillon de la préparation particulaire est déposé au sommet du gradient; dans les gradients de glycogène, un échantillon identique est incorporé dans les solutions servant à préparer le gradient [6].

La centrifugation est effectuée dans le rotor SW39 de l'ultracentrifugeuse préparative Spinco L-HV pendant 2h ½ à 39·000 tours/min, sauf indication contraire. Après centrifugation, le tube est découpé en sections grâce à l'appareil décrit par de Duve *et al.* [1] et chaque fraction est recueillie et traitée d'une façon semblable à celle décrite par Beaufay *et al.* [6]

Mesures enzymatiques

Les mesures enzymatiques se font suivant les méthodes auxquelles nous nous sommes référés précédemment [4]. Toutefois, pour les hydrolases acides, comme les temps d'incubation sont longs (environ 20 h), nous ajoutons, aux substrats, du thiomersalate de sodium à 0·01% pour éviter la contamination bactérienne [5].

Présentation des résultats

Les distributions après centrifugation sont représentées sous forme d'histogrammes, dans lesquels chaque fraction est représentée par un rectangle. La base de ce rectangle représente la hauteur de la section de la colonne liquide centrifugée, occupée par la fraction, et exprimée en pourcentage de la hauteur totale de cette colonne (% de H), la hauteur du rectangle représente la concentration relative du composé, *C/Ci*, soit le rapport de sa concentration *C* dans la fraction à la concentration *Ci* qu'il aurait s'il se distribuait de façon homogène dans la colonne liquide [6]. Les fractions sont représentées dans l'ordre de densité croissante, soit du ménisque à gauche au fond du tube à droite. L'histogramme est souvent caractérisé par sa médiane, c'est-à-dire la valeur d'abscisse où aboutit la verticale divisant la surface de l'histogramme en deux parties égales. Dans le mode de représentation que nous utilisons, elle indique un certain niveau dans la colonne centrifugée et devrait s'exprimer en pourcentage de la hauteur totale du gradient: nous la transformons toutefois en une valeur de densité, en nous référant à la densité correspondant au niveau trouvé.

RESULTATS

Equilibration de densité dans un gradient de saccharose

La Fig. 1 rapporte les distributions de plusieurs enzymes observées après centrifugation isopycnique dans un gradient de saccharose (0, 78–3,42 molal) d'une fraction mitochondriale $M+L$ d'hépatome HW. Il s'agit d'enzymes qu'on retrouve principalement dans cette fraction: la cytochrome oxydase, caractéristique des mitochondries, et plusieurs hydrolases acides associées vraisemblablement aux lysosomes de la tumeur [4].

La cytochrome oxydase est concentrée dans une région relativement étroite du gradient, sa densité médiane d'équilibration étant d'environ 1,18. Les courbes de distribution des hydrolases acides sont plus étalées; leur densité médiane d'équilibration est de l'ordre de 1,21; une proportion appréciable de ces enzymes se retrouve dans le culot de centrifugation, en dehors des limites du gradient. La distribution des protéines est caractérisée par un pic de concentration correspondant à celui de la cytochrome oxydase, et qui est dû probablement aux protéines mitochondriales. Ce sommet est toutefois inférieur à celui que l'on trouve pour l'enzyme; le reste des protéines est principalement récupéré dans le culot de centrifugation.

A la Fig. 2 sont illustrées les distributions de la glucose-6-phosphatase et de la catalase, après équilibration de densité d'une fraction microsomiale *P*, fraction dans laquelles ces enzymes se retrouvent principalement dans l'hépatome HW [4]. La catalase se localise dans la zone du gradient centrée sur une densité de 1,17 à 1,18. Que le temps de centrifugation soit de 150 min ou de 16 h, les résultats sont semblables, ce qui semble bien prouver que les structures portant l'enzyme ont atteint leur position d'équilibre dans le gradient. L'activité glucose-6-phosphatasique se distribue suivant un profil tout différent et se retrouve dans les régions les plus légères du gradient; le profil de distribution des protéines est semblable à celui de cette enzyme, une proportion plus importante de protéines se retrouve néanmoins dans le culot de centrifugation.

Equilibration de densité dans un gradient de glycogène

Trois types de gradients ont été utilisés pour analyser la fraction mitochondriale $M+L$. La

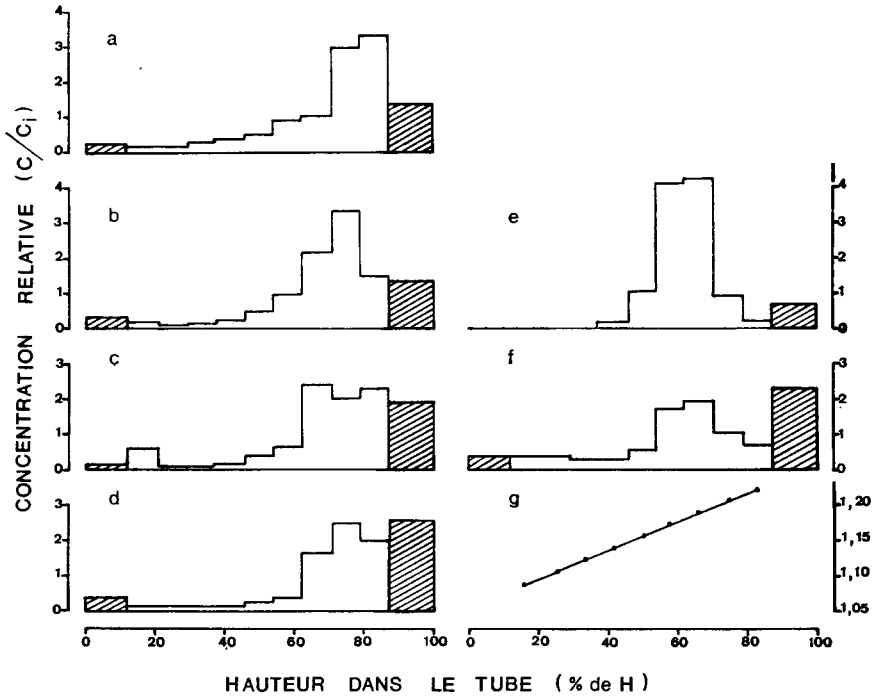


Fig. 1. *Profil de distribution des enzymes après centrifugation isopycnique d'une préparation mitochondriale* (M+L) *d'un hépatome HW, dans un gradient de saccharose dans l'eau* (0,78–3,42 *molal en saccharose*). *La préparation de granules a été initialement déposée au sommet du gradient. Les blocs hachurés ont été utilisés pour les sous-fractions du sommet et du culot et indiquent que celles-ci contiennent des éléments récupérés en dehors des limites du gradient.* (*a*) *phosphatase acide,* (*b*) *cathepsine,* (*c*) β-*galactosidase* (*d*) *ribonucléase, acide,* (*e*) *cytochrome oxydase,* (*f*) *protéines,* (*g*) *densité moyenne des sous-fractions. Les récupérations étaient dans l'ordre cité ci-dessus de* 111,0, 103,2, 91,9, 102,4, 89,6 *et* 116,0%.

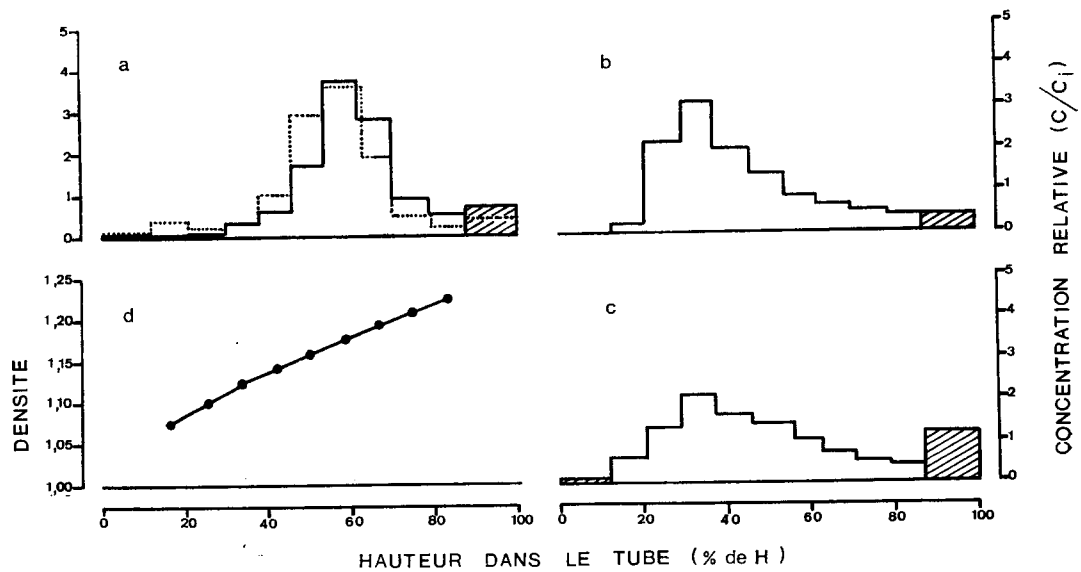


Fig. 2. *Profil de distribution des enzymes après centrifugation isopycnique d'une préparation microsomiale* P *d'un hépatome HW, dans un gradient de saccharose dans l'eau* (0,78–3,42 *molal en saccharose*). *La préparation de granules a été initialement déposée au sommet du gradient,* (*a*) *catalase,* (*b*) *glucose-6- phosphatase* (*c*) *protéines,* (*d*) *densité moyenne des sous-fractions. En pointillé, distribution de la catalase après* 2 *h* 30 *min de centrifugation; les autres distributions ont été obtenues après* 16 *h de centrifugation. Les récupérations étaient respectivement de* 101,5, 102,0, 114,0 *et* 108,0%.

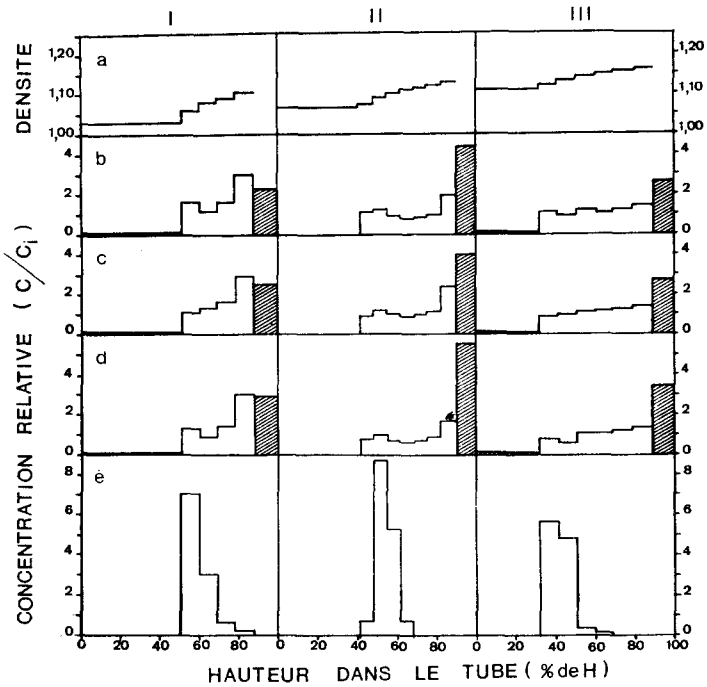


Fig. 3. *Profil de distribution des enzymes après centrifugation isopyncique d'une préparation mitochondriale* (M+L) *d'hépatome HW dans un gradient de glycogène* (0–20%; *poids/poids*), *avec, comme solvant, du saccharose dissous dans l'eau à une concentration* 0,262 (*I*), 0,561 (*II*) 0,988 (*III*) *molale. La préparation de granules a été initialement distribuée de façon homogène dans le gradient.* (*a*) *densité moyenne des fractions,* (*b*) *phosphatase acide,* (*c*) *cathepsine,* (*d*) *ribonucléase acide,* (*e*) *cytochrome oxydase. Pour les récupérations, voir Tableau* 4.

Tableau 1. *Résultats complémentaires des Fig.* 3 *et* 4. *Récupérations des enzymes après centrifugation en gradient de glycogène. I, II et III correspondent aux trois types de gradients utilisés* (*voir Fig.* 3 *et* 4)

	I	II	III
Phosphatase acide	106,3	98,7	112,2
Cathepsine	98,3	95,1	101,5
Ribonucléase acide	101,6	96,6	102,8
Cytochrome oxydase	115,0	100,9	104,2
Catalase	116,2	97,2	105,1
Glucose-6-phosphatase	124,2	108,9	106,6

concentration de glycogène s'étend, dans chacun d'eux, de 0 à 20% (poids/poids) mais les solutions de saccharose, dans lesquelles le polysaccharide est dissous sont respectivement 0,262, 0,561, 0,988 molales. Ont été mesurées: la cytochrome oxydase et trois hydrolases acides: la phosphatase acide, la cathespine et la ribonucléase acide. Les résultats sont illustrés à la Fig. 3. La cytochrome oxydase et les hydrolases acides se comportent tout à fait différemment, l'oxydase s'équilibrant dans les régions les plus légères du gradient, tandis que les hydrolases sont avant tout présentes dans les zones les plus denses de celui-ci. Comme l'indique la Fig. 5, la densité des granules, auxquels les enzymes sont associées, croît en fonction de la densité de la solution de saccharose dans laquelle les granules se trouvent; la fonction est raisonnablement linéaire pour la cytochrome oxydase, elle ne l'est par pous les hydrolases acides. Notons cependant que, pour la cytochrome oxydase, l'estimation de la densité médiane d'équilibration en saccharose 0,262 molal ne peut être qu'approximative, l'enzyme étant concentrée, en majeure partie, dans la zone de distorsion maximale du gradient, là où la densité du milieu change de façon abrupte.

Nous avons établi les distributions de la catalase et de la glucose-6-phosphatase dans les mêmes types de gradient en utilisant une fraction microsomiale P. Quoique les enzymes viennent s'équilibrer toutes deux dans les régions les moins denses du gradient, on peut constater que l'activité glucose-6-phosphatasique est associée à des éléments plus légers que ceux porteurs de catalase (Fig. 4). Comme l'illustre la Fig. 5, la densité d'équilibration des structures portant la catalase croît linéairement en fonction de la densité de la solution de saccharose. L'estimation d'une densité médiane d'équilibration n'est pas possible pour la glucose-6-phosphatase, du moins dans les deux premier gradient, l'enzyme étant, en majeure partie, localisée dans la zone discontinue du

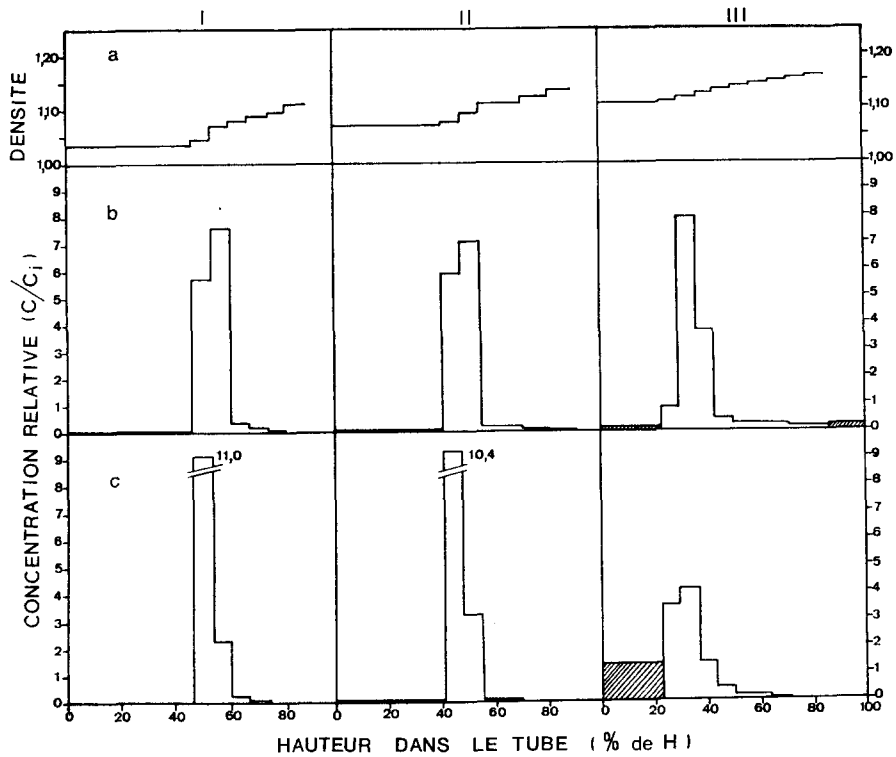


Fig. 4. *Profil de distribution des enzymes après centrifugation isopycnique d'une préparation microsomiale* P *d'hépatome HW dans un gradient de glycogène* (0–20%: *poids/poids avec, comme solvant, du saccharose dissous dans l'eau à une concentration* 0,262 (*I*) 0,561 (*II*) 0,988 (*III*) *molal. Les particules ont été initialement distribuées, de façon homogène, dans le gradient; (a) densité moyenne des fractions, (b) catalase, (c) glucose-6-phosphatase. Pour les récupérations, voir Tableau* 1.

gradient. On remarque néanmoins qualitativement que la densité d'équilibration des structures portant l'enzyme dépend de la densité de la solution de saccharose dans laquelle elles se trouvent.

DISCUSSION

La distribution de la cytochrome oxydase de la tumeur, après équilibration de densité en gradient de saccharose est très semblable à celle que l'on obtient pour l'enzyme hépatique [2, 5, 6]; mais, à l'inverse de ce que l'on observe pour une fraction mitochondriale ($M+L$) de foie de rat, une proportion importante des protéines de la fraction n'accompagne pas l'enzyme, et n'est donc pas d'origine mitochondriale. Ces protéines, que l'on retrouve surtout dans le culot de centrifugation, proviennent, croyons-nous, principalement de noyaux qui contaminent la fraction mitochondriale; elles étaient récupérées dans la fraction mitochondriale lourde M après centrifugation différentielle et contribuaient à diminuer l'activité spécifique de la cytochrome oxydase associée à cette fraction [4].

Tenant compte de ces résultats, et supposant une distribution homogène de la cytochrome oxydase dans la population mitochondriale, on peut facilement estimer que 8–10% seulement des protéines de l'hépatome HW sont mitochondriales. C'est un chiffre environ trois fois inférieur à celui que l'on trouve pour les mitochondries de foie de rat [6].

Le comportement de la cytochrome oxydase dans les gradients de glycogène est d'un intérêt tout particulier. Tout d'abord, il permet d'estimer que la densité moyenne des mitochondries de l'hépatome HW, en saccharose istonique, est de l'ordre de 1,07 (Fig. 5), et est, par conséquent, nettement inférieure à celle des mitochondries du foie de rat qui vaut environ 1,10 [2, 8]. Cela pourrait rendre compte, au moins partiellement, de la valeur plus faible du coefficient de sédimentation des mitochondries de la tumeur [4]. D'autre part, la densité médiane d'équilibration de l'enzyme croît linéairement en fonction de la densité de la solution de saccharose dans laquelle les granules se trouvent (Fig. 5). Ce comportement correspond à celui que Beaufay et Berthet [2] prévoient pour une enzyme localisée dans un granule de volume constant, constitué d'une matrice et d'un espace librement accessible au saccharose du

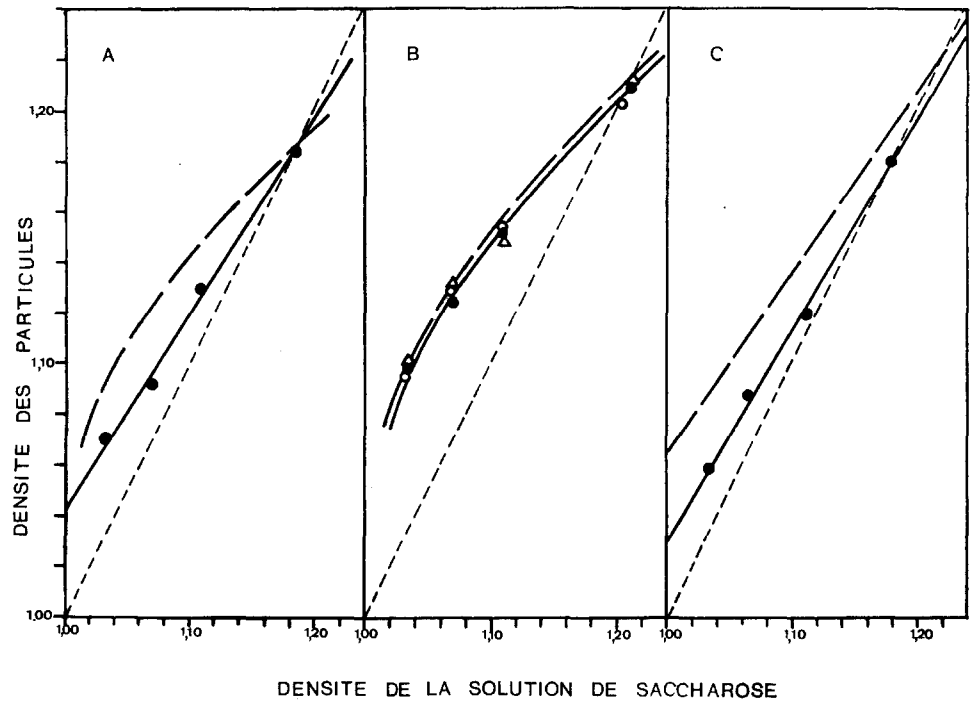


Fig. 5. Variation de la densité médiane des structures portant (a) la ctyochrome oxydase, (b) la phosphatase acide (●), la cathepsine (o) et la ribonucléase acide (Δ), (c) la catalase, en fonction de la densité de la solution de saccharose dans laquelle les granules se trouvent. Les trois premiers points proviennent des expériences d'équilibration en gradient de glycogène, le quatrième de l'expérience d'équilibration en gradient de saccharose, et se trouve à l'intersection de la diagnale avec les courbes. Pour les hydrolases acides, une seule courbe a été tracée vu la similitude de localisation des points correspondent à chacun des trois enzymes. En larges traits discontinus (– – –), nous représentons l'évolution des densités médianes des structures portant (a) la cytochrome oxydase, (b) la phosphatase acide, (c) la catalase dans le foie de rat, en nous référant aux données publiées par Beaufay et Berthet (2).

milieu et de volume constant; un tel granule diffère de la mitochondrie hépatique par l'absence d'espace osmotique dans le saccharose aqueux, et donc par sa perméabilité beaucoup plus grande au saccharose. On peut d'ailleurs se rendre compte, en consultant la Fig. 5, de la différence nette qui existe entre les mitochondries du foie et de la tumeur, quant au comportement de leur cytochrome oxydase dans le saccharose aqueux. Nous ne pouvons cependant préciser si, *in vitro*, la perméabilité de la membrane mitochondriale est plus grande dans l'hépatome que dans le foie, ou si cette membrane est plus facilement altérée *in vitro*, dans le case de la tumeur.

Après centrifugation différentielle, la glucose-6-phosphatase et la catalase présentent des profils de distribution du même type [4]; les résultats obtenus, après centrifugation isopycnique, suggèrent fortement des localisations subcellulaires différentes pour les deux enzymes.

La distribution de la glucose-6-phosphatase des microsomes de l'hépatome HW, après équilibration de densité dans un gradient de saccharose, est comparable à celle que l'on trouve pour l'enzyme du foie de rat [10 et résultats personnels non publiés], et il est vraisemblable que, dans la tumeur, cette activité, enzymatique soit associée, comme dans le foie, au reticulum andoplasmique. Nous n'avons pas de point de comparaison en ce qui concerne le comportement de l'enzyme en gradient de glycogène.

Les structures qui portent la catalase de l'hépatome HW posent un problème tout particulier. Elles se retrouvent dans la fraction des microsomes *P* [4], alors que les perosysomes de foie de rat sédimentent avec les fractions mitochondriales [10]; le coefficient de sédimentation des structures porteuses de catalase dans l'hépatome est donc nettement inférieur à celui des peroxysomes de foie de rat. Leur densité médiane d'équilibre (environ 1,18) dans un gradient de saccharose est aussi largement inférieure à celle des peroxysomes de foie de rat (environ 1,23); de même, leur densité dans le saccharose isotonique vaut environ 1,06, comparée à 1,09 pour les peroxysomes de foie de rat [2, 6, 8]. L'évolution linéaire de leur densité, en fonction de la densité de la solution de saccharose du milieu (Fig. 5), suggère néanmoins que ces structures sont librement perméables au saccharose comme les peroxysomes [2]. Existe-t-il, dès

lors, un rapport entre les structures porteuses de catalase dans l'hépatome HW et dans le foie de rat? Il est intéressant de rappeler ici que, dans le foie de rat, un certain pourcentage de la catalase de l'homogénat se trouve dans la fraction *P*. D'après Higashi et Peters [11, 12], cette catalase microsomiale sédimente principalement avec les microsomes obtenus entre des densités en saccharose de 1,15 à 1,18, et l'enzyme peut être libéré des structures qui le portent, par l'action des détergents, mais non par des congélations et décongélations répétées. On trouve donc là des analogies entre les structures porteuses de catalase dans l'hépatome HW et celles auxquelles l'enzyme est associée dans les microsomes de foie de rat. Or, des expériences à l'aide d'isotopes indiquent que la catalase microsomiale est la première à être synthétisée dans le foie de rat [12]. Ajoutons à cela que plusieurs observations morphologiques suggèrent une participation du réticulum endoplasmique à la formation des peroxysomes; ces granules pourraient provenir de bourgeonnements, à certains endroits du réticulum [13], et la catalase être progressivement incorporée dans les granules, pendant leur maturation. Aussi est-il tentant de supposer que les structures portant la catalase dans la fraction microsomiale correspondent à des 'pré-peroxysomes' ou peroxysomes en voie de formation et que, dans l'hépatome, la catalase soit intégralement localisée dans ces structures. Il est intéressant de rappeler, à ce propos, les observations morphologiques de Dalton [14], qui indiquent la présence, dans un hépatome moyennement différencié, de 'microbodies' beaucoup plus petits que ceux du foie de rat. L'existence de petits 'microbodies' dans l'hépatome HW pourrait d'ailleurs rendre compte du taux faible de catalase, trouvé sous forme non sédimentable; de petits 'microbodies' seraient nettement moins sensibles aux forces de cisaillement mises en jeu lors du broyage des tissus; ils seraient par conséquent détruits en moins grand nombre lors de l'homogénéisation et libéreraient moins de catalase dans la partie soluble de l'homogénat; il semble en effet, d'après de Duve et Baudhuin [13], que l'activité catalasique non sédimentable des homogénats de foie soit avant tout due à une destruction de 'microbodies', lors du broyage de l'organe. L'examen, en microscopie électronique, de fractions riches en catalase, isolées par centrifugation isopycnique en gradient de saccharaose, présenterait, à ce point de vue, un intérêt tout particulier.

Les résultats obtenus sur les hydrolases acides, après centrifugation isopycnique de fractions mitochondriales, confirment la présence, dans l'hépatome HW, d'un système lysosomial comparable à celui du foie de rat, comme le suggèrent les données de la centrifugation différentielle [4]. Les distributions observées pour ces enzymes dans un gradient de saccharose sont du même genre que celles décrites pour les hydrolases acides du foie de rat [2, 5, 6]. L'analyse du comportement de ces ferments en gradient de glycogène suggère, de plus, une structuration des lysosomes de l'hépatome HW, semblable à celle des lysosomes hépatiques. En effet, comme le montre la Fig. 5, les densités médianes d'equilibration des trois hydrolases de référence, la cathepsine, la phosphatase acide, la ribonucléase acide, sont affectées d'une façon très semblable par la densité de la solution de saccharose dans laquelle les granules se trouvent; la fonction n'est plus linéaire, comme pour la cytochrome oxydase ou pour la catalase; elle est du type de celle proposée par de Duve et ses collaborateurs [1, 2, 6] pour catactériser un granule composé d'une matrice, d'un espace de volume constant librement perméable au saccharose, et d'un espace osmotique; les lysosomes du foie se comportent comme un tel granule [2,6 et voir Fig. 5], les lysosomes de l'hépatome également.

Remerciements—Nous remercions M. F. Dubois, Mlle A Jadin et Mme M. Libion pour leur excellente aide technique.

RESUME

1. *Les fractions mitochondriale et microsomiale d'un hépatome transplantable chez le rat ont été analysées par centrifugation isopycnique. Deux sortes de gradients ont été utilisées: l'un de saccharose aqueux pur, l'autre de glycogène dissous dans des solutions de concentration variable en saccharose.*

2. *Après équilibration de densité, dans un gradient de saccharose, d'une fraction mitochondriale, la cytochrome oxydase montre une distribution sensiblement homogène, tandis que les hydrolases acides présentent des courbes de distribution plus étalées dans les régions de haute densité du gradient. Après centrifugation isopycnique dans des gradients de glycogène, les profils de distribution de la cytochrome oxydase sont totalement différents de ceux des hydrolase acides. De plus, les densités d'équilibre des mitochondries et des lysosomes,*

déduites respectivement du comportement de la cytochrome oxydase et des hydrolases acides, sont fort affectées par la concentration en saccharose du milieu, quoique différemment pour les deux espèces de particules.

3. *La centrifugation isopycnique d'une fraction microsomiale dans un gradient aqueux de saccharose, et dans des gradients de glycogène, suggère fortement que la glucose-6-phosphatase et la catalase sont associées à des éléments distincts de la fraction microsomiale.*

4. *La comparaison de nos résultats avec ceux obtenus par de Duve et ses collaborateurs sur le foie de rat* [2, 5, 6] *suggère que des différences significatives existent entre les mitochondries de l'hépatome HW et les mitochondries de foie de rat, aussi bien qu'entre les structures portant la catalase dans les deux tissus; tout au contraire, les lysosomes semblent fort comparables dans la tumeur et dans le foie.*

SUMMARY

1. *Mitochondrial and microsomal fractions from a rat transplantable hepatoma have been analysed by isopycnic centrifugation. Experiments were performed with two kinds of gradients, one made with pure aqueous sucrose, the other with glycogen dissolved in aqueous sucrose solutions of different concentrations.*

2. *After density equilibration in a sucrose gradient of a mitochondrial fraction, cytochrome oxidase shows a relatively homogeneous distribution while acid hydrolases exhibit more flattened distribution curves in higher density regions of the gradient. After isopycnic centrifugation in glycogen gradients, distribution patterns of cytochrone oxidase are quite different from those of acid hydrolases. On the other hand, the equilibrium density of mitochondria and of lysosomes as ascertained respectively from cytochrome oxidase and from acid hydrolases behaviour, is greatly affected by the sucrose concentration of the medium although differently for the two kinds of particles.*

3. *Isopycnic centrifugation of a microsomal fraction in an aqueous sucrose gradient and in glycogen gradients strongly suggest that glucose-6-phosphatase and catalase are associated with distinct elements of the microsomal fraction.*

4. *Comparison of our results with those obtained by de Duve and his co-workers on rat liver* [2, 5, 6] *suggest that significant differences exist between mitochondria of the hepatoma HW and those of rat liver as well as between structures bearing catalase in both tissues; in contrast, lysosomes seem to be very comparable in the tumour and in the liver.*

ZUSAMMENFASSUNG

1. *Mitochondrische und mikrosomale Fraktionen eines transplantablen Ratten-Hepatoms sind mit Hilfe der isopyknischen Zentrifugierung untersucht worden. Man verwendete zwei Arten von Gradienten: wässirige Saccharoselösung und Glykogen in Rohrzuckerlösungen verschiedener Konzentrationen.*

2. *Nachdem das Dichtegleichgewicht einer Mitochondrienfraktion im Rohrzuckergradienten hergestellt ist, weist die Zytochromoxydase eine beinahe homogene Verteilung auf, während sich für die sauren Hydrolasen in den Regionen von hohem Dichtegradienten abgeflachte Verteilungskurven ergeben. Nach isopyknischer Zentrifugierung in Glykogengradienten, erscheinen die Verteilungsprofile der Zytochromoxydase gegenüber denjenigen der sauren Hydrolasen vollkommen verschieden. Andererseits besteht eine starke Beeinflussung der Dichtegleichgewichte der Mitochondrien und der Lysosomen, (welche aus dem Verhalten von Zytochromoxydase, bzw. den sauren Hydrolasen ableitbar sind), durch die Rohrzuckerkonzentration der Lösung: allerdings verhalten sich die beiden Partikelarten verschieden.*

3. *Die isopyknische Zentrifugierung einer mikrosomalen Fraktion in einem wässerigen Rohrzuckergradienten und in Glykogengradienten legt die Vermutung nahe, dass die Glukose-6-Phosphatase und die Katalase mit unterschiedlichen Elementen der mikrosomalen Fraktion verbunden sind.*

4. *Ein Vergleich zwischen unseren Resultaten und denjenigen, die von de Duve und seinen Mitarbeitern bei der Untersuchung der Rattenleber erzielt wurden* [2,5,6] *lässt vermuten, dass signifikante Unterschiede sowohl zwischen den Mitochondrien des HW Hepatoms und denjenigen der Rattenleber existieren als auch zwischen den Katalaseträgern der beiden Gewebe; im Gegensatz dazu sind die Lysosomen in Hepatom und Leber vergleichbar.*

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Immunothérapie Adoptive Locale de la Leucémie L 1210 Ascitique. Analyse des Facteurs de son Efficacité*

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Nous appelons immunothérapie adoptive d'affections néoplasiques leur traitement par des cellules immuno-compétentes, que celles-ci soient greffées, car tolérées en raison du conditionnement de l'hôte ou des relations d'histo-compatibilité hôte-greffon, ou non greffées, car non tolérées, que les cellules soient libres ou placées en chambres de diffusion, que les donneurs soient immunisés ou non.

Nos premières expériences, menées sur la leucémie greffée L 1210 [1, 2], la leucémie spontanée AkR [3, 4] et la leucémie induite par le virus de Charlotte Friend [5, 6], nous ont permis de développer le concept de ce moyen thérapeutique nouveau, et de l'appliquer à l'homme dans le traitement de leucémies aiguës, sous la forme de greffes de moelle osseuse [7–11] ou de transfusions de leucocytes [12, 13]. Ces essais cliniques ont confirmé le pouvoir antileucémique puissant de cette thérapeutique et la corrélation entre l'obtention de cet effet et l'induction du syndrome secondaire, syndrome secondaire beaucoup plus difficile à contrôler chez l'Homme que chez les Rongeurs [14, 15], et qui représente encore la pierre d'achoppement majeure à l'emploi étendu de la greffe de moelle osseuse dans le traitement des leucémies.

Nos études expérimentales avaient, jusqu'ici, porté sur l'immunothérapie adoptive par des cellules immuno-compétentes greffées, et nos essais cliniques avaient montré que les résultats enregistrés étaient très supérieurs, dans les cas de greffe, à ce qu'ils étaient dans les cas de simple transfusion sans greffe. Or, Alexander *et al.* [16] viennent de publier de remarquables effets obtenus sur certaines tumeurs expérimentales, après simples transfusions, sans greffe, de cellules immuno-compétentes de donneurs hétérologues immunisés par les éléments néoplasiques. Nous nous sommes demandés si cette méthode, si efficace entre les mains de ces auteurs sur des tumeurs porteuses de néo-antigènes spécifiques puissants, pourrait s'appliquer à une tumeur dont l'antigénicité spécifique est beaucoup plus réduite, la leucémie L 1210, ce qui en fait un excellent modèle pour l'étude des thérapeutiques applicables aux leucémies aigués humaines, dans les cellules desquelles il est difficile de mettre en évidence des antigènes spécifiques [17].

Le propos du présent travail est donc de comparer les effets antileucémiques de cellules immunocompétentes, selon qu'elles sont tolérées ou non par l'hôte, libres ou en chambres de diffusion, et selon qu'elles proviennent de donneurs immunisés contre les cellules leucémiques ou les cellules normales de l'hôte, ou de donneurs non immunisés.

MATERIEL ET METHODES

I. *Traitement de la leucémie L* 1210 (*leucémie DBA*/2) *greffée sur des receveurs F*1(*DBA*/2 ×

*Ce travail a été effectué grâce à une aide de l'INSERM, contrat n° C5. 66–213.

C57Bl6), par des cellules lymphoïdes semi-allogéniques C57Bl/6 donc tolérées par l'hôte, provenant de donneurs immunisés contre les cellules leucémiques ou les cellules DBA/2 normales, ou de donneurs non immunisés

Deux cent vingt-neuf souris F1 (DBA/2 × C5Bl/6) âgées de trois mois, ont reçu, par voie péritonéale, 10^4, cellules de la leucémie L 1210. Elles ont été réparties au hasard (randomisation) en 5 groupes. Les 45 souris du groupe I servent de témoins. Les souris des quatre autres groupes sont irradiées *in toto* à la dose de 300 rads, au moyen d'une source de Cobalt radioactif dont le débit au plan moyen est de 13,50 r/min (distance: 755mm). Cette irradiation, insuffisante pour permettre la greffe des cellules lymphoïdes incompatibles, favorise, comme nous l'avons montré [18], la prolifération des cellules lymphoïdes parentales chez des hybrides FI. Les 34 souris du groupe II sont seulement irradiées; les 49 souris du groupe III reçoivent, par voie péritonéale, 2, $5 \cdot 10^7$ cellules ganglionnaires de souris C57Bl/6 adultes et du même sexe que les receveurs; aux 51 souris du groupe IV, on a injecté le même nombre de cellules ganglionnaires provenant de souris C57Bl/6, immunisées par des cellules de la leucémie L 1210; cette immunisation a été réalisée par 5 injections de 10^5, $5 \cdot 10^5$, 10^6, $5 \cdot 10^6$ respectivement, séparées les unes des autres par des intervalles d'une semaine. Les cellules ganglionnaires de ces donneurs ainsi immunisés, ont été prélevées une semaine après la dernière injection. Aux 50 souris de groupe V, on a injecté le même nombre de cellules ganglionnaires provenant de souris C57Bl/6, immunisées par des cellules ganglionnaires et thymiques de souris DBA/2 normales; les modalités de l'immunisation sont les mêmes que précédemment.

II. *Même traitement, les cellules lymphoïdes étant placées en chambres de diffusion*

Cent soixantedix souris ont été consacrées à une expérience ne différant de la précédente que par le fait que les cellules lymphoïdes C57Bl/6 ont été placées en chambre de diffusion; le groupe I (témoins leucémiques) comporte 39 animaux; le groupe II (témoins leucémiques irradiés), 34 animaux; le groupe III (souris leucémiques traitées par 2, $5 \cdot 10^7$ cellules ganglionnaires en chambre de diffusion), 34 animaux; le groupe IV (souris leucémiques traitées par 2, $5 \cdot 10^7$ cellules ganglionnaires en chambre de diffusion, et provenant de donneurs immunisés par des cellules leucémiques), 34 animaux; le groupe V (souris leucémiques traitées par 2, $5 \cdot 10^7$ cellules ganglionnaires en chambre de diffusion et provenant de donneurs normaux), 29 animaux.

Un expérience témoin a été destinée à vérifier que les cellules C57Bl/6 ne sont pas sorties de la chambre de diffusion. Nous avons comparé, chez des animaux normaux F1 (DBA/2+C57Bl/6), les courbes des poids des deux ganglions inguinaux, après les avoir irradiés *in toto* à la même dose que précédemment, et avoir placé dans le peritoine 2, $5 \cdot 10^7$ cellules ganglionnaires C57Bl/6, soit libres, soit en chambre de diffusion. Tandis que, dans le premier cas, le ploids ganglionnaire s'élève entre le troisième et le septième jour pour redescendre à la normale le onzième, dans le second cas, le poids des deux ganglions ne subit aucune modification.

III. Traitement de la leucémie L 1210, *greffée sur des receveurs DBA/2, par des cellules lymphoïdes allogéniques C57Bl/6*

L'expérience est ici réduite à 4 groupes: groupe I, temoins (18 animaux); groupe II, traitement par les cellules ganglionnaires de donneurs non immunisés (28 animaux); groupe III, traitement par des cellules ganglionnaires de donneurs immunisés par les cellules leucémiques (28 animaux); groupe IV, traitement par des cellules ganglionnaires de donneurs immunisés par des cellules DBA/2 normales (29 animaux). L'expérience ne diffère de l'éxpérience I que par le fait que les animaux traités sont des souris DBA/2.

IV. *Traitement de la leucémie L* 120 *greffée sur des receveurs F1(DBA/2 × C57Bl/6), par des cellules lymphoïdes de rats immunisés ou non, les cellules étant libres ou en chambre de diffusion*

Cent cinquante-huit souris FI(DBA/2 × C57Bl/B) ont été consacrées à cette expérience qui ne diffère des précédentes que par la source des cellules ganglionnaires utilisées pour l'immunothérapie adoptive: celles-ci proviennent de rats Wistar CF. Les animaux du groupe I (témoins leucémiques) sont au nombre de 20; ceux du groupe II (témoins leucémiques irradiés) de 20; ceux du groupe III (souris traitées par 2, $5 \cdot 10^7$ cellules ganglionnaires de rats nonimmunisés) de 20; ceux du groupe IV (souris traitées par des cellules ganglionnaires de rats immunisés par les cellules leucémiques), de 20; ceux du group V (souris traitées par des cellules ganglionnaires de rats immunisés par des cellules DBA/2 normales), de 20; ceux du groupe VI (souris traitées par des cellules ganglionnaires placées en chambre de diffusion

et provenant de rats non immunisés), de 18; ceux du groupe VII (souris traitées par des cellules ganglionnaires placées en chambre de diffusion et provenant de rats immunisés par des cellules leucémiques), de 20; ceux du groupe III (souris traitées par des cellules ganglionnaire placées en chambres de diffusion provenant de rats immunisés par des cellules DBA/2 normales), de 20. L'immunisation des rats donneurs de cellules ganglionnaires a été identique à celle des souris, mentionnée plus haut.

RESULTATS

L'expérience sur le traitement des souris F1 (DBA/2 × C57Bl/6), porteuses de la leucémie L 1210 (DBA/2), par des cellules ganglionnaires C57Bl/6 (expérience I, Fig. 1): (a) confirme d'abord nos résultats, antérieurement publiés, [1, 2] en ce qui concerne l'effet des cellules lymphoïdes provenant de donneurs non immunisés (groupe III), celles-ci prolongeant la survie des animaux de façon significative (S à 1%), et la moitié d'entre eux mourant de syndrome secondaire lié à la réaction du greffon contre l'hôte; (b) elle montre que l'usage des cellules ganglionnaires C5Bl/6 provennant de donneurs immunisés par des cellules leucémiques n'améliore pas les résultats; (c) elle montre, en revanche, que l'emploi des cellules ganglionnaires C57Bl/6, provenant de donneurs immunisés par des cellules DBA/2 normales, donne un résultat nettement supérieur: 7 souris sur 38 sont guéries, et la grande majorité des souris qui meurent, meurent de syndrome secondaire, et non de leucémie.

Un résultat dans l'ensemble assez semblable, mais un peu moins favorable, a été obtenu en plaçant les cellules C57Bl/6 en chambre de diffusion (expérience II, Fig. 2) [19]. L'expérience témoin, destinée à vérifier que les cellules C57Bl/6 ne sont pas sorties de la chambre de diffusion, a montré que celles-là sont bien demeurées à l'intérieur de celle-ci, puisque le poids des ganglions inguinaux n'a pas suivi la courbe, d'abord ascendante, puis descendante, qu'il suit lorsque les cellules C57Bl/6 sont libres, phénomène que nous avons publié en 1962 (20).

L'expérience III (Fig. 3) montre que l'effet obtenu chez des receveurs DBA/2 par des cellules lymphoïdes C57Bl/6 qui ne se greffent

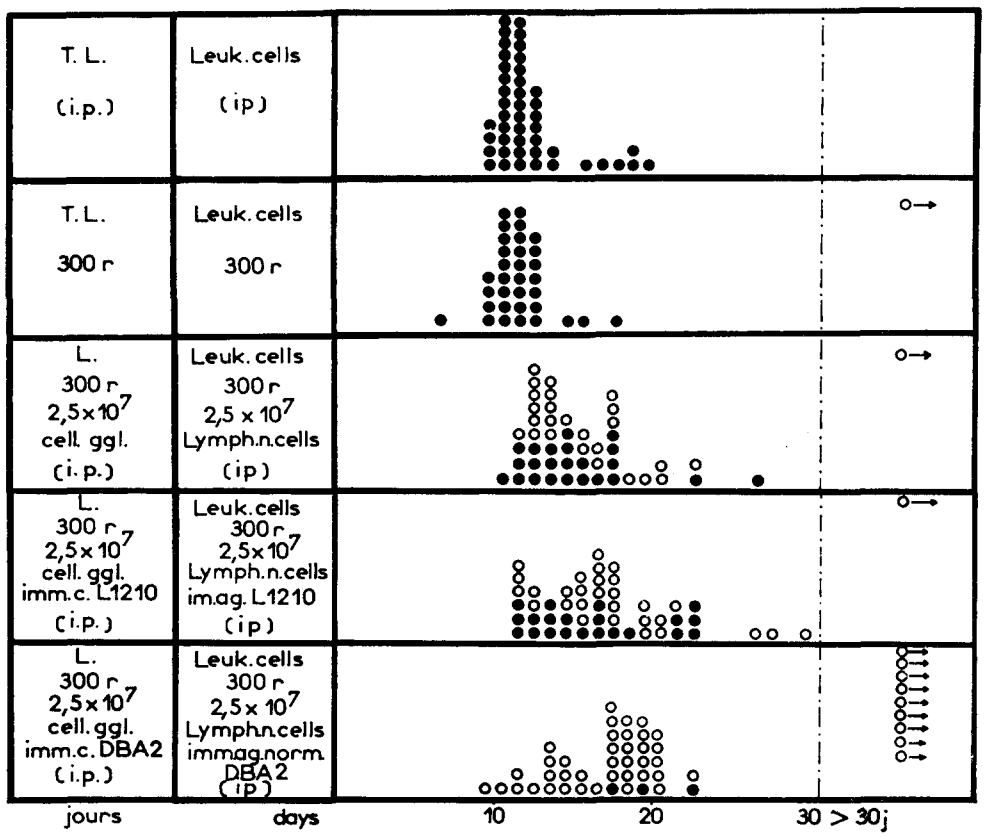


Fig. 1. *Traitement de souris ((DBA/2 × C57Bl/6) porteuses de la leucémie L* 1210, *par une irradiation totale à la dose de* 300 *rads, suivie d'une greffe de cellules ganglionnaires C57Bl/6 de donneurs non immunisés ou immunisés par des cellules leucémiques ou des cellules DBA/2 normales.*

● : *souris mortes de leucémie.* ○ : *souris mortes de syndrome secondaire.*

T.L. = témoins leucémiques.

L. = leucémies.

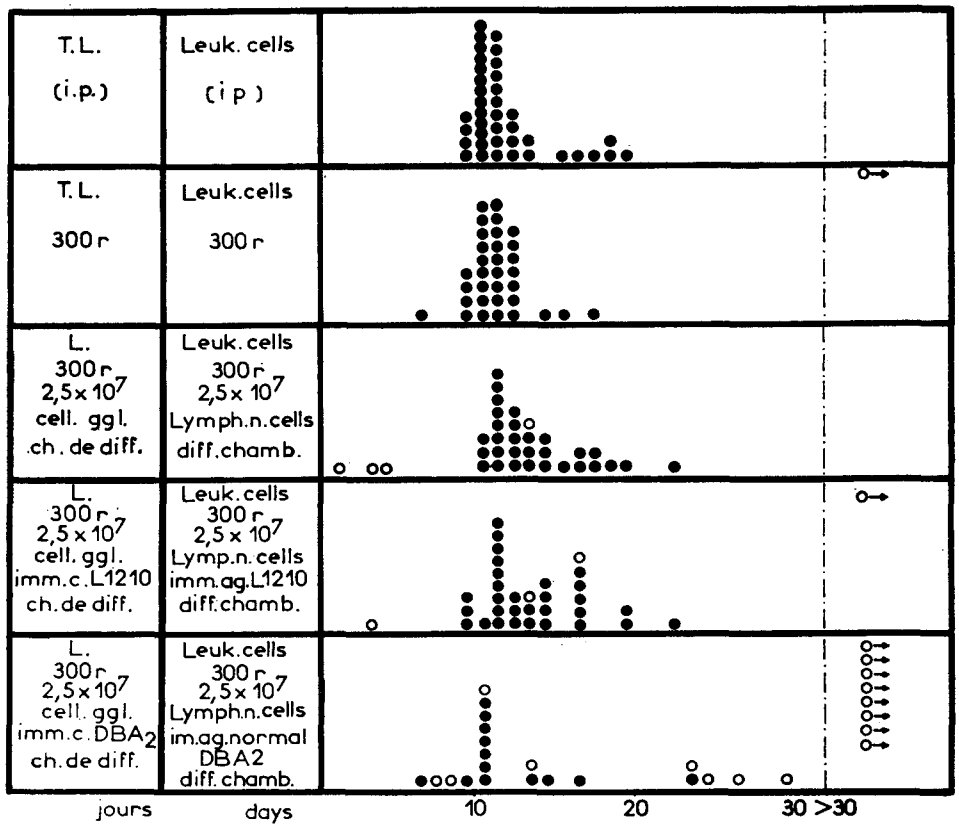


Fig. 2. L'expérience est la même que celle décrite à la figure 1, mais les cellules C57Bl/6 sont en chambre de diffusion. A noter que la leucémie est injectée par voie intrapéritonéale.
● : *souris mortes de leucémie.*
○ : *souris mortes de syndrome secondaire.*

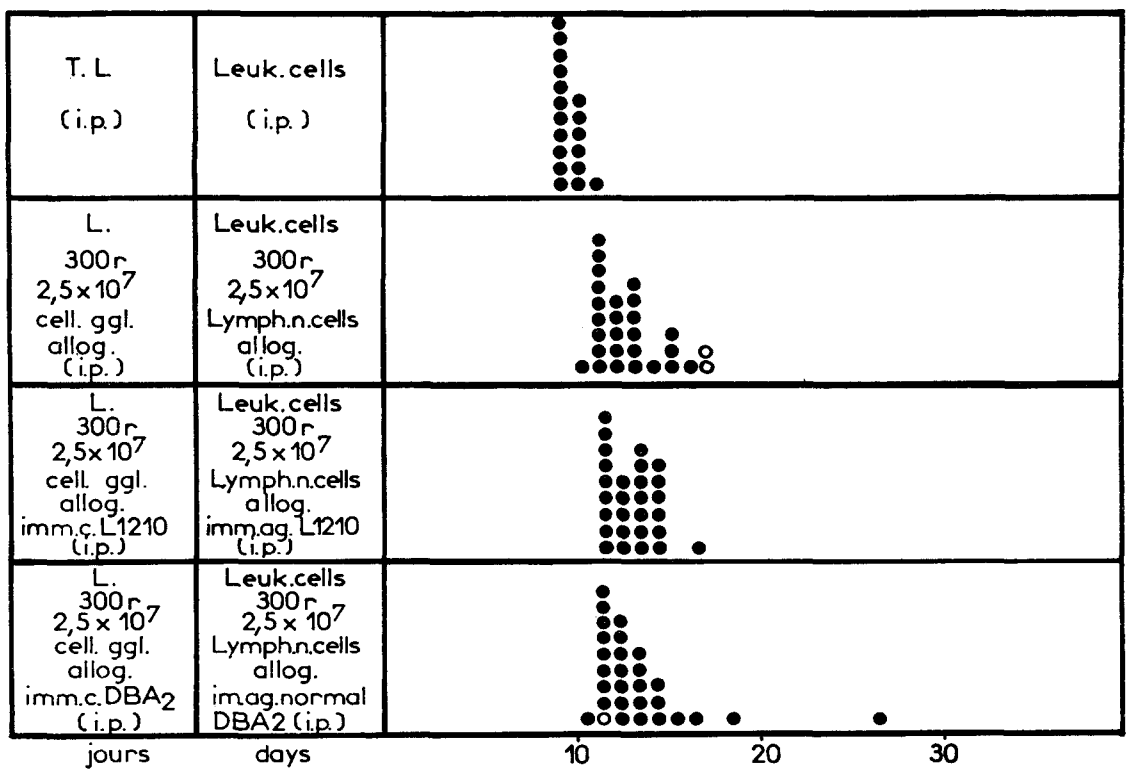


Fig. 3. Traitement de souris DBA/2 porteuses de la leucémie L 1210 par une irradiation totale de 300 rads, suivie d'une greffe de cellules ganglionnaires C57Bl/6 de donneurs non immunisés ou immunisés par des cellules leucémiques, ou par des cellules DBA/2 normales.

pas puisque allogéniques, bien que significatif, est nettement moindre que celui obtenu chez les F1 (DBA/2 × C57Bl/6) chez lesquels les cellules lymphoïdes C57Bl/6 se greffent: la plupart des souris recevant des cellules ganglionnaires de donneurs non immunisés meurent de leucémie; enfin, l'immunisation des donneurs, par des cellules leucémiques ou normales, n'ajoute rien à cet effet.

L'expérience IV (Fig. 4) montre enfin qu'aucun effet antileucémique n'est obtenu par les cellules ganglionnaires de rats, que les donneurs soient immunisés ou non, que les cellules soient libres ou en chambre de diffusion.

DISCUSSION

Trois notions sont donc révélées par cet ensemble d'expériences: (a) les cellules lymphoïdes C57Bl/6 sont d'autant plus efficaces qu'elles sont tolérées; (b) leur action se manifeste même lorsqu'elles sont placées en chambre de diffusion; (c) elle n'est pas amplifiée par l'immunisation des donneurs par les cellules leucémiques, alors qu'elle l'est par l'immuni-

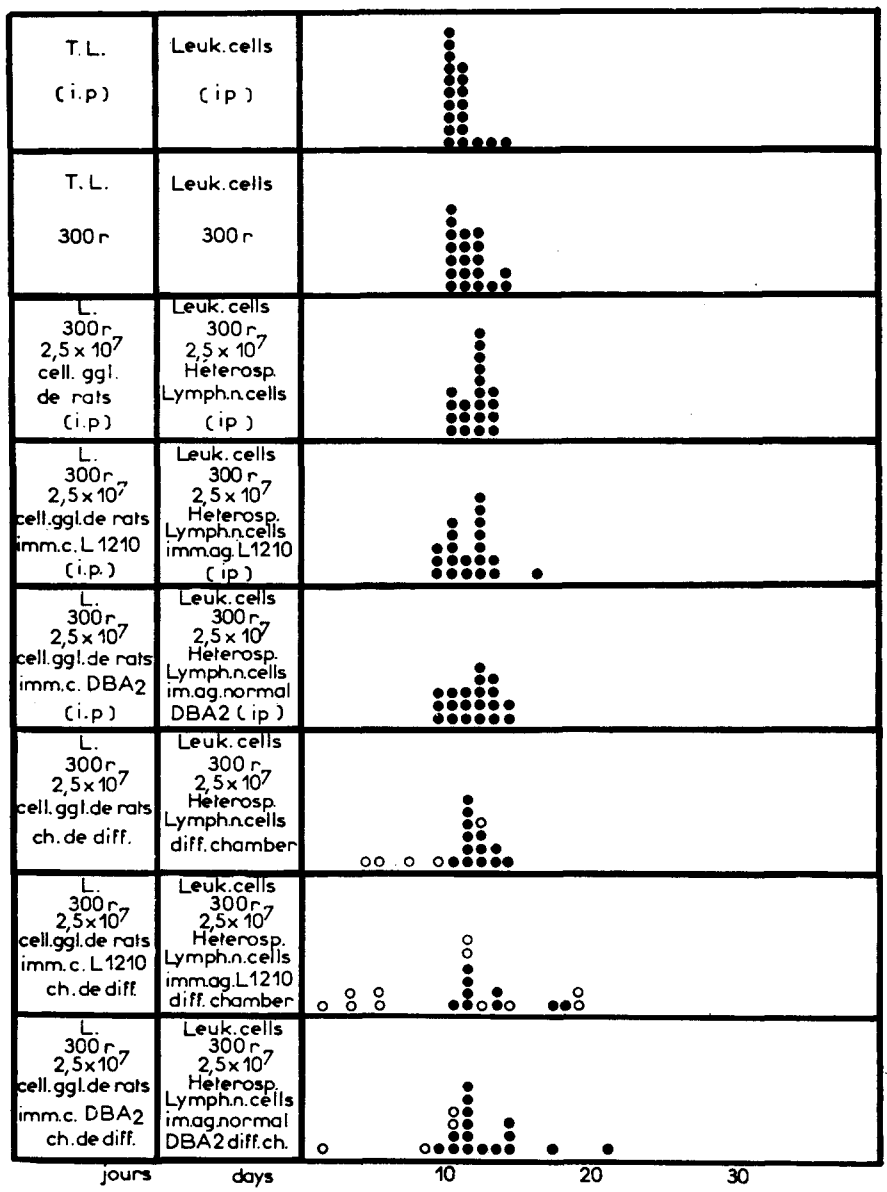


Fig. 4. *Traitement de souris F1 (DBA/2+C57Bl6), porteuses de la leucémie L* 1210, *par une irradiation totale de* 300 *rads, suivie de transfusion de cellules ganglionnaires de rats non immunisés ou immunisés par des cellules leucémiques ou des cellules DBA/2 normales, injectées par voie intraveineuse ou placées en chambre de diffusion.*

● : *souris mortes de leucémie.*

○ : *souris mortes de syndrome secondaire.*

sation des donneurs par des cellules normales de la lignée des cellules leucémiques.

La première notion n'est pas pour surprendre; les cellules lymphoïdes, lorsqu'elles sont tolérées: (a) peuvent prolonger leur action pendant 7 à 10 jours [18]; elles ont donc le temps, lorsqu'elles proviennent de donneurs non immunisés, de s'immuniser et de manifester leur action contre les cellules leucémiques; (b) elles prolifèrent considérablement pendant ces 7 à 10 jours, stimulées par la réaction du greffon contre l'hôte, comme nous l'avons montré [18], ce qui, non seulement peut multiplier leur action immunitaire, mais peut introduire un autre effet antileucémique lié à un effet de compétition.

La seconde notion concerne l'action des cellules ganglionnaires placées en chambre de diffusion; elle indique que cette action s'accomplit, au moins en partie, par l'intermédiaire de facteurs humoraux. Cela ne nous surprend pas, puisque nous avons montré que la mortalité de souris F1 (DBA/2 × C57Bl/6) produite par la réaction de cellules lymphoïdes C57Bl/6 contre les antigènes DBA/2 ne diffère pas notablement selon que les cellules sont libres dans le péritoine ou placées en chambre de diffusion [19].

La troisième notion est beaucoup plus surprenante : l'action des cellules lymphoïdes C57Bl/6 n'est pas amplifiée par l'immunisation des donneurs par les cellules leucémiques, mais elle l'est considérablement par leur immunisation par les cellules DBA/2 normales. Ce phénomène n'est pas, aujourd'hui, explicable de façon satisfaisante: on remarquera qu'il a lieu, que les cellules soient libres ou en chambre de diffusion. Deux notions doivent être ici mentionnées : (a) les cellules leucémiques L 1210 possèdent certes une néo-antigénicité *tumorale* particulière, indiquée par le fait que l'on peut immuniser des receveurs spécifiquement contre elles; mais cette antigénicité n'est que très faible, car l'immunisation de receveurs à leur égard est difficile et ne peut être mise en évidence que par des techniques très sensibles [21]; (b) l'apparition de la néo-antigenicité *tumorale* des cellules semble réduire l'antigénicité liée aux antigènes d'histo compatibilité normaux [22]; ces deux notions conduisent à penser que la réaction des cellules lymphoïdes C57Bl/6 contre les antigènes DBA/2 normaux des cellules leucémiques joue un rôle plus important que leur action contre les antigènes spécifiques.

Nos résultats sur cette leucémie L 1210 sont donc opposés à ceux d'Alexander *et al.* [16] qui obtiennent, avec la leucémie 5178 Y, des résultats par immunothérapie adoptive, sans que les cellules lymphoïdes ne soient tolérées par l'hôte, résultats qu'ils n'enregistrent que si les donneurs sont immunisés par des cellules leucémiques. Nous avons nous-mêmes, dans le cas de la tumeur BP8 ascitique, obtenu d'excellent résultats par des cellules lymphoïdes hétérospécifiques provenant de donneurs immunisés [23]. La raison de ces différences réside peut-être dans le fait que la leucémie 5178 Y est très antigénique (il est facile d'immuniser des souris DBA/2 contre elle), de même que l'est la tumeur BP8, tandis que la leucémie L 1210 l'est très peu.

Etant donné le difficulté à demontre une néo-antigénicité propre aux cellules leucémiques humaines, on peut raisonnablement penser qu'elle est faible et que, pour cette raison, la leucémie L 1210 est un bon modèle expérimental pour étudier le traitement immunitaire des leucémies humaines. Les essais d'immunothérapie adoptive que nous avons conduits chez l'Homme [24, 25] nous ont d'ailleurs déjà révéle que l'action antileucémique d'une greffe de moelle osseuse allogénique est très supérieure, en cas de repopulation médullaire, totale et de longue durée, par le greffon, à ce qu'elle est en cas de repopulation seulement partielle et de courte durée. Ces notions doivent donc inciter à poursuivre les recherches expérimentales et cliniques sur les essais de greffes de moelle osseuse allogénique, dont le syndrome secondaire représente encore un obstacle majeur pour la pratique.

RESUME

Le traitement de la elucémie L 1210 (*leucémie DBA*/2), *dans sa forme ascitique, par des cellules lymphoïdes C*57*B*1/6 *injectées par voie péritonéale, est plus efficace lorsque la leucémie est greffée chez des souris F*1 (*DBA*/2 × *C*57*Bl*/6), *c'est-à-dire lorsque les cellules lymphoïdes C*57*Bl*/6 *sont tolérées, que lorsqu'elle est greffée chez des souris DBA*/2, *c'est-à-dire lorsque les cellules lymphoïdes C*57*Bl*/6 *ne sont pas tolérées; les cellules lymphoïdes hétérospécifiques* (*de rats*) *ne sont d'aucune efficacité.*

Les cellules lymphoïdes C57*Bl*/6 *sont efficaces sur la leucémie L* 1210 *portée par des hôtes F*1 (*DBA*/2 × *C*57*Bl*/6), *même lorsque ces cellules sont placées en chambre de diffusion.*

L'action des cellules lymphoïdes C57Bl/6 sur la leucémie L 1210, *portée par des hôtes F*1 (*DBA/2*×*C57Bl/6*), *n'est pas amplifiée par l'immunisation des donneurs par les cellules leucémiques, alors qu'elle l'est considérablement par l'immunisation des donneurs par des cellules normales DBA/2.*

La signification de ces faits est discutée.

SUMMARY

The treatment of L 1210 *acute leukaemia* (*DBA/2 leukaemia*) *in its ascitic form, by intraperitoneal injection of C57Bl/6 lymphoid cells, is more effective when the leukaemia is grafted on F*1 (*DBA/2*×*C57Bl6*) *mice, i.e. when C57Bl/6 lymphoid cells are tolerated than when it is grafted on DBA/2 mice, i.e. when C57Bl/6 lymphoid cells are not tolerated. Heterospecific lymphoid cells* (*from rats*) *are not effective.*

C57Bl/6 lymphoid cells are effective on L 1210 *leukaemia carried by F*1 (*DBA/2*× *C57Bl/6*) *mice, even when these cells are located in a diffusion chamber.*

The effect of C57Bl/6 lymphoid cells on L 1210 *leukaemia carried by F*1 (*DBA/2*× *C57Bl/6*) *mice is not enhanced by immunization of the donors by leukaemic cells while it is considerably increased by immunization of donors by normal BBA/2 cells.*

The significance of these facts is discussed.

ZUSAMMENFASSUNG

Die Behandlung der Leukämie L 1210 (*DBA/2-Leukämie*) *in ihrer aszitischen Form mit Hilfe von peritonäal injizierten C57Bl/6-Lymphzellen ist erfolgreicher, wenn die Leukämie auf F*1- (*DBA/2*×*C57Bl/6*)-*Mäuse transplantiert wurde, d.h. wenn die C57Bl/6-Lymphzellen toleriert werden, als wenn sie auf DBA/2-Mäuse transplantiert wurde, d.h. wenn die C57Bl/6-Lymphzellen nicht toleriert werden; die Behandlung mit heterospezifischen* (*Ratten*)-*Lymphzellen ist nicht wirksam.*

Die C57Bl/6-Lymphzellen sind bei L 1210-*Leukämie von F*1- (*DBA/2*×*C57Bl/6*)-*Mäusen wirksam, selbst dann, wenn sie sich in einer Diffusionskammer befinden.*

Die Wirksamkeit von C57Bl/6-Lymphzellen auf die L 1210-*Leukämie bei F*1-(*DBA/2*×*C57Bl/6*)-*Mäusen wird durch die Immunisierung der Spender mit Leukamiezellen nicht verstärkt, während sie durch die Immunisierung mit normalen DBA/2-Zellen erheblich intensiviert wird.*

Die Bedeutung dieser Befunde wird besprochen.

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Relation entre Activité Cancérogène et Structure Moléculaire chez des Isostères Soufrés et des Isomères des Benzacridines

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INTRODUCTION

LES RECHERCHES sur l'activité cancérogène des composés hétérocycliques isostères des hydrocarbures polycycliques aromatiques présentent un intérêt fondamental, car elles permettent de soumettre à la vérification expérimentale certains des concepts de base de la théorie électronique de la cancérisation. Cette théorie repose principalement sur les deux notions suivantes [1–5].

(1) *L'activité cancérogène des hydrocarbures aromatiques polycycliques serait étroitement liée à la présence des réseaux de doubles liaisons conjuguées (et par conséquent d'électrons* Π*), que constituent les cycles benzéniques dont sont formés les squelettes moléculaires de ces composés.*

Ce concept a été validé par le fait que la destruction d'un ou de plusieurs de ces réseaux, par hydrogénation, entraîne la diminution ou la suppression de l'activité biologique, dans les cas ou l'organisme animal en jeu est incapable d'hydrogéner à nouveau la molécule [6]. Un corollaire important de ce concept est qu'il doit être possible de substituer un ou plusieurs cycles benzéniques de la molécule cancérogène par un ou plusieurs cycles isoélectroniques (c'est-à-dire équivalents quant au nombre et à la répartition des électrons Π), sans que les propriétés cancérogènes en soient affectées; à condition toutefois que cette substitution n'empêche pas les réactions biochimiques entre le composé hétérocyclique obtenu et les molécules cellulaires impliquées dans le processus de cancérisation. Ce corollaire a été amplement vérifié par la découverte des benzacridines et des thiéno (*b*) phénanthrènes [8] cancérogènes, qui sont des analogues des benzo (*a*) anthracènes en série pyridique et en série thiophénique.

(2) *Les zones mésophénanthréniques (dites zones K selon la terminologie proposée par A. et B. Pullman) [3] joueraient un rôle important dans les réactions biochimiques invoquées ci-dessus*

La grande majorité d'hydrocarbures cancérogènes possède, en effet, une ou plusieurs zones *K*. Chaque fois que la métabolisation de ces composés a pu être élucidée [9], on a trouvé des modifications chimiques au niveau de ces zones (hydroxylation, oxydation en orthoquinones, et même en acides). Il est donc intéressant de savoir ce que deviendra l'activité cancérogène des hydrocarbures munis de zone

K, si l'on remplace, dans ces dernières, la liaison —C(H)=C(H)— par une liaison N=CH ou un atome de –S–. En particulier, le pouvoir cancérogène des benzo (*a*) anthracènes sera-t-il supprime si l'on remplace le cycle C, soit par un cycle pyridine, soit par un cycle thiophène? La réactivité chimique sera devenue alors forcément très différente de celle du cycle benzénique.

Les premiers travaux, de nature à fournir des éclaircissements à ce sujet, sont dus à Tilak [10]*. On savait que le remplacement du cycle A par un cycle thiophène (Tableau 1), dans molécule du 7, 12-diméthyl (*a*) anthracène (1), conduit à des substances qui ne sont que légèrement moins actives (par exemple le composé II).

Poursuivant leurs études, Tilak et Beremblum [11]* ont soumis au test de cancérogénicité le 4,9-diméthyl-2,3-benzo-thiophenanthrène (Tableau I—composé III), dans lequel le cycle *C* est remplacé de telle sorte que la zone *K* fait place à l'hétèroatome de soufre. Ce corps a été trouvé totalement inactif chez la Souris, en injections sous-cutanées, et très faiblement actif en applications sur la peau. Cette confirmation du rôle prépondérant de la zone *K* est encore renforcée par le fait de la réapparition d'un pouvoir cancérogène important quand, dans ce même composé, on fait naître une nouvelle zone *K* par accolement d'un noyau benzénique supplémentaire (Tableau 1 — composés IV et V).

Le présent travail est une étude analogue à celle de Tilak *et al.* [11], effectuée dans une famille chimique à laquelle nous nous intéressons depuis de nombreuses années, celle des benzacridines. Il a porté sur deux catégories de corps:

(1) les isostères soufrés de benzacridines et de dibenzacridines angulaires dont une zone *K* est remplacée par un hétéro-atome de soufre;

(2) les isomères de benzacridines angulaires,

Tableau 1

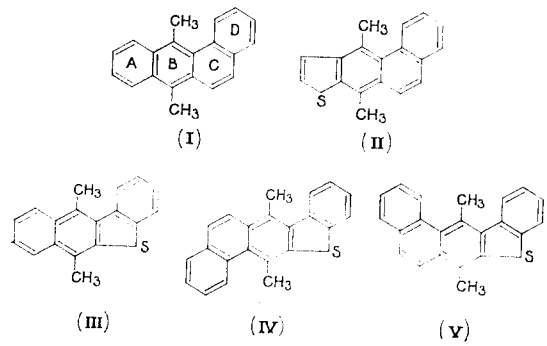
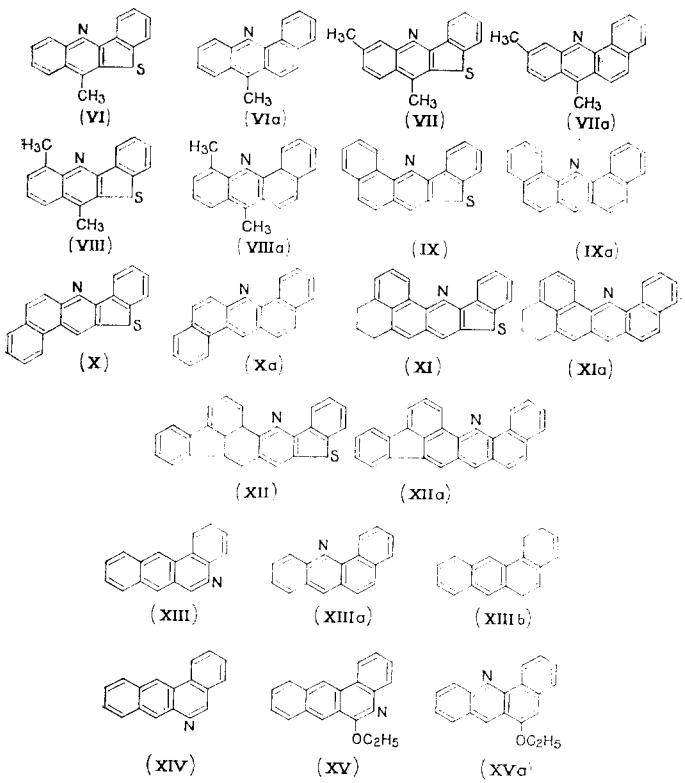


Tableau 2



6, Méthylthiaquindoline (VI)
6, 9-Diméthylthiaduindoline (VII)
6, 10-Diméthylthiaquindoline (VIII)
Naphto (2, 1-*e*) thianaphténo (3, 2-*b*) pyridine (IX)
Naphto (1, 2-*e*) thianaphténo (3, 2-*b*) pyridine (X)
Acénaphténo (4, 5-*e*) thianaphténo (3, 2-*b*) pyridine (XI)
Fluoranthéno (5, 4-*e*) thianaphténo (3, 2-*b*) pyridine (XII)
Benzo (*j*) phénanthridine (XIII)
Benzo (*b*) phénanthridine (XIV)
6-Ethoxybenzo (*j*) phénanthridine (XV)
7-Méthylbenzo (*c*) acridine (VIa)
7, 10-Diméthylbenzo (*c*) acridine (VIIa)
7, 11-Diméthylbenzo (*c*) acridine (VIIIa)
Dibenzo (*c*, *h*) acridine (IXa)
Dibenzo (*a*, *h*) acridine (Xa)
9, 10-Acédibenzo (*c*, *h*) acridine (XIa)
Fluoranthéno (5, 4-*e*) naphto (2, 1-*e*) pyridine (XIIa)
Benzo (*c*) acridine (XIIIa)
Benzo (*a*) anthracène (XIIIb)
6-Ethoxybenzo (*c*) acridine (XVa)

*Les composés synthétisés par cet auteur ont été essayés par I. Beremblum (résultats non publiés).

Tableau 3

Composé	Nombre de souris	Lignée	Sarcomes	Survies en jours	Temps de latence tumorale en jours
(VI)	7 ♂ et 7 ♀	XVIInc/ZE	0	de 220 à 602 (0)	
(VII)	7 ♂ et 7 ♀	idem	0	de 190 à 602 (0)	
(VIII)	7 ♂ et 7 ♀	idem	0	de 260 à 570 (0)	
(IX)	7 ♂ et 7 ♀	idem	1	267 (0) 157 (*S*) 267 (0) 196 (0) 274 (0) 279 (0) 269 (0) 349 (0) 346 (0) 402 (0) 357 (0) 402 (0) 402 (0) 402 (0)	132
	14 ♂ et 14 ♀	Swiss	2	202 (0) 225 *S* 252 *S*	190 201
				270 (0) 362 (0) 368 (0) douze sacrifiées à 460 (0) dix sacrifiées à 460 (0)	
(X)	7 ♂ et 7 ♀	XVIInc/ZE	1	173 (0) 208 (0) 222 *S* 241 (0)	152
				224 (0) 312 (0) 302 (0) 343 (0) 362 (0) 402 (0) 398 (0) 402 (0) 402 (0) 402 (0)	
	14 ♂ et 14 ♀	Swiss	3	192 *S*	152
				228 *S* 252 (0)	168
				278 *S*	210
				306 (0) 362 (0) 322 (0) 392 (0) treize sacrifiées à 460 (0) neuf sacrifiées à 460 (0)	
(XI)	14 ♂ et 14 ♀	XVIInc/ZE	0	de 315 à 602	
(XII)	14 ♂ et 14 ♀	idem	0	de 290 à 602	
(XIII)	14 ♂ et 14 ♀	idem	0	de 307 à 602	
(XIV)	14 ♂ et 14 ♀	idem	0	de 215 à 602	
(XV)	14 ♂ et 14 ♀	idem	0	de 275 à 602	

(*S*) Sarcome
(0) Absence de sarcome.

dans la molécule desquels l'hétéro-atome d'azote est situé sur la zone *K*. De tels isomères peuvent également être considérés comme des isostères azotés de benzo (*a*) anthracènes.

MATERIEL ET METHODES

(1) *Composés chimiques*

Une dizaine de substances, utilisées pour la présente étude, avaient été synthétisées *ad hoc* dans nos laboratoires respectifs [12]:

A — Sept isostères soufrés (formules de VI à XII du Tableau 2) des benzacridines et dibenzacridines (formules VIa à XII), dont le pouvoir cancérogène a été étudié précédemment (7).

B — Trois isoméres suivants de benzacridines: (a) la benzo (*j*) phénanthridine (XIII), isomère

de la benzo (*c*) acridine (XIIIa), et isostère azoté du benzo (*a*) anthracène (XIIIb); (b) la benzo (*b*) phénanthridine (XIV), également isomère de la benzo (*c*) acridine, et isostère azoté du benzo (*a*) anthracène.
(c) la 6-éthoxybenzo (*j*) phénanthridine (XV), isomère de la 6-éthoxybenzo (*c*) acridine (XVa), et isostère azoté du 6-éthoxybenzo (*a*) anthracène.

(2) *Animaux*

Le pouvoir cancérogène de ces dix substances a été testé chez les souris de la lignée XVII nc/ZE de l'Institut du Radium. Cette lignée, développée à notre Institut, est utilisée systématiquement depuis de nombreuses années pour tous les tests de cancérogènes chimiques, à cause de l'absence complète (sur une population de plus de 70.000 animaux autopsiés) de tumeurs sous-cutanées spontanées. Deux produits (IX et X) ont été essayés également sur des souris Swiss de Carshalton (Surrey Angleterre).

(3) *Technique*

Les animaux (des deux sexes), âgés de 3–4 mois au début de l'expérience, ont reçu trois injections sous-cutanées, à un mois d'intervalle, d'un de ces composés, contenant chacune 0,6 mg dissous dans 0,21 d'huile d'olive neutre.

RESULTATS

Ils sont consignés dans le Tableau 3.

DISCUSSION

Il ressort de l'examen du Tableau 3 que:
(a) Les isostères soufrés (VI, VII et VIII) des trois benzacridines cancérogènes (VIa, VIIa et VIIIa) [7] ont été trouvés totalement inactifs; le remplacement de la zone *K* par un hétéro-atome de soufre fait donc disparaître le pouvoir cancérogène.
(b) Le composés IX et X sont faiblement sarcomogènes.

Il est à noter, comme fait très significatif, que ces deux composés, tout en ayant la région *K* modifiée comme les composés VI, VII et VIII, disposent à la suite de l'accolement d'un noyau benzénique supplémentaire d'une nouvelle zone *K* véritable.

Ces résultats sont donc tout à fait parallèles à ceux obtenus par Tilak et Berenblum, dans une série chimique pourtant toute différente. Ils confirment à nouveau le rôle important que doit jouer la zone *K* dans le processus de cancérisation par les hydrocarbures polycycliques.

La raison de l'inactivité des composés tels que les produits XI et XII s'explique vraisemblablement par l'absence d'une région *K* véritable. En effet la seule liaison qui pourrait éventuellement jouer le rôle d'une zone *K* est encombrée stériquement par un cycle saturé, ce qui empêche probablement ces molécules d'atteindre les récepteurs biologiques impliqués dans la cancérisation. Mais on sait par ailleurs que des molécules totalement dépourvues de zones *K* peuvent montrer une activité cancérogène, faible mais certaine, comme les homologues du dibenzo (*a*, *c*) anthracène [13]. Dans ces cas rares, une zone de la molécule (non identifiée jusqu'à présent), de densité électronique favorable, se substituerait à la zone *K* véritable.

Enfin, l'inactivité des phénanthridines (XIII et XIV) n'est pas surprenante; le modèle moléculaire dont elles dérivent, c'est-à-dire le benzo (*a*) anthracène (XIIIb) et la benzo (*c*) acridine non substituée (XIIa) sont eux-mêmes inactifs. Quant au dérivé éthoxylé (XV), il est difficile de faire des comparaisons, son analogue (XVa), en série benzo (*c*) acridine, n'ayant pas encore été étudié.

Nous nous proposons d'étudier prochainement des analogues de ces produits méthylés en position 7 qui favorise le pouvoir cancérogène.

Remerciements — Ces recherches ont été subventionnées par le S.E.I.T.A. (Directeur des recherches, M. Vesperini) et l'Institut National de la Santé et de la Recherche Médicale (Directeur général, le Pr. Aujaleu).

RESUME

Afin de vérifier l'importance des zones K *dans le processus de la cancérisation par les composés polycliques, on a recherché, chez la souris, l'activité sarcomogène d'une dizaine de substances hétéro-polycycliques, dont* 7 *isotères soufrés et* 3 *isomères de benzacridines et dibenzoacridines. De tous les corps examinés, seuls se sont montrés sarcomogènes ceux qui possèdent une zone* K *véritable. Ce résultat fournit un argument expérimental supplémentaire au concept de l'importance de cette zone dans la cancérisation chimique.*

SUMMARY

In order to verify the importance of the K*-zones in the process of cancerization by polycyclic compounds, the carcinogenic activity of* 10 *heteropolycyclic substances, of which* 7 *were sulfur-containing isosters, and* 3 *were isomers, of benzacridines and dibenzacridines, has*

been investigated. Of these substances, only those possessing a true K-*zone proved carcinogenic. This result leads further experimental support to the concept of the importance of the* K-*zone in chemical carcinogenesis.*

ZUSAMMENFASSUNG

Um die Bedeutung der K-*Zone der polyzyklischen krebserzeugenden Kohlenwasserstoffe besser festzustellen, wurden sieben Schwefelisostere und drei Isomere von Benz-und Dibenzakridinen synthetisiert und ihre sarkomerregende Wirkung am subkutanen Gewebe der Maus untersucht..*

Nur die Verbindungen, die eine K-*Zone besitzen, sind sarkomogen.*

Diese Resultate erbringen einen zusätzlichen Beweis, dass die K-*Zone in der chemischen Kanzerogenese eine wichtige Rolle spielt.*

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Chemotherapy of Myosarcoma (Leio- and Rhabdomyosarcoma)

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INTRODUCTION

UNTIL recently, there has been little investigation into the natural history of myosarcoma, but it is evident that widespread metastases or local recurrence after radical surgery frequently occurs. Following recurrence, the prognosis is very poor, with less than 10% of the patients (adults and children) surviving one year or more. See Creech *et al.* [1], and Potter [2]. Some patients with recurrent leiomyosarcoma may survive up to 3 years Spiro *et al.* [3] and Wiltshaw [4].

Radiotherapy is generally considered only mildly effective, especially for primary invasive bone lesions and metastatic deposits; soft part primary involvement, on the other hand, is known to regress rapidly. However, relapse usually occurs after radiotherapy, even with tumour doses ranging from 4000 to 6000 rad or more [5, 6].

So one may draw the conclusion that many such patients require some form of medical treatment.

Chemotherapy results have been published of small series or single patients, and many of the relevant papers are rather difficult to find: indeed, some cases of myosarcoma are recorded under headings like *Effectiveness of Actinomycin D in Cancer in Children* and so on.

Twenty-seven cases of leiomyosarcoma and about 200 cases of rhabdomyosarcoma, treated by various forms of chemotherapy, have been collected here, both from the available literature and from personal experience, for comparative evaluation of the drugs available.

Similarity of drugs useful in these two conditions (i.e., human leio- and rhabdomyosarcoma) justifies cumulative reporting. Further similarity of chemosensitivity has also been found in our preliminary experiences with various cytotoxic drugs in transplantable myosarcoma in rodents [7, 8].

Report of cases collected from literature

In collecting cases from the literature to assess the effectiveness of various chemotherapeutic agents in leiomyosarcoma and/or rhabdomyosarcoma, many had to be excluded for one or more of the following reasons:—

The patients were lost to follow up shortly after treatment was given; the type of drug and dosage was not adequately recorded, or the patient received only very small amounts of the drug; sometimes radiotherapy was given to all the known deposits at the same time as chemotherapy was being administered [9].

Because we wanted to assess the usefulness of the drugs to the patients, very brief regression or subjective improvement alone were not considered of value and are listed as 'not improved'. Other cases are listed as 'improved' when objective regression of masses occurred and lasted for one month or more or when a symptom or sign related to the presence of the tumour disappeared completely for several years.

Report of our cases

A brief account is given here of the age, sex, histology, primary tumour location and site of

Table 1.

References*	Age and location†	No. of treatments‡	Drug(s) §	No. improved‖	Histology and/or notes¶
[11] Albores-Soavedra 1963	2 young adults and 4 children	6	DACT	3	The two responders were children. 1 of these had DACT+Cytoxan
		1	ThioTEPA and Cytoxan	0	
		1	TEM+Mtx	1	
[12] Ansfield 1959	Adult	1	5 Fu	0	Leiomyosarcoma
[13] Atkins 1962	Children	3	Cytoxan	0	1 patient with embryional rhabdomyosarcoma, 1 myoblastoma and 1 leiomyosarcoma
[14] Bateman 1958	Random age	19	ThioTEPA	4	Sarcomas; not specified how many of the 19 were myosarcomas. Of the remitters 2 were rhabdo- and 2 leiomyosarcomas
[15] Bailey 1961	Children	5	DACT	3	The responders were embryonal type; the non responders 1 embryonal and 1 alveolar
[16] Blanchard 1957	Child; middle ear	1	HN2	0	
[17] Colebatch 1960	Child	1	DACT	0	
[18] Cramblett 1959–60	Child	1	Cytoxan	1	Low dose, complete but unmaintained remission
[19] Di Pietro	Boy	1	Cytoxan	1	This case previously proved resistant to Sarcolysin and Daunomycin
		5	DACT	1	One case, originated from nasopharynx, alive and well 5 years after treatment of pulmonary metastasis by DACT+X Ray (6000 rads tumour dose)
[6] Edland 1965 and [10] Edland 1967	All children	2	VCR	2	One alive and well after 10 months (primary in the liver)
		1	VELBE	0	
		1	HN2	0	
		1	Sarcoysin	0	
		1	Cytoxan+Meticorten	0	
[20] Guazzelli	Child 8 yrs old	1	Cytoxan	1	Long remission
[21] Gumport 1958	Adult	1	Chlor.	0	Leiomyosarcoma
[22] James 1966	All children	6	VCR+DACT	6	This is a particularly valuable association also for other childhood tumours
[23] Karnofsky 1966	All children	10	Daunomycin	3	
[24] Karnofsky 1965	Child, 5 yrs old	1	DACT+Mtx+Cytoxan	1	After 5 years, living and well
[25] Keidan 1966	Children	2	DACT	0	
[26] Manheimer 1966	Adult, stomach	1	Mit.	0	Leiomyosarcoma
[27] Moore 1957	Adult, retroperitoneal	1	Gammaglobulin	1	Leiomyosarcoma

Table 1.—*contd.*

References*		Age and location†	No. of treatments‡	Drugs(s) §	No. improved‖	Histology and or notes¶
[28] and [29]	Pinkel 1962, 1959	All children	6	DACT	1	In the hands of the same author Cytoxan proved more promising than DACT (see below)
[29]	Pinkel 1959	All children	7	Cytoxan	3	
[30]	Shaw 1960	All children	5	DACT	3	
[31]	Steinberg 1963	All children	4	Cytoxan	1	5 years old boy had a bladder rhabdomyosarcoma
[32]	Stobbe 1950	Child, middle ear	1	2,6-diaminopurine	0	
			25	Alkylating agents (except cytoxan)	3	The largest series ever reported of rhabdomyosarcomas treated by chemotherapy, stresses the superiority of cytoxan over other alkylating agents and of vincristine over DACT
			14	Cytoxan	7	
[33]	Sutow 1965	All children	11	VCR	7	
			11	DACT	1	
			21	Miscellaneous	2	
[34]	Sweeney 1962	All children	3	Cytoxan	1	
[35] [37]	Tan 1965, in press	Children and one adult	13	Daunomycin	3	All responders were children
[36]	Tan 1959	All children	7	DACT	2	1 of the responders had improved with Mustine and with Mtx. before DACT
[38]	Wilson 1958	Adult	1	5 Fu	0	Leiomyosarcoma
[39]	Wiltshaw	Adults	1	Melphalan	0	Leiomyosarcoma
			1	Aminochlorambucil		
[4]	Wiltshaw 1967	Adults	11	Mtx.	3	Leiomyosarcoma
			1	Mtx.	0	Rhabdomyosarcoma

*See below. †When reported. ‡Fit for evaluation of chemotherapy.
§*Abbreviations:* Cytoxan=cyclophosphamide; Chlor=chlorambucil; DACT=actinomycin D; 5 Fu=5 fluorouracil; HN2=mechlorethylamine; Mit.=mitomycin; Mtx.=methotrexate; VCR=vincristine; VELBE=vinblastine.
‖See in the text criteria for improvement (we have accepted not less than 1Cl of Karnoksfy [40].
¶Histology, when not specified, is rhabdomyosarcoma.

metastases of 11 patients suffering from myosarcoma and treated by anticancer drugs in our department during the last three years.

For the last five patients final outcome may not be predicted so far; however the clinical course during and after our therapeutic trials is reported in Table 2.

Case 1: woman, aged 67 (No. 7002)
Rhabdomyosarcoma of left kidney; pulmonary, cerebral and osseous metastases.

Case 2: woman, aged 53 (No. 6602)
Leiomyosarcoma of uterus; local recurrence after surgery and radiation therapy.

Case 3: woman, aged 64 (No. 8065)
Rhabdomyosarcoma of left thigh. Pulmonary metastases.

Case 4: woman, aged 38 (No. 8738)
Leiomyosarcoma of uterus; abdominal widespread metastases.

Case 5: man, aged 49 (No. 10100)
Rhabdomyosarcoma of leg; pelvic bone metastases.

Case 6: woman, aged 65 (No. 11006)
Leiomyosarcoma of left thigh. Thyreotoxicosis and myocardial sclerosis.

Case 7: woman, aged 46 (No. 10698)
Leiomyosarcoma of uterus; local recurrence, after surgery and radiation therapy.

Case 8: man, aged 64 (No. 9651)
Leiomyosarcoma of oesophagus; very large abdominal masses, with bowel obstruction.

Table 2.

CASES	5Fu	CYTO-XAN	VCR	DACT	MTX	CHLOR	DAUNO-MYCIN	VELBE	RESULTS ⊕ OF TREATMENTS I	II	III
1		▨							$1C^{3}$		
2	□	▨							0	$1C^{12}$	
3		▨		▨	▨	▨			$1C^{6}$	$1C^{4+}$	+ RADIATION $1C^{10+}$
4		▨						□	$1C^{4}$	0	
5		□		▨	▨	▨			0	$1C^{1+}$	
6		?	?				?		death		
7		□	▨	□	?				0	$1A^{2}$	?
8		▨	▨						$1C^{2+}$		
9		▨	▨	□	?				$1A^{2}$	?	
10		▨	▨						$1C^{1}$		
11		□	?						0	?	

FOR NAMES OF DRUGS, SEE TABLE 1. ⊕ACCORDING TO (40) KARNOFSKY (1961)

THE NAMES OF DRUGS FOLLOW EACH OTHER IN SUCH A WAY THAT VARIOUS TREATMENTS ARE RECORDED IN THE SAME CHRONOLOGY AS THEY HAVE BEEN ADMINISTERED TO OUR PATIENTS -

□ THE DRUG PROVED NOT REWARDING

? THE DRUG'S EFFECT MAY NOT BE ASSESSED

▨ THE DRUG HAS PRODUCED SOME IMPROVEMENT

2 OR 3 DRUGS HAVE BEEN USED IN COMBINATION

1. Extensive previous radiotherapy may have caused early leucopenia, so that treatment was forcedly interrupted at the third elapse.
2. 5 Fu, given to toxicity, resulted completely uneffective. Viral hepatitis compelled to interrupt maintenance Cytoxan treatment, apparently causing terminal relapse.
3. The only deposit persisting after chemotherapy was irradiated on; patient is now in full unmantained remission after 10 months.
4. An attempt to perform radical surgery after chemotherapeutic improvement caused a general decay that proved not reversible.
5. Patient lost to follow-up after dismission.
6. Treated intra-arterially by VCR and daunomycin; intramuscularly by Ctyoxan. Probaly died from reabsorption toxicity from necrotized tumour.
7. Triple therapy, according to Li is now running after secondary resistance to Vincristine.
8. Was moribund and now is walking, after 11 mg VCR+ 4000 mg, Cytoxan. Objective improvement was dramatic: Abdominal masses completely disappeared.
9. In spite of subjective improvement, the mass had grown. Endoscopic resection specimens however, did no more evidence sarcoma after triple therapy.
10. The girl is still under treatment. Objective regression seems more correlated to the Cytoxan than to the Vincristine injections.
11. She failed to respond to Cytoxan, and responded with diffuse necrotic changes to the first vincristine injection (3 mg). She has been operated on immediately after chemotherapy.



Fig. 1. Case 1. *Abdomen röntgenogram March* 29*th* 1965 *the shadow pertaining to the left kidney is enlarged, and no elimination of the contrast medium is evident.*



Fig. 2. Case 1. *Abdomen röntgenogram August* 8*th* 1965 *the shadow of left kidney has shrunk; this film has been taken without contrast media.*

(to face p. 228)

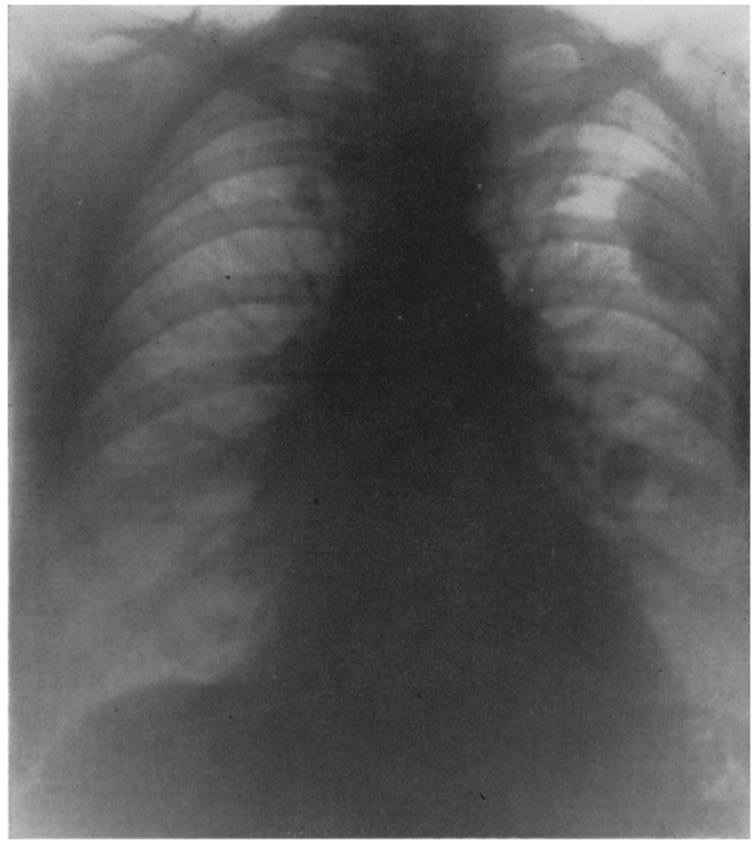


Fig. 3. Case 3. *Chest röntgenogram November* 19*th* 1965.

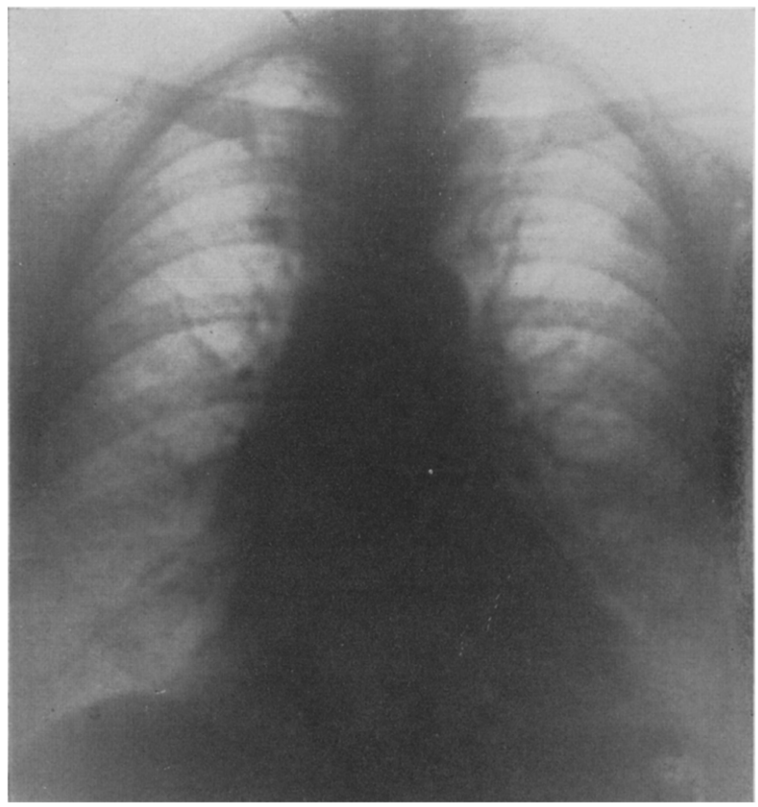


Fig. 4. Case 3. *Chest röntgenogram May* 13*th* 1966.

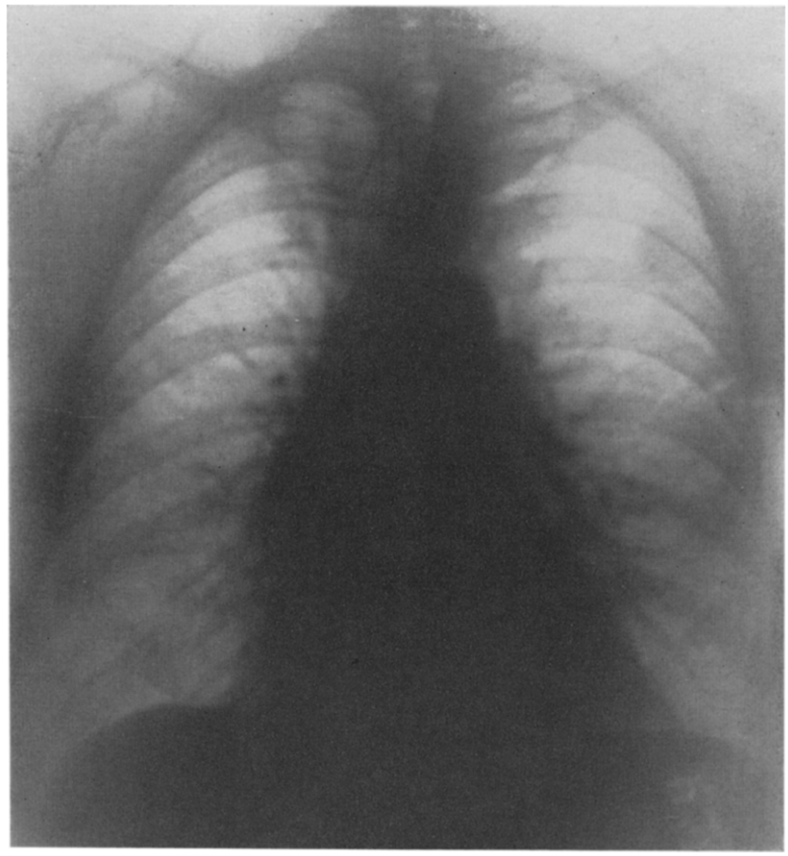


Fig. 5. Case 3. *Chest röntgenogram September 9th* 1966. *Patient alive and well November* 1967.

Case 9: man, aged 79 (No. 10828)
Botryoides sarcoma of bladder.

Case 10: girl, aged 7 (No. 10863)
Embryonal rhabdomyosarcoma of middle ear. Lymph node and pulmonary metastases.

Case 11: woman, aged 60 (No. 11037)
Praesternal rhabdomysarcoma (radiation induced?).

We can finally summarise our observations as follows

Number of subjects improving, by drug

5 Floruouracil	0/1
Cytoxan	4/8
Vincristine	1/1
Combined Cytoxan+vincristine	3/3
Li triple association*	2/4
(2 not clearly assessed)	
Daunomycin (Rubidomycin)	not assessed
VELBE	0/1

The long survivors

No. 1. Rhabdomyosarcoma treated by cyclophosphamide (improved); died 1 year from beginning of chemotherapy.

No. 2. Leiomyosarcoma treated by cyclophosphamide (improved); died 20 months from beginning of chemotherapy.

No. 3. Rhabdomyosarcoma; living and well two years from beginning of chemotherapy (improved). Then irradiated on one single chemoresistant deposit. She is in unmaintained remission since 10 months.

The most dramatic remission

Case No. 8, leiomyosarcoma:
treated since the beginning by the combination of alternating doses of vincristine and cyclophosphamide, administered at weekly intervals. Large palpable abdominal masses, (seen also at laparatomy) completely melted and faded in a few weeks.

DISCUSSION

Two hundred and forty courses of clinical chemotherapy suitable for evaluation are reported here: the courses were given to a little under 230 cases, because 4 of our cases and presumably some of those reported by other authors have been treated by different drugs at different times.

*Li triple association is a combination therapy using an alkylating agent (usually chlorambucil 10 mg a day) plus an antimetabolite (usually methotrexate 7·5 mg a day) with intravenous actinomycin D. The last drug being given in three five days courses beginning from the 3rd, 13th and 25th day of the oral therapy [41].

Although the following agents may not have been prescribed in large enough doses, no claim can be made for the efficacy of aminochlorambucil, chlorambucil, melphalan, mitomycin C, vinblastine, 2,6-diaminopurine or 5-fluorouracil in the treatment of myosarcomas.

Some clinical improvement has been reported with daunomycin (3 cases improving out of 16) Mustine (1 case) methotrexate (4 cases out of 13) and thioTEPA (2 cases). Up to now methotrexate alone has proved its effectiveness in leiomyosarcomata, 3 out of 11 patients improving [4].

The real value of methotrexate when combined with TEM or with chlorambucil and dactinomycin is difficult to assess.

Some activity may be claimed for *dactinomycin* (actinomycin D) with 14 cases improving out of 48 (approximately 29%) and for *cyclophosphamide,* 15 improving out of 37 (more than 40% and *vincristine,* 10 out of 14 (nearly 70%).

It may be of interest that one reported case and one of our patients responded to cyclophosphamide, the former after having failed to improve with sarcolysin and daunomycin, and the latter after fluorouracil in full doses.

The best results up to now have perhaps been obtained by combined chemotherapy with dactinomycin plus vincristine. With 6 out of 6 cases improving [22] or triple association according to Li: this latter method of treatment, i.e., methotrexate plus chlorambucil (or cyclophosphamide) and dactinomycin, has not only produced remissions in 6 out of 6 cases, but 1 of these cases fulfills the common requirements for chemotherapeutic cure [24].

Two other cases, which improved with Li triple association, had formerly proved or become resistant to cyclophosphamide.

One case also improved with combined thioTEPA+cyclophosphamide [11]; one with dactinomycin + cyclophosphamide [1] and one after TEM + methotrexate [1].

We could find no published records of the combined use of vincristine and cyclophosphamide in myosarcoma, though it has been used by James, Hustu and Wrenn [22] with great success in childhood neuroblastomas.

This combination, if tolerated, may be highly recommended in many childhood tumours as well as for myosarcomata, both in children and adults. Our claim is based on the general usefulness of combined treatment in myoblastic tumours, and on the fact that the two suggested drugs are most active when used separately.

It seems clear that any patients suffering

from inoperable myosarcoma now has a chance of a reasonable remission if given vincristine or cyclophosphamide. For these reasons, we always gave this combination of drugs since case 7.

The second patient so treated (No. 8) had a dramatic remission; three patients underwent only partial remission; one is not fit for evaluation.

We suggest that these two drugs be used in combination, for instance on alternating weekly doses; one week vincristine up to 2 mg/m^2 and the next week intravenous cyclophophamide up to 800 mg/m^2 in one intravenous dose.

In case of failure of this or a similar treatment schedule, one may still expect some improvement by Li triple association (chlorambucil+methotrexate+dactinomycin) at the usual doses.

Other drugs as daunomycin and thioTEPA may be tried only as third and fourth choice.

A trial of chemotherapy in selected cases is worthwhile even before giving radiotherapy, as the latter is rarely effective.

The fear of producing some toxicity should not be allowed to interfere with giving the maximum tolerated doses of chemotherapy, in view of the very rewarding results that may accrue.

Acknowledgements—I am indebted to Drs. D. A. G. Galton and E. Wiltshaw for constructive criticism of the manuscript.

RESUME

Onze patients, atteints de myosarcomes (5 léio- et 6 rhabdomyosarcomes), ont été traités par chimiothérapie, soit générale (10 cas), soit intra-artérielle (1 cas).

Nos résultats sont comparables à ceux rapportés par d'autres auteurs qui ont administré la vincristine, le cyclophophamide et la dactinomycine. Ces substances se sont montrées, par ordre décroissant, les agents les plus efficaces sur les myosarcomes.

Le taux de réponse favorable fut de 70% dans notre série, puisque 4 rhabdomyosarcomes et 3 léiomyosarcomes ont été améliorés, après la chimiothérapie, sur 10 que nous pouvons évaluer.

On a constaté que l'association de deux ou trois de ces médicaments était particulièrement efficace.

Les résultats de nos essais de chimiothérapie, portant sur les rats atteints de rhabdomyosarcome transplanté, provoqué par le méthylcholanthrène, ont montré que ces tumeurs sont sensibles aux mêmes médicaments qui se sont révélés efficaces sur les myosarcomes humains (D'Ancona et Fiorentino).

C'est donc sur ces données qu'on propose:

(1) de traiter les cas de myosarcome, chez l'homme, par une association de cyclophosphamide et de vincristine, et de réserver une thérapeutique d'association triple, selon Li (dactinomycine+méthotrexate+chlorambucil), aux cas résistants au traitement par l'association vincristine–cyclophosphamide.

(2) d'utiliser les rhabdomyosarcomes transplantés du rat comme test préliminaire pour essayer les médicaments efficaces dans le traitement du myosarcome humain.

SUMMARY

Eleven patients with myoblastic sarcomas (5 leio- and 6 rhabdomyosarcomas) have been treated by chemotherapy, either systemic (10 cases) or mixed, systemic and intra-arterial (1 case).

Our results agree with other reported cases, where vincristine, Cytoxan and dactinomycin have proved, in decreasing order, the more effective agents in both leio- and rhabdomyosarcoma.

The overall good response rate in our patients, has been 70% with 4 rhabdomyosarcomata and 3 leiomyosarcomata improving (out of ten evaluable cases) after chemotherapy.

Whenever two or three drugs have been given together they have been particularly useful.

Our preliminary experimental results with the chemotherapy of transplanted methylcholanthrene induced rat rhabdomyosarcoma showed that these tumours were sensitive to the same drugs which had proved useful in human myosarcoma (D'Ancona & Fiorentino).

It is accordingly suggested that:

(1) human cases of myosarcoma should be treated with combined Cytoxan and vincristine, and that the triple association therapy according to Li (dactinomycin+methotrexate+

chlorambucil) should be reserved for those cases who fail to respond to the vincristine–cytoxan combination.

(2) *transplantable rat rhabdomyosarcoma may serve as a preliminary screening test for useful drugs in human myosarcoma treatment.*

ZUSAMMENFASSUNG

Sechs Patienten mit Rhabdomyosarkom und 5 *Patienten mit Leiomyosarkom wurden einer generaliserten* (10 *Fälle*) *bzw. einer kombinierten generalisierten und intra-arteriellen* (1 *Fall*) *Chemotherapie unterzogen.*

Unsere Resultate decken sich mit anderen Berichten, wonach Vincristin, Endoxan und Actinomycin D—in dieser Reihenfolge abnehmender therapentischer Wirkung—bei Leio- und Rhabdomyosarkomen die wirksamsten Cytostatica sind.

Siebzig prozent unserer 10 *auswertbaren Patienten sprachen auf die Behandlung an, nämlich* 4 *Rhabdo- und* 3 *Leiomyosarkome.*

Die Kombination von 2 *oder* 3 *Medikamenten hat sich als besonders wirkungsvoll erwiesen.*

Unsere vorläufigen experimentellen Erfahrungen mit der Chemotherapie eines transplantierten Methylcholanthren-induzierten Rhabdomyosarkoms der Ratte ergaben, dass dieser Tumor gegenüber den gleichen Medikamenten empfindlich ist, die auch die Myosarkome des Menchen beeinflussen (*D'Ancona und Fiorentino*).

Es ergeben sich aus diesen Beobachtungen folgende Schlussfolgerungen:

(1) *Myosarkome werden am besten mit der Kombination Endoxan + Vincristin behandelt. Die Dreierkombination nach Li* (*Actinomycin D+Methotrexat+Chlorambucil*) *sollte für Fälle reserviert bleiben, die sich gegenüber Endoxan+Vincristin als resistent erwiesen haben.*

(2) *Das transplantierte Rhabdomyosarkom der Ratte kann als Testmodell für die vorklinische Prüfung wirksamer Substanzen verwendet werden.*

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Carcinogenic Properties of certain Rubber Additives

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INTRODUCTION

UNEQUIVOCAL evidence is now available to show that there is an increased incidence of tumours of the bladder amongst workers in the rubber industry [1–3]. The original data, presented by Case and Hosker [1], were "consistent with the hypothesis that the risk was introduced with a certain antioxidant in 1928 which was known to cause bladder tumours in those workmen who manufactured it." The antioxidant in question was a formaldehyde condensation product of α- and β-naphthylamine which contained approximately 2·5% of uncombined α-naphthylamine and 0·25% β-naphthylamine. Use of this material in the rubber industry was stopped in 1949 but whether this was the only carcinogenic substance responsible for this disturbing situation is unknown.

In the course of manufacture of rubber products, various substances are added to the raw materials to modify its physicochemical properties; such compounds are numerous and the processes in which they participate are complex but four main groups of additives may be considered: *organic accelerators, carbon-black reinforcing agents, organic anti-oxidants,* and a miscellaneous group which includes *plasticisers, softeners and dyestuffs* [1]. The groups of organic accelerators and antioxidants overlap to some extent and comprise a wide variety of chemicals including aromatic amines, nitrosamines and their derivatives. The relationship between aromatic amines and tumours of the bladder has long been familiar [4–6] but it is now recognised that nitrosamines can also induce bladder tumours [7–9]. It seemed, therefore, desirable to examine the carcinogenic properties of some of the compounds used as organic accelerators or antioxidants and, in the present paper, we describe experiments with four derivatives of nitrosamines.

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MATERIALS AND METHODS

Rats. One hundred and twenty male CB stock rats were used, aged 6–7 weeks and weighing 200–230g. They were kept in metal cages, 6 animals in each, and were maintained on standard cubed diet no. 86 (Withers Ltd., Godstone, Surrey) and water *ad libitum.*

Rubber additives. The following rubber additives were examined:

1. *N-nitrosodiphenylamine* ("Vulcatard")
2. *N,N-dinitrosopentamethylenetetramine* ("Vulcacel") Both kindly supplied by I.C.I. Ltd., England.
3. *Polymerised N-nitroso* 2,2,4-*trimethyl*-1,2-*dihydroquinoline.*
4. *Nimethyl-N,4-dinitrosoaniline* Both kindly supplied by Monsanto Chemicals Ltd., England.

Conduct of experiment

The rats were divided into 5 equal groups, composed of 24 animals. Each of the 4 rubber additives was freshly dissolved in polyethylene glycol 400 and 0·25 ml was injected intraperitoneally at weekly intervals for six months. One group of animals acted as solvent controls and were injected with 0·25 ml polyethylene glycol only. The doses of additives which were administered were as follows:

Group 1. *Polymerised N-Nitroso* 2,2,4-*trimethyl*-1,2-*dihydroquinoline*—25 mg/week; total 650 mg.

Group 2. *N-methyl-N*,4-*dinitrosoaniline*—5 mg/week; total: 130 mg.

Group 3. *N,N-dinitrosopentamethylenetetramine*—25 mg/week; total: 650 mg.

Group 4. *N-nitrosodiphenylamine*—2·5 mg/week; total: 325 mg.

The rats were examined daily and all sick animals were killed at once. The experiment was terminated after two years. A standard post-mortem examination [10] was performed and all tumours and tissues showing macroscopic abnormalities were fixed in Bouin's solution. Paraffin sections were cut at 5μ and were stained with haematoxylin and eosin and, in some instances, with haematoxylin and Van Gieson, Masson's trichrome, Gordon and Sweet's Silver technique for reticulin and the McManus Periodic acid–schiff (PAS) technique.

RESULTS

1. *Intraperitoneal tumours*

The number of intraperitoneal tumours which developed in the four experimental groups is shown in Table 1. Eight tumours were seen—six in group 1 and two in group 2—in rats treated with *polymerised N-nitroso* 2,2,4-*trimethyl*-1,2-*dihydroquinoline* and with *N-methyl-N*,4-*dinitrosoaniline*, respectively. No tumours were found in rats in groups 3, 4 or 5. The times of death of tumour-bearing animals are also recorded in Table 1 but, since intraperitoneal neoplasms are rarely detectable during life, the time of induction of these lesions remains uncertain.

Apart from the presence of tumours, certain pathological features were common to rats in all four test groups, irrespective of which rubber additive had been injected. At autopsy, rats from all groups showed fibrous adhesions in the peritoneal cavity, binding the liver, diaphragm, stomach, spleen, small intestine, omentum and the anterior abdominal wall. Retroperitoneal fibrosis was sometimes apparent around the kidneys and pancreas. Such changes varied in extent in different rats and in different experimental groups but they appeared to be most consistent and most intense in animals in the group (Group 4) treated with *N-nitrosodiphenylamine*. Traces of injected material were frequently visible in visceral adhesions and small amounts of ascitic fluid were occasionally present.

On microscopic examination, the adhesions were found to be infiltrated with inflammatory cells consisting mainly of lymphocytes and macrophages (Fig. 1). Plasma cells and also polymorphs were occasionally seen but no discrete abscesses were found. Injected material was present in variable amounts; it was predominantly intracellular, lying within macrophages which sometimes accumulated in large numbers (Fig. 2). There was no evidence of granuloma formation. Dilated lymphatic vessels were sometimes seen, especially in the omentum and in thickened hepatic and splenic capsules. Comparable but less intense changes were also observed in several control rats, injected with polyethylene glycol only, and it appeared that repeated intraperitoneal injections frequently led to a low-grade chronic peritonitis, irrespective of the material administered.

In two of the experimental groups, intraperitoneal sarcomas were also encountered. The tumours were somewhat different in the two groups and they are therefore discussed separately:

(A) Of 24 rats injected with *N-methyl-N*,4-*dinitrosoaniline*, one developed a fibromyxosarcoma and one developed a spindle cell sarcoma (Figs. 3–5). The fibromyxosarcoma showed extensive myxoid degeneration together with zones of haemorrhage, necrosis and ectopic calcification. Viable tumour elements consisted mainly of spindle cells with occasional multinucleate giant cells. Mitoses were inconspicuous. The second tumour was a large spindle cell sarcoma which was adherent to the body wall and had invaded the caecum. Spindle cells predominated but more pleomorphic cells and mitoses were common; nuclear chromatin, arranged in a coarse stippled fashion, was unusually prominent. Both tumours showed a moderate amount of collagen formation and had a well-developed reticulin framework. Neither tumour contained any of the injected material. No metastases were seen.

(B) The six intraperitoneal sarcomas which were observed in rats treated wtih *polymerised N-nitroso* 2,2,4-*trimethyl*-1,2-*dihydroquinoline* differed in several respects from those just described. The tumours varied in size (some of them measuring up to 4 cm dia.) and many appeared to be multifocal. Their gross pattern was also variable, ranging from solid masses to flat plaques which tended to infiltrate muscle planes and grow along peritoneal membranes. The tumours were composed mainly of pleomorphic polygonal and spindle cells with moderate numbers of mitoses (Fig. 6). Multinucleate giant cells of an unusual type

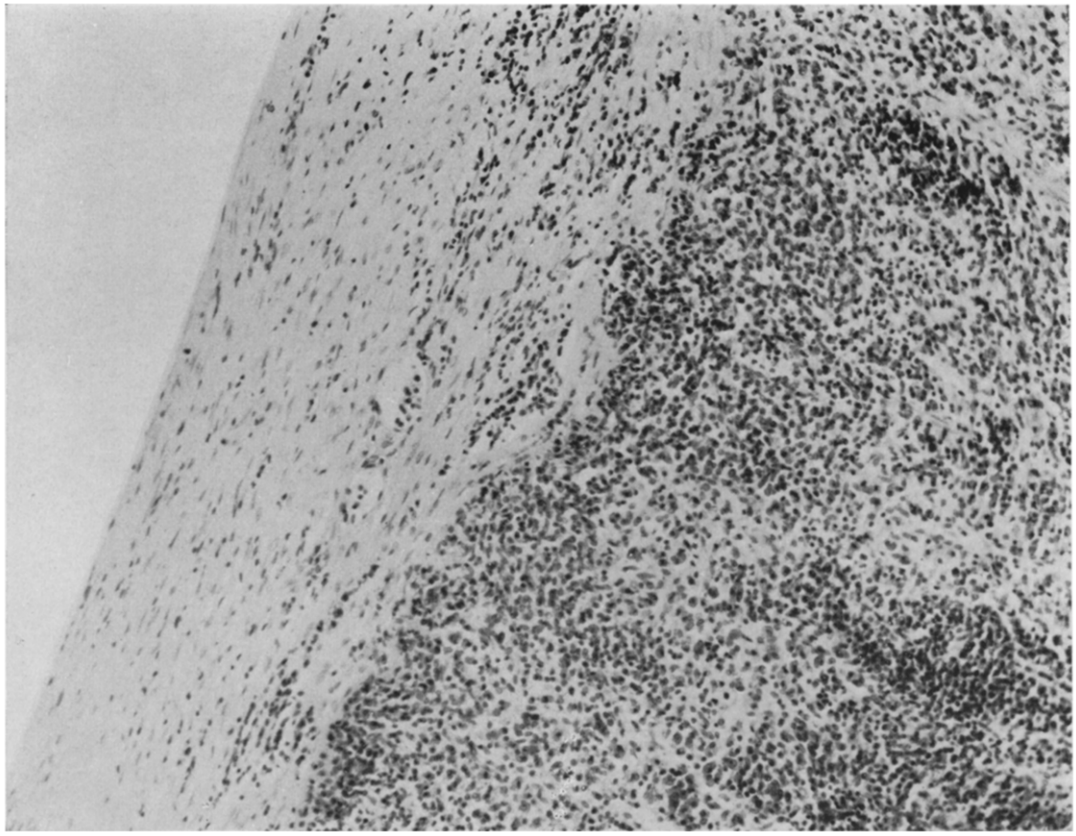


Fig. 1. *Spleen from rat injected with* N-nitrosodiphenylamine. *The capsule is thickened and shows a diffuse inflammatory infiltrate and dilated lymphatic channels. (N.B.—All photomicrographs are shown at a magnification of* × 160 *and stained with haematoxylin and eosin unless otherwise stated).*

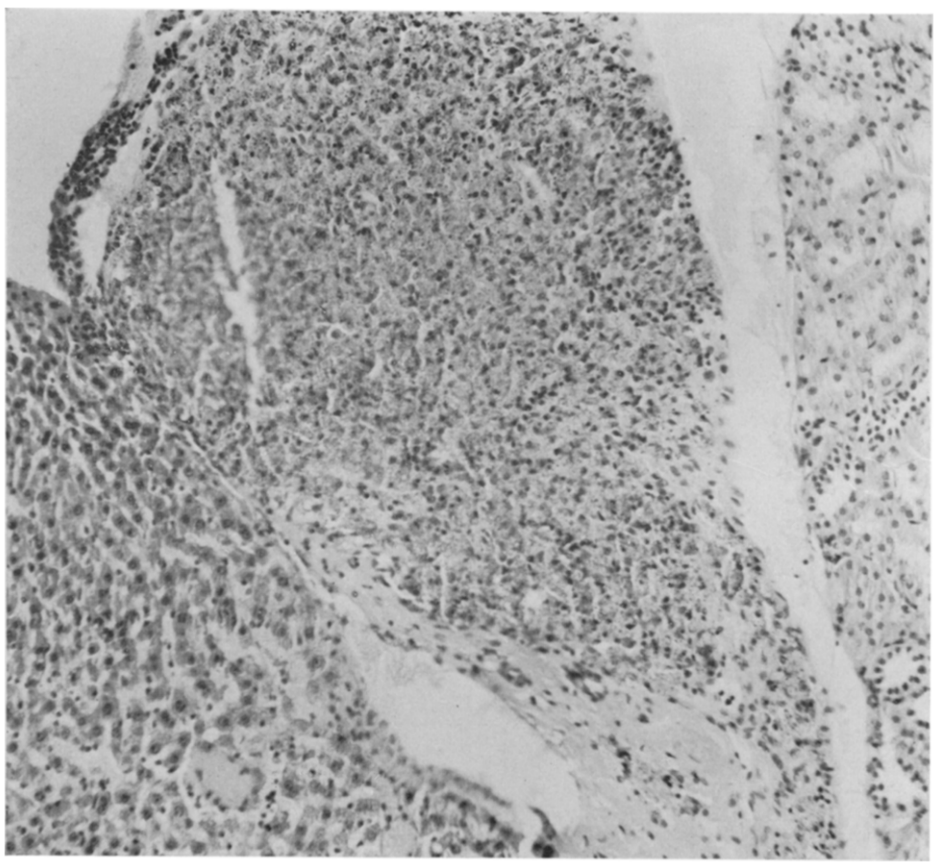
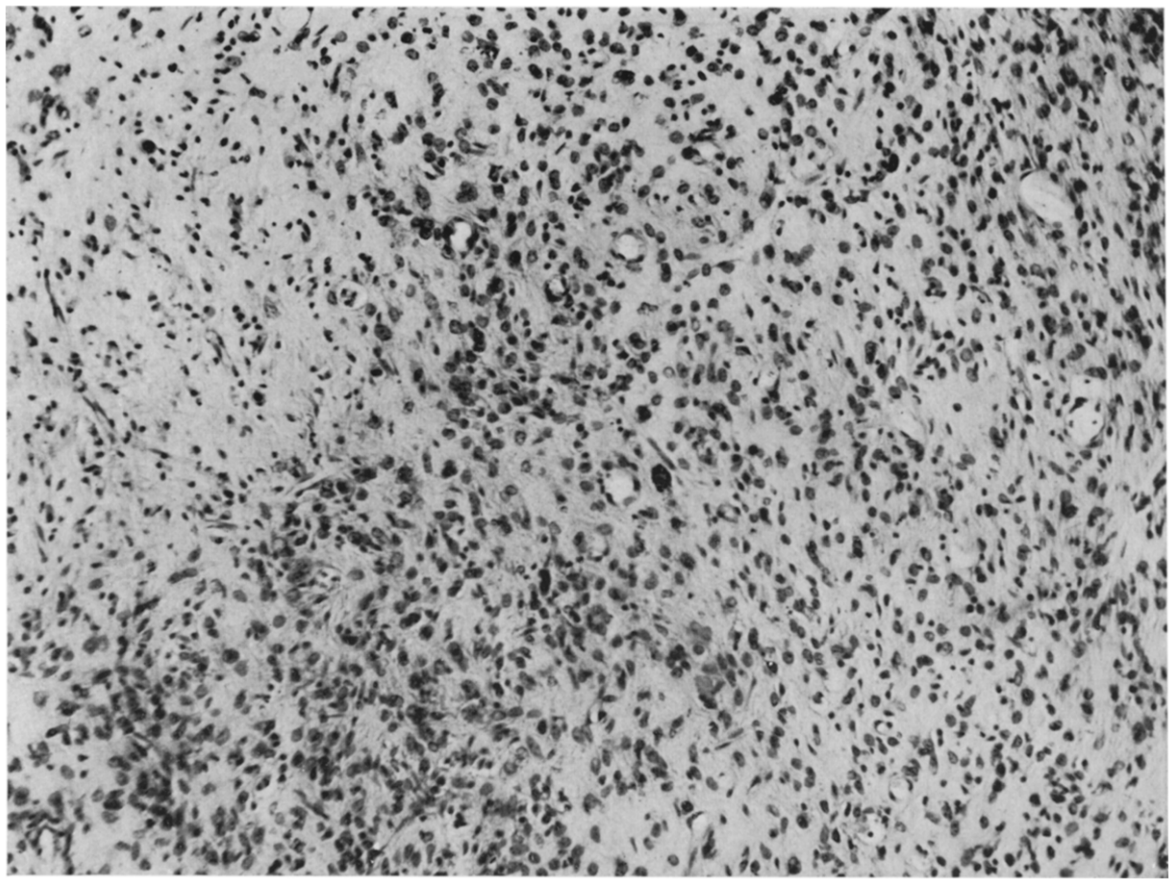
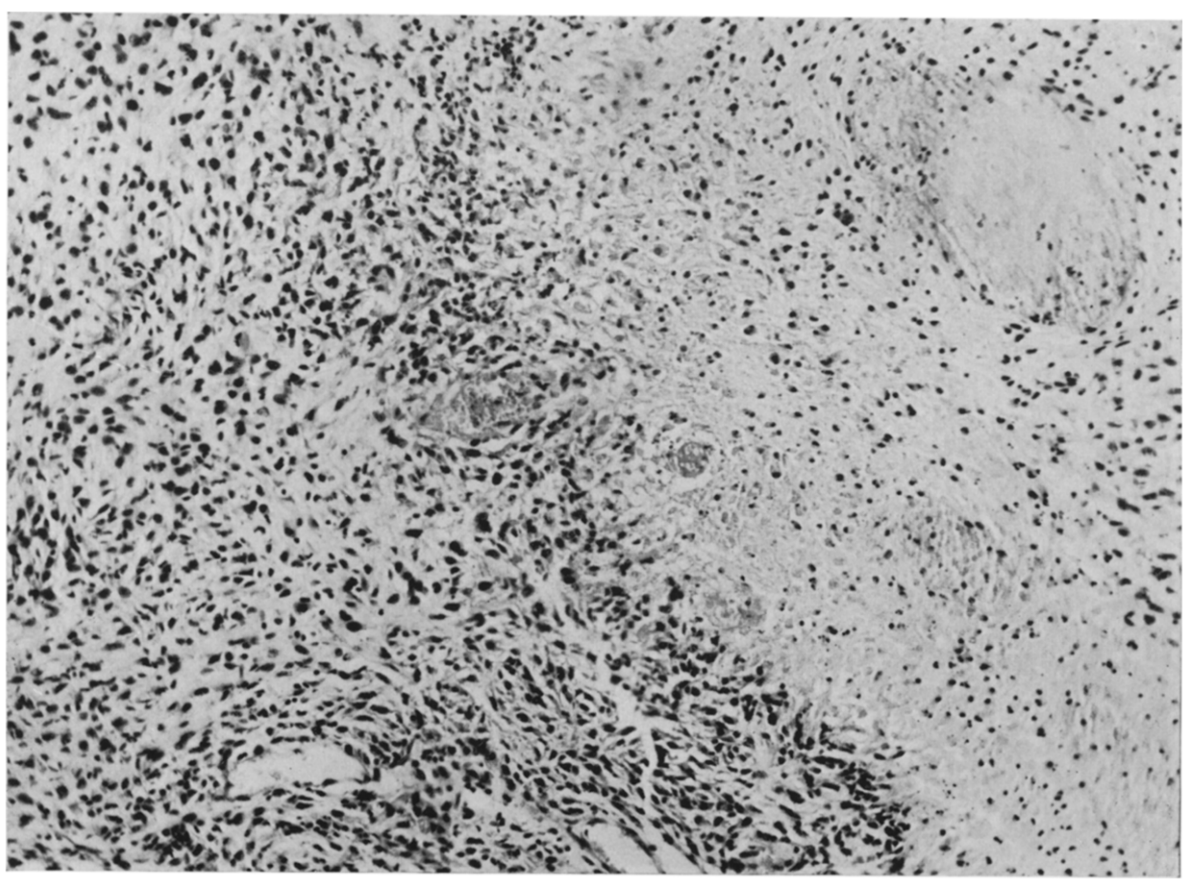


Fig. 2. *Adhesions binding together the liver and kidney from a rat injected with polymerised* N-nitroso 2,2,4-trimethyl-1,2-didydroquinoline. *There is a dense accumulation of macrophages in which ingested material is clearly seen.*

(to face p. 234)



Figs. 3 *and* 4. *Intraperitoneal myxosarcoma from rat injected with* N-methyl-N,4-dinitrosoaniline.



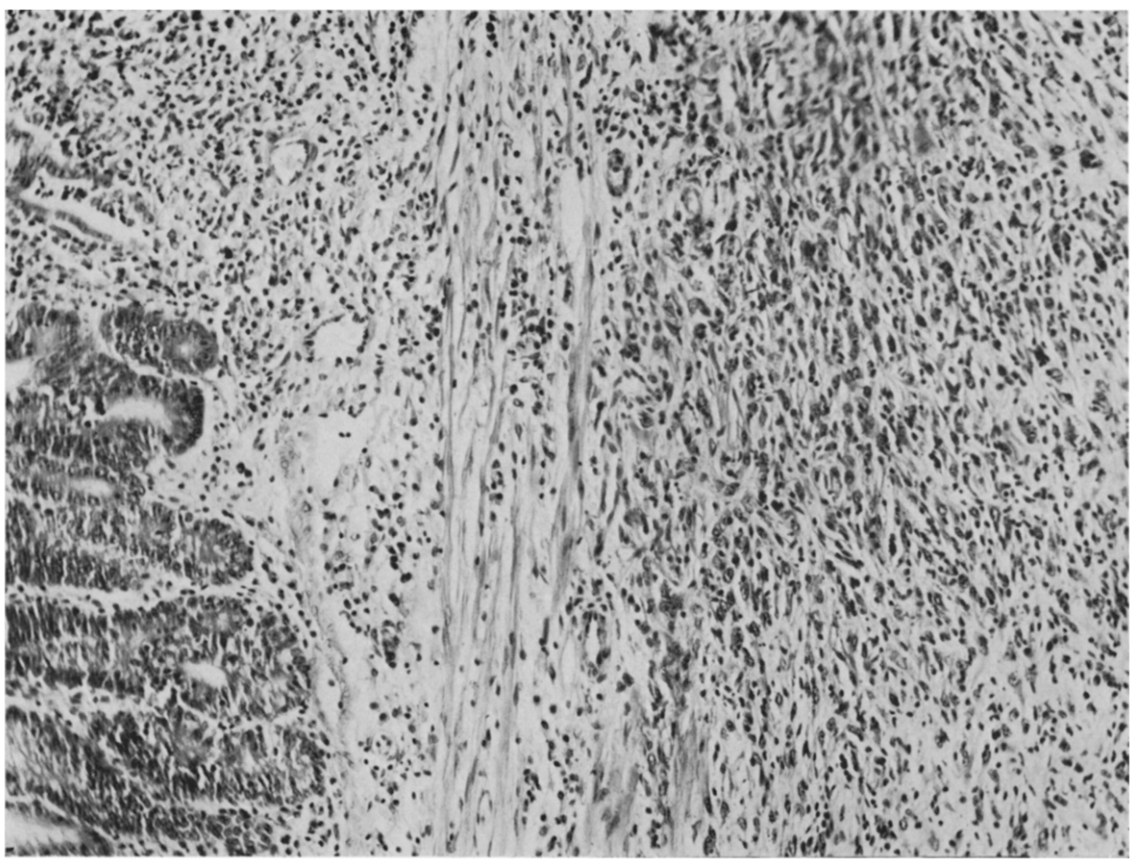


Fig. 5. *Spindle cell sarcoma from rat injected with* N-methyl-N,4-dinitrosoaniline. *The tumour is invading the wall of the caecum.*

Figs. 6, 7 *and* 8. *Intraperitoneal sarcomas from rats injected with polymerised* N-nitroso 2,2,4-trimethyl-1,2-dihydroquinoline.

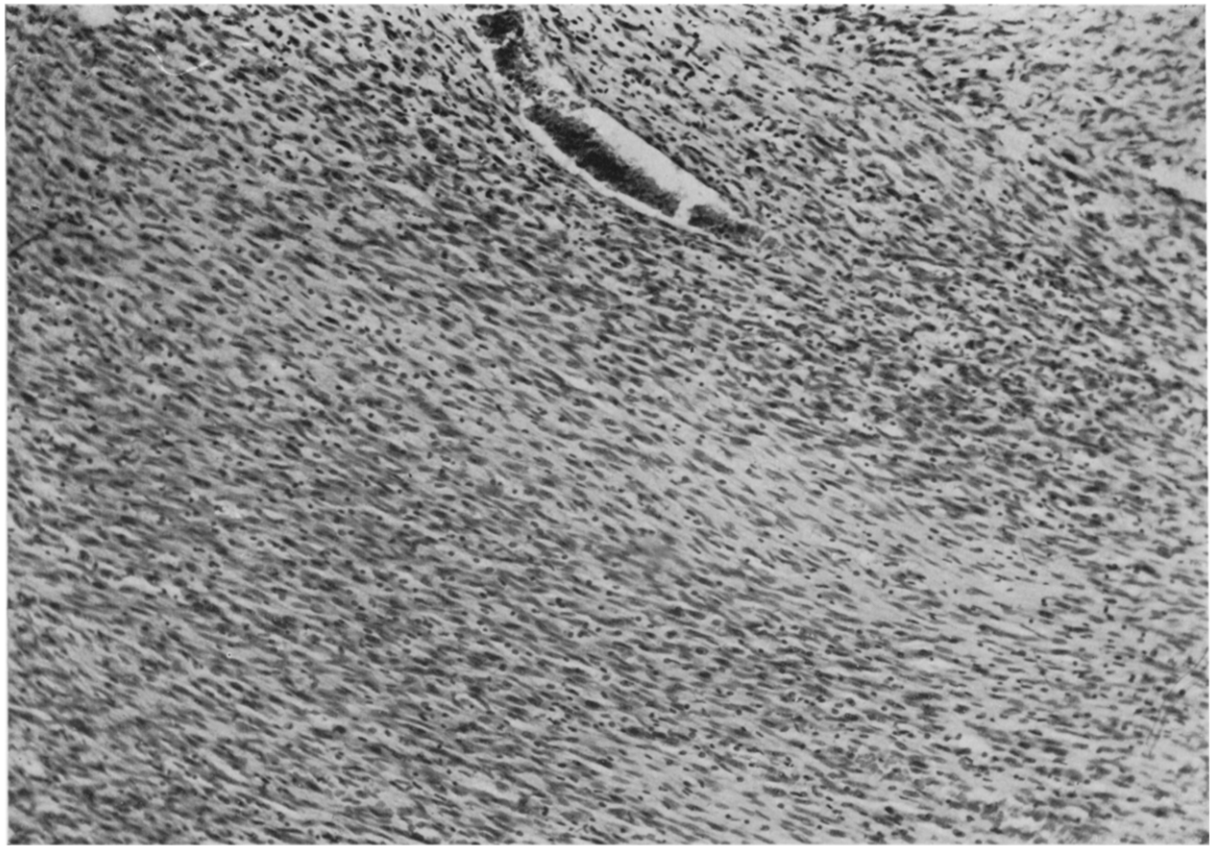


Fig. 6. *A sarcoma composed predominantly of spindle cell elements.*

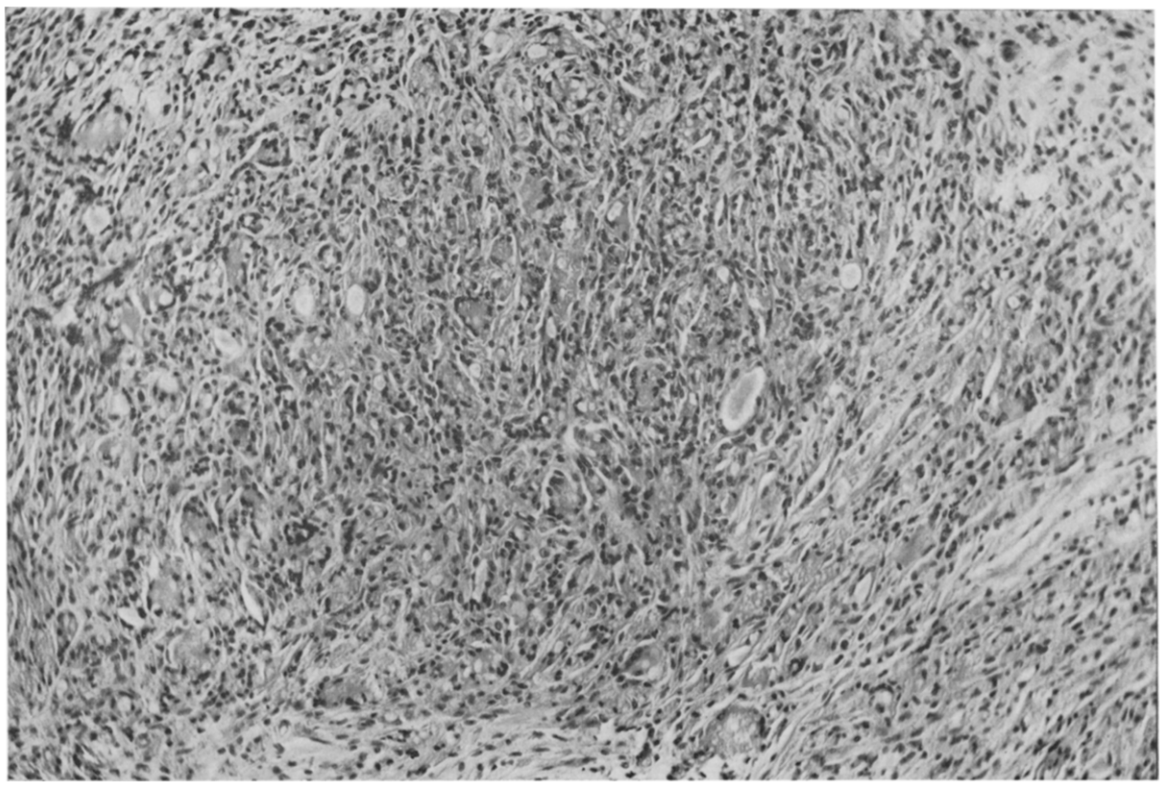


Fig. 7. *A more pleomorphic tumour containing numerous multinucleate giant cells.*

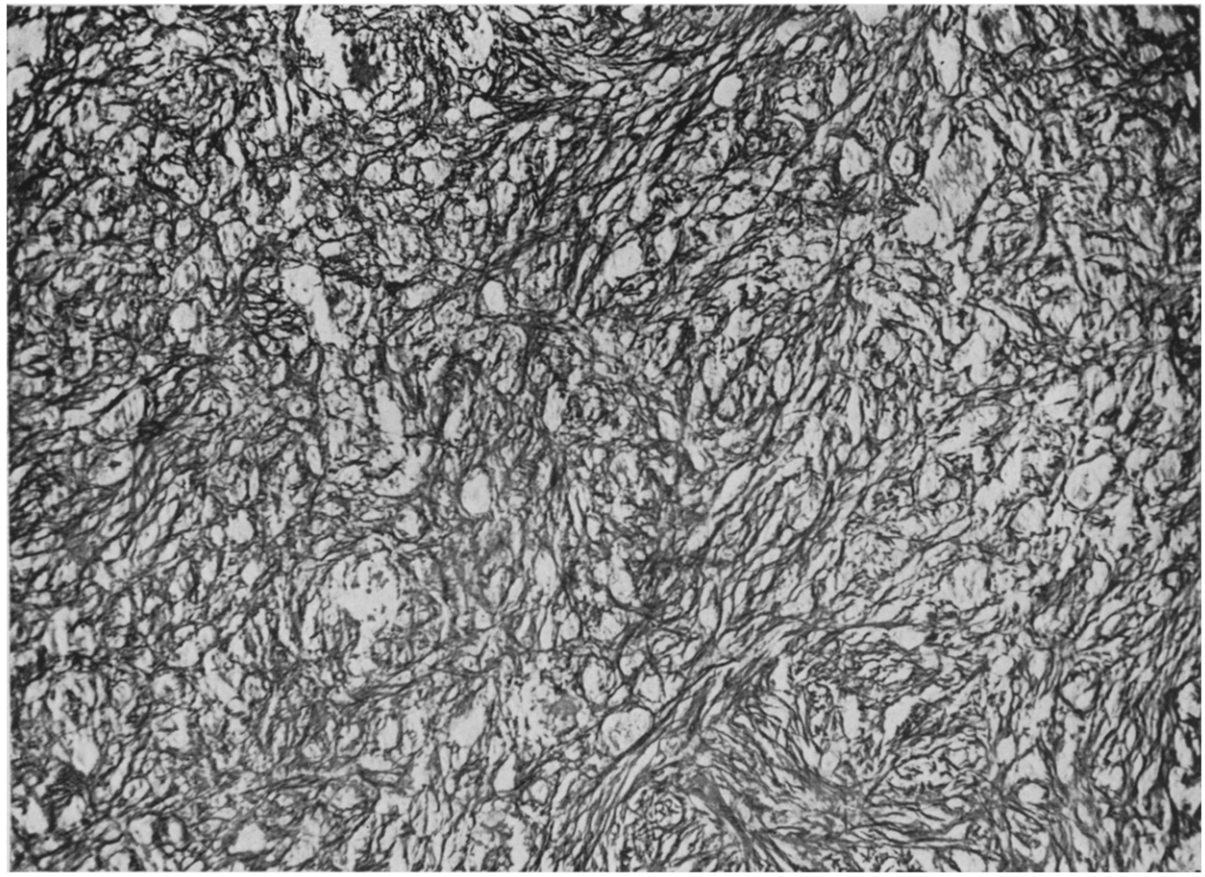


Fig. 8. *Reticulin preparation showing the dense reticulin framework typical of these tumours.*

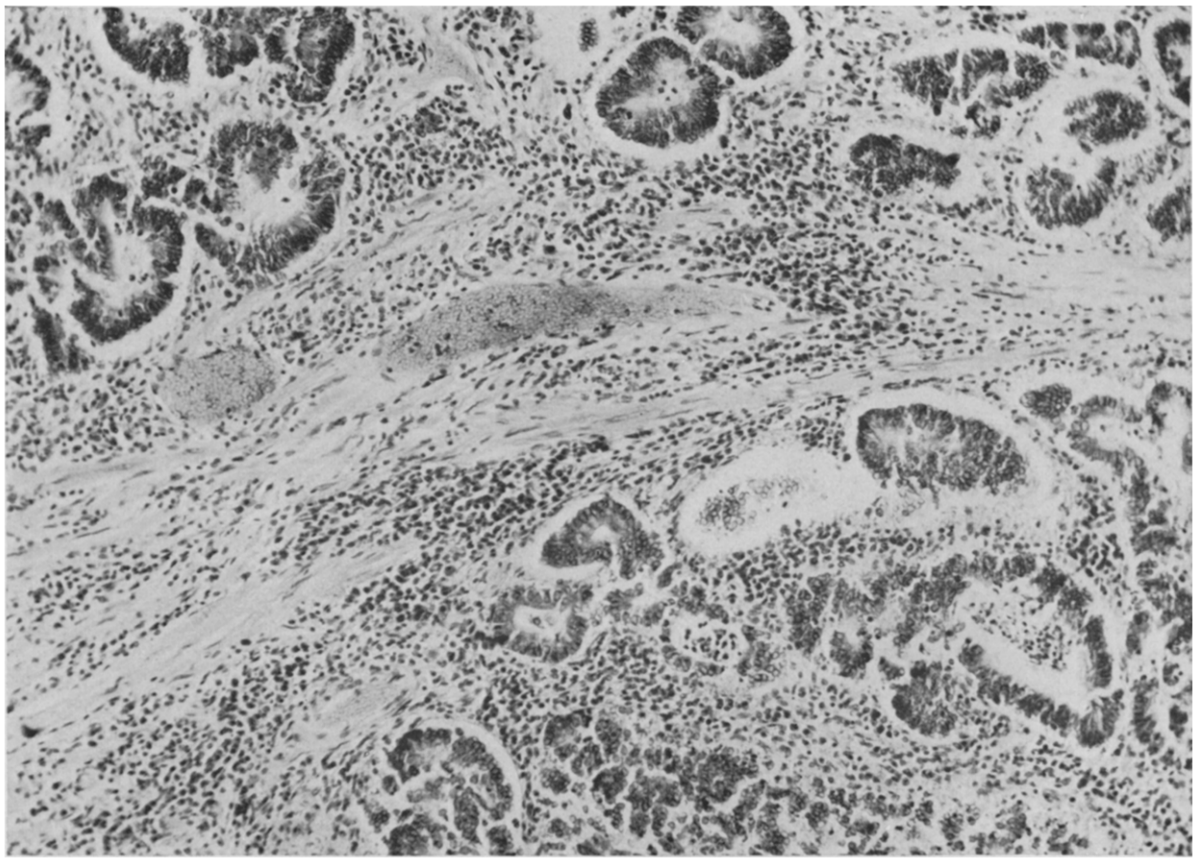


Fig. 9. *Adenocarcinoma of rectum from rat injected with polymerised* N-nitroso 2,2,4-trimethyl-1-2-dihydroquinoline.

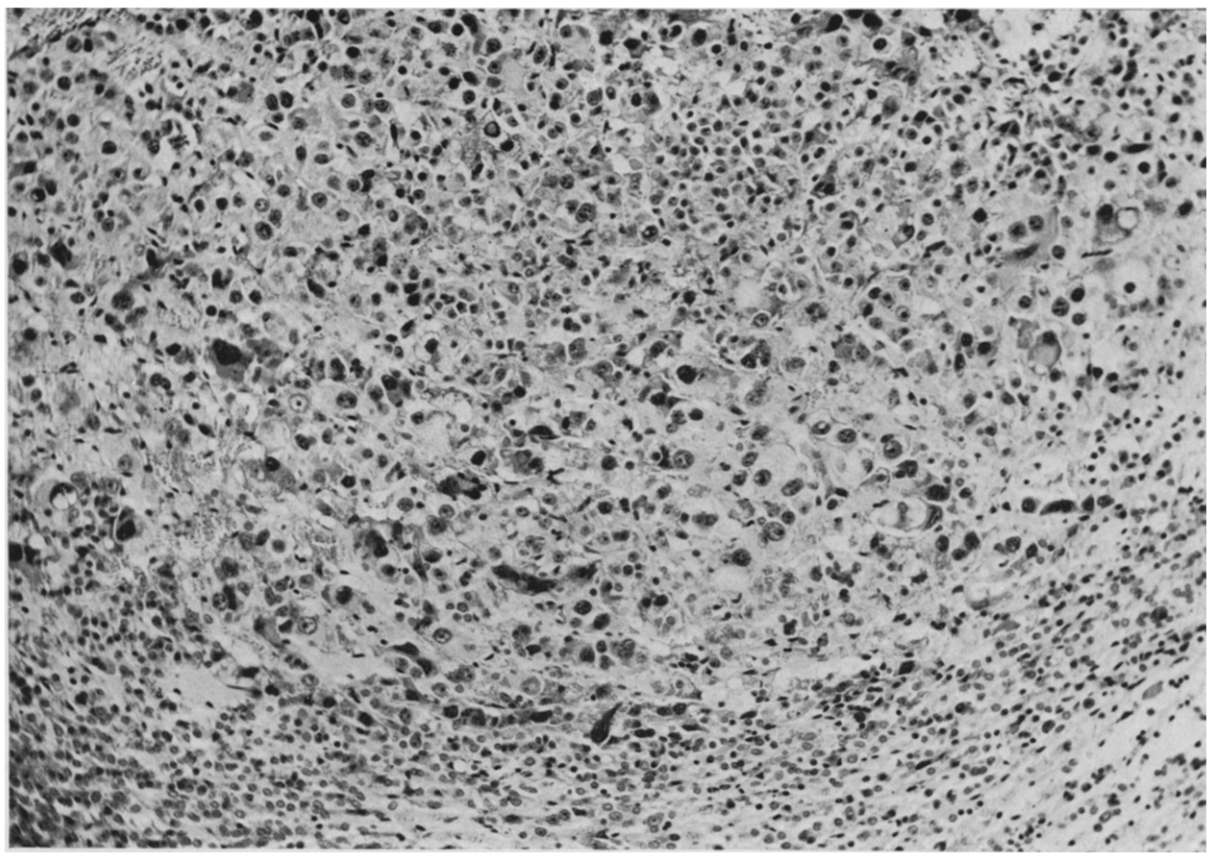


Fig. 10. *Chromophobe adenoma of pituitary gland from rat injected with* N-nitroso-diphenylamine.

Table 1. Tumours in male CB stock rats following intraperitoneal injections of four rubber additives

EXPERIMENT GROUP	No. OF RATS	TREATMENT (INTRAPERITONEAL INJECTIONS IN 0·25 ml. POLYETHYLENE GLYCOL, ONCE WEEKLY FOR 6 MONTHS)	TIME OF DEATH (months)						No. OF INTRAPERITONEAL TUMOURS	No. OF TUMOURS AT OTHER SITES
			0–9	10–12	13–15	16–18	19–21	22–24		
I	24	POLYMERISED N-NITROSO 2,2,4,-TRIMETHYL-1,2-DIHYDROQUINOLINE 25 mg.	○○○	■■?	■■○○ [R]	○○○○ ○	■■○○ ○○	○○	6	2
II	24	N-METHYL N,4-DINITROSOANILINE 5 mg.		○	○○○○ ○○○○ ○	■○○○ [P][T]	■○○○ ○○[E][H]		2	4
III	24	N,N-DINITROSOPENTAMETHYLENETETRAMINE 25 mg.	○	○○○○ [H]	○○○○ ○	○○○○ ○○	○[P]	○○○○ ○	0	2
IV	24	N-NITROSODIPHENYLAMINE 25 mg.	○○○[H] ??	○○○	○○○○	○○○○ ○?	○○[P]	○○	0	2
V	24	POLYETHYLENE GLYCOL 400 (CONTROLS)	○○	○○○○	○○○○ ○	○○○	○○○○ ○○○	○○[H]	0	1

○ = RATS WITHOUT TUMOURS ■ = RATS WITH INTRAPERITONEAL SARCOMATA

E = Exocrine adenoma of pancreas H = Hepatoma P = Pituitary tumour

A = Adenocarcinoma of rectum S = Squamous carcinoma of skin T = Thymoma

? = Full post-mortem examination precluded by decomposition

were frequently observed with a peripheral ring of darkly-staining pyknotic nuclei encircling a large volume of foamy cytoplasm (Fig. 7); no cell inclusions were seen. Numerous thin-walled blood vessels were observed in many tumours. There was quite marked formation of collagen fibres and silver preparations showed a well-developed reticulin framework (Fig. 8). Recognition of metastases from multifocal tumours of this kind is obviously difficult but no tumour masses were seen outside the abdominal cavity.

2. *Tumours other than intraperitoneal sarcomas*

Other tumours encountered in the present study are listed in Table 1. A low incidence was found in all four test groups and there is no evidence that treatment with any of the test materials influenced either their frequency or variety. Certain of these lesions are, however, of interest. Adenocarcinoma of the rectum (Fig. 9) is rare in rats and so is exocrine adenoma of the pancreas—the latter has already been described elsewhere [11]. Chromophobe adenomas of the pituitary gland are more frequently encountered but the considerable cellular atypia in one of them (Fig. 10) is worth recording. It is noteworthy that no tumours of the urinary bladder were encountered.

(3) *Effects of certain rubber additives in mice*

It was originally planned to extend these observations and to study the effects of the same and other rubber additives in young C57 × DBA2 mice. Three substances were tested: *N-methyl-N,4-dinitrosoaniline*; 6-*ethoxy*-2, 2,4 - *trimethyl* - 1,2 - *dihydroquinoline* ("Santoflex AW"); and 6-*dodecyl*-2,2,4-*trimethyl*-1,2-*dihydroquinoline* ("Santoflex DD"). These additives were injected intraperitoneally in arachis oil at weekly intervals for six months and the mice were subsequently observed at regular intervals thereafter. A low incidence of intraperitoneal sarcomas was observed in animals from all three test groups but this results in uninterpretable since similar tumours also appeared in 3 mice injected with solvent alone. This unexpected finding in mice treated with arachis oil is being investigated and the effects of rubber additives (in other solvents) on mice is to be re-examined in the near future.

DISCUSSION

Two of the four rubber additives tested by repeated intraperitoneal injections in rats have been shown to be carcinogenic: treatment with *N-methyl-N,4-dinitrosoaniline* induced two intraperitoneal sarcomas and treatment with *polymerised N-nitroso* 2,2,4-*trimethyl*-1,2-*dihydroquinoline* produced six intraperitoneal tumours. The latter appears to be the stronger carcinogen but it was administered in a larger dose and it is, in any case, a good deal less potent than other nitrosoamines [7–9]. The weak carcinogenic effect of *N-methyl-N,4-dinitrosoaniline* which was found in the present survey confirms similar observations by Weisburger and his associates [12]. No tumours were produced in rats treated with *N,N-dinitrosopentamethylenetetramine* or *N-nitrosodiphenylamine*, or in animals injected with solvent alone.

Certain pathological changes may be emphasised. First, the additives tended to persist in the peritoneal cavity for long periods and could be readily recognised macroscopically at post-mortem examination, many months after the last injection. Secondly, intraperitoneal injections induced a low-grade peritonitis in a high proportion of cases including rats which were injected with solvent only. No association was seen between the intensity of the inflammatory changes and the subsequent appearance of sarcomas; the most extensive inflammation was seen in rats treated with *N-nitrosodiphenylamine* and none of these animals developed intraperitoneal tumours. Thirdly, it is clear that the neoplasms induced by polymerised *N*initroso 2,2,4- *trimethyl*-1,2-*dihydroquinoline* presented certain unusual features. They were frequently multifocal and showed considerable pleomorphism. Their cell of origin is obscure: in some cases a diagnosis of mesothelioma was considered but, in the absence of any positive evidence, non-committal terms such as "spindle cell" or "pleomorphic" sarcomas have been preferred. Lastly, it should be noted that although the number of spontaneous neoplasms was not increased by treatment with rubber additives, certain unusual lesions were encountered. In this context, it is interesting that, in the one other report on the carcinogenic effects of *N-methyl-N,4-dinitrosoaniline* [12], one most uncommon tumour—a carcinoma of the thyroid—was found. Some of these pathological findings indicate the shortcomings of the intraperitoneal route for testing carcinogenic substances, particularly the high incidence of low-grade chronic infection.

It is unfortunate that the data from tests using C57 × DBA 2 mice cannot be interpreted at the present time (see results). A low level of carcinogenic activity was found in all three substances which were examined but these results are vitiated by the development of similar intraperitoneal sarcomas in mice treated with solvent alone.

Despite their rather low activity, there is no doubt that two of the four rubber additives which have been tested are carcinogenic in rats. This results, together with the epidemiological findings which were noted earlier [1–3], emphasise the need for a systematic examination of the carcinogenic activity of the materials used as rubber additives. The scope of such an investigation is very large but some of the possible carcinogens which should (in our opinion) be examined in the future are listed in Table 2.

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Table 2. Ingredients for rubber which have not been tested for carcinogenic activity or are suspect

(*a*)	*Aromatic amine derivatives*
	Aldol-1-naphthylamine
	N-Phenyl-2-napththylamine
(*b*)	*Benzidine dyes*
	3,3-Dichlorobenzidine coupled with 2, 4-acetoxylidide
	3,3-Dichlorobenzidine coupled with acetoacetalinide
(*c*)	*Nitrosamines*
	N,N-Dinitroso N,N-dimethyltereph-thalamide
	Dinitrosopentamethylenetetramine
	N-Methyl-N,4-dinitrosoaniline
	Polymerized N-nitroso 2,3,4-trimethyl-1,2-dihydroquinoline
	1,-2-dihydroquinoline
	Nitrosofurfuramide
(*d*)	*Thiourea derivatives*
	Trimethylthiourea
	Diethylthiourea
(*e*)	*Cross linking agents*
	bis (2-Chloroethyl) formaldisulphide
(*f*)	*Acid anhydrides*
	Phthalic anhydride
(*g*)	*Hydrazides*
	pp′, Hydroxy bis (benzenedisulphonyl-hydrazide)
	Benzenesulphonylhydrazide
(*h*)	*Nitrosophenols*
	Ferric complex of 1-nitroso-2-naphthol
(*i*)	*Inorganic materials*
	Asbestos
	Cadmium sulphide
	Selenium

RESUME

Cent-vingt rats mâles du stock CB reçurent des injections intrapéritonéales de divers additifs du caoutchouc, dissous dans le polyéthylène glycol: la N-nitroso 2,3,4, trim-thyl-1,2–dihydroquinoline polymérisée, *la* N-méthyl-N,4-dinitrosoaniline, *la N,N-dinitosopenta methylène-tétramine et la N-nitrisodiphénylaminé. Ces substances furent administrées une fois par semaine, pendant* 6 *mois, et les animaux furent ensuite maintenus en observation pendant* 2 *ans. Aucune tumeur ne fut observée chez les rats traités par la* N,N-dinitrosopentaméthylène tétramine, *par la* N-nitrosodiphénylamine, *ou par le* polyéthylène glycol *seul. Des sarcomes intrapéritonéaux*

apparurent chez 2 *rats traités par la* N-méthyl-N,4-dinitrosoaniline *et chez* 6 *rats traités par la* N-nitroso 2,2,4-triméthyl-1,2-dihydroquinoline *polymérisée. Ces derniers avaient des tumeurs multifocales qui présentaient divers caractères histologiques inhabituels. Des signes discrets de péritonite chronique furent observés chez certains rats de tous les groupes.*

La N-méthyl-N,4-dinitrosoaniline, *ainsi que le* 6-éthoxy-2,2,4-triméthyl-1,2-dihydroquinoline *et la* 6-dodécyl-2,2,4-triméthyl-1,2-dihydroquinoline, *furent également testés, selon les mêmes modalités, chez des souris* C57 × DBA2. *Un petit nombre de tumeurs intrapéritonéales furent observées dans les* 3 *groupes traités, mais ces résultats ne sont pas interprétables, parce que* 3 *tumeurs de même type apparurent dans le groupe témoin traité par le solvant.*

Ces observations montrent la nécessité d'une étude détaillée des additifs du caoutchouc quant à leur éventuelle cancérogénicité. Elles indiquent aussi que, pour des études ultérieures, la voie d'administration intrapéritionéale n'est peut-être pas la plus favorable.

SUMMARY

One hundred and twenty male CB stock rats were injected intraperitoneally with the following rubber additives dissolved in polyethylene glycol-polymerised N-nitroso 2,3,4-trimethyl-1,2-dihydroquinoline; N-methyl-N,4-dinitrosoaniline; N,N dinitrosopentamethylenetetramine; N-nitrosodiphenylamine. *These substances were given once weekly for six months and the animals were then observed for up to* 2 *years. No tumours were seen in rats treated with* N-N-dinitrosopentamethylenetetramine *or* N-nitrosodiphenylamine *or with polyethylene glycol alone. Intraperitoneal sarcomas developed in two rats treated with* N-methyl-N,4-dinitrosoaniline *and in six rats treated with* polymerised N-nitroso 2,2,4-trimethyl-1,2-dihydroquinoline. *The latter were multifocal tumours which showed a number of unusual histological features. Evidence of a low-grade chronic peritonitis was found in rats in all groups.* N-methyl-N,4-dinitrosoaniline, *together with* 6-ethoxy-2,2,4-trimethyl-1,2-dihydroquinoline *and* 6-dodecyl-2,2-4-trimethyl-1,2-dihydroquinoline, *was also tested in male* C57 × DBA2 *mice in a similar fashion. A small number of intraperitoneal tumours developed in all three test groups but these results were uninterpretable as three similar tumours appeared in the solvent controls. The present observations emphasize the need for detailed investigation of potential carcinogens in rubber additives but the efficacy of the intraperitoneal route, for future testing, is questioned.*

ZUSAMMENFASSUNG

Man injizierte 120 *männlichen CB-Ratten folgende, in Polyäthylenglykol gelöste Gummizusätze intraperitonäal:* polymerisiertes N-nitroso 2,2,4-trimethyl-1,2-dihydrochinolin; N-methyl-N,4-dinitrosoanilin; N,N-dinitrosopentamethylentetramin; N-nitrosodiphenylamin. *Diese Substanzen werden während sechs Monaten einmal pro Woche injiziert und die Tiere sind während dieser Zeit und bis zu zwei Jahren unter Beobachtung. Es wurden keine Tumoren bei den Ratten festgestellt, die mit* N, N-dinitrosopentamethylentetramin *oder mit* N-nitrosodiphenylamin *oder mit* Polyethylenglykol *allein behandelt wurden. Bei zwei mit* N-methyl-N,4-dinitrosoanilin *und dei sechs mit polymerisiertem* N-nitroso-2,2,4-trimethyl-1,2-dihydrochinolin *behandelten Ratten entwickelten such intraperitonäal Sarkome. Letztere waren multifokale Tumoren mit einer Reihe ungewöhnlicher histologischer Charakteristika. Bei Ratten aller Gruppen fand man Anzeichen einer leichten chronischen Peritonitis. Versuche mit* N-methyl-N,4-dinitrosoanilin *und* 6-Aethoxy 2,2,4-trimethyl-1,2-dihydrochinolin *und* 6-Dodecyl-2,2,4-trimethyl-1,2-dihydrochinolin *wurden auch in ähnlicher Weise bei männlichen* C57 × DBA/2-*Mäusen gemacht. Einige intraperitonäale Tumoren wurden in allen drei Versuchsgruppen festgestellt, doch konnten diese Befunde nicht befriedigend interpretiert werden, da drei gleiche Tumoren in der Kontrollgruppe beobachtet wurden. Die gemachten Beobachtungen weisen einmal mehr auf die Notwendigkeit hin, Gummizusätze auf ihren Gehalt an potenziellen Karzinogenen zu untersuchen, wobei die Zweckmässigkeit der beschriebenen intraperitonäalen Methode in Frage gestellt werden darf.*

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Studies on Lymphocytes IX. The Survival of Autotransfused Labeled Lymphocytes in Chronic Lymphocytic Leukemia*

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INTRODUCTION

EXTRACORPOREAL irradiation of the blood (ECIB) has been proposed as a potential therapeutic measure in the treatment of leukemia [1–3]. ECIB has been used in the treatment of bovine lymphocytic leukemia [3, 4], chronic lymphocytic leukemia (CLL) and other leukemias of man [5, 6]. Since ECIB is capable of killing a large fraction of the radiosensitive cells in an extracorporeal circuit, the rate of exchange of leukemic lymphocytes in the blood with those in the tissues is of paramount importance. If there is substantial recycling of leukemic cells from blood to tissues in man, as Gowans *et al.* [7, 8] have established for normal rat lymphocytes, one could anticipate that ECIB would cause depletion of the leukemic cells from extravascular depots. Furthermore, ECIB has been shown to deplete lymphocytes from the thymus, the spleen and the lymph nodes of the normal calf [9].

The purpose of the present work was to estimate, using a method initiated by Fliedner [10, 11], the exchange of blood lymphocytes with the extravascular pool of lymphocytes in CLL.

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METHODS

Eight patients with CLL were studies (*M*, *S*, *W*, *B*, *A*, *SH*, *L* and *R*). In each of 5 studies (*M*, *S*, *W*, *B*, and *A*) (see Table 1), approximately 500 ml of venous blood was collected in a Fenwal (*R*) plastic bag containing 75 ml ACD formula A. In some experiments the red blood cells were allowed to sediment, and the supernatent lymphocyte-rich plasma was decanted for incubation. In other studies the whole blood was incubated. One or 2 μc tritiated cytidine (^{3}H-Cyt) (Schwarz Bio-Research, specific activity 2·6 c/mMole) were added to each ml of the lymphocyte-rich plasma or whole blood and then incubated at room temperature (23° C) for 1 hour. The cells were autotransfused either immediately after incubation (*M*) or after washing twice (*S*, *W*, *B*, *A*) with approximately 100 ml tissue culture medium (NCTC-109). Autotransfusions required 2 min–1 hr. For patient *SH* (Table 1), 500 ml of blood was incubated for 30 minutes with ^{3}H-Cyt (1 μc/ml) and autotransfused; the procedure was repeated 7 additional times within the next 7 hours.

Following reinjection, serial blood samples for autoradiography (ARG) were obtained for several days. After sedimentation of the red cells of 2 ml of blood with 0·5 ml of an anticoagulant mixture the white cells were concentrated by centrifuging the supernatant

Table 1. *Methodologic data on six patients with chronic lymphocytic leukemia*

Patients	Blood lymphocytes per mm²	Blood vol. (l.)	³H-Cyt μc/ml of incubation mixture	Washing of incubated cells	Total No. injected labeled lymphocytes × 10¹⁰	Tissue samples
SH	68,000	5·0*	1	No	Not measured	Bone marrow
M	40,000	6·25	1	No	1·2	Bone marrow
S	285,000	5·25	2	Yes	10	Lymph node
W	350,000	5·25	2	Yes	11·3	Lymph node
B	55,000	5·24*	2	Yes	0·82	—
A	50,000	4·8*	2	Yes	2·4	—

*Estimated on basis of body weight.

(0·7% saline, 0·5% EDTA, and 3% dextran). Bone marrow aspirations were obtained from patients *SH* and *M*. Biopsies of inguinal lymph nodes were performed with a Menghini needle in patient *S* and *W*.

Autoradiographs of the blood, bone marrow smears, and lymph node sections were done by the standard methods using NTB2 Kodak liquid emulsion, exposed for 30 to 50 days, and stained with Giemsa. The labeling index (L.I.) of the injected mixture and all samples was determined by counting the proportion of cells with more than 5 grains overlying the nucleus and cytoplasm in all cases except *SH*, in whom a threshold of 10 grains was chosen. For patients *S*, *W*, *B* and *A*, the volume, leukocyte count, differential count, and the L.I. of the injection mixture lymphocytes were determined in order to calculate the total number of labeled lymphocytes returned to the patient. The blood volume was measured for patients *S*, *M*, and *W* using ^{131}I human serum albumin and was estimated on the basis of body weight for patients *A* and *B*.

In two of the studies, the significance of free ³H-Cyt injected was evaluated by incubating the supernatant from the second washing with an equal volume of fresh blood from the donor for one hour at room temperature. The L.I. was 0·1% in both cases, compared with 96 and 99% in the original ³H-Cyt incubation mixture.

In patient *L*, measurements of tritium radioactivity in a Packard Tricarb Liquid Scintillation Spectrometer Model 4322, using a method described elsewhere [12], showed that less than 5% of the activity used for *in vitro* incubation was left in the cell suspension after two washings. More than 70% of this activity was in the cells. In patient *R* (150,000 leucocytes mm³, 98% lymphocytes) the white cells were partially isolated by dextran sedimentation and distribution of their volumes was determined by a Coulter Model B Blood Counter with an automatic recording distribution plotter. The *in vitro* procedure (labeling and subsequent washings) lasted approximately 4 hr. The viability of CLL lymphocytes after such manipulations was estimated by the Trypan Blue exclusion test in 7 procedures in 5 patients not included in this study. This test showed an average of 89.0% viability with a range of 80·0–98·3%.

RESULTS

Figure 1 shows that in patients *B* and *W*, in whom the autotransfusion was performed rapidly, 100% of the injected cells were recovered in the circulation a few minutes after the end of autotransfusion, whereas only approximately 50% were recovered in patient *A* in whom the autotransfusion took more than 30 minutes. Figure 1 also shows that the concentration of labeled lymphocytes diminishes

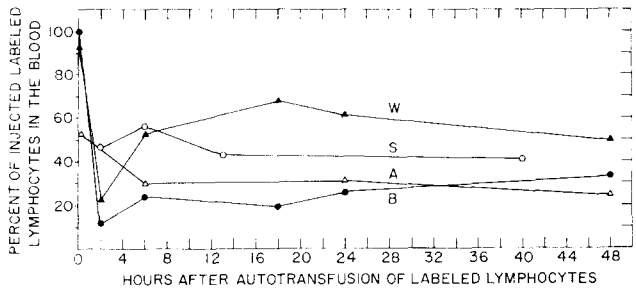


Fig. 1. *The percent of injected labeled lymphocytes found in the blood as a function of time. Time* 0 *represents the end of the autotransfusion.*

rapidly, reaching a nadir at about 2–6 hr. From 6 to 48 hr, the fraction of the labeled circulating lymphocytes appears relatively constant.

Figure 2 shows that for patients *M* and *S* the per cent of labeled lymphocytes is considerably

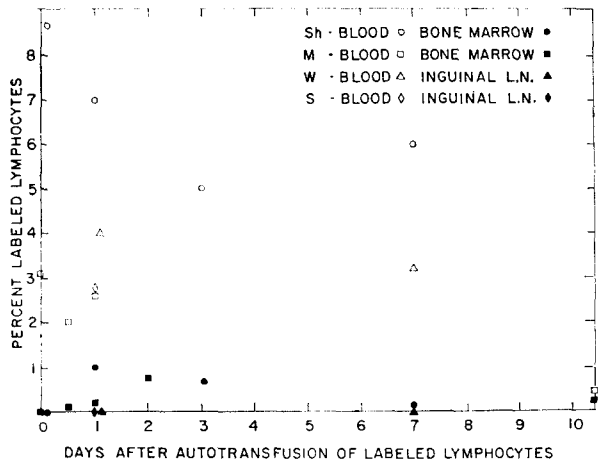


Fig. 2. The percent of labeled blood lymphocytes at various times in 4 patients with chronic lymphocytic leukemia (SH, M, W, S) *is indicated by the open symbols. The closed symbols represent the percent of labeled lymphocytes in tissue samples at the same times as in the blood.*

lower in the bone marrow than it is in the peripheral blood at the same time. No significant number of labeled lymphocytes could be seen in the inguinal lymph nodes of patient *W* at days 1 and 7 and patient *S* at day 1 after autotransfusion. In patient *S*, in whom the number of labeled cells injected was the highest, only 6 labeled cells were seen in 10 sections of a lymph node biopsy. Three of these cells were in the mass of extravascular small lymphocytes, and three were in the lumen of small blood vessels. The proportion of extravascular space to intravascular space determined by point analysis [13] was 33:1. Since the lymphocytes were always less densely packed in the vascular spaces than in the extravascular spaces, the observed ratio must be a minimum ratio of extravascular to intravascular lymphocytes.

DISCUSSION

The validity of the label as a lymphocyte marker, in the conditions of the experiment, must be considered. The half time of disappearance of the label from the lymphocytes of patients (*B*, *S*, *W*, *G*) by RNA turnover was found to vary between 87 and 280 hours [14]. Therefore, the loss of labeled cells by this turnover is considered negligible during the 48 hours of this study. For the same reason, it is assumed that there is negligible *in vivo* transfer of ^{3}H-Cyt to other cells. The control studies, presented under *Methods*, rule out *in vivo* labeling of lymphocytes by free ^{3}H-Cyt injected with labeled cells. To rule out with more certainty *in vivo* labeling of lymphocytes by free Cytidine or by Cytidine liberated by the turnover of labeled RNA, the threshold used to define a labeled cell was kept high (5 grains). In patient *SH*, in whom the labeling procedure was done 8 times, the risk of *in vivo* labeling was much higher.Therefore, a threshold of 10 grains was chosen.Moreover, Fliedner has shown that 500 μc ^{3}H-Cyt injected intravenously in a patient with CLL does not label his blood lymphocytes when the slides are exposed for 30 to 60 days.

The curves in Fig. 1 show two main components, an initial rapid decrease and a subsequent plateau reached after approximately 6 hr and maintained, at least, for the subsequent 2 days. Only a part of the initial decrease can be attributed to removal from the circulation of autotransfused lymphocytes killed during *in vitro* manipulations. The plateau mainly represents rapid equilibrium with a pool of noncirculating lymphocytes. The size of the pool of dilution can be estimated as follows:

$$TPD = \frac{CBLP}{FLL}$$

TPD = total lymphocyte pool of dilution at 24 hours.

CBLP = total circulating blood lymphocyte pool (blood lymphocytes/mm^3 × 10^3 × blood volume in ml).

FLL = fraction of labeled lymphocytes remaining at 24 hours in the circulation.

The *TPD* was $1{\cdot}25 \times 10^{12}$ lymphocytes for patient *B*, $3{\cdot}3 \times 10^{12}$ for patients *S* and *W* and $0{\cdot}76 \times 10^{12}$ for patient *A*. The *CBLP* represents approximately 25–60% of these numbers. Although the anatomical location of the noncirculating part of the *TPD* was not determined, it may be either the extravascular pool (*EVP*) from which lymphocytes can return to the circulation via the lymphatics such as the thoracic duct (*TD*), as shown in normal subjects [15], or a sequestered intravascular pool communicating with the circulating portion and comparable to the marginating pool described for granulocytes [16].

In the case of equilibrium with the *EVP* the following alternatives must be considered: (1) a rapid equilibrium of all blood lymphocytes with the whole EVP, (2) a rapid equilibrium of only a fraction of the blood lympho-

cytes with the whole *EVP*, (3) a rapid equilibrium of all blood lymphocytes with only the part of the *EVP* of lymphocytes which is easily accessible to the blood, and (4) a combination of (2) and (3) in which a portion of the blood lymphocytes equilibrates with a part of the *EVP*.

In alternative (1), the *TPD* would represent all the lymphocytes in the blood. The findings in patient *S* show that this alternative must be false. His *TPD* was $3 \cdot 3 \times 10^{12}$ cells, of which 2×10^{12} were noncirculating. The average blood lymphocyte voluem was measured in patient *R* and found to be $250\mu^3$. Using this figure and the volume of one of the multiple lymph node masses of patient *S*, measured with a caliper (315 cm^3), it appears that this single mass alone could contain approximately 1×10^{12} cells. In addition, the biopsy of this mass showed that it was packed by small lymphocytes and contained practically no labeled cells.

In alternative (2), only a fraction of the blood lymphocyte population is able to equilibrate rapidly with the whole extravascular pool. The rapidly recycling population would then represent respectively 40 and 60% of the whole blood lymphocytes in patients *W* and *S* (79 and 85×10^{10} cells) of which 6 and 4×10^{10} are labeled). Assuming that the leukemic cells represent 10% (10^{13} cells) of the total number of cells of the body, a reasonable estimate in these patients, the L.I. in the lymph nodes would be approximately 0·5% if there were a homogeneous distribution of the rapidly recycling cells. The exact L.I. in the nodes was not obtainable because of the scarcity of labeled cells, but was certainly less than 1 in 10,000.

In alternative (3), all the blood lymphocytes leave the circulation rapidly to only a small part of the *EVP*, and the plateau obtained would then represent a rapid return of some cells from the *EVP* to the blood. In the normal calf, radioactivity appears in the lymph ½–2 hr after intravenous injection of ^{51}Cr, ^{75}Se, or ^{65}Zn labeled cells [17]. Perry has shown that when male patients with malignancies other than CLL were transfused with TD lymphocytes obtained from female donors, lymphocytes with a female karyotype were observed as early as 10 hours in the TD lymph [14]. A rapid return through the TD in CLL patients is not supported by Binet's finding that the label first appears in the TD cells 4 days after the injection of ^{3}H-Cyt labeled cells [18]. This argument, however, does not rule out an equilibrium between the EVP and the blood through other pathways (e.g., in the spleen). Bierman *et al.* [14] have shown that the daily output of lymphocytes from the TD in 3 patients with CLL varied between $1 \cdot 08$ and $2 \cdot 81 \times 10^{10}$. This figure is lower than the average output in the normal individual estimated by Yoffey $3 \cdot 5 \times 10^{10}$ [19]. Therefore, if the plateaux represent cells which returned to the circulation (138×10^{10} in *W* and $22 \cdot 3 \times 10^{10}$ in *S*) one must conclude that approximately 97 and 85%, respectively, of the blood lymphocytes of *W* and *S* return to the circulation through other pathways than the TD.

In alternative (4), only a fraction of the blood lymphocytes would spread inhomogeneously in the lymphoid organs, and the plateaux would then represent a long disappearance time from the blood of the rest of the lymphocytes. This explanation is compatible with other observations: (a) Bierman *et al.* found that TD drainage produced a slower rate of decrease of the blood lymphocytes in patients with CLL than in normal individuals [20]; (b) we have shown a more rapid depletion in the blood than lymphoid organs with ECIB in these patients [21].

Complete equilibrium with the *EVP* (alternatives 1 and 2) is not supported by the present findings. The present study is, however, compatible with inhomogeneous distribution (Alternatives 3 and 4) so that the L.I. would vary with the area sampled. Alternative (4) is compatible with our observations and those of others. A fifth alternative is that almost no communication exists between the blood lymphocytes and the extravascular lymphocytes and that the rapid initial decrease of labeled cells is due to dilution in an intravascular, noncirculating pool of lymphocytes comparable to the marginal pool described for the granulocytes. This would, as in alternative (4), imply long vascular transit for CLL lymphocytes.

The question then arises as to why some lymphocytes stay in the blood circulation for a long period of time. Gowans *et al.* have shown that lymphocytes migrate from the blood stream to the lymph nodes through the post-capillary venules [8]. Gesner's work implicates the surface coating of the lymphocytes in this process [22]. Although the number of labeled cells seen is obviously too small to be significant, the preferential localization of these cells in the vascular space in the lymph node of patient *W* suggests that the lymphocytes have difficulty leaving the circulation, perhaps because of a defect in the leukemic lymphocytes or in the post-capillary venules. The few labeled cells seen in the bone marrow may result from con-

tamination by peripheral blood at the time of the bone marrow aspiration. The L.I. of the bone marrow is also lower than that of the blood in the normal rat after injection of isologous labeled lymphocytes [7].

If a lymphocyte receives a lethal dose of radiation during each transit through the irradiator during ECIB, then the efficacy of this treatment is dependent on the rate of exchange between the vascular and extravascular pools. The present study indicates a poor exchange.

RESUME

1. *Les lymphocytes de* 4 *patients, atteints de leucémie lymphoïde chronique, ont été marqués,* in vitro, *par la cytidine* ^3H. *Vingt-quatre à* 48 *heures après autotransfusion de ces lymphocytes marqués, on a retrouvé, dans le sang circulant,* 25–60% *des cellules marqués. L'index de marquage était considérablement plus bas dans la moelle osseuse et dans les ganglions qu'il ne l'était, au même moment, dans le sang.*

2. *On a observé, dans les premières heures, une disparition rapide d'une fraction des cellules marquées du sang, et on a interprété cette disparition comme résultant d'un équilibre partiel des lymphocytes du sang avec un pool extravasculaire, ou d'un équilibre total avec un pool intravasculaire non circulant.*

3. *Une partie des lymphocytes marqués resta dans le sang, pendant toute la période d'observation. Ceci semble indiquer un séjour prolongé, dans le sang, d'une fraction, ou de la totalité, des lymphocytes de la leucémie lymphoïde chronique.*

4. *Ces résultats sont discutés en raison d'une éventuelle application de l'irradiation extracorporelle au traitement de la leucémie lymphoïde chronique.*

SUMMARY

1. *In four patients with chronic lymphocytic leukemia,* 24 *to* 48 *hours after autotransfusion of lymphocytes labeled with* ^3H*-Cyt* in vitro, 25–60% *of the labeled cells were still in the circulating blood, whereas considerably lower labeling indices were seen in the bone marrow and lymph nodes.*

2. *A rapid initial decrease of the circulating labeled lymphocytes was seen and may be interpreted as the result of equilibrium with an extra or intravascular sequestered pool.*

3. *A plateau was observed for the subsequent* 2 *days of the study and was interpreted as the result of a long blood transit time for a fraction of lymphocytes of chronic lymphocytic leukemia.*

4. *The implication of the resvlts of this study in treating chronic lymphocytic leukemia with extracorporeal irradiation of the blood is discussed.*

ZUSAMMENFASSUNG

1. *Bei* 4 *Patienten mit chronisch.-lymphatischer Leukämie konnten* 24–48 *Stunden nach einer Autotransfusion von mit H*3 *Cytidin* in vitro *markierten Lymphocyten* 25–60% *der markierten Zellen im zirkulierenden Blut nachgewiesen werden. Im Knochenmark und in den Lymphknoten dagegen war der Anteil der markierten Zellen bedeutend niedriger.*

2. *Es wurde ein rascher initialer Abfall von zirkulierenden markierten Lymphocyten festgestellt. Dieser kann als Ausdruck eines Gleichgewichtszwischen den Blutlymphozyten und einem extra-oder intravaskulären, aber nicht zirkulierenden Pool gedeutet werden.*

3. *In den folgenden* 2 *Tagen wurde eine konstante Konzentration festgestellt. Dies könnte auf eine längere Verweildauer der Lymphocyten von chronisch-lymphatischer Leukämie im Blut hinweisen.*

4. *Die Bedeutung dieser Resultate in Bezug auf die Behandlung der chronisch-lymphatischen Leukämie mit extracorporealer Bestrahlung des Blutes wird diskutiert.*

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Announcement

Oettlé Memorial Fund

DURING his all too brief, but highly productive, life Dr. George Oettlé demonstrated brilliantly not only to South Africa but to the world, what could be done in the field of Demographic Pathology in South Africa. Many before him had noted differences in disease patterns in the race groups of South Africa but it took Oettlé to apply this to the field of cancer. His researches over a brief period of sixteen years strengthened the theory that environment might be all important in the causation of cancer. Together with his colleague Higginson he determined cancer rates in the Bantu population of Johannesburg. This survey, planned with great care, and carried out with meticulous attention to detail, has become the standard pattern for similar surveys in underdeveloped countries in other parts of the world where accurate vital statistics are not available. As a result of this work he became convinced that at least 80 per cent of cancers are induced by environmental factors and are, therefore, at least theoretically, preventable.

Despite many highly attractive and financially rewarding offers to work in overseas institutions he firmly refused to leave South Africa. He believed that the multiracial nature of South Africa's population, together with the infinite spectrum of slow transition in the Bantu from tribalism to Western sophistication offered opportunities for this type of work which were not available anywhere else in the world.

Unfortunately, throughout his career in cancer research, he was dogged by a chronic lack of assistance in the field of medical statistics. Mathematicians and statisticians in academic and industrial fields were generous in the assistance they gave him and he, by force of circumstance, trained himself in the basic essentials of the science, but a full time medically qualified person trained in epidemiology and medical statistics was never available to him. His lack was part of South Africa's shortage of skilled personnel.

Realising the way in which Oettlé's work, despite its brilliance, was handicapped by this lack and realising also, that if his work is to be continued in this land of infinite opportunity, skilled personnel of this type must be trained, his colleagues are seeking to set up a fund for the purpose. With the aid of this fund, promising young people would be trained in medical statistics and particularly in cancer epidemiology.

We, his colleagues and admirers, appeal for support for the establishment of such a fund. In view of Oettlé's many international contacts and the wide circle of friends he established in all quarters of the globe, we venture to appeal to overseas as well as to South African colleagues to support the fund. Cheques should be made payable to the "Oettlé Memorial Fund" c/o The National Cancer Association of South Africa, P.O. Box 2000, Johannesburg.

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Grafting of HeLa Cells in Young Chick Blastoderms*

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INTRODUCTION

THE ASSOCIATION of malignant and normal tissues in organotypic culture [1] affords ample demonstration of the invasive properties of malignant cells. The differentiated tissues fail to show any resistance towards the neoplastic cells which invariably kill and destroy their associates.

As far as we know, associations of cancer cells with early embryonic tissues have not been reported. One may ask whether malignant cells would show the same invasive and destructive properties towards these cells. It would also be interesting to learn whether the associated embryonic tissues would also exhibit no reaction towards the cancer cells.

MATERIAL AND METHODS

Young chick blastoderms, from commercial stock were used as hosts. They were incubated through stage 4 or stage 7 of Vakaet [2] after about 12 and 20 hr of incubation respectively. They were then cultivated further *in vitro* according to a modification of New's technique [3] by Gallera and Castro-Correia [4]. A small incision in the entophyll wall, at one of its posterior horns allowed the placing of a graft underneath it, i.e. between the overlying endoblast and the ectophyll (Fig. 1a).

The grafts consisted either of HeLa cells from laboratory mono layer stock, or of testis fragments from full-term mouse foetuses, Control cultures of HeLa cells and testis fragments were made on vitelline membranes, using egg-white only as nutrient medium.

The cultures were incubated at 38°C for 24hr. Then they were fixed successively in Zenker's solution, coloured *in toto* with Unna's methyl Green-pyronin at pH 4·66, photographed *in toto*, embedded in paraffin, sectioned at 8μ and recoloured with Haemalumeosin or Unna's stain.

RESULTS

1. *Grafting of HeLa cells (Host stage* 4 : 33 *cultures. Host stage* 7 : 10 *cultures).*

The HeLa cells. At the moment of fixation, the HeLa cells formed a distinct nodule, with vital appearance. If not too large, no signs of necrosis were visible from outside.

This could be confirmed microscopically, as mitoses were abundant, and central necrosis found only in rare, large nodules. This was in striking contrast to the fate of HeLa cells in control cultures: they formed a small layer of dead cells after 24 hr incubation on vitelline membrane and egg-white.

The HeLa cells assumed a somewhat organized structure which could be compared with the aspects of HeLa cells cultured on chick mesonephros [5]. It was quite different from the monolayer that was transplanted.

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No invasiveness was shown by the cancer cells towards the blastoderm cells. No HeLa cells could be traced over the blastoderm, even after thorough histological examination. This was made possible by the distinct morphological difference between the cell types of host and graft.

The host blastoderms. The host blastoderms developed in the same normal way as did control blastoderms without graft. After incubation, the site of graft recovery was rather variable. This variability was less on stage 7 than on stage 4 hosts. In the former, the graft was either found near its site of implantation, or in the cephalic region in contact with the neurectoblast. Grafts on stage 4 blastoderms showed a much greater relative shift: they were either recovered in the cephalic region (Fig. 1b) in contact with the nervous system, or laterally at the border of area opaca and area pellucida (Fig. 1c) suggesting a relative standstill, or mediocaudally (Fig. 1d) due to a broad caudal glide.

In these last cases of mediocaudal transposition, several (5 out of 7 caudal migrations) blastoderms showed an elongated condensation following the graft rather like a meteor's track, from its original site close to the site where the graft is finally found (Fig. 2). The host germ was bent towards this structure. In some cases it sometimes appeared less developed at the side of the graft.

Microscopically, there was a noteworthy fact to be observed in the endoblast. This layer never covered the upper side of the nodule, although in most cases it surrounds it closely (Fig. 3). Mesoblast cells were usually crowded against it, at least when it was found in a lateral or posterior position. There was a greater proportion of mesoblast on the side containing the graft. The graft was always found resting on the ectoblast, which never seemed to react directly to it. It was never ruptured by HeLa cells. In blastoderms showing an elongated condensation, this was never in direct contact with the grafted HeLa cells. On the contrary, the elongated conden-

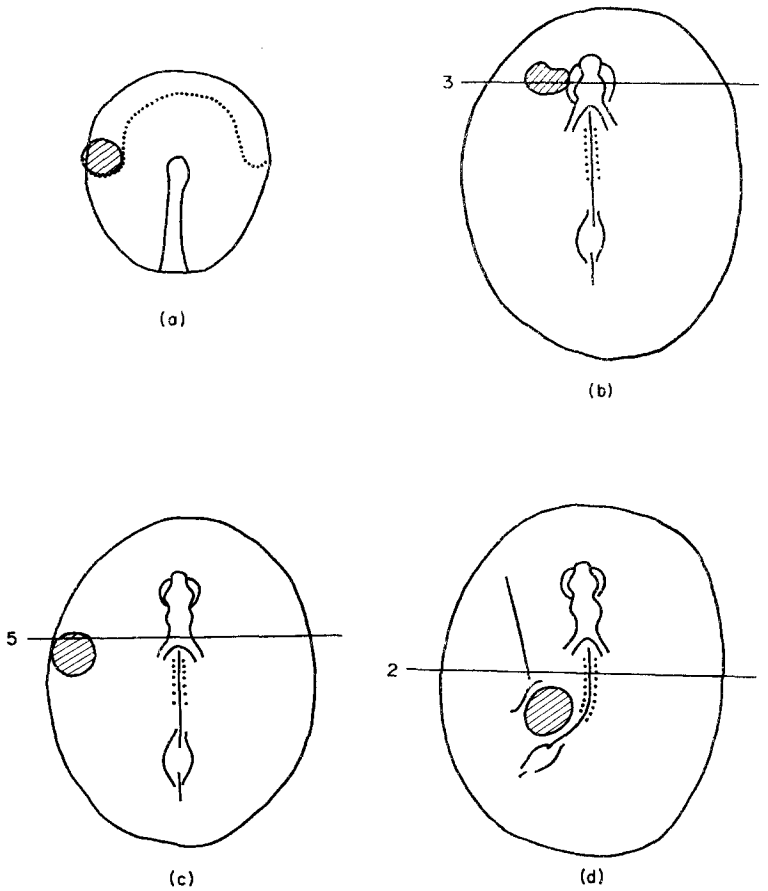


Fig. 1. *Outlines of grafting experiments.*
(*a*) *Stage* 4 *blastoderm with graft at posterior horn of endophyll wall, before incubation.*
(*b*) *Cephalad shift of graft after* 24 *hr incubation.*
(*c*) *Relative standstill of graft during incubation.*
(*d*) *Caudally directed shift of graft and situation of streak-like reaction, which may occur with HeLa grafts.*

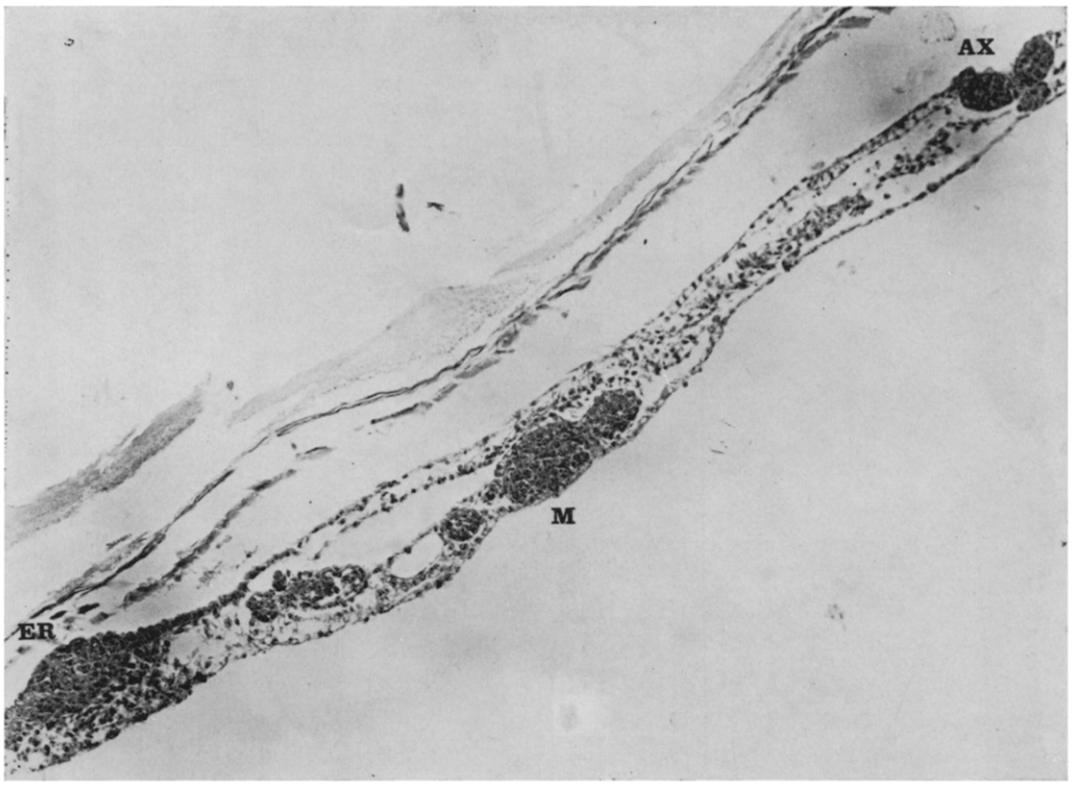


Fig. 2. *Result of grafting HeLa cells.* (*cf. Fig.* 1 *d; section following* 2). *Note the ectophyll reaction* (*ER*), *the mesoblast condensation* (*M*) *and the axial parts of the host-germ* (*AX*). *The graft is situated more caudally. H. and E. Obj. Zeiss planapochr. X*10.

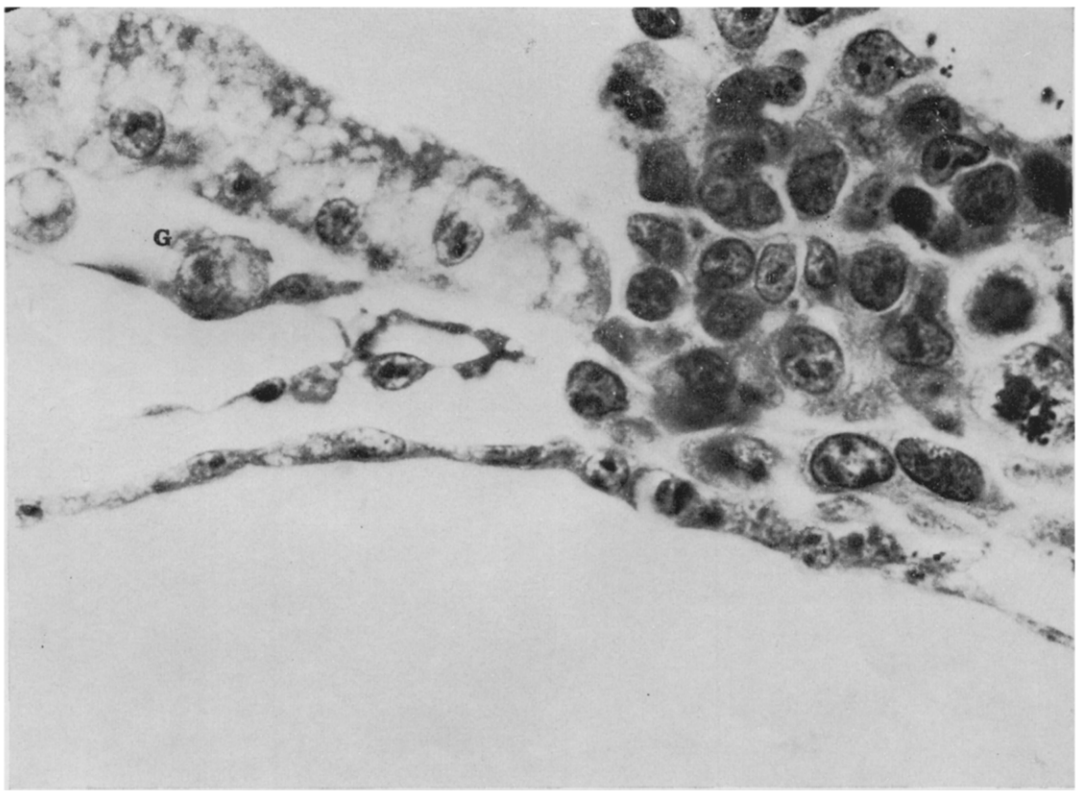


Fig. 3. *Illustration of relationship between HeLa nodule, ectoblast and endoblast* (*cf. Fig.* 1, *b; section following* 3). *Note mutual contact inhibition of HeLa cells and endoblast. A primordial germ cell* (*G*) *is visible. H. and E. Obj. Wild Fluotar X*50, *imm.*

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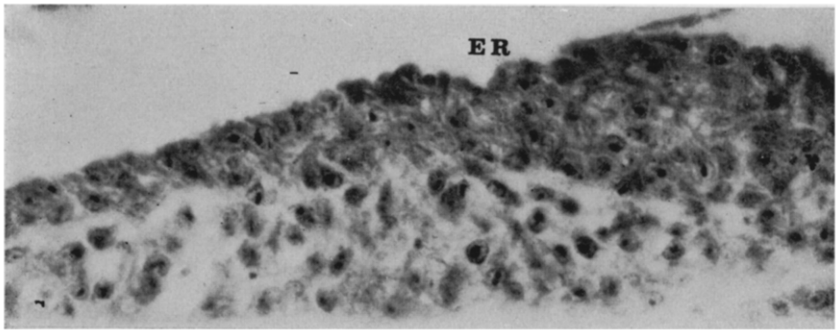


Fig. 4. *Higher magnification of the ectophyll reaction* (*ER*), *suggesting invaginating activity. H. and E. Obj. Zeiss planapochr. X*20.

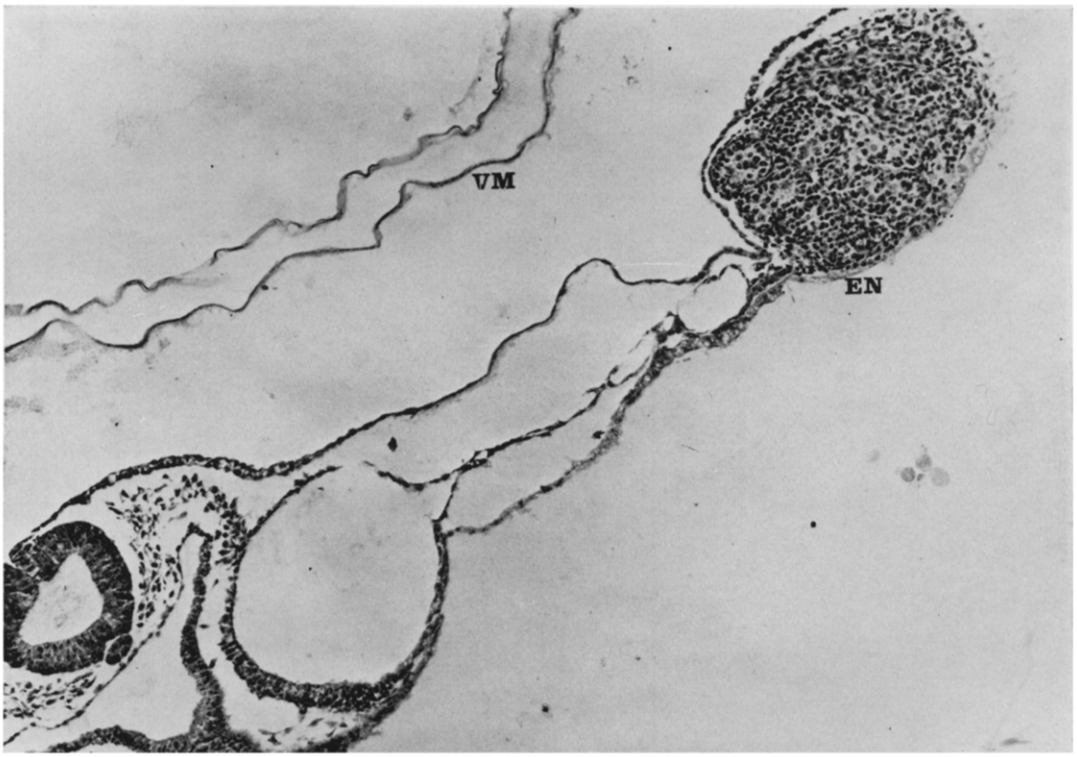


Fig. 5. *Result of grafting a piece of mouse testis* (*cf. Fig.* 1 *c; section made following* 5). *Note endoblast* (*EN*) *covering completely the nodule. V.M.=vitelline membrane. H. and E. Obj. Zeiss planapochr. X*10.

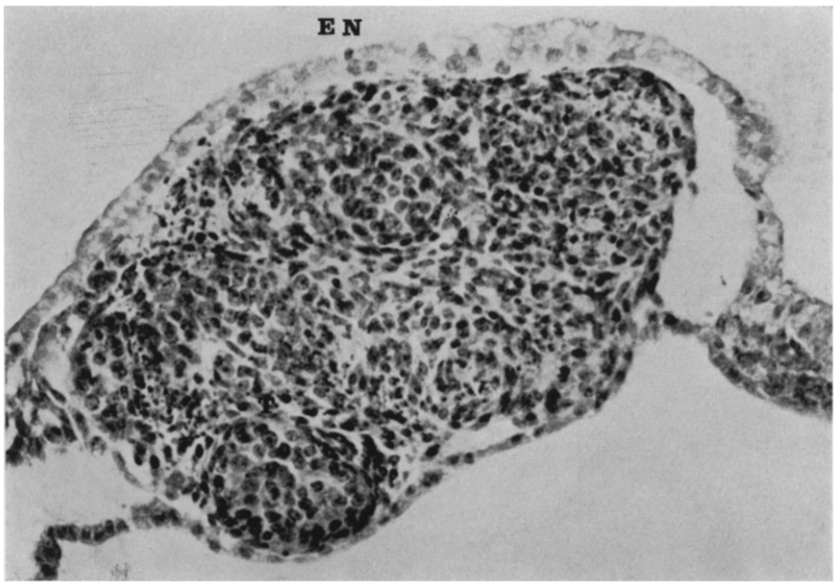


Fig. 6. *Higher magnification of endoblast covering testing grafts. Endoblast (EN) closed; testis (T) organized. H. and E. Obj. Zeiss planapochr. X*20.

sation had, deep to it, cells which, together with the ectophyll thickening, were often very suggestive of a process similar to the one observed on sections of normal primitive streak (Fig. 4). In the latter case, a process of invagination is very probable, although direct proof of it is still lacking. The cells under the streak-line thickening did not fuse with the axial parts of the host, and indeed were limited to the lower side of the ectophyll presenting the reaction.

2. *Grafting of mouse testis.* (*Host stage* 4 : 22 *experiments*)

Mouse testis grew very well in organ culture. It did so on vitelline membrane with egg-white as food, as well as on the young chick blastoderms that serve as hosts. Testis fragments formed a nodule, in which the tubules were well preserved after the short incubation time used. Mitoses were present and spermatogonia started swelling in the seminiferous tubules.

The blastoderms grew very well, and imposed the same relative displacements on the testis as they did on the HeLa nodules.

However, no concomitant reactions in the blastoderm appeared. Histological examination confirmed this impression (Fig. 5). Contrary to what happened with HeLa grafts, the endoblast always covered the testis (Fig. 6). Mesoblast crowded up against it: it was impossible to distinguish this reaction from that observed with HeLa cells. Enlargement of the coelomic cavity could be present in either case, and seemed to be due essentially to mechanical factors. The ectoblast did not show any significant reaction. Mechanical folds, sometimes observed, were entirely different from the reaction evoked by HeLa cells.

DISCUSSION

HeLa cells and mouse testis fragments grow very well as grafts on young chick blastoderms. The difference in their behaviour, when cultured on vitelline membrane alone, is probably due to nutritional reserves, present in testis but absent in HeLa cells.

It is therefore clear that the HeLa cells feed on metabolites from the blastoderm. Are the metabolites akin to those, which Wolff and Wolff [6] try to separate from chick mesonephros cells?

In spite of the short incubation time, a space of 24 hr is enough for the HeLa cells to evolve from a layer one cell thick, into a nodular structure. This reorganisation might possibly be another example of the influence of the physico-chemical composition of the medium on the behaviour of cultured tissues.

In our short-term experiments, HeLa cells do not invade the blastoderm. HeLa cells from the same stock grow into chick mesonephros within 24 hr [5] but not into blastoderms. Therefore the short incubation time may not be a valid explanation for the non-aggressive behaviour of the HeLa cells, neither can the mesoblast cells of the host be held responsible for the practical segregation and nodular transformation of the HeLa cells. In fact the rearrangement of HeLa cells also occurs in grafts found in the head region, at a time when mesoblast is not present. An acceptable explanation for this phenomenon is now under investigation.

The movements of the grafts may easily be understood from the knowledge of the known morphogenetic movements within the chick germ, which remain unhindered by grafts. The direction of the shifts depend on the presumptive fate of the ectophyll on which the graft is layed down [7].

Mesoblast cells heap up in contact with the grafts. This accumulation is probably passive, and not a kind of leucocytic reaction.

The endoblast layer shows a different behaviour toward testis and HeLa grafts. It heals up quickly over a grafted testis nodule (Fig. 6), while on the contrary, it never covers a grafted HeLa cell mass (Fig. 3). We have been able to demonstrate [8] that chick endoblast may regenerate very rapidly. Therefore we feel that the repulsion which prevents the endoblast layer from growing over the HeLa cells may be an interesting problem for future study.

The ectophyll may show a streak-like reaction in stage 4 hosts carrying a caudad migrating HeLa nodule. Although there are some indications that this might be the homologue of a true blastopore, the problem needs further investigation. If this structure is a real primitive streak, it remains to be explained how it has been evoked.

We feel that in this formation the absence of entoblast regeneration also plays a major role.

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RESUME

Des cellules HeLa et des fragments de testicule de souris ont été greffés dans de jeunes blastodermes de poulet, et cultivés in vitro.

Nous avons observé que les blastodermes se développent d'une manière normale. L'ectoblaste reste intact, le mésoblaste est en contact avec les bords des greffes. L'endoblaste recouvre les fragments de testicule, ce qui n'est jamais le cas avec les cellules HeLa.

Le déplacement relatif des greffes est attribué aux mouvements morphochorétiques des blastodermes.

Les deux sortes de greffes sont bien conservées in vitro; *les cellules cancéreuses HeLa ne sonta pas invasives. Dans les cas où les greffes HeLa sont transportées dans une direction médio-caudale, une réaction, comparable à une ligne primitive, se montre fréquemment dans l'ectoblaste.*

Les résultats sont discutés, et les auteurs essayent de trouver une corrélation entre le caractère non invasif des cellules HeLa, le comportement de l'ectoblaste, les mouvements des greffes et la réaction du type "ligne primitive".

SUMMARY

HeLa cells and mouse-testis fragments were grafted into young chick blastoderms and further cultivated in vitro.

The blastoderms developed normally. The ectoblast was undamaged, the mesoblast crowded up against the grafts. The endoblast covered the testis, but never the HeLa cells. The relative displacements of the grafts were brought about by the morphochoretic movements of the blastoderm. Both grafts developed well, the HeLa cells being strikingly non-invasive.

When HeLa grafts, uncovered by endoblast, were carried medio-caudally, a streak-like reaction was frequently evoked in the ectoblast.

These phenomena are discussed, and an attempt is made to find a correlation between non-invasiveness of HeLa cells, endoblast behaviour, graft movements and streak-like reaction.

ZUSAMMENFASSUNG

HeLa-Zellen wurden zusammen mit Fragmenten von Maus-Testis auf junges Hühnerblastoderm transplantiert und anschließend in vitro *kultiviert.*

Das Blastoderm entwickelte sich normal. Der Endoblast blieb intakt. Der Mesoblast staute sich an den Rändern des Implantats. Der Endoblast überwuchs den Testis, jedoch nie die HeLa-Zellen. Die relative Verlagerung der Implantate wurde durch morphochoretische Bewegungen des Blastoderms verursacht. Beide Implantate entwickelten sich gut, wobei die HeLa-Zellen auffallend noninvasiv blieben.

Verschob man die vom Endoblasten unbedeckten HeLa-Zellen nach medio-caudal wurde häufig eine primitivstreifenartige Reaktion im Ektoblasten ausgelöst.

Diese Phänomena werden diskutiert und es wird versucht eine Beziehung zu finden zwischen dem nichtinvasiven Verhalten der HeLa-Zellen, der Reaktion des Endoblasten, der Wanderung des Implantats und den primitivstreifenartigen Effekten.

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Serological Studies of Antigenic Changes Occurring in Two Lines of Ehrlich's Ascites Tumor after Long Periods of Cultivation *in vitro*

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INTRODUCTION

IN A PREVIOUS publication [1], the gradual loss of transplantability of two lines of Ehrlich's ascites tumor (ELT and ELD) during cultivation *in vitro* was described. The antigenicity of these two cell lines was revealed by comparative studies of their transplantability in adult and newborn mice, and by immunization experiments which showed the establishment of resistance to *in vivo* propagated Ehrlich's ascites tumors (ELTa and ELDa) by pre-immunization with the two *in vitro* propagated cell lines (ELTc and ELDc). However, no answer was obtained as to whether the loss of transplantability during cultivation was due to antigenic changes or to other factors. The present investigation was carried out in order to elucidate this question by a serological analysis of the antigenic structure of the ELT and the ELD cells propagated *in vivo* and *in vitro*.

MATERIAL AND METHODS

The morphology and transplantability [1] as well as the growth pattern and metabolism [2] of the cell lines under investigation have previously been described. The near-tetraploid (ELT) and the near-diploid (ELD) line of Ehrlich's ascites tumor were originally obtained from Professor Klein, Karolinska Institutet in Stockholm. In this laboratory, they have been carried as ascites tumors in inbred mice of the ST/a, C3H and DBA/2 strains under the names ELTa and ELDa. The ELT cells obtained from a ST/a mouse were adapted to *in vitro* conditions in this laboratory in January 1964. Since then they have been propagated *in vitro* under the name ELTc. The ELD cells were adapted to *in vitro* conditions by Dr. Holmberg at the Research Division of the Raduim Center in Stockholm in January 1962. This cell line has been propagated *in vitro* in this laboratory since June 1962 under the name ELDc. The culture age of the ELTc and ELDc cells at the time of the present experiments was $3\frac{1}{2}$ and $5\frac{1}{2}$ year respectively.

The cells were grown on glass in 1 l. Roux bottles under our FIB 14B medium which is composed of 20% fetal bovine serum and 80% Eagle's MEM [3] modified to contain twice the concentration of amino acids and four times the recommended concentration of glutamine and vitamins.

The ELTa and the ELDa cells used were collected from C3H and ST/a mice, spun down at 500 **g**, and washed twice in Tyrode's solution. When used for pre-immunization the cells were finally exposed to 0·2 mM iodo-acetate for 1 hr at 37°C as described by Apffel *et al.* [4], in order to prevent progressive growth on subsequent inoculation, and then resuspended in

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Tyrode's solution at a concentration of 5×10^6 cells per 0·1 ml.

The ELTc and the ELDc cells were harvested after trypsinization with 0·25% trypsin (Difco, Detroit, Michigan, U.S.A.) solution. After centrifugation at 500 **g** for 10 min, the cells were washed once in Tyrode's solution and finally resuspended in the same solution at a concentration of 5×10^6 cells per 0·1 ml.

Antisera were produced by five subcutaneous inoculations of 5×10^6 cells into 2–3 months old C3H and ST/a mice. The interval period between the two first inoculations was two weeks, while the remaining three inoculations were given at weekly intervals. The animals were bled from the retro-orbital sinus with a glass capillary pipet 5 days after the last inoculation. Antisera, which were not used immediately, were stored at −20° C.

Absorption of antisera was carried out by mixing 0·25 ml of serum at various dilutions with 2×10^8 packed cells. After thorough mixing, the sera were incubated with the cells for 1 hr at 37°C and then left overnight at 4°C. After absorption, the cells were removed by centrifugation for 20 min at 1000–1500 **g**. The absorbed sera were tested against the cell line used for absorption and discarded if they gave a trypan blue positive test. Sera, which in this way were found to be completely absorbed, were tested against all the remaining cell lines — as will be described later. In one series of experiments, a second absorption cycle was run. The absence of any additional effect showed that absorption had been complete.

The cytotoxic effect of the antisera was tested according to the trypan blue method described by Gorer and O'Gorman [5] with minor modifications. The cells were harvested and washed as described above and resuspended in Tyrode's solution at a concentration of 1–2 millions per ml. Fifty µl of the cell suspension were mixed with equal volumes of antisera at various dilutions and fresh guinea pig serum which served as a complement. After incubation for 1 hr at 37°C 100 µl of a 0·25 per cent trypan blue solution were added and the percentage of stained and unstained cells was finally determined by counting 200 cells at 200 × magnification.

Table 1. *Cytotoxic effect of antisera produced by ST/a mice after the inoculation of Ehrlich's ascites tumor cells propagated* in vivo *and* in vitro

Antisera Cell line used for immunization	Dilution	Cytotoxic effect Percentage of trypan blue stained target cells			
		ELTa	ELTc	ELDa	ELDc
ELTa	1:1	<10	<10	>90	>90
	1:4	<10	<10	>90	>90
	1:16	<10	<10	>90	>90
	1:64	<10	<10	>90	25
	1:128	<10	<10	45	<10
	1:256	<10	<10	<10	<10
ELTc	1:1	<10	<10	>90	>90
	1:4	<10	<10	>90	>90
	1:16	<10	<10	>90	>90
	1:64	<10	<10	>90	30
	1:128	<10	<10	>90	<10
	1:256	<10	<10	<10	<10
ELDa	1:1	<10	<10	>90	>90
	1:4	<10	<10	>90	>90
	1:16	<10	<10	>90	64
	1:64	<10	<10	>90	<10
	1:128	<10	<10	75	<10
	1:256	<10	<10	<10	<10
ELDc	1:1	<10	<10	>90	>90
	1:4	<10	<10	>90	>90
	1:16	<10	<10	>90	>90
	1:64	<10	<10	36	81
	1:128	<10	<10	<10	<10
	1:256	<10	<10	<10	<10
ST/a normal serum	1:1	<10	<10	<10	<10
	1:4	<10	<10	<10	<10

RESULTS

Table 1 shows the cytotoxic effect of antisera produced in ST/a mice with the four cell lines under investigation. As is seen, neither the ELTa nor the ELTc cells showed a trypan blue positive test in any of the four antisera, not even in the anti-ELTa and anti-ELTc sera. On the other hand with the ELDa and the ELDc cells, a trypan blue positive test was obtained in all four antisera. The ELDa cells were more sensitive to the anti-ELDa, anti-ELTa, and anti-ELTc sera than the ELDc cells, but not to the anti-ELDc serum which was found to be just as toxic or, perhaps, even a little more toxic to the ELDc cells than to the ELDa cells.

Table 2 shows the results of similar experiments carried out with sera produced in C3H mice. As is seen, all four cell lines gave a positive trypan blue test in all four antisera. The ELDa cells were more sensitive than the ELTa cells—even when exposed to anti-ELTa and anti-ELTc sera. It is also seen from the table that all four antisera were cytotoxic at higher titers to the *in vivo* propagated cells than to the *in vitro* propagated cells with the only exception of the anti-ELTc serum, which proved to be equally toxic to ELTc and the ELTa cells.

From these experiments, it seems clear that the ELTa, ELTc, ELDa and ELDc cells share more than one antigen. In order to clarify whether these cells also contain individual antigens, absorption experiments were carried out as described. Table 3 shows the cytotoxic effect of C3H antisera after absorption. As it is seen, all four cell lines were able to absorb all the anti-ELTa and anti-ELTc activity from these sera. However, complete absorption of the anti-ELDa and the anti-ELDc activity was only obtained with the corresponding cell lines, i.e. ELDa and ELDc.

Table 2. Cytotoxic effect of antisera produced by C3H mice after the inoculation of Ehrlich's ascites tumor cells propagated in vivo *and* in vitro

Antisera: Cell line used for immunization	Dilution	Cytotoxic effect: Percentage of trypan blue stained target cells			
		ELTa	ELTc	ELDa	ELDc
ELTa	1:1	>90	>90	>90	>90
	1:4	>90	>90	>90	>90
	1:16	>90	65	>90	31
	1:64	70	<10	>90	<10
	1:128	<10	<10	80	<10
	1:256	<10	<10	<10	<10
ELTc	1:1	>90	>90	>90	>90
	1:4	>90	>90	>90	>90
	1:16	>90	>90	>90	26
	1:64	71	56	>90	<10
	1:128	<10	<10	63	<10
	1:256	<10	<10	<10	<10
ELDa	1:1	>90	>90	>90	>90
	1:4	>90	>90	>90	>90
	1:16	>90	25	>90	>90
	1:64	35	<10	>90	35
	1:128	<10	<10	54	<10
	1:256	<10	<10	<10	<10
ELDc	1:1	>90	>90	>90	>90
	1:4	>90	31	>90	>90
	1:16	47	<10	>90	>90
	1:64	<10	<10	>90	63
	1:128	<10	<10	35	<10
	1:256	<10	<10	<10	<10
C3H normal serum	1:1	<10	<10	<10	<10
	1:4	<10	<10	<10	<10

Table 3. Absorption experiment — the cytotoxic effect of antisera produced in C3H mice by the inoculation of Ehrlich's ascites tumor cells propagated in vivo *and* in vitro *and subsequently absorbed by the same cells*

Antisera: Cell line used for immunization (Dilution)	Cell line used for absorption	Residual activity*			
		ELTa	ELTc	ELDa	ELDc
ELTa	ELTa	0	0	0	0
(1:4)	ELTc	0	0	0	0
	ELDa	0	0	0	0
	ELDc	0	0	0	0
ELTc	ELTa	0	0	0	0
(1:4)	ELTc	0	0	0	0
	ELDa	0	0	0	0
	ELDc	0	0	0	0
ELDa	ELTa	0	0	+	0
(1:4)	ELTc	0	0	+	0
	ELDa	0	0	0	0
	ELDc	0	0	+	0
ELDc	ELTa	0	0	0	+
(1:4)†	ELTc	0	0	0	+
	ELDa	0	0	0	+
	ELDc	0	0	0	0

*+ indicates more than 90% trypan blue positive cells and 0 less than 10 per cent.

†In the test against the ELTc cells undiluted serum was used.

Provided that the absorption was exhaustive, these observations indicate that the ELDa and the ELDc cells contain individual antigens which they do not share with any of the other three cell lines. Considering the large number of cells used for the absorption of small volumes of antisera and the long duration of the absorption period, there was little reason to suspect the absorption not to be complete. However, in order to obtain direct evidence for the adequacy of the absorption technique used, the anti-ELDa and the anti-ELDc sera were submitted to a second absorption cycle carried out under exactly the same conditions as the first. Table 4 presents the results of one of these experiments which all showed the same. As is seen, double absorption did not change the results described above.

DISCUSSION

Previous immunization and cross-immunization experiments carried out in this laboratory [1] indicated that the ELTa, ELDa, and ELDc cells, but not the ELTc cells, shared at least one antigen which could be demonstrated by transplantation into ST/a mice. In the same system, the ELTa and the ELTc cells were found to contain at least one common antigen. Furthermore, by transplantation into pre-immunized C3H mice, all four cell lines were found to contain at least one common antigen.

The present experiments have shown that an antiserum, which is active against the ELDa and the ELDc cells, but not against the ELTa and the ELTc cells, can be produced in ST/a mice by the inoculation of all four cell lines. This observation indicates that the four cell lines share a common substance which is antigenic to ST/a mice, but which is not present in ELTa and in ELTc cells in sufficient concentrations to give a positive trypan blue test.

The experiments carried out with the C3H antisera showed that all four cell lines shared at least one substance which is antigenic to the C3H mice. The concentration of this antigen seemed to be higher in the ELD cells than in the ELT cells; but, in contrast to the antigen revealed by ST/a antisera, it was sufficiently high even in the ELTa and the ELTc cells to give a positive trypan blue test.

Table 4. The effect of repeated absorption with ELDa cells on the cytotoxic activity of C3H anti-ELDc sera

Antisera	Percentage of trypan blue stained target cells			
	ELTa	ELTc	ELDa	ELDc
Unabsorbed	>90	>90	>90	>90
Absorbed once	<10	<10	<10	>90
Absorbed twice	<10	<10	<10	>90

The results obtained both with the ST/a anti-ELDa and the C3H anti-ELDa sera indicated that the concentration of surface antigens in the ELD cells decreased after cultivation *in vitro*. A similar decrease after cultivation *in vitro* was seen in experiments with C3H anti-ELTa.

The decreased concentration of surface antigens during cultivation *in vitro* may be due to the increase in cell size previously reported [1]. However, the sensitivity of the ELTc and the ELDc cells to their own antisera produced in C3H and ST/a mice, respectively, was greater than their sensitivity to the corresponding antisera produced by ELTa and ELDa cells, and this indicates that other changes might have occurred too. This possibility was investigated in the absorption experiments which showed that both the ELDa and the ELDc cells contained at least one antigen which was not present in the three other cell lines. However, in the ELTa and the ELTc cells, only common antigens shared by all four cell lines were revealed by these experiments. This finding is in agreement with observations made by Hauschka *et al.* [6, 7] and Kaziwara [8] which indicated that the ELT cells are less antigenic than the ELD cells.

The absence in the ELDc cells of an antigen specific for the ELDa cells may be considered further evidence in favor of the assumption that cultivation *in vitro* leads to antigenic deletion. However, the significance of the demonstration of an antigen in the ELDc cells, which is not found in the ELDa cells, is less clear. It may indicate that a transformation of the ELD cells had taken place during cultivation *in vitro*, and this may account for the loss of transplantability previously described [1]. The contamination with bovine protein from the culture medium is a theoretical possibility which, in all probability, can be ruled out as being of any significance since a similar change would then have been expected in the ELTc cells.

Further studies of the antigenic properties acquired by the ELD cells during cultivation *in vitro* are needed before their influence on the transplantability of these cells can be assessed. However, the results obtained with the ELT cells in the present experiments show that the changes of the transplantability of tumor cells during cultivation *in vitro* cannot be explained by changes in their reaction with humoral antibodies alone.

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RESUME

On a étudié la structure antigénique de deux souches de tumeur ascitique d'Ehrlich, propagées in vivo *(ELTa et ELDa) et* in vitro *(ELTc et ELDc), à l'aide d'antiséra produits dans des souris C3H et ST/a. Les expériences ont montré que les quatre lignées cellulaires contenaient un facteur commun qui est antigénique dans les souris ST/a, mais qui n'est pas présent dans les cellules ELTa et ELTc en concentrations suffisantes pour donner une réaction de bleu-trypan positive. En outre, on a constaté que les quatre lignées cellulaires contiennent au moins une substance commune qui est antigénique dans les souris C3H, et qui se trouve en concentrations suffisantes dans les quatre lignées cellulaires pour donner une réaction de bleu-trypan positive.*

Les expériences d'absorption ont montré la présence d'au moins un antigène dans les cellules ELDa, qui ne se trouvait pas dans les trois autres lignes cellulaires, et des observations semblables ont été faites pour les cellules ELDc.

Les résultats des expériences mentionnées ci-dessus portent à croire que, au cours de la

propagation in vitro *des deux souches cellulaires, il s'est produit une diminution de la concentration d'antigènes de surface.*

On discute la diminution, ou la perte, de transplantabilité des cellules ELTc et ELDc, rapportées antérieurement [1], *et on arrive à la conclusion que les altérations antigéniques démontrées dans le présent travail ne peuvent pas, à elles seules, expliquer les modifications de transplantabilité.*

SUMMARY

The antigenic structure of two strains of Ehrlich's ascites tumor propagated in vivo (*ELTa and ELDa*) *and* in vitro (*ELTc and ELDc*) *was studied with antisera produced in C3H and ST/a mice. The experiments showed that all four cell lines share at least one common substance which is antigenic to ST/a mice, but which is not present in ELTa and ELTc cells in sufficient concentrations to give a positive trypan blue test. Furthermore it was found that all four cell lines contain at least one common substance which is antigenic to C3H mice, and which is present in sufficient concentrations in all four cell lines to give a positive trypan blue test.*

Absorption experiments suggested the presence of at least one antigen in ELDa cells which was not found in the other three cell lines, and similar observations were made with the ELDc cells.

Finally a decreased sensitivity to the action of cytotoxic antibodies has been found after in vitro *cultivation of both ELD and ELT cells. This effect might possibly be explained by a decrease of the concentration of surface antigens involved in the cytotoxic reaction and argues against the view that the concomitant loss of transplantability of the cultured cells is due to factors linked to the host immune reactivity of the non-cellular type.*

ZUSAMMENFASSUNG

Die Antigenstruktur von 2 in vivo (*ELTa und ELDa*) *und* in vitro (*ELTc und ELDc*) *propagierten Stämmen des Ehrlich Ascites Tumors wurde mit Antisera von C3H und ST/a Mäusen studiert. Die Versuche haben gezeigt, dass es in allen* 4 *Stämmen wenigstens eine gemeinsame Substanz gibt, die sich in ST/a Mäusen antigenisch zeigte. Die Konzentration dieser Substanz in den ELTa und in den ELTc Zellen ist aber zu niedrig um eine positive Trypanblau Reaktion zu geben. Es wurde auch gefunden, dass sich in allen* 4 *Zellstämmen eine gemeinsame Substanz befindet, die sich in den C3H Mäusen antigenisch zeigte. Die Konzentration dieser Substanz war hoch genug in allen* 4 *Zellstämmen um eine positive Trypanblau Reaktion zu geben.*

Absorptionsversuche zeigten die Gegenwart von wenigstens einem Antigen in den ELDa Zellen, das nicht in den anderen Zellstämmen gefunden werden konnte. ähnliche Beobachtungen wurden mit den ELTc Zellen gemacht.

Die Resultate deuteten darauf hin, dass die Züchtung sowohl von ELT als von ELD Zellen in vitro *eine Verminderung der Konzentration von Oberflächenantigenen herbeiführt.*

Die Verminderung oder der Verlust der Transplantabilität der ELT und ELD Zellen während der Züchtung in vitro, *die schon früher mitgeteilt wurden, werden diskutiert. Die Autoren kommen zum Schluss, dass die Antigenenveränderungen, die in dieser Arbeit aufgezeigt wurden, nicht als genügende Erklärung für die Änderung in der Transplantabilität betrachtet werden können.*

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Further Biochemical Studies on a Dialysable Polypeptide Obtained from Tumor Fluids

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INTRODUCTION

IN PREVIOUS publications, the biological effects of a dialysable polypeptide mainly derived from human tumor effusions have been described. During short-time incubations in the presence of dialysate from tumor ascites fluids strain L cells were found to detach from the glass surface [1]. Purified polypeptide was observed to cause a cell growth inhibiting effect on four exponentially growing cell lines, an inhibition which was proportional to the amount of added polypeptide [2]. The cell growth inhibiting effect was found to be due to a decrease in the mitotic index as judged by the depressive effect of added polypeptide on the mitotic frequency in partially synchronized cell populations. The polypeptide was previously isolated by means of a method involving gel filtration and column chromatography [3]. This method was assumed to give a biochemically homogeneous material which contained the amino acid residues alanine, arginine, cysteine, glutamic acid, glycine, leucine, tyrosine, and valine. The same amino acids were found in all polypeptide preparations tested irrespective of their origin from mouse, rat or human sources. The present article deals with the electrophoretic behaviour and the estimation of the apparent molecular size of the polypeptide as well as with the isolation and the composition of its chymotryptic and tryptic digestion products. The polypeptide was for the present experiments purified by gel filtration and column chromatography of a mercury–polypeptide complex, crystallized from alcoholic extracts of lyophilized dialysates of tumor ascites fluids. The present results indicate a biochemically well defined material.

MATERIALS AND METHODS

Crystallization

Freshly collected ascites fluid from tumor patients was centrifuged as soon as possible for removal of cells and cell debris. The fluid was then dialysed against 5 times the volume of ion-free water. The diffusate was lyophilized to complete dryness. The resulting white to white-yellow powder contained ninhydrin-positive impurities absorbing light in the lower ultraviolet region as well as inorganic salts.

The freeze-dried diffusate was extracted in the proportion of 25–30% (w/v) with 80% ethanol for 2 hr at room temperature. The brownish extract was centrifuged and cooled to refrigerator temperature. To the clear supernatant $HgCl_2$ was added to a final concentration of 1×10^{-3} M. The mixture was stored for a period of about 24 hr and then centrifuged to remove any possible precipitate. The clear supernatant was inoculated with microcrystals of the mercury–polypeptide complex from previous preparations and was stored at refrigerator temperature. Within some weeks the complex appeared as colourless crystals (Fig. 1).

Further purification

Crystals of the complex were collected, washed twice with small amounts of cold absolute ethanol and once with cold ethyl ether and redissolved in 0·5–1·0 ml 1×10^{-3} M HCl. The complex was completely soluble in hydrochloric acid, giving an u.v. spectrum with an absorption maximum around 280 mμ, which moved towards 290 mμ in 0·1 M NaOH, and with a relatively low light scattering at wavelengths above 310 mμ. The hydrochloric acid solution was applied to a thiolated [4] Sephadex G 25 fine (Pharmacia, Upsala, Sweden) column of size 1·0 × 15 cm and eluted at room temperature with 0·05 M acetic acid, pH 3·0 containing 0·5 M KCl. The thiolated polysaccharide particles have been demonstrated to separate radiomercury completely from serum albumin [4]. In order to test the capacity of the thiolated Sephadex G 25 for binding mercury ions, the mercury–polypeptide complex dissolved in HCl was mixed with 10 μc [^{203}Hg] $HgCl_2$ (Amersham Radiochemical Centre, England) and applied to the column after some hours standing at room temperature. No radioactivity was found in the eluted fraction containing the u.v. absorbing material at the wavelength 280 mμ; all the radioactivity was retained in the upper part of the column.

The mercury-free polypeptide fraction was freeze-dried and applied on a DEAE–Sephadex A 25 column for further purification. Stepwise elution was performed with phosphate buffers as described earlier [3].

Electrophoresis

The migration of the polypeptide during electrophoresis was studied in an LKB-apparatus model 3276 B. The samples were applied on 300μ thick layers of MN-cellulose 300 (Macherey, Nagel & Co., Germany) spread on glass plates of size 11 × 16 cm. The plates were connected to the electrode vessels by means of 12 cm Whatman No. 1 paper strips. The following buffers were used: 0·05 M citrate at pH 3·8, 0·05 M phosphate at pH 7·0, and 0·05 M veronal at pH 8·4. Electrophoresis was performed during 2 hours, after which time the plates were dried and sprayed with 0·25% ninhydrin in acetone containing 4% acetic acid.

Estimation of the apparent molecular size

Estimation of the molecular size of the polypeptide was performed by gel filtration on Sephadex G 25 (Pharmacia, Upsala, Sweden) using 0·05 M acetic acid containing 0·5 M KCl and acidified to pH 2·5 by means of HCl. As model substances bovine serum albumin (Kabi Ltd., Stockholm, Sweden), RNase (crystallized, Worthington Biochem. Corp., USA), A- and B-chains of insulin (Novo, Copenhagen), bacitracin (Astra, Södertälje, Sweden), and oxytocin (Ciba, Basle) were used. The substances were eluted separately on the same column and the sampled 300 μl fractions were analysed by u.v.-absorption at 280 mμ or at 570 mμ after ninhydrin coupling to 100–150 μl hydrolysed samples using a Beckman DU spectrophotometer.

According to Andrews [5], the logarithms of the molecular weights of gel filtrated substances are, within certain limits, proportional to the elution volumes (V). This has been assumed also to be the case for Sephadex G 25 and for the actual range of molecular weights [6], although there have been difficulties in obtaining proteins and polypeptides with elution characteristics good enough for a detailed calibration of the column bed used.

In a separate gel filtration experiment, the polypeptide was also compared with the insulin chains, glucagon (Lillie & Co., USA) and with oxytocin on Sephadex G 50 using the same eluant. The molecular weights of the model substances were found in Table workings [7–9].

Peptide fragments from chymotryptic and tryptic digestion

Enzyme digestion was performed on performic acid oxidized polypeptide in ion-free water containing added Ca^{++}-ions controlled at pH 9·0 at 37°C overnight with α-chymotrypsin (3x-crystallized, Worthington Biochem. Corp., U.S.A.) and with trypsin (2x-crystallized, Worthington Biochem. Corp., U.S.A.). Separation of the chymotryptic and tryptic peptides was achieved by gel filtration on Sephadex G 14 columns of size 1·5 × 21 cm equilibrated with 0·05 M acetic acid at pH 2·5 containing 0·5 M KCl. The subpeptides were identified by ninhydrin determinations on 100–150 μl hydrolyzed samples of each fraction. The collected fractions were 300 or 500 μl in volume. The presence of tyrosine in the fractions was demonstrated by the u.v. spectrum in acid and alkaline medium [10]. For qualitative amino acid analyses subpeptides from several preparations were pooled and submitted to further purification on Sephadex G15.

In a separate experiment, the proportion of liberated free carboxyl groups during digestion of the oxidized polypeptide with chymotrypsin and trypsin was determined by means of a Radiometer SBR 2 Titrigraph-unit connected

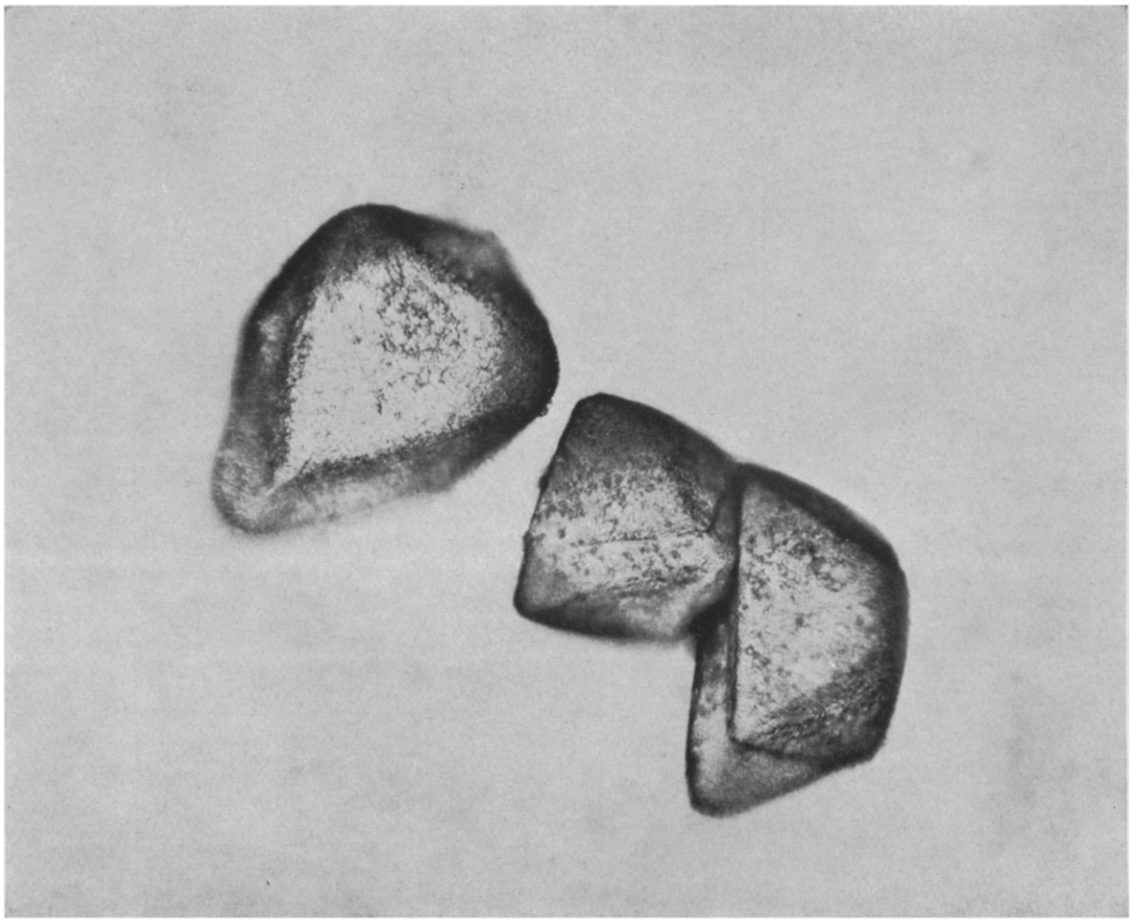


Fig. 1. *Crystals of the mercury–polypeptide complex. Linear magnification* × 300.

(to face p. 264)

to an automatic TTT 11 Titrator, pH-statted at 8·5 using 0·1 M NaOH as titrant. The reaction mixture contained polypeptide dissolved in 0·2% NaCl to which the enzymes, dissolved in water containing Ca^{++}-ions, were added separately until a final concentration of 50 µg enzyme per ml reaction mixture. The proportion of consumed volumes of alkali was estimated from the titrigraph records for each enzyme. The amounts of polypeptide available for this experiment were not sufficient for reliable ninhydrin determinations which could confirm the titration results.

Amino acid analysis

The qualitative amino acid composition was determined on samples hydrolyzed for 16 hours with 6 M HCl at 105° C and/or on samples digested overnight with carboxypeptidase A-DFP (3x-crystallized, Worthington Biochem. Corp., USA). The amino acids were separated on 300µ thin layers of MN-cellulose by combined electrophoresis and ascending chromatography. Electrophoresis was performed in 0·7 % formic acid by applying a current of 2 mA and 220 V for 7 min [11]. As eluant during chromatography *n*-butanol: acetic acid: water (250: 60: 250) was used. The amino acid spots were detected by spraying with ninhydrin-collidine [12]. The presence of tyrosine was confirmed by using α-nitroso-β-naphtol-spray and the presence of arginine was ascertained by Sakaguchi's test [13]. Chromatographically pure amino acids were processed in electrophoresis and chromatography as described above as references. The N-terminal of the polypeptide was determined by coupling to phenylisothiocyanate [14] and subsequent one-dimensional ascending thin-layer chromatography (TCL) using 350 µ silica HF_{254}-layers (Merck, Germany) with chloroform: methanol (9:1) [15] as eluant. The N-terminal thiohydantion derivate was identified under a u.v. mineral lamp. Synthesized PTH-derivates of pure amino acids were run parallel to samples for comparison. The C-terminal of the polypeptide was identified after one-dimensional ascending TLC with *n*-butanol: acetic acid: water as eluant on the material digested with carboxypeptidase. The C-terminal analysis was confirmed by hydrazinolysis [16] and subsequent one-dimensional TLC as mentioned above for the free amino acids.

RESULTS

The qualitative composition of the polypeptide

The polypeptide prepared by mercury chloride crystallization from alcoholic extract of lyophilized dialysates of tumor ascites fluids, and the subsequent passage through thiolated Sephadex G 25 particles could be eluted from a DEAE–Sephadex column by 0·05 M phosphate buffer at pH 7·0 (Fig. 2). This fraction contained the amino acids alanine, arginine, cysteine, glutamic acid, glycine, leucine, tyrosine, and valine. Determination of the N-terminal by coupling the polypeptide to phenylisothiocyanate, cleavage and separation by chromatography on silica layer revealed the presence of the phenylthiohydantoin of glycine. The first appearing free amino acid after carboxypeptidase treatment was leucine. By hydrazinolysis of the polypeptide the only non-converted amino acid was found to be leucine, thus confirming the result of the carboxypeptidase method for C-terminal analysis.

The growth inhibiting activity of the purified polypeptide as well as its depressive effect on the mitotic index was ascertained on four different cell types [2].

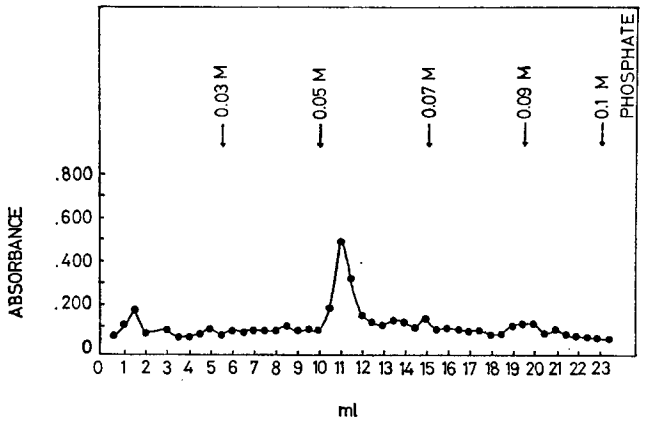


Fig. 2. Ion-chromatography of mercury-free polypeptide on DEAE–Sephadex A 25 medium. Bed size: 0·5 × 3cm. Stepwise elution with ion-free water and phosphate buffers at pH 7·0. Absorption measured at 280 mµ.

Table 1. *Migration of polypeptide and bovine serum albumin during* 2 *hours' thin layer electrophoresis*

Migration of polypeptide	Migration of serum albumin	pH	Buffer	mA	V
2·0 cm towards the cathode	2·7 cm towards the cathode	3·8	0·05 M citrate	10	200
2·3 cm towards the cathode	1·8 cm towards the anode	7·0	0·05 M phosphate	6	320
2·5 cm towards the cathode	2·6 cm towards the anode	8·4	0·05 M veronal	10	240

Electrophoresis

During electrophoresis, the polypeptide behaved as a positively charged substance at the three pH's investigated (Table 1). Sufficient amounts of the polypeptide for a more detailed electrophoretic analysis were not available.

Estimation of the apparent molecular size

From Fig. 3 the V of the purified polypeptide can be compared to the V's of model substances. The dead volume (V_0) was determined with serum albumin, which substance is completely excluded from Sephadex G 26 particles. Figure 4 plots the logs of the mol. wt against the V/V_0 [17]. From the line drawn, the V/V_0 for the polypeptide intersects with the line corresponding to a mol. wt. of 1900. In a separate experiment, where a Sephadex G 50 was used for an estimation of the molecular size using RNase, insulin B, glucagon and oxytocin as model substances, the mol. wt. of the polypeptide was found to be 2000. This value is in agreement with that obtained on Sephadex G 25.

Titrigraphic measurements

The titration curve of the free carboxyl groups of the performic acid oxidized polypeptide during digestion by chymotrypsin and trypsin at a constant pH 8·5 maintained by automatic titration with 0·1 M NaOH can be seen in Fig. 5. The consumption of alkali after trypsin addition was 2·1 times greater than the alkali consumption during the chymotrypsin splitting of the molecule, a result compatible with an arginine/tyrosine ratio of 2: 1.

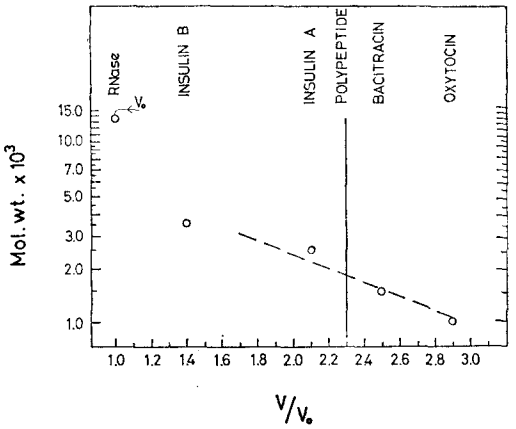


Fig. 4. *The logarithms of molecular weights of known substances plotted as a function of the* V/V_0*-ratio compared to the* V/V_0*-ratio of the polypeptide. Values taken from the inflexion points of the curves in Fig.* 3.

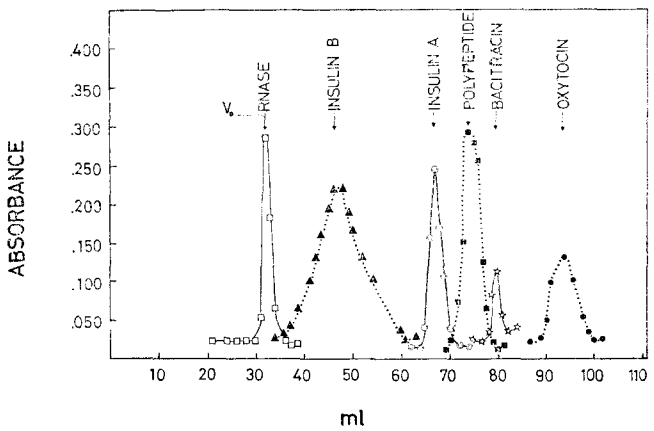


Fig. 3. *Gel filtration of mercury-free polypeptide and model substances with known mol. wt. on Sephadex G* 25. *Bed size:* 1·5 × 52 *cm.* 0·05 M *acetic acid containing* 0·5 M *KCl and acidified to pH* 2·5 *was used as eluant. Absorption measured at* 280 mμ (*RNase, A- and B- chains of insulin*) *or at* 570 mμ *after acid hydrolysis and coupling to ninhydrin reagent* (*bacitracin, polypeptide, oxytocin*).

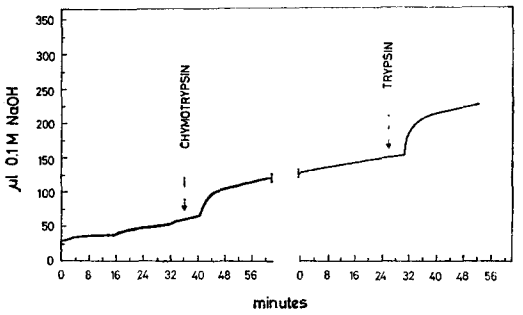


Fig. 5. The consumption of 0·1 M NaOH at pH 8·5 after addition of chymotrypsin and trypsin to purified polypeptide dissolved in 0·2 % NaCl. Reaction mixture thermostatted at 37°C. The proportion of liberated free carboxyl groups corresponds to a ratio of 1: 2·1.

Chymotryptic and tryptic peptides

Digestion of performic acid oxidized polypeptide by chymotrypsin and subsequent separation by gel filtration of the digest on Sephadex G 15 revealed the presence of two separable sub-peptides (Fig. 6). The qualitative amino acid composition of the chymotryptic peptide A_{ct} was found to be: alanine, arginine, cysteine, glutamic acid, glycine, leucine, tyrosine, and valine. The chymotryptic peptide B_{ct} contained alanine, arginine, cysteine, glycine, and leucine.

Separation of the tryptic sub-peptides on Sephadex G 15 revealed the presence of three fractions (Fig 7). The amino acid composition of the three subunits was as follows: A_t: alanine, arginine, cysteine, glycine, leucine, and valine; B_t: alanine, arginine, cysteine, glutamic acid, glycine, leucine, tyrosine, and valine; and C_t: glycine and leucine. The small amounts of material used did not permit N- or C-terminal analysis, nor was it possible to test the biological activity of any of the subfractions.

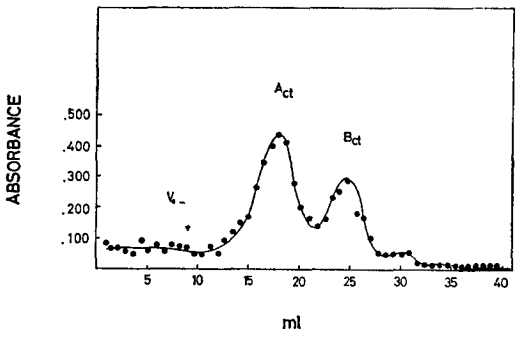


Fig. 6. The separation of sub-peptides from a chymotryptic digest of mercury-free oxidized polypeptide on Sephadex G 15. Bed size: 1·5×21 cm. 0·05 M acetic acid containing 0·5 M KCl, acidified to pH 2·5, was used as eluant. Absorption measured at 570 mµ after acid hydrolysis and coupling to ninhydrin reagent.

Formation of aggregrate

During some gel filtration procedures on thiolated Sephadex G 25, the polypeptide appeared in two fractions, one occurring at $V/V_0=1·1$, one at the V/V_0-ratio corresponding to that expected for a mol. wt. of 1900–2000 ($V/V_0=2·3$). This behaviour was sometimes also found on ordinary Sephadex G 25 with polypeptide solutions previously made free from mercury. When the two fractions were isolated separately, concentrated by freeze-drying and independently applied on two identical Sephadex G 25 columns and eluted with acetic acid, it could be shown that still two fractions occurred. Thin layer electrophoresis of the two fractions at pH 7·0 indicated that they possessed the same electrophoretic mobility. It was not possible to identify subunits of either fraction by electrophoresis. Analyses of the qualitative amino acid composition revealed no difference between the two forms. The biological activity was also found to be equal.

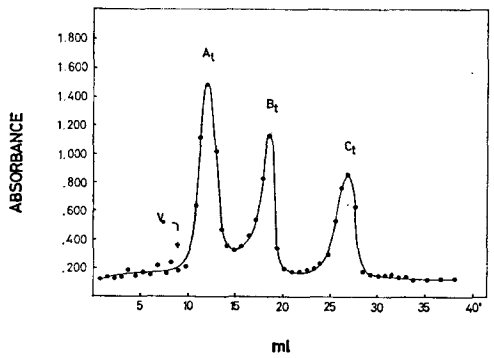


Fig. 7. The separation of sub-peptides from a tryptic digest of mercury-free oxidized polypeptide on Sephadex G 15. Bed size: 1·5×21 cm. 0·05 M acetic acid containing 0·5 M KCl acidified to pH 2·5 was used as eluant. Absorption measured at 570 mµ after acid hydrolysis and coupling to ninhydrin reagent.

DISCUSSION

The preparation method involving crystallization of the polypeptide as a mercury complex seems to be an improvement compared with that published earlier [3]. Firstly, the extraction of the dry substance obtained by freeze-drying the dialysate and the formation of crystals from the alcoholic extract can be regarded as a purification step in itself, and, secondly, the longer storing time with little or no risk for inactivation by oxidation is an advantage.

The behaviour of the polypeptide during electrophoresis and during the gelfiltration and column chromatography procedures indicated that no peptide impurities were present. This

conclusion is further supported by the reproducible results of the qualitative amino acid analyses, including the N- and C-terminal determinations as well as by the results of analyses and separation of the chymotryptic and tryptic peptides. It should also be added that mercury-polypeptide crystals were obtained from a dialysate of ELD mouse ascites fluid; a polypeptide-complex, which upon further purification revealed the same qualitative amino acid composition (see also reference 3), including the N-terminal, as did the polypeptide of human origin. One may thus possibly claim the general existence of biochemically well-defined polypeptides in all the tumor ascites fluids so far sampled which have structural identity at least in the biologically active part of the molecule.

The proportion of free carboxyl groups released by chymotrypsin and trypsin indicates the presence of twice as many arginine residues as there are tyrosine residues. The separation of two peptides after chymotrypsin digestion supports the idea that only one tyrosine residue exists in the polypeptide molecule. The interpretation of the tiration curve would thus lead to the conclusion that the molecule contains two arginine residues, a conclusion which is supported by the isolation of three peptides by gel filtration of the tryptic digest. The charge of the molecule, as shown by the electrophoresis experiments, is positive at at neutral and acid pH indicating that the arginine residues predominate over the glutamic acid ones. Thus, one would expect the existence of only one glutamic acid residue in the polypeptide molecule. This supposition has been supported by the detection of glutamic acid only in the tryptic peptide B_t.

The qualitative amino acid analysis of the tryptic peptides reveals the presence of 6 different amino acids in the tryptic peptide A_t which appears first in the eluate from Sephadex G 15 columns. The second subpeptide B_t contains 8 different amino acid residues and the third, C_t, 2 different residues. As no quantitative determinations have been made the possibility cannot be excluded that A_t contains more than one residue of some of the observed amino acids which would then give A_t a molecular size corresponding to its early appearance in the eluates. The assumption that some amino acids in A_t are doubled would be in agreement with the molecular weight of 1900–2000 found for the entire polypeptide. On the other hand, the explanation for the appearance of the A_t-peptide in the eluates can well be its probably low solubility, a conclusion drawn from the high light scattering of the u.v. spectrum as observed in the A_t fraction.

Gel filtration of the polypeptide on G 25 may reveal the presence of two interconvertible fractions. As no differences in electrophoretic behaviour, in qualitative amino acid composition, nor in the biological activity, have been found, the two fractions are probably to be regarded as the monomeric form and its aggregate. This aggregration phenomenon warrants further study to determine the apparent molecular size of the aggregrate, the number of monomers it contains and the nature of the bonds involved. This information could help give an insight into the three-dimensional structure of the polypeptide molecule and its mode of action *in vivo*.

Acknowledgements—The author wishes to express his gratitude to the Professors B. Sylvén, O. Lindberg, and O. Snellman for their great interest and advice during this work. He is also indebted to L. Nathorst-Westfelt, Astra Ltd., for valuable help with the freeze-drying procedure and to Dr. J. A. Forrester for revising the English text. The gift of the A- and B-chains of insulin from Dr. K. Brunfeldt (Nordisk Insulinlaboratorium, Gentofte, Denmark) is gratefully acknowledged. This work was supported by grants from the Swedish Cancer Society and the Stockholm Cancer Society.

RESUME

L'auteur décrit une méthode de purification, à partir de liquide d'ascite néoplasique humain, d'un polypeptide dialysable, capable d'inhiber la croissance cellulaire. La méthode comporte la cristallisation d'un complexe mercurique. Le polypeptide purifié se comporte, au cours de la filtration sur gel, comme une substance ayant un poids moléculaire de 1900 *à* 2000. *Il est constitué de* 8 *acides aminés différents, avec la glycine comme N terminal, et la leucine comme C terminal. Il migre, à l'électrophorèse, comme une bande ninhydrine positive, chargée positivement, et se comporte comme un composé unique, lors de la chromatographie sur échangeur d'ions. La digestion par la chymotrypsine sépare* 2 *fractions, tandis que la digestion par la trypsine sépare* 3 *fractions . La composition en acides animés de ces sous-peptides a été déterminée. L'apparition de* 2 *fractions polypeptidiques, au cours de certaines méthodes de filtration sur gel, a été observée, et l'interprétation qui peut en être donnée est discutée.*

SUMMARY

A method for purifying from human tumor ascites fluid a dialysable polypeptide demonstrated to possess cell growth inhibiting effects is described. The process involves crystallization as a mercury complex. The purified polypeptide behaves during gel filtration as a substance with a mol. wt. of 1900–2000. *The polypeptide is built up of* 8 *different amino acids and has glycine as N-terminal and leucine as C-terminal. It migrates in electrophoresis as one positively charged ninhydrin-positive band and behaves as one component during ion-exchange chromatography. By separation of the sub-peptides obtained by chymotryptic digestion* 2 *fractions were found. Tryptic digestion produced* 3 *separable fractions. The qualitative amino acid composition of the sub-peptides is given. The occurrence of two polypeptide fractions during some gel filtration procedures and its possible interpretation is described.*

ZUSAMMENFASSUNG

Es wird eine Methode beschrieben, nach der aus menschlichem Tumorascites ein dialysierbares Polypeptid gereinigt wird, das zellwachstumshemmende Wirkung besitzt. Ein Quecksilber-Komplex wird kristallisiert. Das gereinigte Polypeptid verhält sich bei der Gelfiltration wie eine Substanz vom Molgewicht 1900–2000. *Es besteht aus* 8 *verschiedenen Aminosäuren und hat Glykokoll als N-terminale und Leucin als C-terminale Gruppe. Es wandert bei der Elektrophorese als eine positiv geladene Bande (ninhydrin-positiv) und verhält sich bei der Jonen-Austauschchromatographie wie eine einheitliche Verbindung. Durch chymotryptische Verdauung entstehen* 2 *trennbare Fraktionen; durch tryptische Verdauung dagegen entstehen* 3 *trennbare Fraktionen. Die qualitative Zusammensetzung der Teilpeptide wird angegeben. Das Auftreten zweier Polypeptid-fraktionen bei einigen Gelfiltrationsprozessen und eine mögliche Erklärung hierfür werden beschrieben.*

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The Effects on Cell Multiplication *in vitro* of a Dialysable Polypeptide Derived from Tumor Fluids

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INTRODUCTION

A DIALYSABLE polypeptide fraction derived from tumor fluids has been found to reduce the frequency of attachment of strain L cells to a glass substratum during short-term incubations in Carrel flasks [1]. The loss of adhesion was accompanied by a decrease in the proportion of cells bearing pseudopodia. The withdrawal of pseudopodia was taken as an indication of a rapid reaction between the polypeptide and the cell surface. Preliminary observations on the cell multiplication rate of strain L cells in suspension culture over a longer period of time showed that, in the presence of dialysate from Ehrlich ascites tumor fluid, there was an inhibition of cell multiplication and an increase in the occurrence of irreversibly damaged cells.

In the present communication, a growth inhibition of four different types of cells in exponential growth, in presence of purified polypeptide, is described. Inhibition of cell multiplication was also observed as a marked depression in mitotic frequencies in partially synchronized strain L populations treated with polypeptide. Experiments have also been done in an attempt to detect an interaction between the polypeptide and human red blood cells and Chang liver cells by means of free cell electrophoresis. The polypeptide material used for this investigation was prepared according to a method involving crystallization as a mercury–polypeptide complex, gel filtration on thiolated Sephadex and subsequent chromatography. This method was found to give a homogeneous and well-defined material as judged from biochemical studies [2].

MATERIALS AND METHODS

Stock cultures

Strain L (clone 929), Chang liver cells and HeLa (Gey) cells were grown in suspension in Eagle's glutamine-supplemented medium [3] continuously gassed with humidified air containing 5% CO_2. The medium for strain L and Chang cells was supplemented with 10% horse serum and the medium for HeLa cells with 10% calf serum. All sera were heated at 56°C during 3 hours to diminish the risk of PPLO-contamination; 100 μg/ml each of the antibiotics sodium benzylpenicillin and dihydrostreptomycin sulfate was added to the culture media.

MB 64E malignant lymphoid cells, derived from an MB III lymphoblast strain, were cultivated in Hank's solution containing 0·1% glucose, 0·5% lactalbumin hydrolysate (Sigma Chem. Co., U.S.A.) and 20% non-heated human serum. Antibiotics were added as mentioned above. The lymphoid cells grew spontaneously in suspension if the flasks were shaken regularly at least twice a day.

Stock cultures were regularly tested for PPLO-contamination by serological and by biochemical tests [4].

Asynchronously growing cells

Cells were harvested for growth studies from stock cultures at end of the logarithmic phase of growth, when aliquots of the cell suspension were diluted with fresh medium to a starting cell concentration between $0{\cdot}20$–$0{\cdot}30 \times 10^6$ cells/ml. Freshly prepared polypeptide material dissolved in a small volume of culture medium, was added at the time of medium dilution. The first sample for cell counts was taken 1/2 hr after addition of polypeptide (zero day sample) and then regularly at 24 hr interval during 3 subsequent days. Control cultures were treated identically without addition of polypeptide material.

Cell titres were based upon counts in a Buerker hemocytometer of duplicate samples from each cultured flask. The frequency of irreversibly injured cells was based on counts of at least 300 cells from each flask exposed to lissamine green [5].

The growth curves were obtained from the mean values from two parallel suspension cultures. The difference between such parallel cultures was not greater than the accuracy of counting, about 5%.

Partially synchronized cells

For studies on the effect of added polypeptide on the mitotic frequency, partially synchronized strain L cells were used. Strain L cells were harvested from suspension stock cultures during logarithmic phase of growth and were seeded out on Petri dishes (7 cm dia.) with a total of 1×10^6 cells/dish in 10 ml medium. The cells were incubated at 37°C for 24 hours in order to reach the logarithmic phase again. The medium was then replaced by a medium containing thymidine (TdR) (P-L Biochemicals Inc.) at a final concentration of 2 mM [6]. To half the number of dishes freshly prepared polypeptide was added as soon as possible after medium replacement. The dishes were then incubated for 24 hours at 37°C in humidified air containing 5% CO_2, after which time the cells were washed twice with thymidine-free medium and re-incubated. Prewarmed and pregassed media were used at every medium change. The zero time was set when half the number of dishes were reincubated. At hourly intervals the medium from one dish of the TdR-treated series and from one of the TdR+polypeptide-treated series was centrifuged. The cells growing on the dish were trypsinized, harvested and centrifuged together with those in the medium. After centrifugation the cells were fixed in ethanol: acetic acid (3:1) for at least 30 min and then smeared on microscope slides. The cell smears were dried at room temperature and stained with haematoxylin. The frequency of mitoses was based on the counts of 1000 cells.

Polypeptide

The polypeptide was purified according to a method involving crystallization as a mercury complex [2]. The complex was gel filtrated on a thiolated Sephadex column and further purified by DEAE–Sephadex chromatography. The resulting fraction was desalted and concentrated by lyophilization. Polypeptide concentrations were determined on non-hydrolyzed material after coupling with a commercial hydrindantin and measurements in a Beckman DU spectrophotometer. Doses are expressed as μM leucine equivalents [7]. The polypeptide solutions were mixed with small amounts of culture medium and sterilized by filtration through bacterial filters before addition to cell cultures. Inactivation was performed by irradiation of polypeptide solutions with u.v. light in plastic tubes at room temperature in presence of atmospheric oxygen for at least 18 hr.

Cell electrophoresis

Cell electrophoretic mobility determinations were made in a cylindrical cell apparatus as described by Bangham *et al.* [8] at 25°C. Determinations were made on human erythrocytes and on Chang liver cells with and without preincubation with polypeptide. Electrophoretic measurements were made in a medium $0{\cdot}144$ M NaCl and $0{\cdot}006$ M KCl adjusted to pH $7{\cdot}0$. The results are expressed in μ/sec/V/cm and are the mean values with standard errors based on measurements on twenty individual cells in alternate directions. The samples were coded.

Erythrocytes were thrice washed in Tyrode's solution and preincubated with polypeptide in physiological saline for periods of from 2 to 24 hr at +2° or +37°C. After incubation the cells were gently centrifuged, resuspended in the electrophoresis medium and allowed to equilibrate at 25°C before measurement. Control cell suspensions were treated identically but without the polypeptide present.

Chang cells were measured after cultivation in the presence of growth inhibiting amounts of polypeptide. The cells were harvested during

the logarithmic phase of growth (after 2 days) and during the stationary phase (after 4 days). As control cells, the parallel polypeptide-free culture was harvested after the same periods of culture. The cells were washed thrice in Tyrode's solution before suspension in the electrophoresis medium for measurement.

RESULTS

Population growth curves

After addition of various doses of polypeptide, cell multiplication was studied on four types of *in vitro* cultivated asynchronously growing cell lines. The polypeptide doses varied between 0 and 80 μM final concentration. Growth was followed by changes in the cell count. The effect of the polypeptide on the cell vitality was followed by the frequency of occurrence of cells permeable to lissamine green. Figure 1 (bottom diagram) shows the population growth

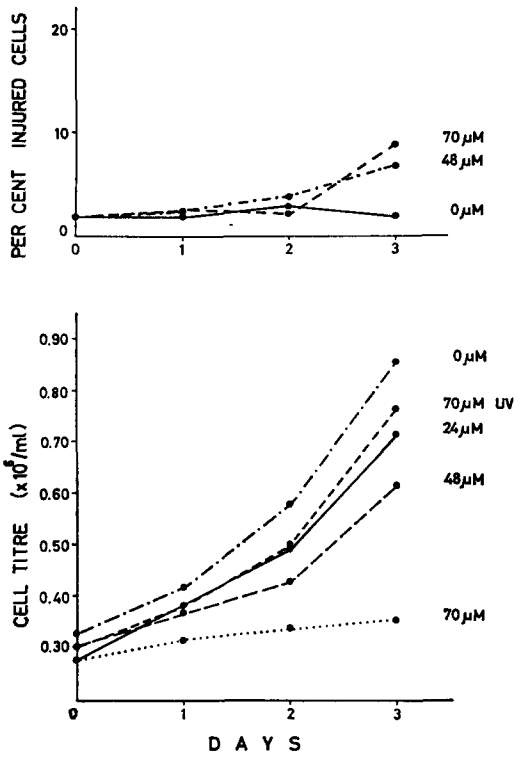


Fig. 1. Cultivation experiment with MB 64E cells in suspension in absence and in presence of polypeptide at various final concentrations expressed as leucine equivalents. Top diagram shows the changes in the frequency of injured cells with incubation time. Bottom diagram shows variations in cell count with time. Cell counts are mean values of two parallel cultures. The polypeptide was derived from the ascites fluid of a patient with advanced ovarian carcinoma.

curves of a representative experiment with MB 64E lymphoid cells in the presence of added polypeptide derived from the ascites fluid of a patient carrying an ovarian carcinoma. Each point represents the mean cell counts from two flasks, which did not differ more than about 5%. The population growth rate was dependent on the dose of polypeptide added, the lowest dose of polypeptide showing a significant retardation of the growth rate being 24 μM. The polypeptide could be inactivated by u.v. irradiation (see curve labelled 70 μM u.v., Fig. 1, bottom diagram). At the higher concentration of polypeptide, the frequency of injured cells increased (Fig. 1, top diagram) and if the period of culture was extended to more than 3 days without change of culture medium then the proportion progressively increased until most of the cells had degenerated.

Figure 2 illustrates the cell multiplication for all cell lines tested as the cell titre at 3rd day plotted as a function of the polypeptide concentration. The polypeptide was prepared from the ascites fluids of human patients. As can be seen, the polypeptide was active at the same dose levels irrespective of the cell line tested. During these series, the cell titres at the start of incubation were kept at $0{\cdot}20$–$0{\cdot}30 \times 10^6$ cells/ml. The zero counts in Fig. 2 are marked on the ordinate with an arrow.

Gross observations of cell populations given higher polypeptide doses revealed a blebbing of cell membranes and granulation of cells in those cultures where an increase in the frequency of injured cells was found. Such morphological changes were usually seen in cell populations undergoing a progressive cell injury leading to total degeneration of the cells [5].

Depression of mitoses

The frequency of mitoses in control asynchronous populations was generally low. At higher polypeptide doses, this frequency was depressed especially at the end of the experiments at which time the cell mortality was increased. In order to study a significant depressive effect on the mitotic frequency during conditions at which no measurable increase in the cell mortality could be expected the variations in the percentage of mitoses was followed in partially synchronized populations. Experiments performed in order to study the variations in the frequency of injured cells in polypeptide-treated strain L cell suspensions after the release of a TdR-blockage showed no measurable increase in the proportion of lissamine green-stained cells over the normal range of 2–5% within 24 hours.

Strain L cells growing in Petri dishes were subjected to TdR-treatment for 24 hours. Upon release of the TdR-blockage one mitotic

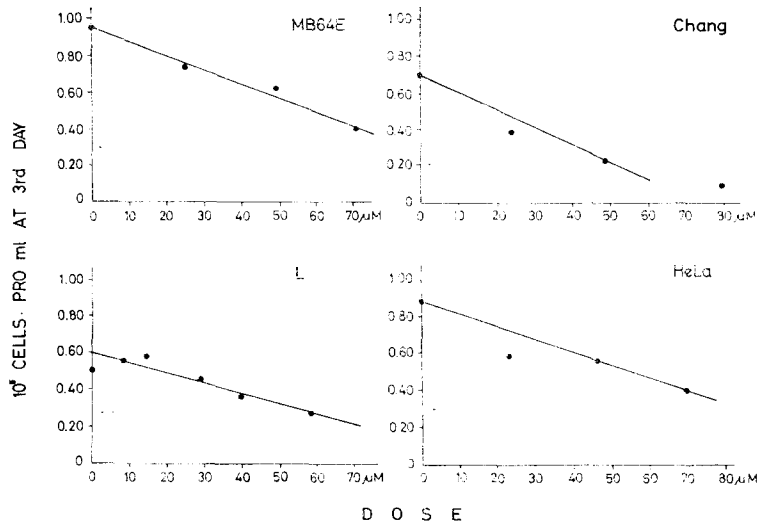


Fig. 2. The cell titres at 3rd day of incubation plotted as a function of final polypeptide concentration expressed as leucine equivalents for MB 64E, Chang liver, L, and HeLa cells in suspension. The polypeptide was prepared from ascites fluid of human patients. Arrow indicates the cell count at zero time.

burst with a maximum at 11 hr after reincubation was obtained (Fig. 3). In a cell population where polypeptide was present at a final concentration of 40 μM during the entire period of TdR-treatment, the mitotic wave was depressed (Fig. 3) although still present. By addition of increasing amounts of

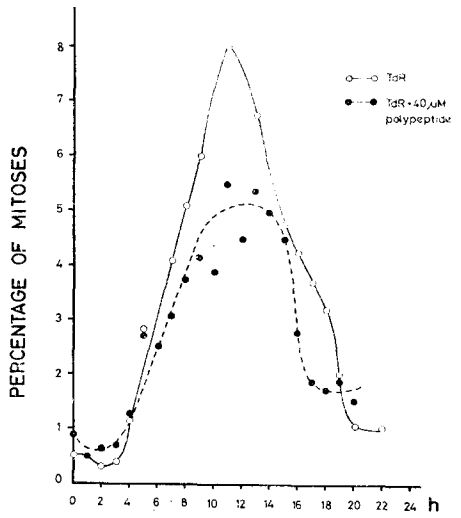


Fig. 3. Mitotic frequency of partially synchronized strain L cells in a Petri dish experiment at various times (hr) after TdR-treatment. o—o treated with TdR for 24 hours. ●—● treated with TdR+40μM polypeptide for 24 hours. Each point represent estimates of 1000 cells.

polypeptide to parallel series of cell populations subjected to partial synchronization, it could be shown that the size of the subsequent mitotic wave was decreasing. It could also be shown that u.v. irradiation of polypeptide solutions abolished the depressing effect on the mitosis. A depressive effect on the mitotic frequency was also seen in partially synchronized HeLa and Chang cells. No abnormal or arrested mitoses were seen in polypeptide treated cells so far.

Electrophoresis

The results of measurements on human red blood cells and on Chang cells after various times of incubation with the polypeptide are found in Table 1. The polypeptide was derived from ELD mouse ascites fluid. So far no significant difference in mobilities have been observed between control and polypeptide-treated red blood cells even after 24 hr incubation. The measurements on Chang cells did show a slight reduction in the electrophoretic mobility of cells cultivated for 4 days in the presence of growth inhibiting amounts of polypeptide, a reduction which is at the limit of significance due to the heterogeneity of the population.

DISCUSSION

The results presented above show inhibition of the cell population growth rates of four cell lines when they are cultivated in the presence of added amounts of a dialysable polypeptide, which was purified from pooled and from different tumor ascites fluids. The observed growth inhibition was proportional to the final concentration of polypeptide expressed as μM of a leucine standard determined by a ninhydrin method. Inactivation of the polypeptide was obtained by u.v. irradiation, which is known to destroy protein structures [9].

Parallel with the cell growth inhibiting effect, a tendency to a progressive increase in

the frequency of cells permeable to lissamine green was observed at higher polypeptide doses. The increase in the frequency of injured cells occurred towards the end of the experiments and the toxic effect of the polypeptide was thus not of an immediate lethal nature. The absence of early lethal effects of added polypeptide, at the dose ranges tested, was further confirmed in the partially synchronized strain L cell populations, where the frequency of injured cells was normal during 24 hours, following the release of the TdR-blockage in polypeptide-treated cells. The depression of mitoses observed is thus not due to cell loss but rather to an impairment in some processes essential for mitosis. In the experiments with partially synchronized populations, the cells were washed in order to remove the added TdR. The washing process had apparently no effect on the action of the polypeptide previously added, and it may therefore be suggested that the polypeptide molecules are irreversibly bound to their site of action.

The cell electrophoretic measurements did not show significant changes in mobilities of red blood cells after short-time treatment with polypeptide. The measurements on Chang liver cells cultivated in the presence of polypeptide material did, on the other hand, show a slight reduction in mobility which, although at the limits of significance, would merit further studies. As a decrease in cell mobilities has been observed in slowly growing cell populations [10, 11] such studies would involve systematic measurements on control cells at various stages of growth parallel to cells growing in the presence of polypeptide. It may well be that a reduction in cell mobility of polypeptide treated cultures is due to the decrease in the growth rate rather than to a direct effect on the cell membrane. Thus the cell electrophoresis measurements did not reveal any rapid reaction between the polypeptide and the cell surface. This makes a primary effect of the positively charged [2] polypeptide molecule on the cell membrane less likely and one may therefore suggest that the polypeptide exerts its effects within the cell. Experiments are under work in order to investigate the influence of added polypeptide on premitotic phases in the cell life cycle.

Table 1. *Electrophoretic mobilities of cells treated with polypeptide derived from ELD mouse ascites fluid*

Cell type	Treatment	Elecphoretic mobility (μ. sec^{-1}. V^{-1}. $cm^{-1} \pm SE$)
Human red blood cells	Control	$1{\cdot}08 \pm 0{\cdot}03$
	Control	$1{\cdot}08 \pm 0{\cdot}03$
	2 hr incubation +2°C with 30 μM polypeptide	$1{\cdot}08 \pm 0{\cdot}03$
Human red blood cells	Control	$1{\cdot}08 \pm 0{\cdot}03$
	Control	$1{\cdot}08 \pm 0{\cdot}03$
	2 hr incubation +37°C with 30 μM polypeptide	$1{\cdot}05 \pm 0{\cdot}05$
Human red blood cells	Control	$1{\cdot}08 \pm 0{\cdot}03$
	Control	$1{\cdot}08 \pm 0{\cdot}03$
	24 hr incubation +2°C with 30 μM polypeptide	$1{\cdot}08 \pm 0{\cdot}03$
Chang liver cells	Control at 2nd day of exponential growth	$1{\cdot}11 \pm 0{\cdot}12$*
	2nd day of growth with 30 μM polypeptide	$1{\cdot}09 \pm 0{\cdot}10$†
Chang liver cells	Control at 4th day of exponential growth	$1{\cdot}12 \pm 0{\cdot}12$‡
	4th day of growth with 30 μM polypeptide	$0{\cdot}96 \pm 0{\cdot}08$§

*Cell concentration: $0{\cdot}45 \cdot 10^6$/ml.
†Cell concentration: $0{\cdot}40 \cdot 10^6$/ml.
‡Cell concentration: $0{\cdot}91 \cdot 10^6$/ml.
§Cell concentration: $0{\cdot}70 \cdot 10^6$/ml.

The biological activity of the polypeptide preparations is essentially the same for all cell lines tested, as judged from the cell multiplication rates at different dose levels. It may thus be assumed that the effect of the polypeptide is directed towards some general mechanism within the cell essential for the cell division process, a mechanism which can be at least partially inhibited by the polypeptide. Polypeptide prepared from different sources such as mouse ascites and ascites from human cancer patients has been found to possess the cell growth inhibiting activity [12]. This indicates that the preparation method described [2] results in a polypeptide material, the biological active part of which ought to be identical, irrespective of origin.

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RESUME

L'effet inhibiteur sur la croissance cellulaire d'un polypeptide dialysable, isolé de liquides néoplasiques, a été étudié et mis en évidence sur la souche L en croissance exponentielle, sur le foie Chang, sur les cellules HeLa et MB 64E, et en présence d'un matériel polypeptidique, correspondant à des concentrations micromolaires d'un standard de leucine. La fréquence avec laquelle les cellules sont lésées, de façon irréversible, augmente progressivement à la fin des expériences. On en conclut que le polypeptide, dans les limites des concentrations étudiées, n'a pas un effet léthal immédiat. Dans des populations de cellules partiellement synchronisées, le polypeptide diminue la fréquence des mitoses au cours de la culture, même lorsque les cellules sont soigneusement lavées. Il semble donc que les molécules du polypeptide sont liées, de façon irréversible, à leur site d'action. Les mesures de la mobilité électrophorétique des cellules traitées n'ont pas permis de mettre en évidence une action sur la surface cellulaire. On émet l'hypothèse que le polypeptide agit sur un mécanisme général qui est situé à l'intérieur de la cellule, et qui aboutit à l'inhibition de la mitose et à une diminution de la vitesse de croissance.

SUMMARY

The cell growth inhibiting effect of a dialysable polypeptide derived from tumor fluids has been studied. Growth inhibition was obtained in exponentially growing strain L, Chang liver, HeLa and MB 64E cells and in the presence of polypeptide material corresponding to micromolar concentrations of a leucine standard. A progressive increase in the frequency of irreversibly injured cells was observed at the end of the experiments. It was concluded that the polypeptide, at the dose range tested, did not cause an immediate lethal effect. In partially synchronized cell populations a depressive effect on the mitotic frequency was obtained in polypeptide-treated cultures, although such cells were carefully washed. The polypeptide molecules were thus suggested to be irreversibly bound to their site of action. Measurements of electrophoretic mobilities of polypeptide-treated cells failed to detect an interaction with the cell surface. It is suggested that the polypeptide affects some general mechanism within the cell leading to mitotic inhibition and a reduced growth rate.

ZUSAMMENFASSUNG

Es wurde die zellwachstumshemmende Wirkung eines Polypeptids untersucht, das aus Tumorflüssigkeiten stammte. Bei exponentiell wachsenden Stamm-L-, Chang-Leber-, HeLa- und MB 64E-Zellen wurde Wachstumshemmung erzielt in Gegenwart des Polypeptids, das auf ein Leucin-Standard bezogen wurde. Fortschreitend wurden irreversibel geschädigte Zellen beobachtet. Es wird der Schluß gezogen, daß das Polypeptid im untersuchten Dosisbereich keine sofortige lethale Wirkung hat. In teilweise synchronisierten Zellpopulationen wurde nach Polypeptidbehandlung ein Absinken der Mitosefrequenz erhalten, obwohl die Zellen sorgfältig gewaschen wurden. Man muß daher annehmen,

daß die Polypeptidmoleküle irreversibel an ihren Wirkungsort gebunden werden. Meßungen der elektrophoretischen Beweglichkeit polypeptidbehandelter Zellen ließen keine Wechselwirkung mit der Zelloberfläche erkennen. Es wird diskutiert, daß das Polypeptid einen Mechanismus in der Zelle angreift, wodurch Mitoserate und Wachstum vermindert werden.

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Cytogenetic and Antigenic Properties of GW-127, a Heterologously Transplantable Tumor*

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SEVERAL publications by Goldenberg *et al.* have drawn attention to a variety of human tumors which can be serially transplanted in unconditioned golden hamsters [1–4]. So far, only a subline of the human adenocarcinoma H.Ad.IU, its original form established by Toolan [5], has proved to heterologously transplantable in unconditioned hosts without losing its human cytogenetic characteristics [6, 7]. One of Goldenberg's tumors, GW-127, derived from a human ovarian carcinoma, was given to us in its 9th pouch generation. The tumor, which at that time histologically presented the picture of an undifferentiated carcinoma (Fig. 1), was then serially propagated in adult, unconditioned golden hamsters. Intraperitoneal implantation of solid material from the 14th pouch generation resulted in the establishment of an ascites tumor line which, at present, is in its 65th generation, yielding 10–15 ml of a haemorrhagic ascites per animal with approximately 80×10^6 ascites tumor cells/ml (Fig. 2).

Upon implantation of ascites tumor cells in the pouch or subcutaneously, solid tumor growth occurs again.

Animals implanted intraperitoneally with approximately 30×10^6 ascites tumor cells die on the 6th or 7th day from a combination of massive loss of blood into the peritoneal cavity and from suffocation, their lungs being almost completely obstructed by tumor cells which fill capillaries and larger vessels. Subcutaneous implantation or inoculation of the same number of ascites tumor cells into the pouch results in a somewhat longer survival time of 10 or 11 days. By this time, not only the lungs, but kidneys, adrenals and the liver are also invaded by tumor cells.

This aggressive behavior, together with the easily attained conversion from the solid to the ascitic form, gave rise to doubts whether the tumor has retained its human nature or whether it has been substituted by a hamster tumor. Chromosome analyses, including late replication studies, and immunological tests are the tools to clarify such problems. The present communication, therefore, deals with cytogenetic constitution and antigenic properties of this originally human ovarian carcinoma.

MATERIAL AND METHODS

1. *Chromosome analyses and late replication pattern*

Hamsters bearing solid or ascitic tumors received intraperitoneally Vinblastine sulfate (Eli Lilly and Co., Indianapolis, Ind., USA) 1·5 μg/g body weight. Four hours later, the animals were sacrificed and the tumor cells processed according to the combined methods of Ockey [8] and Moorhead *et al.* [9], described elsewhere [10]. Chromosome analyses were performed on 81 metaphases of solid tumor material derived from the 9th pouch generation,

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obtained from Goldenberg, and on 100 metaphases from the 1st ascites generation. Karyotypes were arranged by use of the grouping system based on relative chromosome length and position of the centromere introduced for the cytogenetic characterization of HaTu 25, a spontaneous hamster reticulum cell sarcoma [10].

For the labelling experiments, ascites tumor cells were cultivated for three days with Parker 199, supplemented with 20% calf serum and antibiotics. Tritiated thymidine (H^3-TdR) at a concentration of 0·5 μC/ml medium, (H^3-TdR, specific activity 1·9 C/mM, Schwarz Bio-Research), and 10 μg Colcemid (Ciba) per ml medium were added 5½ and 2 hr, respectively, before termination of the culture. Aceto-orcein squash preparations were prepared in the conventional way. The locations of 120 suitable mitotic figures were recorded before the slides were covered with Kodak AR-10 stripping film. The slides were exposed to the autoradiographic film for 10 days, and after developing they were restained with Giemsa. For technical details see Schmid [11, 12]. For control photographs of suitably labeled metaphases, the silver grains were bleached off with potassium ferricyanide.

2. *Antigenic properties*

For the evaluation of the antigenic properties of GW-127 tumor cells, the Ouchterlony gel diffusion technique was employed [13]. Solid tumor material from the pouch was homogenized, and the homogenate diluted with Ringer 1:1. This extract was stored in a deep-freezer, thawed, and emulsified in Freund's complete adjuvant (DIFCO) 1:1 before use. Two rabbits were injected subcutaneously four times at intervals or 2·4 weeks. After the fourth injection, the animals were bled, and their sera tested against the following tissues.

1. Normal human tissue: kidney, gut.
2. Human tumors:
   Primary liver carcinoma (autopsy material).
   H.Ep.2 (tissue culture material and solid, transplantable material).
   H.S.1 (solid, transplantable material).
3. Normal hamster tissues: liver, gut.
4. Hamster tumors: Fortner melanoma HaTu 25 (spontaneous reticulum cell sarcoma).

The results were interpreted after the gels had been dehydrated and stained by the usual procedure.

RESULTS

1. Chromosome analysis and late replication pattern

As presented in Table 1, the 9th pouch generation revealed a modal chromosome number of 61 in 27% of the 81 karyotypes studied. In metaphases of the 1st ascites generation, 5 transfer generations later, the number had shifted to 62. Marker chromosomes were not obvious, minutes were inconsistent. A representative karyotype of an ascites tumor cell is shown in Fig. 3. Results of cytophotometric DNA measurements on 248 Feulgen-stained ascites tumor cells (including 15 meta- and 3 telophases) showed a correlation to the modal chromosome number,

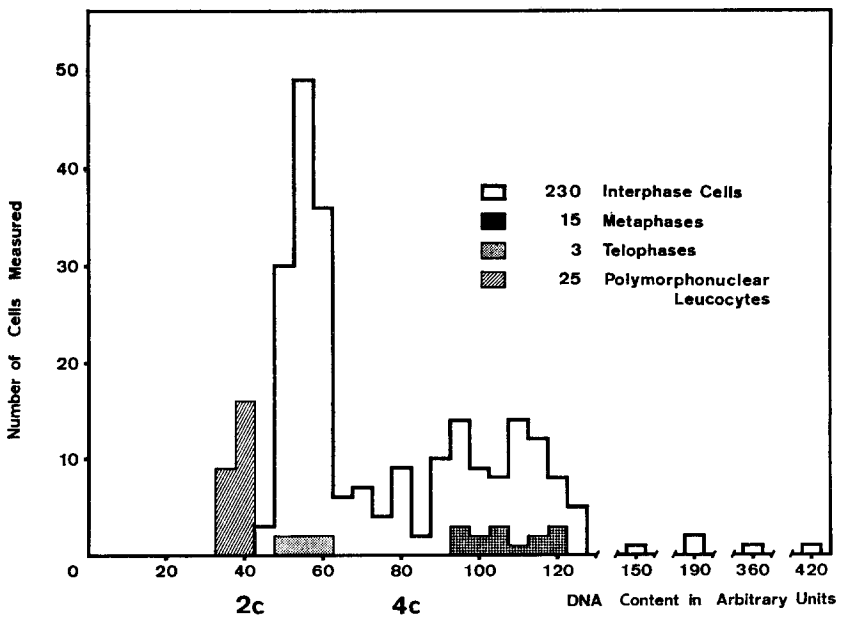


Fig. 4. Relative amount of DNA in tumor cells of the first ascites generation.

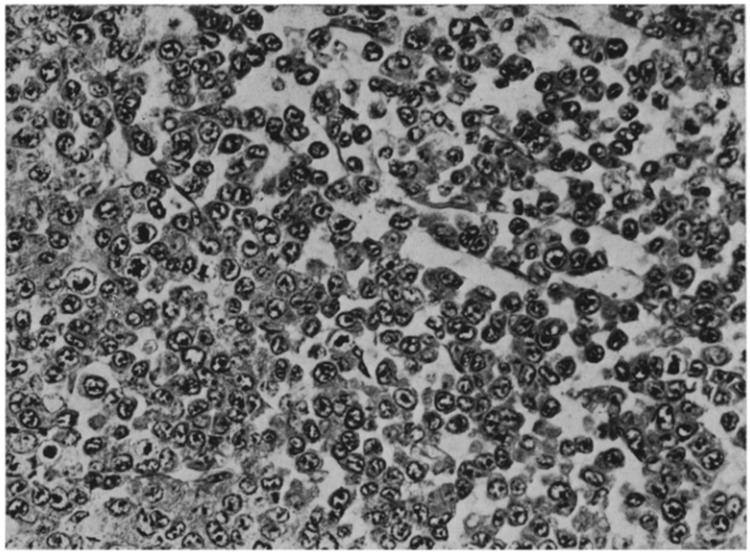


Fig. 1. *GW*-127: *histological appearance of the* 9*th transfer generation in the pouch of an unconditioned golden hamster. H and E* 250 : 1.

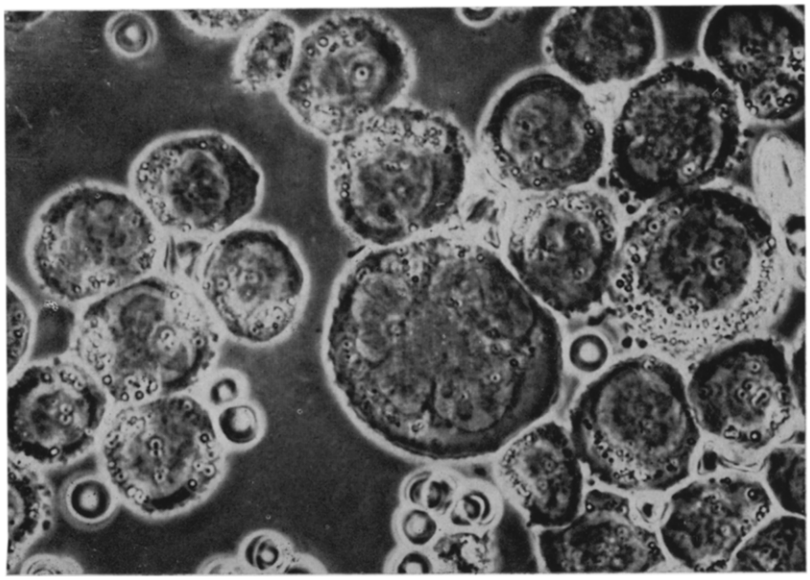


Fig. 2. *Tumor cells of the first ascites generation. Phase contrast* 800 : 1.

(to face p. 280)

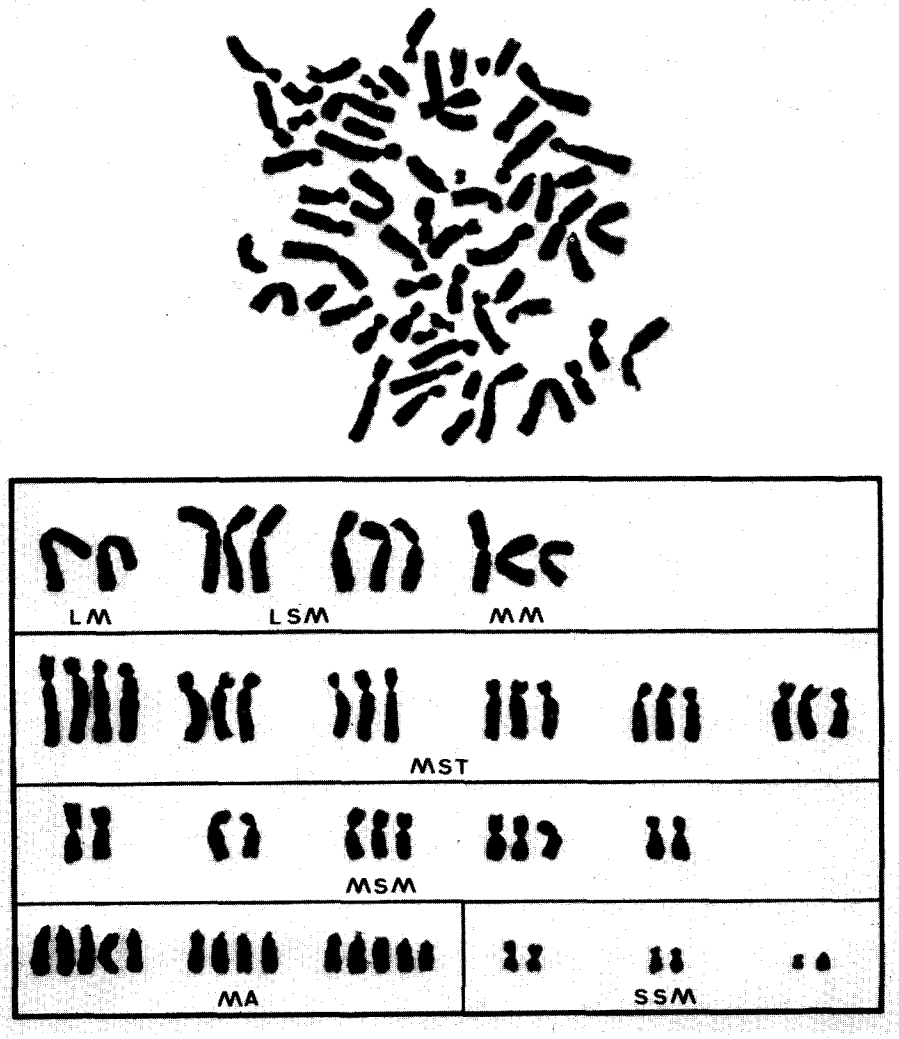


Fig. 3. *Representative karyotype of a metaphase of the first ascites generation with* 62 *chromosomes.*

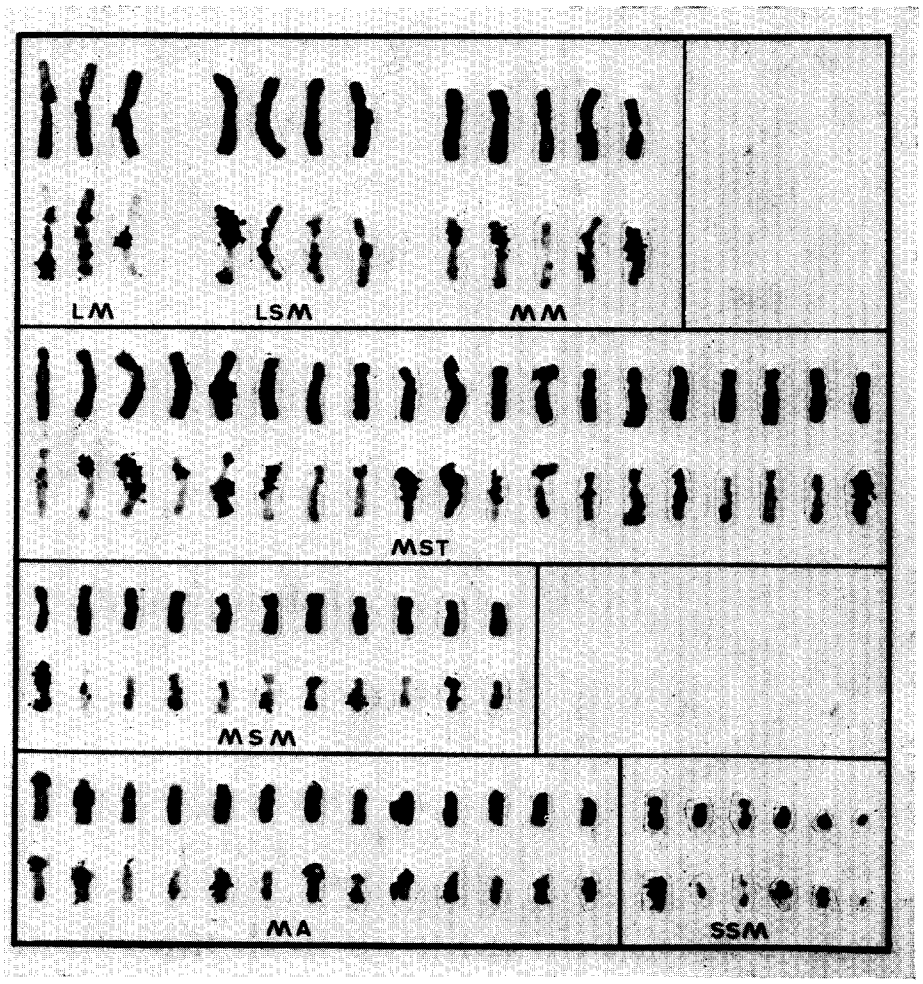


Fig. 5. *Late replication pattern of chromosomes of a cultured tumor cell.*

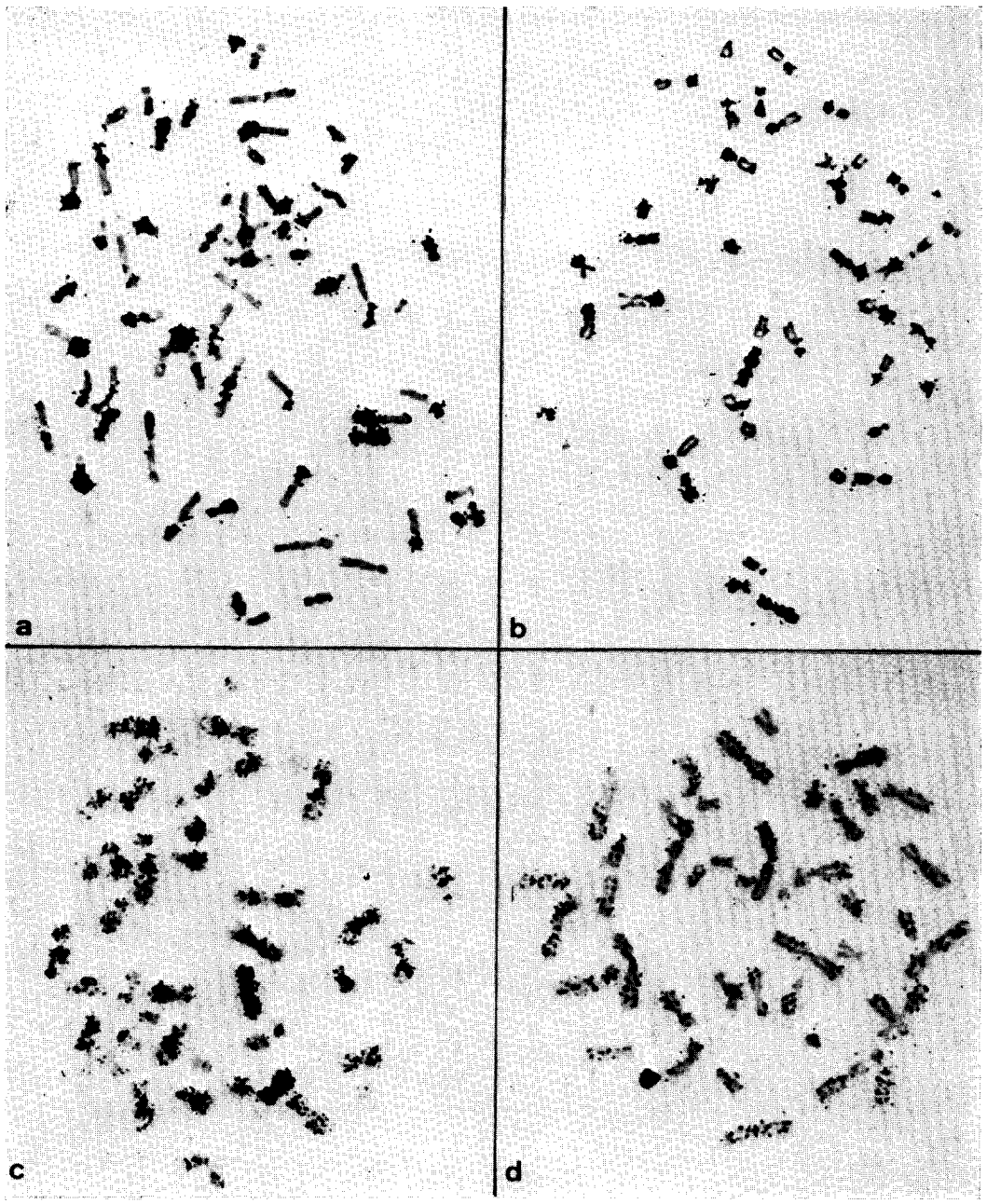


Fig. 6. *a–d Autoradiographs of chromosomes labeled with tritiated thymidine close to the end of the DNA-Synthetic period* (1 μ*C/ml*).
(*a*) *GW*-127 *tumor cell.*
(*b*) *Kidney epithelial cell from a normal male hamster.*
(*c*) *and* (*d*) *Normal human lymphocytes.*
(*The tissue differences are irrevelant for this problem, provided differences in the duration of* G_2*-period are taken into account.*) *The human* (!) *cells were exposed to the autoradiographic emulsion twice as long as the tumor and the hamster cells. Despite this fact the late replicating blocks in the tumor and hamster cells are very heavily labeled, whereas, at the same time, the rest of the chromatin is virtually unlabeled. In human cells heavily labeled chromatin blocks are only found as long as the majority of the chromatin is still lightly labeled. Very late stages* (*d*) *do not show the heavily labeled blocks as do chromosomes from species rich in structural heterochromatin.*

Table 1. *Chromosome distribution in metaphases of GW-127 tumor cells**

Tumor	Chromosome number																									Total
	52	53	56	57	58	59	60	61	62	63	64	69	73	80	85	109	110	117	120	121	122	126	128	163	242	
Solid 9th generation	—	—	—	—	5	13	21	22	12	5	—	—	—	—	—	—	1	1	1	—	—	1	—	—	—	81
Ascites 1st generation	1	1	2	2	2	6	8	24	28	10	1	1	1	1	1	1	1	—	1	3	1	1	1	1	1	100

*Chromosome distribution in GW-127 tumor cells implanted into the pouch and the peritoneal cavity of the golden hamster.

the first peak being located around 3c, the second at 6c (Fig. 4).

Of the 53 metaphases used for the late replication studies, 31 had chromosome numbers between 59 and 62. Seven cells had counts between 49 and 58, 5 from 63 to 66 and 10 cells were in the tetraploid range with numbers between 88 and 120. From 6 autoradiographs cut-out karyotypes were prepared; one is shown in Fig. 5. Late replicating chromatin blocks were found predominantly in the following locations (Figs. 5 and 6a): In the short arms of many long and medium sized submetacentric and subtelocentric chromosomes, in a near-centrometric region of 1–3 of the longer metacentric chromosomes, and in some long and short arms of the smaller metacentric and submetacentric chromosomes. The acrocentric chromosomes generally were lightly labeled, some showed moderately late foci in the proximal regions. In the groups of the very small metacentric chromosomes two out of five or six members exhibited late labeling over one arm.

2. *Antigenic properties*

Several distinct lines of precipitation developed between the rabbit antisera and extracts from the various hamster tissues; some lines, although weaker, appeared also between the sera and extracts of human tissues. In order to decide whether the latter were the result of a cross-reaction between anti-hamster antibodies and human antigens or whether they were due to the presence of specific anti-human antibodies, the following absorption tests were performed: 0·9 ml of anti-GW-127 rabbit serum and 0·1 ml of an extract prepared from pooled hamster tissues (liver, HaTu 25, melanoma) were placed together at 4°C for 5–15 hr and then centrifuged. The supernatant was used for further Ouchterlony tests and for repeated absorption. After the absorption procedure had been repeated 3 times, precipitation lines were no longer visible, neither against any of the human nor against hamster tissues.

DISCUSSION

GW-127, a transplantable tumor, originated by implantation of material from a human ovarian carcinoma into the pouch of unconditioned golden hamsters, has lost its human characteristics. A similar incidence, the 'induction' of a mouse tumor by a heterologously transplanted human tumor, has been reported for HT.37, a squamous-cell carcinoma, by Klausner and Richards [14]. The aggressive behavior in the heterologous host was the decisive factor for questioning the human nature of GW-127 and for investigating its cytogenetic and antigenetic properties. Analyses of the karyotype revealed a modal chromosome number of 61–62 together with definite signs for at least some structural rearrangements (occasional presence of minutes). We are aware that it is risky to decide on purely morphological grounds whether cells of such a grossly aneuploid population are of human or of hamster origin. However, comparison with the normal karyotypes of the two species shows more resemblance to the one of the hamster. As far as the metacentric and submetacentric chromosomes are concerned, one can still argue that pericentric inversions could have changed the picture. A rather strong argument against a human origin is the following: The modal line of the tumor cells contains 12 or 13 acrocentrics, in size corresponding to the three human pairs 13–15 or to the 4 hamster pairs 16–19 (nomenclature used by Schmid *et al.*, reference 12). Those of man possess distinct short, satellited arms, whereas in the hamster these chromosomes look almost telocentric, and this aspect is shared by all the acrocentrics of the tumor cells. To account for a transformation of all the human acrocentrics in this direction one would need a very complicated explanation.

For the biological problem in question—the differentiation between labeling patterns of human versus Syrian hamster chromosomes—the most informative autoradiographs are the very late patterns, i.e. those that arise from the uptake of tritiated thymidine very close to the end of the DNA-synthetic period (S-period), when the greatest part of the chromatin has ceased uptake of DNA-precursor. The great majority of our autoradiographs fulfilled this criterium and permitted an evaluation of the so-called late replicating regions.

The normal karyotype of the Syrian hamster is characterized by the presence and typical distribution of a great amount of structural heterochromatin, a material which, in certain cell types, forms obtrusive chromocenters in interphase nuclei [15]. This component of the karyotype varies to a great extent between different mammalian species; in some it can be almost completely absent [16]. Structural heterochromatin is known to be late replicating since the pioneer studies of Taylor [17] in the Chinese hamster.

The human karyotype possesses considerably less structural heterochromatin than the Syrian hamster. Distinct blocks of it are mainly present in the following positions: close to the

centromere of chromosome No. 1, in the proximal regions of the long arms of chromosomes 16 and two members of the C-group, in the short arms of No. 5 and the distal portions of No. 13 [11]. These late replicating blocks can be demonstrated clearly only as long as there is some DNA replication going on in the euchromatic portion of the complement as well (Fig. 6c). Very late patterns as a rule show a light grain pattern over numerous foci (Fig. 6d) (always provided the same concentration of the isotope and the same exposure time to the autoradiographic emulsion were applied). From the above description and from Fig. 6a–d there remains little doubt that the labeling pattern of the chromosomes of GW 127 is of the type found in the Syrian hamster.

It may be argued that the replication patterns of chromosomes in tumor cells might undergo profound changes. This assumption is not supported by any available evidence. The first line of evidence comes from studies on confirmed malignant human and hamster cells: no fundamental changes in the typical human character of labeling patterns were found in H.Ep.2 cells (Zweidler, in preparation); nor were such changes found in leukemic cells with the Philadelphia chromosome [18, 12, 19]. The typical replication pattern of hamster cells was also found in a spontaneous reticulum cell sarcoma of the golden hamster [10]. The second line of evidence that gross replication patterns do not change easily comes from studies on many different cell types in the Chinese hamster [20, 21] and on very early embryonic stages of the Syrian hamster [22]. In all of these materials no changes in autosomal labeling patterns were demonstrated.

The cytogenetic findings were corroborated by results of the immunological studies. No precipitation lines were formed when anti-GW 127 rabbit serum, absorbed with extract of various hamster organs, was tested against human and hamster tissues. It can thus be assumed that the tumor material used for immunization did not contain human antigens.

We have no knowledge at all of the events which, on the cellular level, took place during the first 8 transplantation passages of GW-127. To explain our findings either of two explanations seems to be the most probable: (1) the hamster tumor arose independently of the original transplantation of human tumor material; (2) a specific or unspecific property of the human tumor material induced a hamster tumour in the pouch. Unlikely explanations are—in our view—some other possibilities mentioned by Goldenberg *et al.* in their last paper: mixture of human and hamster chromosomes (hybridization *in vivo*), replacement of the original human chromosomes by those of the host, and viral oncogenesis "in which case the karyotype of the transplanted tumor would resemble the host's genotype while retaining in many respects its original phenotype" [4]. We want to restrict our comment to the statement that the material from the 9th pouch generation and from the subsequent 1st ascites generation did not belong to a human tumor.

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RESUME

La tumeur GW-127 est une tumeur que Goldenberg et al. *ont rendue transplantable en greffant un carcinome ovarien humain (matériel opératoire) dans l'abajoue de hamsters non conditionnes. Celle tumeur se développe de deux façons: sous forme solide et sous forme ascitique. Elle tue les animaux dans un délai très court: des hamsters meurent 6 à 7 jours après une implantation par voie intrapéritonéale. Ce comportement malin, dans un hôte étranger peut faire douter de l'origine humaine de cette tumeur. C'est pourquoi des recherches ont été entreprises afin de déterminer son caryotype, sa "réplication" et l'antigénicité. Les résultats de ces expériences démontrent que la tumeur GW-127, en tenant compte des propriétés analysées, n'est pas une tumeur humaine, dans sa forme actuelle, mais qu'elle possède les attributs caractéristiques du hamster doré.*

SUMMARY

GW-127, a tumor made transplantable by Goldenberg et. al., *has developed in pouches of unconditioned golden hamsters after the implantation of surgical material from a human ovarian carcinoma. The tumor can be propagated both in the solid and the ascitic form and kills the animals in a short time: hamsters implanted intraperitoneally die within a week. This aggressive behavior in a foreign host made it questionable whether the tumor is of human origin. Experiments, therefore, were undertaken to determine its karyotype, replication pattern and its antigenicity. Results of these investigations indicate*

that with regard to the properties analyzed, GW-127 demonstrates the typical characteristics of the Syrian golden hamster.

ZUSAMMENFASSUNG

GW-127 ist ein Tumor, der von Goldenberg und Mitarbeitern durch Implantation von Operationsmaterial eines menschlichen Ovarialcarcinoms in die Backentasche nichtconditionierter Goldhamster transplantabel gemacht wurde. Der Tumor wächst solid und in der Ascitesform und tötet die Tiere innerhalb kurzer Zeit: intraperitoneal implantierte Hamster sterben am 6. oder 7. Tag. Dieses aggressive Verhalten des Tumors in fremden Wirten liess seine 'menschliche' Natur zweifelhaft erscheinen. Es wurden daher Experimente zur Beurteilung von Karyotyp, Replikationsmuster der Chromosomem und Antigenität durchgeführt. Dabei zeigte es sich, dass GW-127 kein menschlicher Tumor sein kann, da er, im Hinblick auf die untersuchten Eigenschaften, die typischen Merkmale des syrischen Goldhamsters aufweist.

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Cell Proliferation in Human Malignant Lymphomas

Analysis of Labelling Index and DNA Content in Cell Populations Obtained by Biopsy

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THE STUDY of cell proliferation in malignant tumours of the lymphoid system in man is complicated not only by factors common to all solid tumours [1], but also by cell traffic in and out of the tumour and the presence of several different morphological cell types in the tumour. Apart from the frequency of mitotic figures, histological methods can give little information about the proliferative behaviour of the individual cells of the tumour. The technique of multiple sampling after a single injection of tritiated thymidine which has proved to be of value in the analysis of cell proliferation in human bone marrow [2–4] is for practical purposes inapplicable for the study of lymphomas. On the other hand, it usually is possible to obtain one or two biopsies from a lymphoid tumour without difficulty. Recently, it has been shown that the combination of autoradiographic and quantitative cytochemical methods allows the distribution of a population of cells into various stages of interphase to be analysed and this related to the cellular morphology [5, 6]. This technique has been used to study Burkitt tumour and has given information about some of the characteristics of tumour cell proliferation in this neoplasm [7]. It has also been of value in the investigation of changes in the composition of the lymphoblast population in the bone marrow in acute lymphoblastic leukaemia in children [8] that occur during the course of the disease. In the present investigation, these techniques have been applied to analyse the heterogeneous cell populations present in biopsy specimens obtained from a variety of malignant lymphomas, to find out to what extent the cellular composition of the node could be resolved.

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MATERIALS AND METHODS

Lymph node tissue was obtained by surgical excision or aspiration biopsy and the specimen divided into a portion for histology and a portion for cytochemical and cytogenetic analysis. A piece of node was finely divided in tissue culture medium TC 199 and auto-

logous plasma to form a cell suspension. This suspension was incubated with tritiated thymidine (^{3}H-TdR) 2–4 μ C/ml for 15–30 min, the cells were then concentrated by centrifugation (less than 1000 rev/min for 5 min) and spread on grease free slides, air dried and fixed in absolute methanol.

The technique for Feulgen staining, autoradiography and measurement of Feulgen dye absorption by a Deeley pattern microdensitometer [9] has been described elsewhere [7]. When the tumour population was composed of a number of different morphological cell types, the preparation was first stained by the May–Grünwald–Giemsa method and a photographic map prepared of representative areas. The various cells were identified and their position recorded on the map. The May–Grünwald–Giemsa stain was then leached out with cold 5% trichloroacetic acid for 1–2 min followed by methanol. Subsequently the DNA content was measured and ^{3}H-TdR labelling observed. (Labelling index=(LI)= percentage of cells labelled with ^{3}H-TdR; where possible it was estimated by observing 1000 cells). The method for preparing chromosome spreads from lymph node biopsies is described elsewhere [10].

Histological sections were examined by two of us (I.M.E.H. and R.G.M.). A slightly different histological classification was used at each centre. Cases seen in Paris were classified into one of the following groups: lymphoblastosarcome; réticulosarcome histiocytaire; réticulosarcome syncytial and Hodgkin's Disease [11, 12]. Cases seen in London were classified as follows: malignant lymphoma, lymphocytic, well differentiated; malignant lymphoma, lymphocytic, poorly differentiated (lymphoblastic); malignant lymphoma, mixed lymphocytic and reticulum cell; malignant lymphoma histiocytic; malignant lymphoma, reticulum cell or stem cell; Hodgkin's Disease [13, 14].

The patients were seen either at the Royal Marsden Hospital or at the Institut Gustave Roussy and were all of Caucasian origin.

RESULTS

Examples of the cell populations obtained from lymph node preparations in various forms of malignant lymphoma are shown in Fig. 1. The very well differentiated lymphomas are typified by chronic lymphocytic leukaemia where the population is predominantly composed of mature small lymphocytes (1a) interspersed with occasional lymphoblasts. At the other extreme, there is massive replacement of the node by tumour cells of undifferentiated appearance often showing considerable variation in cell size (1g). It was usual to find, even in these cases, a proportion of small lymphocytes and histiocytes scattered among the main tumour cell population. Lymphomas showing intermediate degrees of differentiation contain variable proportions of immature lymphocytic cells, reticulum cells, small lymphocytes and plasma cells (1c).

The labelling index for each preparation is given in the Table. The overall labelling index is a measure of the frequency of DNA synthesising cells in the total cell population present in the cell suspension. In assessing the "tumour cell" labelling index there has been an inevitable subjective discrimination in deciding what constitutes the proliferating or tumour cell population. In obtaining the tumour cell labelling index, small lymphocytes, histiocytes and mature plasma cells were excluded and considered as non-proliferating cells. In most of the preparations the immature cells could be distinguished on morphological grounds although it was not possible to distinguish which of these were the malignant cells and which were the precursors of normally differentiating cells.

The extent of deviation from the normal chromosome content by the tumour cell population was estimated by two techniques. First, the DNA content of those tumour cells not synthesising DNA was compared to that of small lymphocytes (normal diploid mode=2c) by quantitative cytochemistry. In this way, the modal DNA content of the tumour became apparent. Minor changes such as the gain or loss of one or two chromosomes are not detectable by this method. Secondly, in a number of patients, chromosome preparations were made and the modal chromosome number is given for each of these cases. In three cases (Nos. 15, 16, 24), the modal chromosome number was hyperdiploid. The modal DNA content of the tumour cells did not differ significantly from the 2c valve and is therefore written as "2c" in the table. It can be seen that tumours can be broadly divided into three groups, those with both a normal DNA content and chromosome count, those with hyperdiploid and those with hypotetraploid modes. In the latter two situations occasional grossly aneuploid cells were encountered.

Examples of the histograms of DNA distribution in the tumour cell population are shown in Figs. 2–6. Figure 2 (case 7) shows the distribution of DNA content in a poorly differentiated lymphocytic lymphoma. The labelling index in this case was 16·8% and

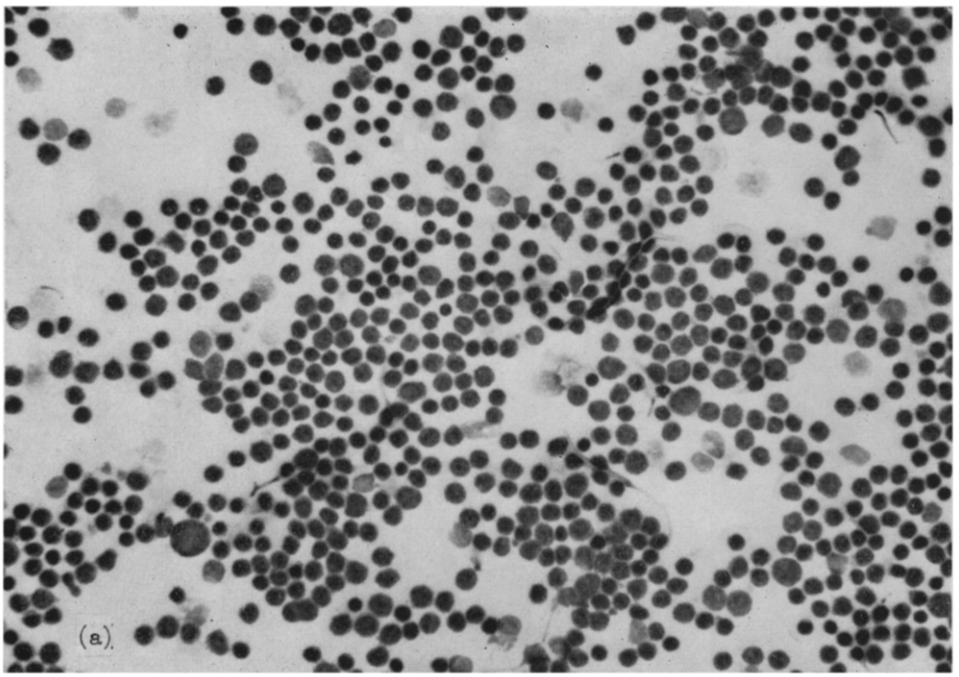
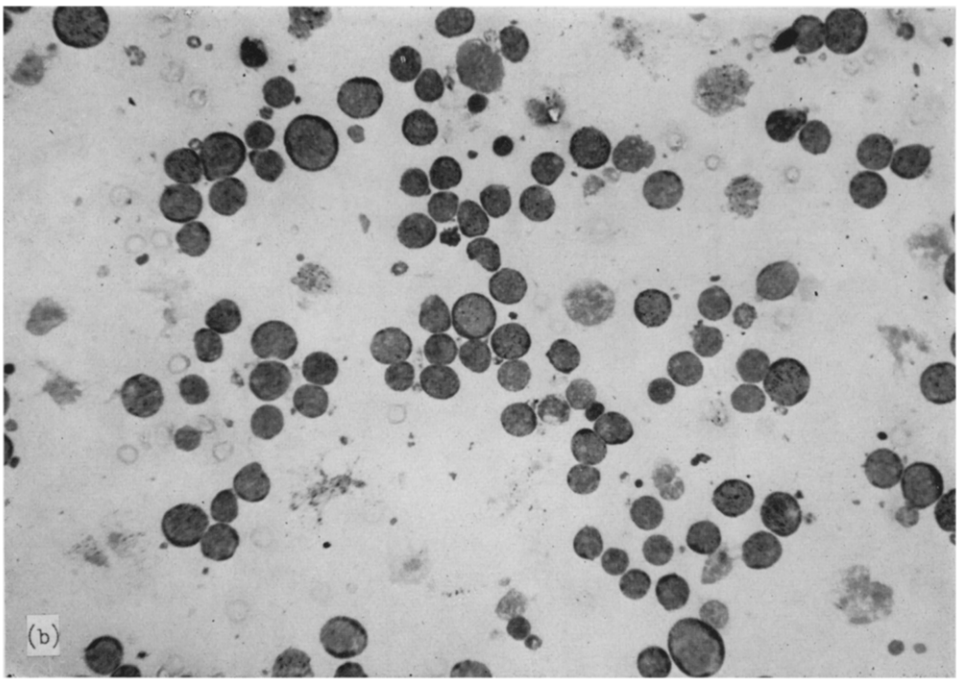
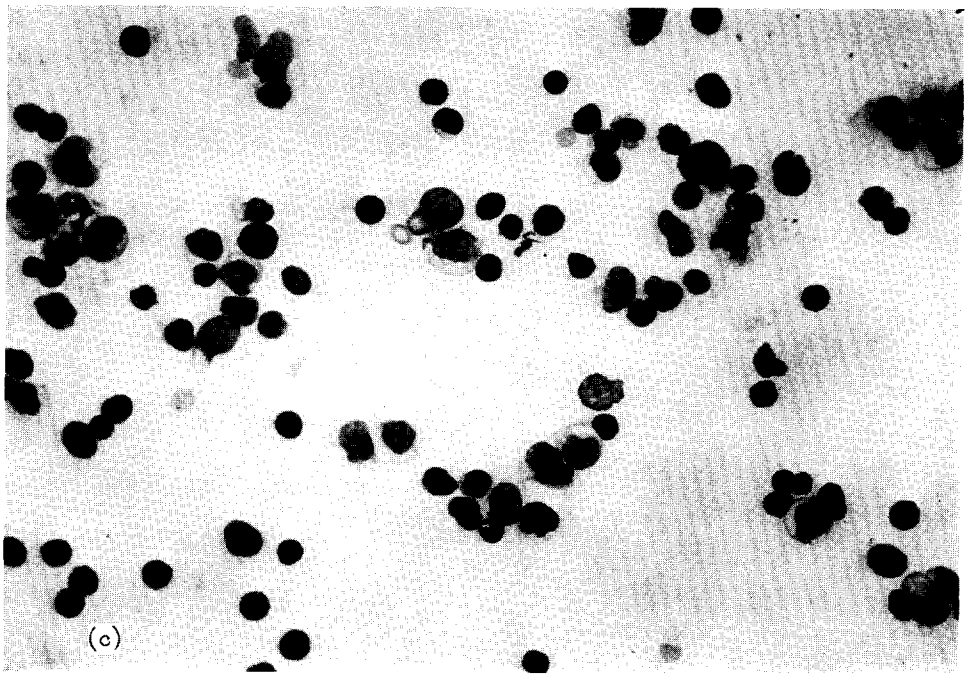


Fig. 1. *Examples of preparations made from lymph nodes in malignant lymphoma.*
(*a*) *Chronic lymphocytic leukaemia* (*case* 5). *The predominant cell type is the small small lymphocyte interspersed with occasional lymphoblasts.*



(*b*) *Poorly differentiated lymphocytic lymphoma* (*case* 19) *showing virtual total replacement of the lymph node with large malignant lymphoblasts.*

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(*c*) *Reticulum cell sarcoma* (*case* 25) *showing large tumour cells admixed with other cell types.*

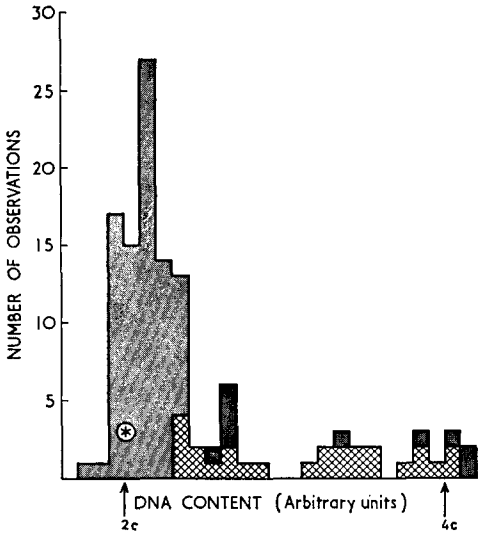


Fig. 2. (*case* 7). *Sixty-five year old male with a lymphoblastic lymphosarcoma presenting as a rapidly enlarging chest wall mass with skin deposits.*

* = *Modal value for small lymphocytes.*
Shaded area = *Cells not synthesising DNA.*
Hatched area = *Cells labelled with* ^{3}*H-TdR.*

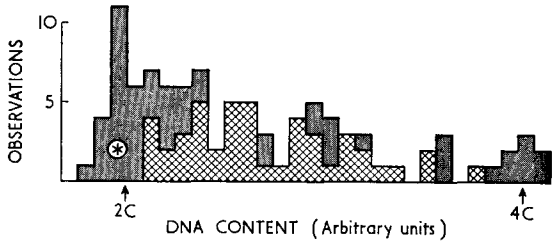


Fig. 3. (*case* 19). *Twenty-two year old male with a poorly differentiated reticulum cell sarcoma presenting as a right tibial deposit and spreading rapidly to involve lymph nodes, liver and spleen and the post nasal space.*

* = *Modal value for small lymphocytes.*
Shaded area = *Cells not synthesising DNA.*
Hatched area = *Cells labelled with H-TdR.*

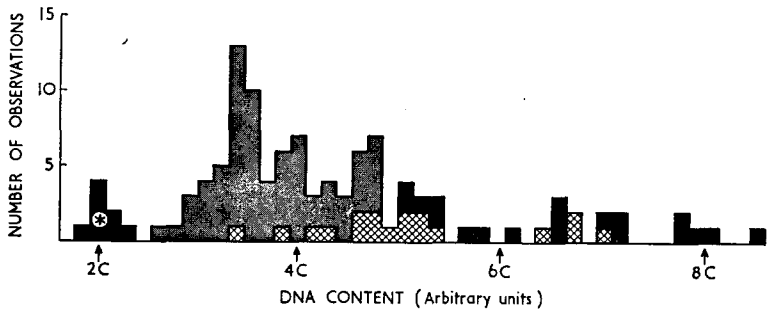


Fig. 4. (*case* 21). *Fifty-eight year old male with a reticulum cell sarcoma presenting with an ulcerating left tonsillar tumour and enlarged cervical nodes.*

* = *Modal value for small lymphocytes.*
Shaded area = *Cells not synthesising DNA.*
Hatched area = *Cells labelled with* ^{3}*H-TdR.*

Case	French histology	English histology	Labelling index % (a) Overall	Labelling index % (b) Blast or tumour cell	Modal DNA Content of tumour cells	Modal chromosome number
1	Reactive (Toxoplasmosis)	Reactive (Toxoplasmosis?)	6·6	43·5	2C	
2	Reactive	Reactive	2·2	16·5	2C	
3	C.L.L.	*Well differentiated lymphocytic	0·4	19·0	2C	
4	C.L.L.	No histology seen	0·3	46·0		
5	C.L.L.	No histology seen	0·9	43·0		
6	C.L.L.	*Well differentiated lymphocytic	1·5	20·0		46
7	Lymphoblastosarcome	*Poorly differentiated lymphocytic (lymphoblastic)		16·8	Hyperdiploid	—
8	Lymphoblastosarcome	*Poorly differentiated lymphocytic (lymphoblastic)	0·4	32·0	2C	—
9	Lymphoblastosarcome	*Poorly differentiated lymphocytic (lymphoblastic)	0·5	5·5	2C	—
10	Lymphoblastosarcome	*Poorly differentiated diffuse (lymphoblastic)? some plasmacytic differentiation	13·0	16·3	Hyperdiploid	—
11	Lymphoblastosarcome	*Poorly differentiated (lymphoblastic)	3·0	21·0	Hypotetraploid	—
12	Lymphoblastosarcome	*Poorly differentiated lymphocytic (lymphoblastic)	2·8	26·0	2C	
13	Lymphoblastosarcome	*Poorly differentiated lymphocytic (lymphoblastic)	3·5	26·0	2C	
14	Reticulosarcome histiocytaire	*Poorly differentiated mainly reticulum cell in type with some "lymphoblasts"	4·6	11·6	2C	—
15	Reticulosarcome	*Mixed lymphoblastic and reticulum cell	14·0	27·0	"2C"	†46 and 50
16	Lymphoblastosarcome	*Lymphoblastic and reticulum cell	0·8	9·5	"2C"	50
17	Reticulosarcome histiocytaire or lymphosarcome	Malignant tumour of uncertain histogenesis, probably malignant lymphoma, lymphoblastic	28·0	28·0	2C	—

18	Reticulosarcome histiocytaire	*Mixed reticulum cell and lymphoblastic	8·0	13·0	2C	47
19	Lymphoblastosarcome reticulosarcome on cytology	*Reticulum cell sarcoma or stem cell sarcoma	46·0	48	2C	—
20	Reticulosarcome histiocytaire	*Reticulo-histocytic	0·4	—	2C	—
21	Reticulosarcome histiocytaire	*Reticulo-histocytic	11·0	12·0	Hypotetraploid	—
22	Reticulosarcome histiocytaire	*Reticulum cell sarcoma	2·0	5·4	Hyperdiploid	—
23	Reticulosarcome syncytial nodulaire	*Histocytic, nodular	10·5	30·0	2C	46
24	Lymphoblastosarcome	Reticulum cell sarcoma or stem cell sarcoma	20·0	24·0	"2C"	49
25	Hodgkin's NCI IV (most probably)	*Reticulum cell sarcoma nodular	5·6	7·2	2C	46
26	Hodgkin's NCI IV	Hodgkin's Disease	0·3	35·0	Mainly diploid few polyploid cells	
27	Hodgkin's NCI IV	?Reticulum cell sarcoma ?Hodgkin's Disease	4·0	32·0	Hypotetraploid	46 and 78–92
28	Hodgkin's	Hodgkin's Disease	1·2	—	2C	
29	Hodgkin's NCI III	Hodgkin's Disease	4·2	19·0	2C	
Normal nodes	8 nodes removed at mastectomy	Histologically normal	0·3 1·1			

*The words "malignant lymphoma" present in the pathologist's report have been omitted.
†Abnormal karyotype.
"2C" No shift of modal value.

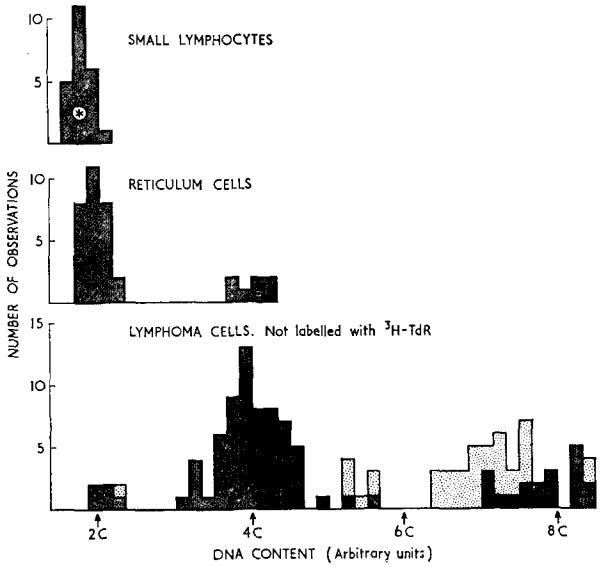


Fig. 5. (*case* 27). *A fifty-eight year old male with a long history of lymphoma (probable reticulum cell sarcoma) presenting with enlarged cervical nodes and becoming generalised after ten years.*

* = *Modal value for small lymphocytes.*
Shaded area = *Cells not synthesising DNA.*
Dotted area = *Naked nuclei not synthesising DNA.*

most of the cells lay at the post mitotic pre-synthetic DNA mode which is hyperdiploid compared to the small lymphocytes. Figure 3 (case 19) is an example of a highly proliferative tumour (48% labelling index) with conservation of ploidy of the tumour cell line close to that of normal lymphocytes. Considerable variation in cell size is seen (Fig. 1b) and this could be mainly accounted for by enlargement of the cell as it passes through the cell cycle.

Figure 4 (case 21) is an example of the distribution of DNA content observed in a grossly aneuploid reticulum cell sarcoma showing a hypotetraploid dominant mode. At the time of biopsy, 12% of the tumour cells were synthesising DNA. Figure 5 (case 27) shows another example of the distribution of DNA content in a reticulum cell sarcoma in which the dominant mode of the tumour cells was hypotetraploid. The tumour cells were clearly distinguishable from the normal elements because of their large size and cytological characteristics. All the other elements including the histiocytes had a normal DNA content. It was noticed that the cells of highest DNA content were extremely fragile and many were seen in the preparation as naked nuclei. In this patient chromosome analysis showed that there were two groups of proliferating cells, those with a normal karyotype and those whose chromosome number varied from 78 to 92. The combination of the morphological identifica-

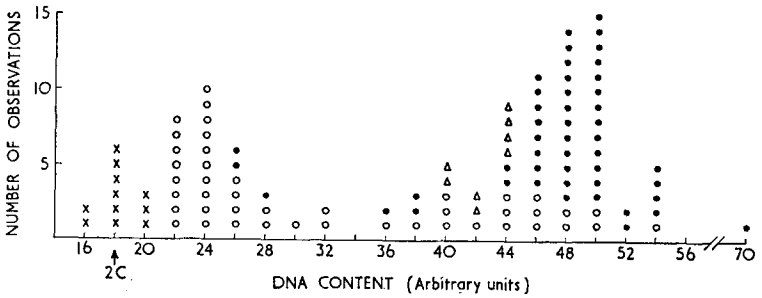


Fig. 6. (*case* 10). *Seventy-eight year old female, presenting with a thyroid mass and a right tonsillar mass which proved to be a lymphoblastic lymphoma. She subsequently developed skin deposits.*

× = *Small lymphocytes.*
o = *Small and medium size tumour cells.*
● = *Large tumour cells.*
Δ = *Mitotic figures.*

tion of the cells and measurement of their DNA content made it reasonably certain that the majority of normal karyotypes were derived from the non-malignant cells in the node, as very few tumour cells were observed with DNA contents closely related to the normal 2c mode. In several of these tumours, where histiocytes were present, the technique of Romanowsky staining followed by Feulgen staining demonstrated clearly that these cells were rarely labelled with ^{3}H-TdR. The majority of them had a diploid DNA content. Figure 6 (case 10) shows in detail the relation between cell size and DNA content. In addition to the increase in size as the cell passes through interphase, there was in this tumour an unusual population of large cells whose DNA content was greater than the modal content of metaphase figures (by an amount greater than could be accounted for by technical reasons alone). It is possible that these large cells were the products of abnormal mitoses resulting in the formation of cells whose genetic content was such that they were unable to continue to proliferate.

DISCUSSION

In broad terms the cell populations obtained from the different types of malignant lymphoma can be considered to be of two kinds. In the first kind, the population consists predominantly of abnormal cells (lymphosarcoma cells, malignant reticulum cells and other atypical lymphoid cells), and, in the second, the population is composed of several different cell types in which abnormal cells may be interspersed with many morphologically normal cells such as small lymphocytes and histiocytes. Chronic lymphocytic leukaemia falls into neither of these categories since in this disease there is a massive accumulation of small lymphocytes in the lymph node with relatively few lymphoblasts; both these cell types are morphologically indistinguishable from their normal counterparts. When abnormal cells made up the bulk of the population both high and low labelling indices of the tumour cells were found despite an often marked cytological similarity between cell populations derived from different cases. The high L.I.'s were similar to those observed in the Burkitt Tumour [7] except that in the latter there was a remarkable conservation of ploidy, whereas several of the lymphomas in this series were grossly aneuploid. Analysis of the distribution of DNA content in cell populations with a low labelling index showed that the major part of the population was at the post mitotic, presynthetic DNA mode. This distribution is similar to that observed in untreated acute leukaemia [8, 15]. As in acute leukaemia it was the smaller of the blast cells which tended to make up the bulk of the population at this mode, whilst the larger cells tended to be those which were synthesising DNA or were at the postsynthetic mode (G_2). Several investigators [4, 8, 16, 17] have found in acute leukaemia that the larger cells synthesise DNA while some of the smaller cells probably represent the non-proliferating progeny of large cells. More recently Foadi *et al.* [8] have shown that in acute lymphoblastic leukaemia the cells which are unlabelled are predominantly at the post mitotic presynthetic mode. The question now arises as to whether unlabelled cells in acute leukaemia and lymphoid malignancy lying at this mode are G_1 cells in cycle, or whether they represent cells permanently or temporarily out of cycle which should not be considered as forming part of the G_1 compartment.

Gavosto and his colleagues [16] and Killman [17] have suggested that in acute leukaemia the large blast cells may mature to become smaller sterile forms. There is however no direct evidence that this process occurs in those lymphomas which have particularly low labelling indices of the tumour cell population. In chronic lymphocytic leukaemia, although the overall L.I. is very low, the L.I. of the lymphoblasts is in the order or 40%. This is similar to the L.I. of lymphoblasts in normal lymph nodes and suggests that the kinetics of this proliferating cell show little deviation from normal although their progeny may be functionally dissimilar to normal small lymphocytes.

In those tumours where several cell types were present and the tumour cells formed only a part of the total cell population, the overall L.I. was often low although the L.I. of the tumour cell population was usually high. This indicates that the majority of the tumour cells were committed to cell division. It is clear that L.I. does not form a reliable basis for the assessment of malignancy, and, in the two cases of reactive hyperplasia which were examined, the L.I.'s were higher than was the case in some of the well differentiated lymphomas. On the other hand, disturbance of karyotype, even though this may be accompanied by little change in the modal DNA content, is a strong indication of malignancy [10].

It is not possible on the basis of the current experiments to predict the generation time of the various cells in the node since the observations have been limited to a single point in time. If generation time and growth rate are to be

related to the L.I. then the growth fraction (i.e. the fraction of cells engaged in proliferation and the duration of DNA synthesis must both be known. Data on the duration of the *S* phase in man are limited. It is in the order of 12–13 hr for normal erythroid and neutrophil precursors [2, 3, 18], and probably similar for leukaemic lymphoblasts [4] although it may be considerably longer for other tumour cells. Clarkson and his collaborators [19] have reported an *S* phase of 17–34 hr for tumour cells in malignant effusions, and Malaise, Frindel and Tubiana [20] found an *S* period of 19 hr in two basal cell carcinomas.

In many of the cases of lymphoma in the present series, it seems likely that the growth fraction was relatively low. Since cells in the G_1 phase of interphase could not be distinguished from out of cycle cells which lie at the post mitotic presynthetic DNA mode, no estimate of generation time was possible. However, in the lymphomas where the labelling index of the tumour cells was high, it is a strong possibility that the major part of the population was engaged in cyclic division. The generation time (G.T.) can be estimated when the L.I. and the duration of *S* are known providing that the total cell population is engaged in proliferation [21]. Thus for a population with a 50% L.I. and an *S* period of 13 hr, the G.T. would be 26 hr and 68 hr if the *S* period were 34 hr. This gives an approximation which is useful in determining the probable G.T. for a lymphoma. Even when the long *S* period is assumed, the G.T. is far shorter than the rate of doubling of the size of the lymph nodes as observed clinically. In various benign conditions, especially in secondary immune responses, it is well recognized that the nodes may increase in size at a rate which is close to the calculated G.T. of the lymphoblasts, although factors other than cell proliferation (such as oedema) may contribute to the increase in size of the nodes. In the immune response, the proliferation may be relatively short lived lasting for a few days or weeks. On the other hand, in the lymphomas, the higher rates of cell division may be sustained for several months, yet in some instances accompanied by only a slow increase of tumour mass. It is likely that cell death occurring within the node largely accounts for the discrepancy. Cell loss into the peripheral blood may become an important feature as a terminal event, but does not appear to occur to any significant degree except in chronic lymphocytic leukaemia and in occassional cases of lymphosarcoma.

The presence of debris-laden macrophages in some of these tumours supports the concept that there is a high intranode cell death rate. These cells were found to be unlabelled and to be at the 2c DNA mode. Thus these cells would not appear to proliferate and to be actively macrophagic at the same time, and their precursor or dividing forms were not morphologically identifiable.

Metcalf, Ishidate and Brumley [22] have reported a low rate of histiocyte proliferation in the AKR murine lymphoma, where a rapid proliferation of the tumour cells is associated with a high cell death rate and a large number of histiocytes in the tumour. They found that the large phagocytic cells divided every 200–300 hr whereas the doubling time of the lymphoma cell population was 24–48 hr.

There is clearly a requirement for more information on the duration of DNA synthesis in lymphoma tumour cell populations. It is possible that grossly aneuploid populations may show a considerable range in the duration of the *S* phase which may vary according to the ploidy of the nuclei.

The present method has measured the labelling index and DNA content and related these to the morphology of the cells. However, the usual Romanowsky staining often fails to give sufficient criteria on which to distinguish one functional cell type from another. Several of these tumours are highly pleomorphic. Part of this pleomorphism is related to the marked increase in size of the cell as it passes through the cycle, but there are almost certainly many other reasons, as yet unknown, for the diversity of morphological appearances found within a particular tumour. Further information about the functional behaviour of cells with differing morphology may be revealed when other histochemical techniques are used in a sequence comparable to that described above.

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RESUME

Le mode de prolifération cellulaire a été examiné dans des ganglions de patients atteints de divers types de lymphomes malins, à la fois par autoradiographie et par des méthodes cytochimiques quantitatives. Les chromosomes ont aussi été étudiés chez certains patients. Dans certains cas, il y avait une bonne conservation de la ploïdie, et les cellules tumorales pouvaient être classées dans les compatiments S *et* G_2 *de l'interphase. Les cellules ne proliférant pas ne pouvaient cependant pas être distinguées des cellules en phase* G_1. *Une variation considérable de la capacité proliférative était observée parmi des tumeurs appartenant à la même forme histologique. Ceci était dû, en partie, à la variabilité de la proportion de cellules tumorales non prolifératives, et, en partie, à la présence, dans le ganglion, de populations de cellules non tumorales. Dans les tumeurs où il semblait vraisemblable que la majorité des cellules participaient à la prolifération, il existait une discordance importante entre le temps de génération estimé et la vitesse de croissance cliniquement observée. Ceci est probablement dû à une mort de cellules à l'intérieur du ganglion.*

L'analyse chromosomique mettait en évidence une hyperploïdie dans certaines tumeurs, alors qu'une modification du contenu modal en ADN n'avait pas été démontrée par l'analyse microspectrophotométrique. Certaines tumeurs avaient une aneuploïdie considérable, et, dans ces cas, il n'était pas possible de déterminer si les cellules en interphase étaient dans la phase G_1 *ou dans la phase* G_2. *Le contenu modal en ADN de ces tumeurs aneuploïdes était soit hyperdiploïde, soit hypotétraploïde.*

SUMMARY

The pattern of cell proliferation has been examined in lymph node material from a group of patients suffering from a variety of malignant lymphomas using a combined autoradiographic and quantitative cytochemical method. In a number of these patients, the chromosomes were also studied. In some cases, there was good conservation of ploidy and tumour cells could be assigned to the S *and* G_2 *compartments of interphase. Cells which were out of cycle could not, however, be distinguished from cells in interphase lying at the presynthetic DNA mode* (G_1). *Considerable variation of proliferative behaviour was seen in tumours which were in the same histological category. This was due in part to variability in the proportion of non-proliferating tumour cells, and in part to the admixture of tumour cells with morphologically different non-proliferating cell populations within the node. In those tumours where it seemed likely that the majority of cells were committed to proliferation, the estimated generation time greatly exceeded the clinically observed tumour growth rate and it is probable that intra-node cell death largely explains this discrepancy. Chromosomes analysis showed a hyperdiploid number of chromosomes in some tumours where no shift of modal DNA content could be detected microspectrophotometrically. Some of the tumours examined showed considerable aneuploidy and in these cases it was not possible to assign cells to either the* G_1 *or* G_2 *compartments of interphase. It was found that the modal DNA content of the aneuploid lymphomas tended to fall into either the hyperdiploid or hypotetraploid position.*

ZUSAMMENFASSUNG

Unter Anwendung einer kombinierten autoradiographischen und quantitativen cytochemischen Methode wurde die Zellproliferation in Lymphknoten einer Gruppe von Patienten untersucht, die an verschiedenen malignen Lymphomen erkrankt waren. In einigen Fällen wurden die Chromosomen untersucht. Bei einem Teil der Fälle war der Ploidiegrad erhalten und die Tumorzellen konnten nach der S *und* G_2 *Phase geordnet werden. Zellen, die nicht mehr den Zellcyclus durchliefen, konnten nicht von Zellen in der* G_1 *Phase unterschieden werden. Auch Tumoren der gleichen histoligischen Kategorie zeigten große Unterschiede in der Proliferation. Dies war teilweise bedingt durch das Mengenverhältnis an nicht-proliferienden Tumorzellen und teilweise durch das Vorhandensein anderer nicht-proliferierender Zellen in dem Knoten. In Tumoren, in denen die Mehrzahl der Zellen Proliferationstendenz hatten, überstieg die gemessene Generationszeit die klinisch beobachtete Wachstumsrate. Wahrscheinlich läßt ein Zugrundegehen vieler*

Zellen im Lymphknoten diese Diskrepanz erklären. Die Chromosomenanalyse zeigte in einigen Tumoren eine hyperdiploide Zahl von Chromosomen an, obschonmi krospektrophotometrisch keine Veränderung des durchschnittlichen DNS-Gehaltes festgestellt werden konnte. Einige der untersuchten Tumoren zeigten beträchliche Aneuploidie und in diesen Fâllen war eine Zuordnung zur G_1 *oder* G_2 *Phase unmöglich. Der mittlere DNS-Gehalt der aneuploiden Zellen lag entweder bei der hyperdiploiden oder der hypotetraploiden Menge.*

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Permanent Changes in the Fine Structure of Rat Hepatocytes Following Prolonged Treatment with 2-Acetylaminofluorene

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INTRODUCTION

MANY previous electron microscope studies of changes in hepatic cells, induced by carcinogens [1–11], have been concerned with the fine structural alterations which occurred while the animals were being fed the carcinogen. Some, at least, of these changes have been considered to be non-specific in nature, owing to their similarity to those produced by non-carcinogens [12, 13]. There is a high probability that the change or changes which may be involved in the carcinogenic process would be observed amongst those which are permanent and irreversible in nature and, therefore, independent of the presence of the carcinogen. It seemed to be of interest, therefore, to examine the fine structure of the hepatic cells of preneoplastic rat liver, at varying intervals, after a period of carcinogen treatment.

Since the size of the sample of a liver which can be examined by electron microscopy is extremely small, it was necessary to treat animals with a carcinogen for a sufficient length of time to ensure that the majority of the hepatic cells had received a carcinogenic dose of the agent. In male Leeds rats, a dosage rate of 0·05% 2-acetylaminofluorene (2-AAF) in the diet, given over a period of 10 months, ensures a subsequent 100% incidence of liver tumours within 8 months after the end of treatment. The hepatic tissue, during the post-treatment period, might be described as "preneoplastic", without necessarily implying that all the hepatocytes are potential tumour cells. 2-Acetylaminofluorene (*N*-2-fluorenylacetamide, 2-acetamidefluorene, or 2-AAF) is a well-established carcinogen [15–17], whose action on the rat liver has been shown to resemble that of other hepatocarcinogens [18, 19].

The fine structure of the hepatocytes of preneoplastic rat liver is examined here and brief consideration is given to the earlier changes produced by 2-AAF.

MATERIALS AND METHODS

Male albino rats of the Leeds strain, bred in this laboratory and weighing approximately 200 g, were fed a standard diet containing 0·05% of 2-acetylaminofluorene (2-AAF). Control animals were fed the identical diet with out the carcinogen. Both experimental and control rats were fed *ad libitum* and maintained under identical, controlled, laboratory conditions.

Groups of animals, consisting of approximately 6 rats per group, were sacrificed at 7–10 days, 3–4 weeks, 8–12 weeks and 8–10 months after the start of feeding the carcinogen diet. The experimental animals remaining after 10 months were returned to the standard diet without further 2-AAF treatment. These animals were sacrificed at intervals from approximately 2 months up to 6 months after the cessation of 2-AAP feeding. By the end of this period, all remaining animals had liver tumours.

All animals were killed at the same time of day, in order to minimise any influence of

diurnal variation of the fine structure of their hepatic cells. The rats were killed by stunning, after which small portions of liver were rapidly removed for processing for electron microscopy. Adjacent samples of tissue were taken for examination by light microscopy. In the case of animals having liver tumours, the immediate vicinity of the tumour nodules was avoided when taking liver samples.

Immediately after removal, the samples of liver tissue were placed in ice-cold fixative and minced into 1 mm^3 blocks, in order to allow rapid penetration of the fixative.

The tissue was fixed for 4 hr at 0–4° in 4% glutaraldehyde, buffered with 0·067 M cacodylate buffer at pH 7·2 according to the modification by Epstein and Holt [20] of the method described by Sabatini, Bensch and Barrnett [21]. The blocks were then washed for at least 16 hr at 0–4° in 0·25 M sucrose in 0·1 M cacodylate buffer, before being post-fixed in Millonig's phosphate-buffered 1% osmium tetroxide [22] for 2 hr at 0·4°. Rapid dehydration was carried out in ethanol at room temperature, and the blocks were embedded in Epon 812 using Luft's technique [23].

Thin sections were cut with glass knives, using a Sorvall MT-1 Porter Blum ultramicrotome. Those sections showing pale gold to silver interference colours were picked up on uncoated copper grids and stained with lead tartrate [24]. Sections were examined and electron micrographs were taken in an RCA EMU-3G electron microscope, using an accelerating voltage of 100 kV.

RESULTS

The changes observed during 2-AAF treatment were considered to be of relatively minor importance to the present study and their major features are, therefore, only briefly summarised here.

7–10 *days of* 2-*AAF treatment*

Macroscopically, the livers of treated rats were normal, and histological examination showed no change, except for some localised round cell accumulation. Electron microscopy of the hepatocytes revealed a proliferation of the agranular endoplasmic reticulum, partial glycogen depletion, some local loss of ribosomes from the granular endoplasmic reticulum and dilatation of the bile canaliculi.

3–4 *weeks of* 2-*AAF treatment*

At autopsy the rat livers were abnormally pale, and light microscopy showed some hepatic cell proliferation in the portal areas. Vacuolation of the hepatic cell cytoplasm was observed. At the ultrastructural level, there was a further increase in the quantity of the agranular endoplasmic reticulum and more extensive glycogen depletion. The granular endoplasmic reticulum was mainly present in the form of single cisternae, and there was marked distortion and dilatation of the bile canaliculi.

8–12 *weeks of* 2-*AAF treatment*

Although the livers showed no gross abnormalities, microscopical examination revealed distortion of the normal pattern of liver structure, owing to nodular hyperplasia of the hepatic cells, the lobules being surrounded by quantities of connective tissue. The ultrastructure of the hepatic cells resembled that of the cells examined after 3–4 weeks of treatment, except for some accumulation of glycogen and the presence of numerous large lysosomes, accompanied by hypertrophy of the Golgi zones. In addition, there were numbers of narrow, tubular, cytoplasmic invaginations in the region of the dilated bile canaliculi.

8–10 *months of* 2-*AAF treatment*

The livers were enlarged, finely nodular in appearance and abnormally friable. Microscopically, their lobular structure had been entirely lost and there was evidence of widespread hyperplasia of the hepatic cells. Electron microscopical examination of the hepatic cells revealed the loss and dilatation of the agranular endoplasmic reticulum, as well as further loss and distortion of the granular form of this organelle. Little glycogen was present and mitochondrial abnormalities were observed, whilst the previously observed cell surface modifications were also found at this stage. The cytoplasm contained abnormally abundant, free ribosomes.

Pre-neoplastic 2-*AAF liver*

At autopsy the livers of all experimental animals were considerably enlarged, pale in colour and coarsely granular in texture. Histological examination (Fig. 1) revealed an irregular pattern of hepatic cells and the presence of numerous cystic channels. Examination revealed no evidence of neoplasia, but the presence of substantial numbers of mitotic figures indicated that there was some hyperplasia of the hepatic tissue.

The present studies showed that there were no discernible differences in structure between the hepatic tissues obtained at different intervals after 2-AAF treatment, whether or not the animals had liver tumours at the time of

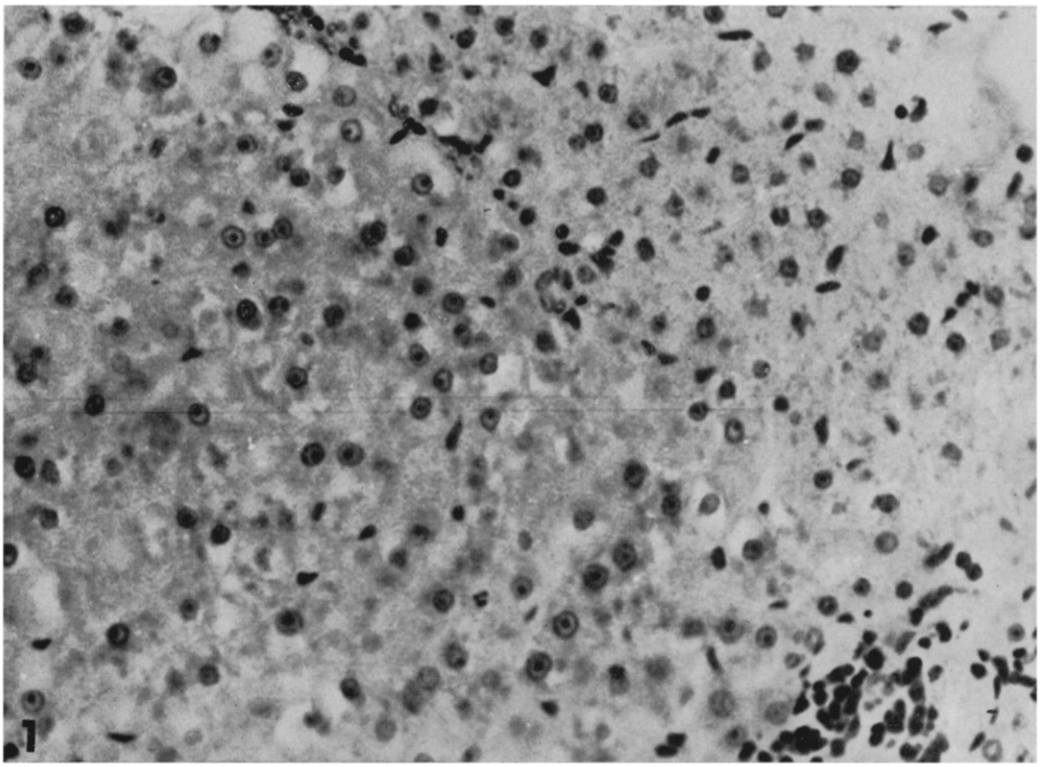


Fig. 1. *Histological section of liver from an animal bearing* 2-*AAF induced hepatomata. Magnification* × 375.

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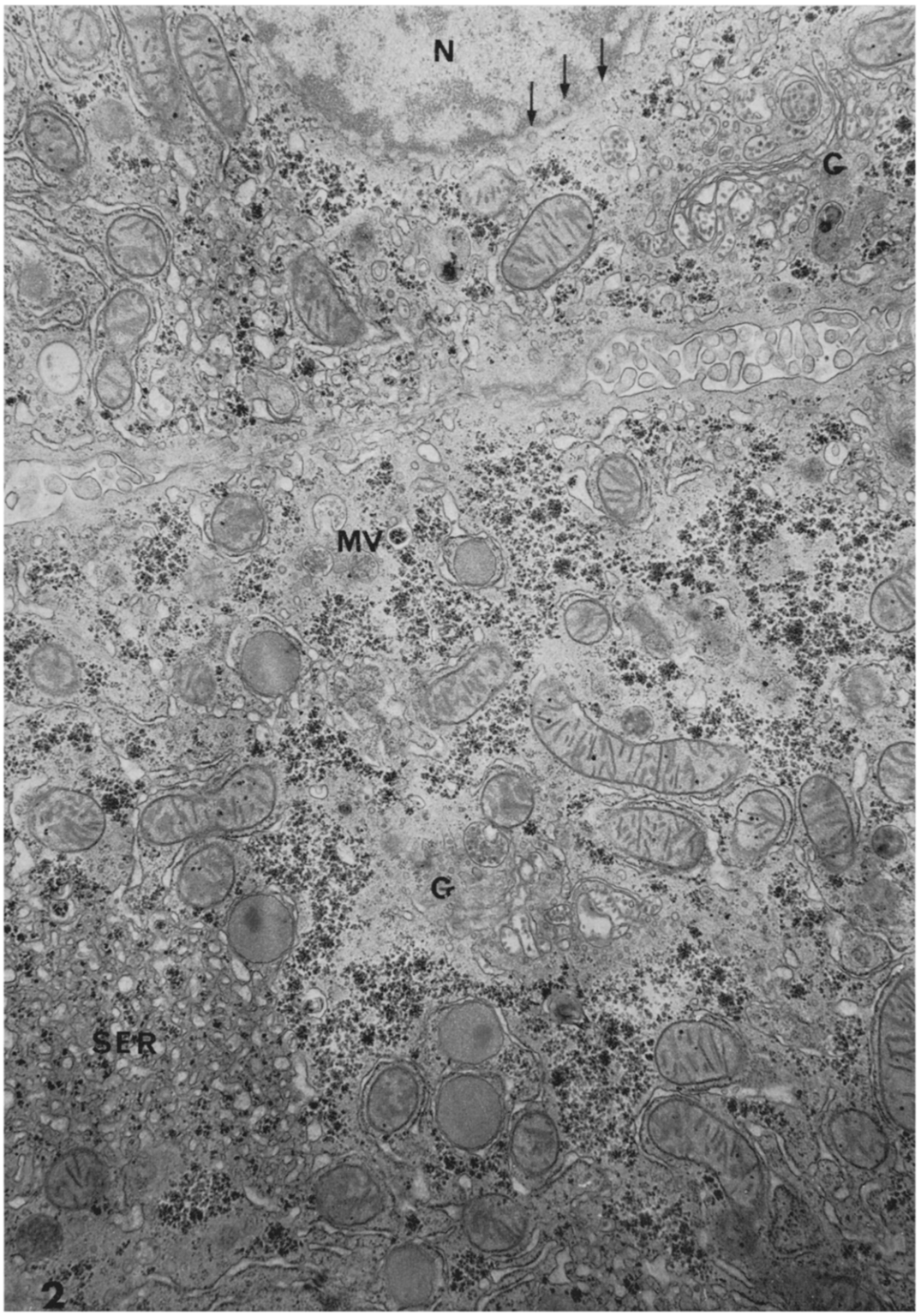


Fig. 2. Pre-neoplastic 2-AAF liver. Portions of two adjacent liver cells are shown. The nuclear membrane of the nucleus (N) *of the uppermost cell has been sectioned tangentially and so reveals the outlines of nuclear pores (arrows). The outer nuclear membrane is irregular. The cytoplasm contains areas of glycogen rosettes, having a normal appearance. Some glycogen is also associated with distended elements of the agranular endoplasmic reticulum* (SER). *The granular endoplasmic reticulum is present as dilated cisternae associated with microbodies and mitochondria. The Golgi zones* (G) *have an active appearance, being extensive and containing numerous granules. A number of multivesicular bodies* (MV) *are present near the surface of the lower cell. The bile canaliculi are large and contain abundant microvilli.*
Magnification × 15,000.

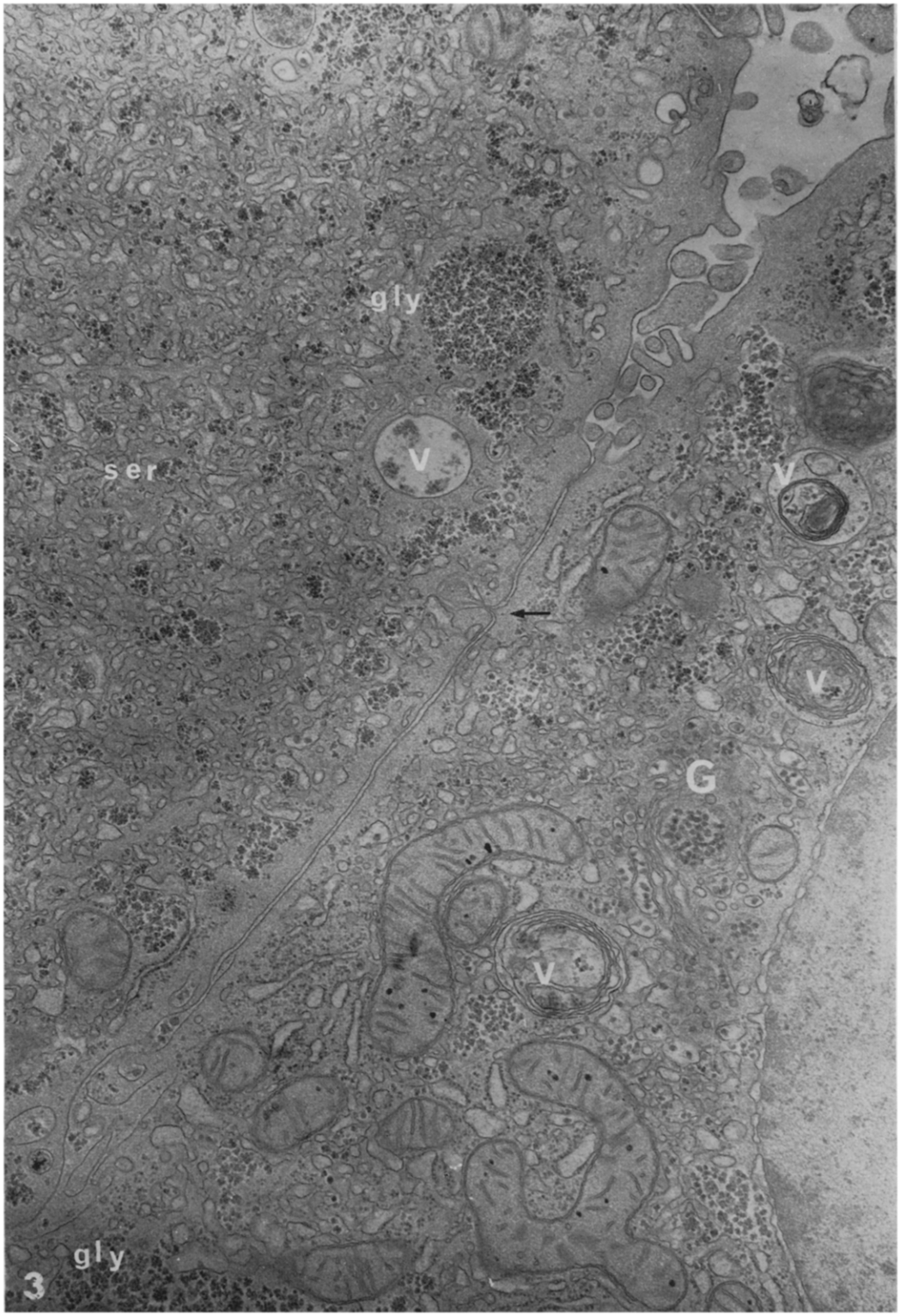


Fig. 3. *Pre-neoplastic* 2-*AAF liver. An extensive area of agranular endoplasmic reticulum is present, interspersed with glycogen. The glycogen also forms local concentrations* (*gly*). *Fragmented profiles of dilated rough-surfaced cisternae are present towards the left of the field. The cytoplasm contains large vacuoles* (v); *some of these contain membranes and/or fragmented material and may represent sites of localised cellular lysis. Their ultrastructure is not, however, that which is normally associated with lysosomes. A large, narrow, branching form of mitochondrion is to be seen at the upper part of the field. A protrusion of the cell membrane of one cell into the cytoplasm of another is indicated by an arrow.*
Magnification × 22,500.

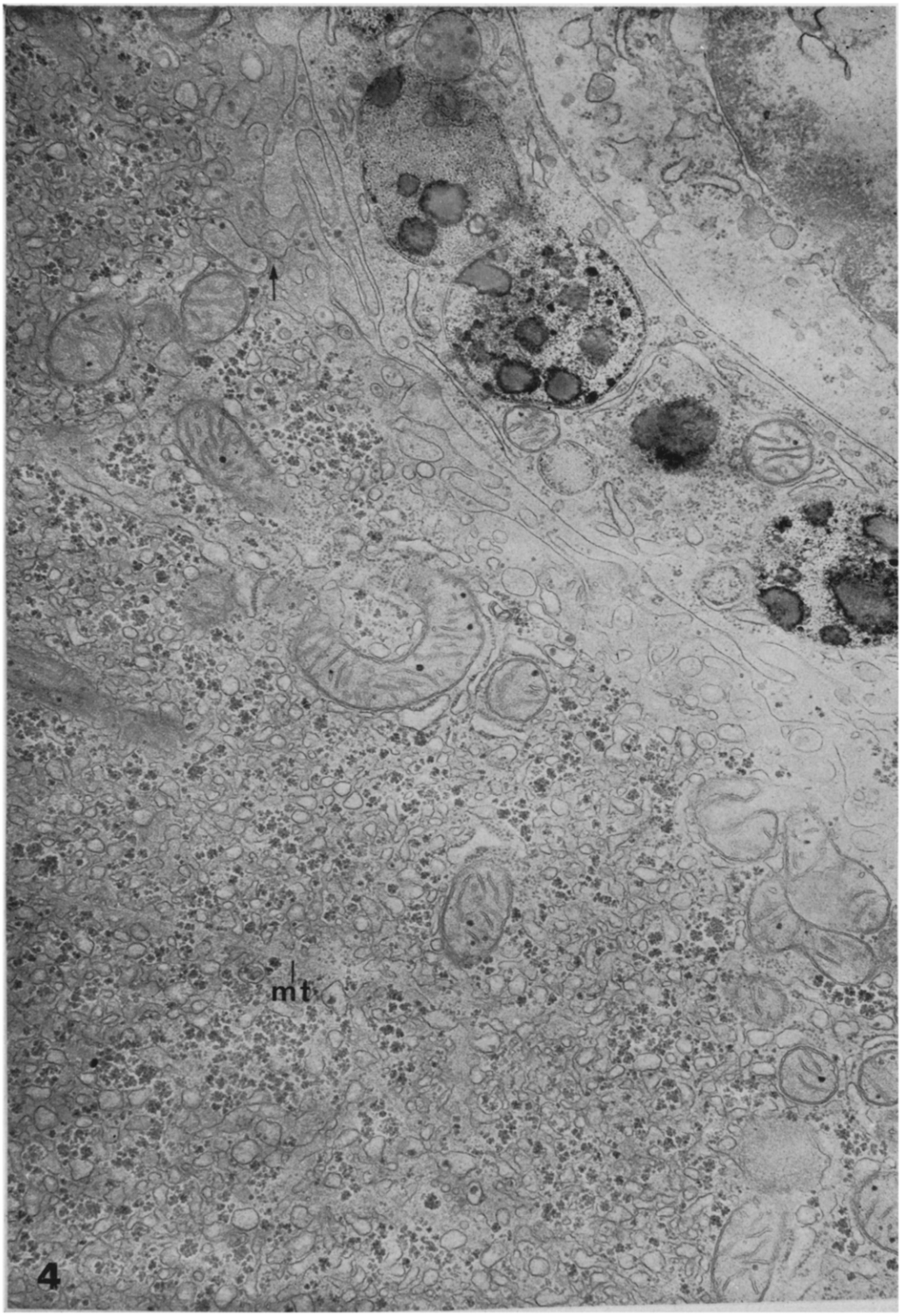
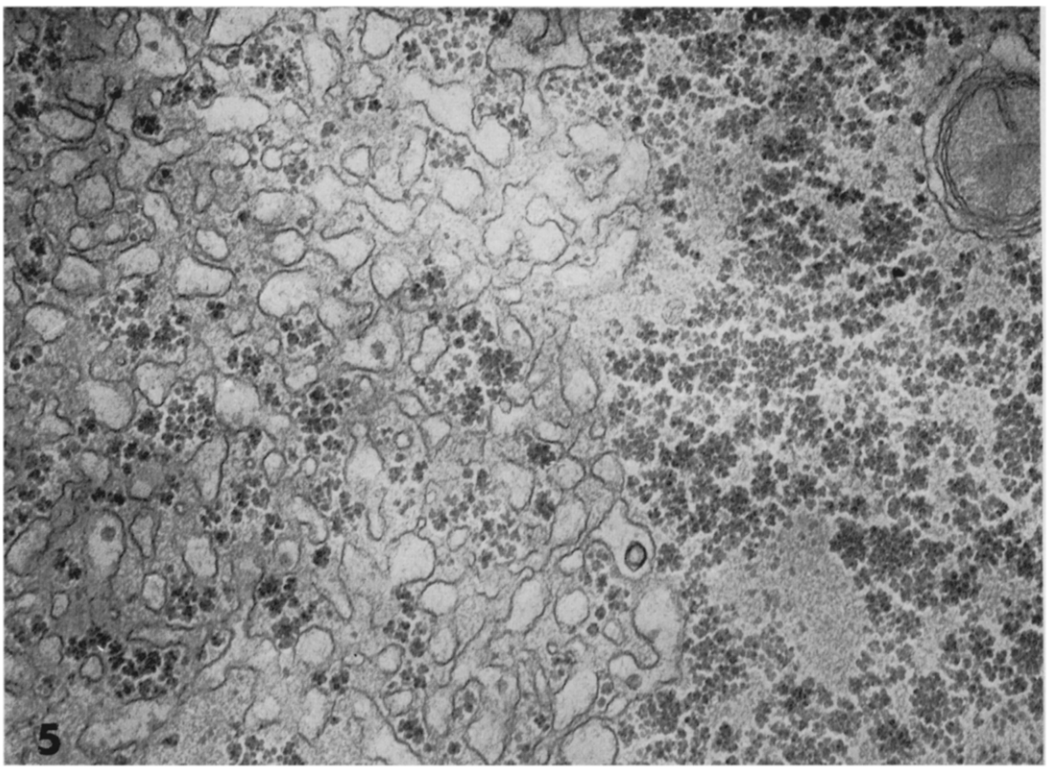


Fig. 4. Pre-neoplastic 2-AAF liver. The electron micrograph shows a portion of a hepatic cell which is facing a liver sinusoid. The cell surface facing the space of Disse is very irregular and it appears (arrow) that small granules are being released into the space from cytoplasmic vesicles. A long microtubule (mt) *is present in the cytoplasm.*
Magnification × 22,500.



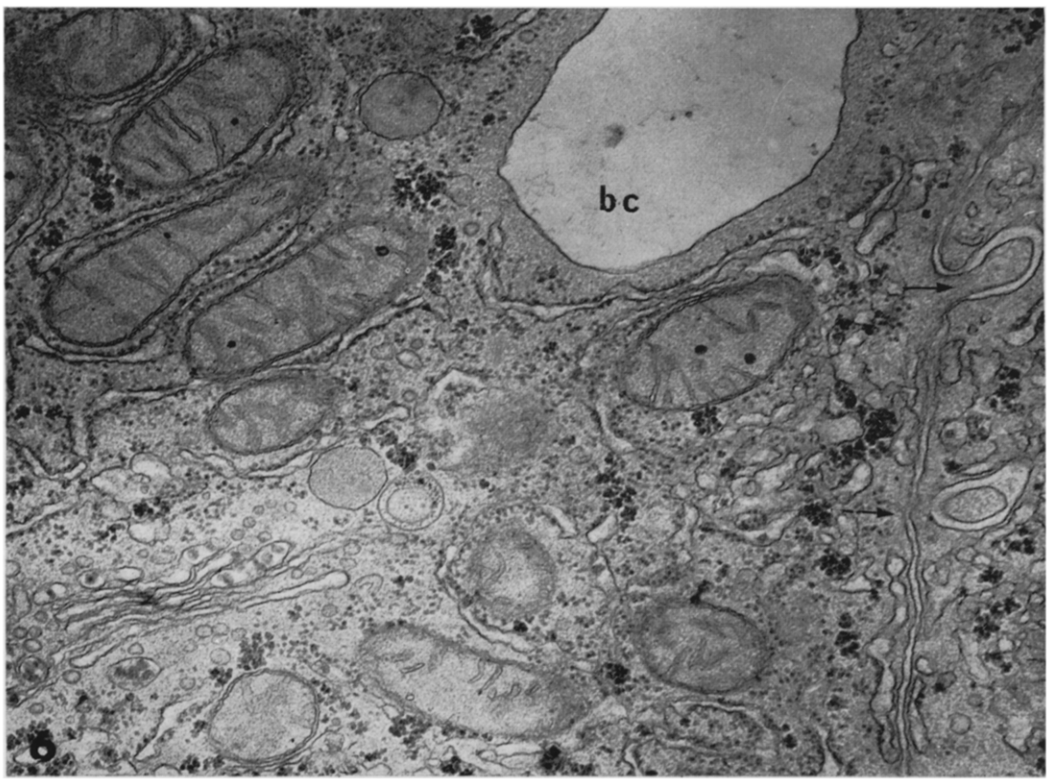


Fig. 5. *Pre-neoplastic* 2-*AAF liver. Detail of agranular endoplasmic reticulum, showing the form of its dilated, branching tubules and vesicles. An adjacent glycogen area is also shown.*
Magnification × 45,000.

Fig. 6. *Pre-neoplastic* 2-*AAF liver. The cell membrane possesses two protrusions* (*arrows*) *and a dilatation canaliculus is present* (bc).
Magnification × 30,000.

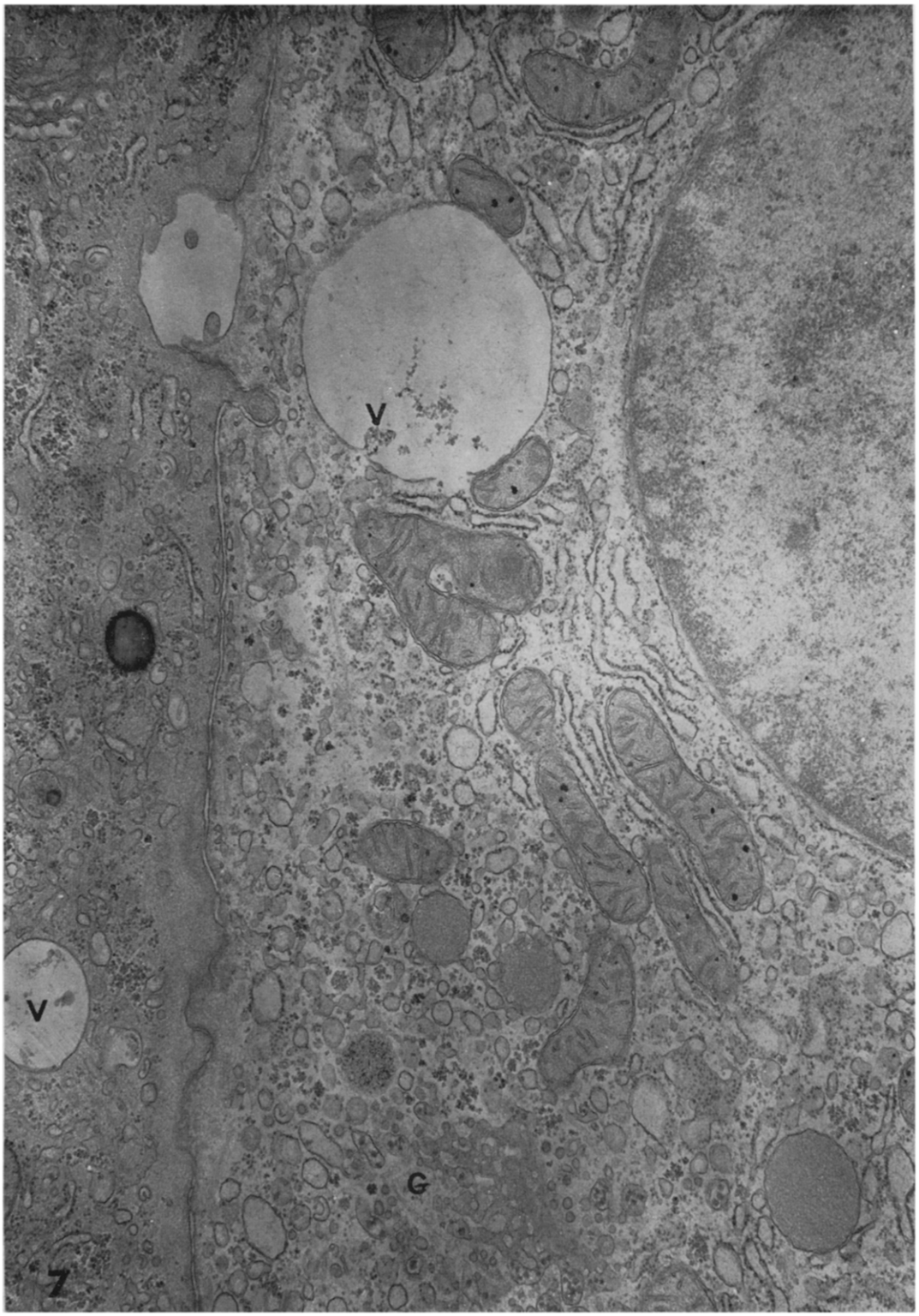


Fig. 7. Pre-neoplastic 2-AAF liver. The cell membranes of two hepatocytes are closely applied, with numerous desmosomes. A cytoplasmic protrusion is present near the bile canaliculus. Two vacuoles are also shown (v) *containing small amounts of granular material. The larger of these has two small mitochondria closely applied to its limiting membrane. The granular endoplasmic reticulum is irregularly deleted; cisternae are sometimes almost circular in outline. A Golgi zone* (G) *is present which reveals the fenestration of its membrane.*
Magnification × 15,000.

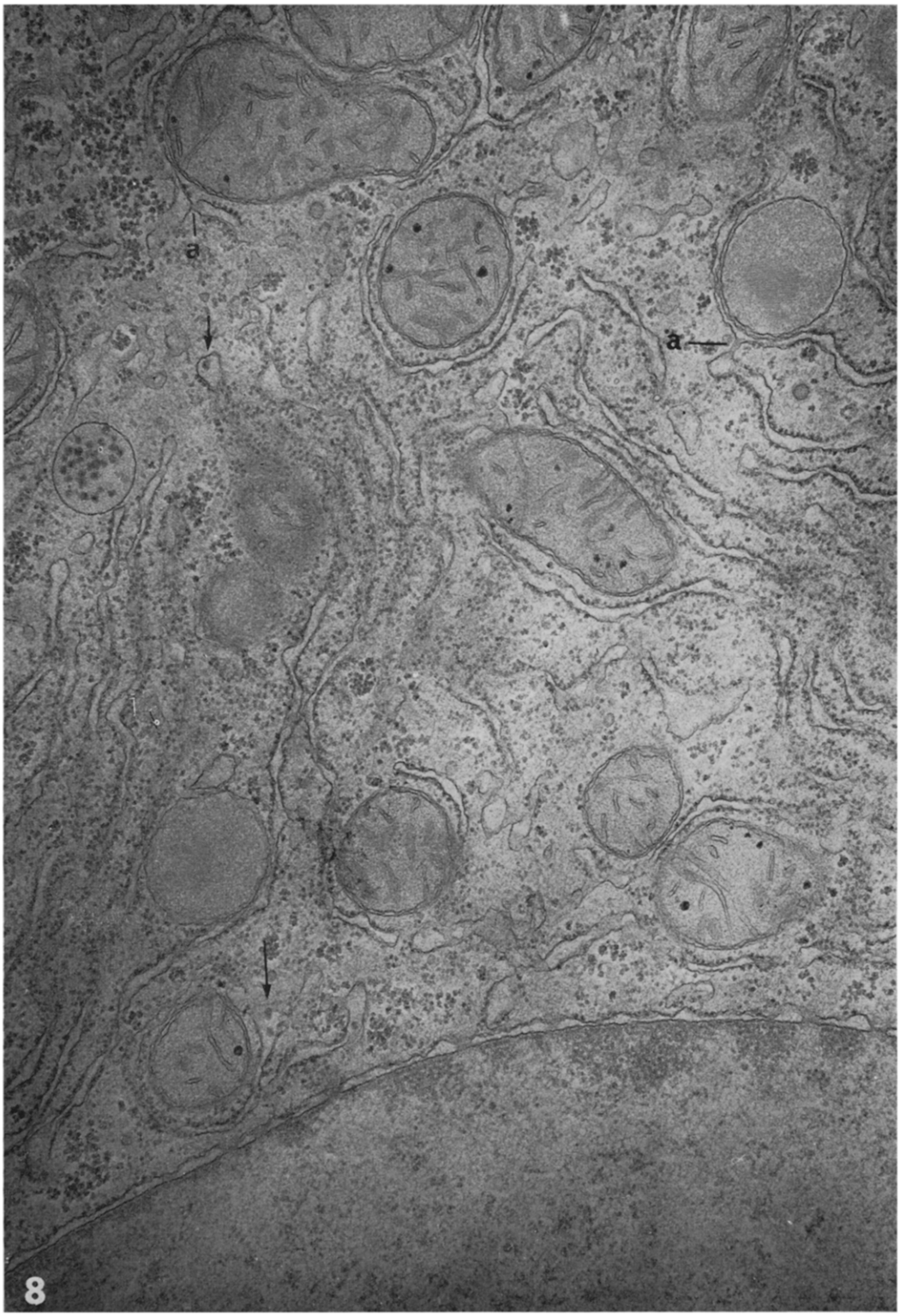


Fig. 8. *Pre-neoplastic* 2-*AAF liver. The granular endoplasmic reticulum contains granules* (*arrows*) *and anstomoses are observed between adjacent cisternae* (a).
Magnification × 30,000.

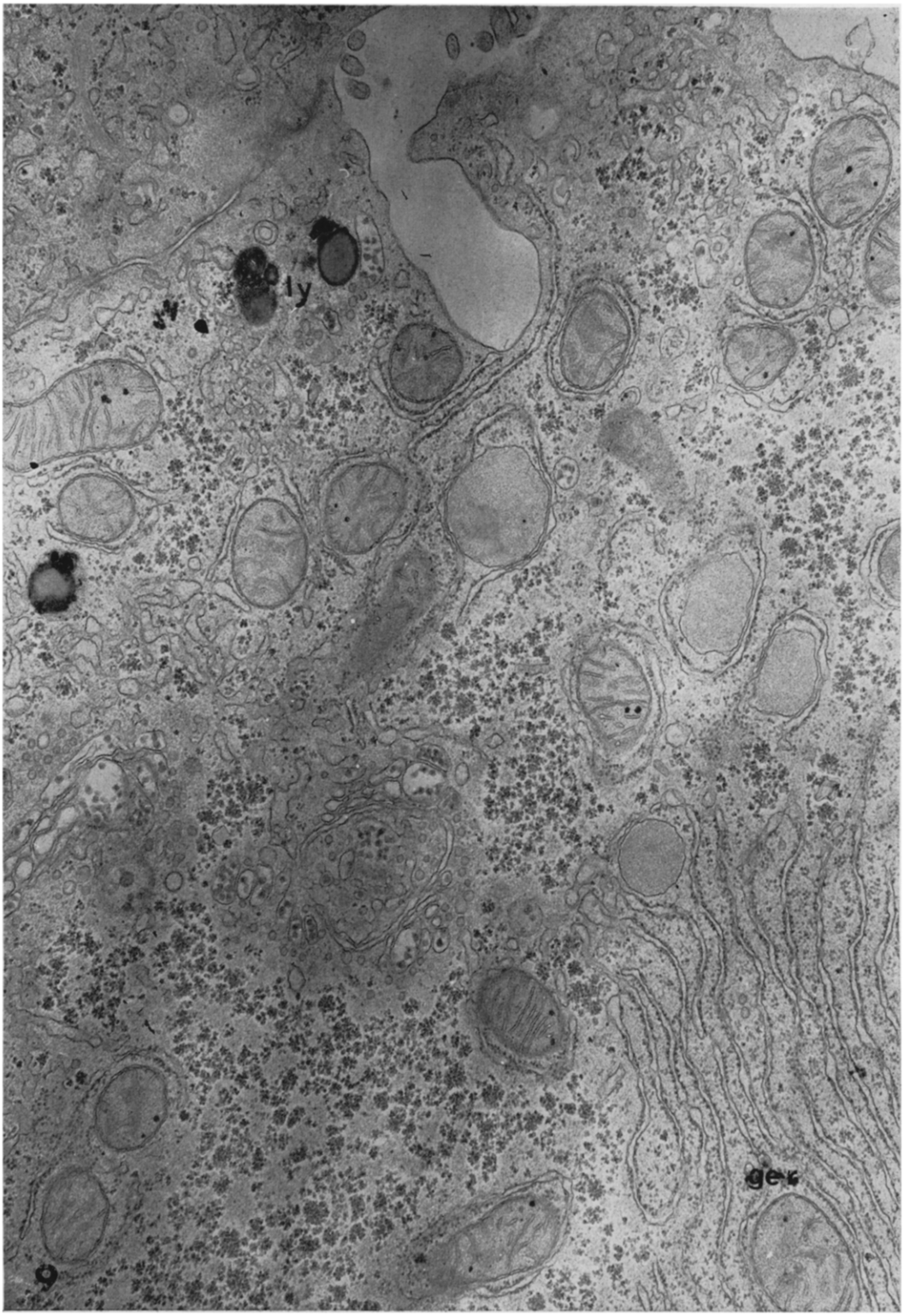


Fig. 9. *Pre-neoplastic* 2-*AAF liver. The upper part of the field shows a dilated bile canaliculus and bodies having the ultrastructure of lysosomes* (ly). *An unusual parallel array of rough-surfaced cisternae, is also present* (ger). *The mitochondria of the* 2-*AAF liver cell, although approximately normal in form are considerably smaller than those of the normal hepatic cell.*
Magnification × 22,500.

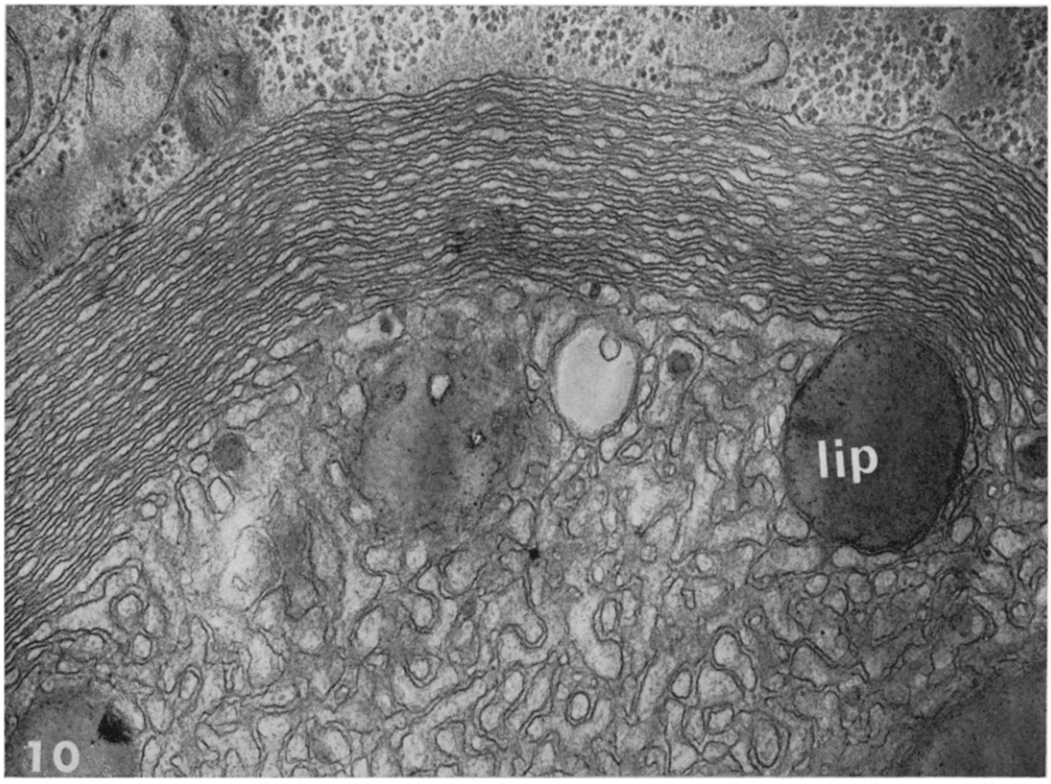


Fig. 10. *Pre-neoplastic 2-AAF liver. Part of a myelin figure of Nebenkern is shown. This is limited by a series of parallel, closely packed membranes, forming cisternae which are continuous with the smooth surfaced, membraneous tubules and vesicles which fill the interior of the structure. Large lipid inclusions are also present, (lip).*
Magnification × 35,000.

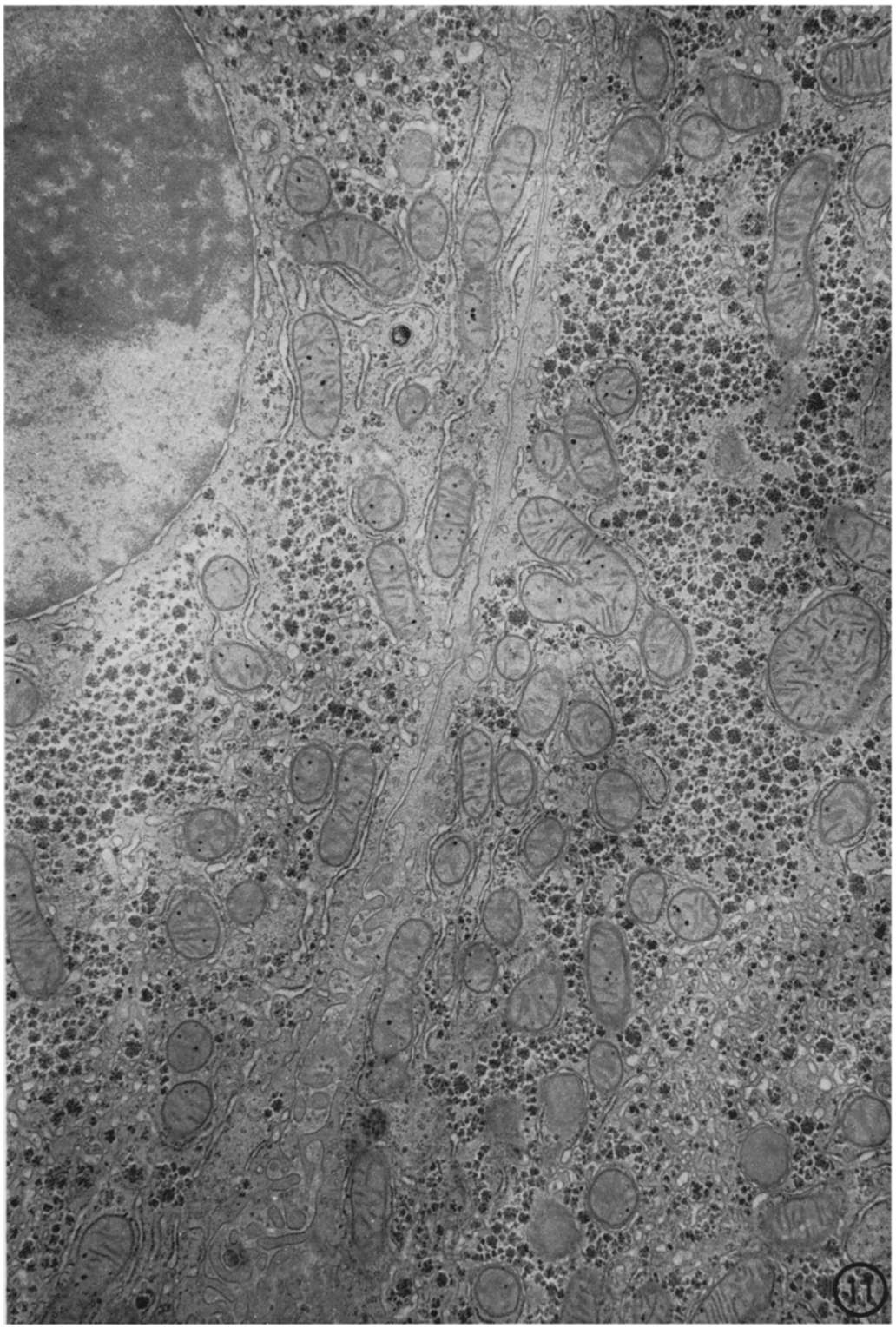


Fig. 11. *Pre-neoplastic 2-AAF liver. Portions of two hepatocytes are shown. The nucleus contains a a diffuse, prominent nucleolus.*
Magnification × 15,000.

sampling. This structural similarity was observed at both histological and ultra-structural levels of organisation and appeared to persist throughout the remaining life-span of these animals.

Electron microscopical study of the hepatocytes from the pre-neoplastic livers of 2-AAF-treated animals (Fig. 2) revealed that there was only partial recovery from the effects of the carcinogen, even up to 6 months following the administration of the last dose of 2-AAF.

The granular endoplasmic reticulum was present in appreciable quantities in the hepatic cells of experimental animals, although it was by no means as abundant as in normal rat hepatocytes. It was usually present in the form of irregularly distended cisternal profiles which, while showing considerable variation in length were usually short. The cisternae were often fenestrated and anastomoses were frequently observed between adjadent profiles (Fig. 8). In some specimens, small numbers of granules were present within the cisternae membranes, perhaps consisting of secretory material. In spite of the considerable cisternal dilatation, the granular endoplasmic reticulum possessed numerous attached ribosomes. Only occasionally were observations made of parallel arrays of rough-surfaced membranes of a size approaching that of the similar arrays which are found in normal liver cells (Fig. 9).

The agranular endoplasmic reticulum was present in abundance, in the form of dilated branching tubules and irregular vesicles, distributed throughout most regions of the cytoplasm (Figs. 3–5). The quantity of this organelle which was present in the hepatocytes of pre-neoplastict appeared to be significantly greater than in normal liver. However, apart from some dilatation, its morphological appearance was similar to that found in the normal rat hepatic cell.

The hepatocytes of experimental animals contained glycogen in the form of rosette-like aggregates of granules, having a normal appearance and normal abundance. The distribution of glycogen within the cytoplasm was also normal, the glycogen being segregated into 'glycogen areas' as well as being interspersed with smooth-surfaced elements of the endoplasmic reticulum.

The mitochondria in general, appeared to possess the ultra-structure of those found in normal hepatic cells, except for the occurrence of elongated and branching forms (Figs. 3 and 7). The cristae mitochondriales were present in numbers which were equal to or greater than those in normal hepatocyte mitochondria. The mitochondria appeared to be substantially smaller than normal. They were often smaller than the microbodies and their average diameter was approximately 60–70% of that of normal mitochondria. For purposes of measurement, comparisons were made between normal and preneoplastic liver cells which had been subjected to identical preparative techniques.

The Golgi zones (Figs. 2, 6, 7, and 9) were well-developed, consisting of smooth-surfaced, flattened cisternae, which were often fenestrated, together with small and large vesicles enclosing numerous granules. There was an abundance of similar granules in the insusoidal spaces, and regions of the hepatic cell surface were not infrequently observed where these granules appeared to be in the process of being released from cytoplasmic vesicles into the space of Disse (Fig. 4).

In addition to lysosomes having a normal ultra-structural appearance (Fig. 9), the cytoplasm frequently exhibited the presence of membrane-bound vacuoles of variable size and appearance (Fig. 3). These were found to enclose variable quantities of disorganized membranes or coarse, granular material or both. In some cases, these vacuoles also contained large osmiophilic granules, presumably lipidic in character, which may possibly have been derived from the breakdown of lipoprotein membranes. The vacuoles may represent sites of localised cytoplasmic breakdown and may, therefore, be more appropriately named autophagic vacuoles or cytolysomes.

The cell membranes of adjacent hepatocytes were closely apposed in regions desmosomes and structures resembling tight junctions occasionally being present. However, the Bile canaliculi were often large and extensive, containing abundant microvilli or were irregularly dilated and possessed few microvilli. The cell membrane of one hepatic cell was frequently invaginated by protrusions of the cytoplasm of another (Fig. 6). Surface relations between cells, therefore, appeared abnormally variable and irregular in character. The hepatic cell surfaces which face the space of Disse were abnormally irregular in outline.

The nuclei of the hepatic cells fron experimental animals frequently contained diffuse hypertrophied nucleoli, and the nuclear membrane was interrupted by abnormally large numbers of nuclear pores (Fig. 3).

The presence was observed, in a few cells, of membraneous structures resembling myelin figures or Nebenkerne (Fig. 10). These were roughly circular in section and consisted of concentric layers of smooth-surfaced mem-

branes enclosing a central space. The central space was filled with closely-packed, smooth, membraneous vesicles and tubules and with large, lipid granules. Close examination revealed that the concentric membranes from cisternae which were continuous with the central, membraneous structures.

In general, the fine structural organisation of the hepatic cells from the pre-neoplastic livers, although differing in many respects from that of normal rat liver cells, showed little evidence of serious disruption, and these cells appear to be functionally active, using morphological criteria alone.

DISCUSSION

The early changes produced by 2-AAF in rat hepatocytes resembled, in general, those produced by various other chemicals, both carcinogenic and non-carcinogenic. They might, therefore, be regarded as non-specific reactions to the toxicity of this agent. During the later stages of 2-AAF feeding, the liver hyperplasia was accompanied by only limited recovery of normal ultrastructures, while further changes in the form of the endoplasmic reticulum and mitochondria had occurred, possibly due to chronic 2-AAF toxicity. From the viewpoint of carcinogenesis, the fine structure of pre-neoplastic liver, uncomplicated by the continued presence of 2-AAF, is perhaps of more direct interest. In contrast to some previous reports [25, 26], the presence of tumours did not seem to affect the liver cell ultrastructure.

The granular endoplasmic reticulum in the hepatocytes of 2-AAF treated liver was observed to be decreased in amount and of an abnormal form, and this did not return to normal when the 2-AAF feeding was stopped. The dilatation of the rough surfaced cisternae could have been the result of an accumulation of protein within their lumina. The frequent occurrence of granular material within the cisternae lends some support to this idea, as do a number of other observations which will be discussed now in relation to their possible significance to protein synthesis. It seems unlikely that the dilatation was due to a fixation artefact, since identically prepared normal liver cells did not possess this feature.

The nucleolar hypertrophy which has been noted in the pre-neoplastic liver is consistent with the report by Rutman, Cantarow and Pashkis [27], that 2-AAF produced an increase in the RNA of the nuclear fraction of rat liver and might be related to increased protein synthesis, since the nucleolus is generally considered to be the main site of ribosomal RNA production. Nucleolar hypertrophy was accompanied by an increase in the number of pores in the nuclear membrane. A similar association between these two changes was observed by Salomon, Salomon and Bernhard [11] in the hepatic cells of rats fed with thioacetamide. However, these changes occurred shortly after giving thioacetamide, whereas 2-AAF did not produce them until after prolonged feeding. The nuclear pores may represent a route of nucleo-cytoplasmic exchange of particulate material [28–31], probably not by simple permeability [32] but by an energy-dependent process [33]. Thus, the present findings would not be inconsistent with an increase in nuclear RNA synthesis.

The presence of hypertrophic Golgi zones, containing numerous granules, in the pre-neoplastic liver cells is further support for the concept of an increased RNA and protein synthesis, in view of the accepted role of this organelle [34–37]. The granules formed in the Golgi zones were similar, with regard to size and electron density, to those found within the granular, endoplasmic reticulum and, in abundance, in the liver sinusoids. They also resembled those which may have been in the process of being released from the sinusoidal surfaces of some hepatocytes as shown in Fig. 4.

Part of the activity of the Golgi zones may have been directed towards the production of lysosomes [38, 39], since numbers of cytolysomes or autophagic vacuoles (Fig. 3) were observed. As these are rare in normal liver cells, they were probably the result of an increased rate of intracellular autolysis [40–42].

It would seem that 2-AAF produces permanent changes in those components of rat hepatic cells which are involved in the synthesis of non-structural or 'export' proteins. The earliest visible indication of this lesion may be alterations of the granular endoplasmic reticulum, whose role is central to 'export' protein synthesis [43–46].

The changes in the granular endoplasmic reticulum which result from prolonged 2-AAF feeding may differ functionally from the early alterations in this organelle. Laws has shown [47, 48] that while short periods of 2-AAF feeding inhibit liver regeneration following partial hepatectomy, longer periods do not. This also indicates that there are qualitative differences between the lesions present at the different stages.

The recovery of normal glycogen stores which was observed, has also been noted by Salomon *et al.* [10, 11] during prolonged

thioacetamide feeding, but in their experiments it occurred whilst treatment was still in progress. In the preneoplastic liver cells glycogen accumulation was accompanied by partial recovery of the normal morphology of the agranular endoplasmic reticulum, although abnormally high amounts of the latter appeared to be present [Figs. 3–5]. This organelle may have a functional relationship to glycogen storage in these cells [13, 49–52], whereas its proliferation during the early stages of 2-AAF feeding is probably related to deoxication [53].

Insufficient biochemical information is available to allow a functional interpretation of the permanent decrease in mitochondrial size. 2-AAF seems to resemble thioacetamide with respect to the bile canalicular changes which it produces [11]. These changes, together with the increased irregularity of the hepatic cell surface and the presence of unusual cytoplasmic invaginations, indicate that the normal type of contact between adjacent cells was permanently altered, although it is by no means clear that any overall loss of adhesion had occurred. However, this finding was not unexpected, in view of the changed histological structure of the liver tissue at this stage.

The occasional myelin figures are, apparently, of a transient nature and have been observed in both hepatic and other cells, following various experimental procedures [3, 7, 9, 10, 54, 55]. Their presence is, therefore, non-specific.

It was somewhat surprising that the appearance of the pre-neoplastic liver tissue, when examined by either light or electron microscopy, should have remained constant throughout the experiment, whether or not the animals were bearing tumour at the time of sampling. This was not entirely consistent with the observations of Ghadially and Parry [25, 26] on the liver cells of the tumour-bearing rat, However, it does not appear that the presence of the tumours *per se* in an animal, necessarily affects the fine structure of its hepatocytes.

The livers of 2-AAF treated rats contained increasing numbers of separate hepatomata with increasing time. The highly differentiated nature of many of these tumours precludes the possibility that they may have arisen by metastasis. In view of this behaviour, it is possible to speculate that most, if not all, the hepatic cells of the pre-neoplastic livers may be capable of producing tumours, given sufficient time. This view could be examined critically by study of the primary hepatomata induced by 2-AAF in the rat.

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RESUME

Des rats albinos mâles, de la lignée de Leeds, ont été soumis, durant une période maximale 10 *mois, à une alimentation contenant* 0,05% *de* 2-*acétylaminofluorène* (2-*AAF*). *Ils étaient remis ensuite à un régime dépourvu de substance cancérigène. Les animaux furent tués, à intervalles, pendant le traitement au* 2-*AAF, et pendant une période de* 2 *à* 6 *mois après l'arrêt du traitement. Les foies, prélevés au cours de cette dernière période, étaient considérés comme prénéoplasiques; à la fin de cette période, tous les animaux étaient porteurs de tumeurs hépatiques.*

L'examen des cellules hépatiques, en microscopie électronique, a montré que les modifications, observées pendant le traitement, étaient probablement non spécifiques. Au contraire, certaines modifications permanentes de l'ultra-structure des cellules hépatiques étaient présentes dans les foies prénéoplasiques. Les altérations les plus remarquables consistaient en une hypertrophie des nucléoles, une dilatation du réticulum endoplasmique granuleux, une diminution de la taille des mitochondries, une abondance du réticulum endoplasmique lisse. On notait aussi des anomalies de la membrane cellulaire. La présence de tumeurs hépatiques ne paraissait pas modifier la structure fine des cellules hépatiques.

Les auteurs discutent la signification des différences d'altérations observées pendant le traitement et après celui-ci. Ils en concluent que certaines modifications permanentes pourraient refléter des altérations du mécanisme de synthèse des protéines dans les cellules des foies prénéoplasiques.

SUMMARY

Male albino rats of the Leeds strain were fed on a diet containing 0·05% *of* 2-*acetylaminofluorene* (2-*AAF*) *for up to* 10 *months, before being returned to a carcinogen-free*

diet. Animals were sacrificed at intervals during 2-AAF treatment and from approximately 2 months up to 6 months after the cessation of treatment. During the post-treatment period, the rat livers were considered to be in a pre-neoplastic state and all animals bore liver tumours by the end of this period.

Electron microscope studies of the hepatic cells showed that, while many of the changes observed during treatment were probably non-specific, there were a number of permanent changes in the fine structure of the hepatic cells of the pre-neoplastic rat livers. The most conspicuous of these included hypertrophy, dilation of the granular endoplasmic reticulum, decreased mitochondrial size and the presence of abundant agranular endoplasmic reticulum. Abnormal modifications of the cell membrane were also present. It was noted that the fine structure of the hepatic cells did not appear to be affected by the presence of liver tumours. The observed changes and their relation to those observed during 2-AAF treatment, have been discussed.

The conclusion has been drawn that at least some of the permanent changes may reflect alterations in the protein-synthesis apparatus of the pre-neoplastic liver cells.

ZUSAMMENFASSUNG

Männliche Albinoratten erhielten bis zu einer Dauer von 10 Monaten eine Diät, die 0,05% 2-Aethylaminofluoren (2-AAF) enthielt, anschliessend wieder carcinogenfreie Normalkost. Die Tiere wurden getötet, in Intervallen, während der 2-AAF Behandlung und in den folgenden 2-6 Monaten. Nach Beendigung der applikation erschienen die Rattenlebern in einem prä-neoplastischen Stadium und zu Ende dieser Periode trugen alle Tiere Lebertumoren.

Elektronenmikrokopsche Studien an d n Leberzellen zeigten neben vielen wahrscheinlich unspezifischen Veränderungen eine Reihe von permanenten Veränderungen in der Feinstruktur der Zellen präneoplastischer Rattenlebern. Die auffallendsten Merkmale waren nukleolare Hypertrophie, Erweiterung des granulären endoplasmatischen Retikulums, verringerte Mitochondriengrösse und das reichliche Vorhandensein von agranulärem endoplasmatischem Retikulum. Abnorme Modifikationen der Zellmembran traten ebenfalls auf. Es wurde festgestellt, dass die Feinstruktur der Leberzellen nicht durch die Gegenwart von Lebertumoren beeinflusst zu sein schien. Die geschilderten Veränderungen und ihre Beziehung zu Zellveränderungen, die während der 2-AAF Behandlung auftraten, wurde diskutiert.

Es wird angenommen, dass mindestens einige der permanenten Veränderungen die Antwort auf Störungen des Proteinsyntheseapparats in den präneoplastischen Leberzellen bedeuten könnten.

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The Lymph Node Barrier against Vx2 Cancer Cells before, during and after Lymphography

A Preliminary Report of Experiments on Rabbits

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LYMPHOGRAPHY is a well-established diagnostic procedure. It is used in searching for lymph-borne metastases of malignant tumours. The reported complications of the procedure include wound infection, lymphangitis and oil embolism to the brain and the lungs [1–4].

The possibility that tumour cells may be released and transported by the oily contrast medium during lymphography was studied by Tjernberg *et al.* [5] in human subjects. After cannulation of the thoracic duct, lymphography was performed *via* pedal lymphatics and lymph was collected from the cannula for cytologic analysis. In a series comprising 4 cases, no evidence was obtained that tumour cells were transported with the contrast medium [6].

The effect of lympography on the barrier function of lymph nodes has received little attention in the literature. It is conceivable that the lymph node barrier against cancer cells is disturbed by lymphography, thus permitting lymphatic dissemination of the tumour during or after lymphography. An investigation of the possible effect of lymphography on the barrier function of lymph nodes seemed therefore to be warranted.

The most appropriate procedure in such an investigation involves cannulation of afferent and efferent lymphatics of a single node, injection of contrast medium and cancer cells into the afferent vessel and collection of efferent fluid for cytologic analysis. Experiments of this type obviously cannot be made on human subjects.

In the present study rabbits were used. Vx2 carcinoma cells were injected into a lymphatic entering the popliteal lymph node before and after lymphography. The barrier function of the node was assessed by studying the efferent lymph for presence of the Vx2 cells.

A description of the technique is now presented along with a preliminary report of findings in 16 rabbits.

MATERIALS AND METHODS

Cannulation of lymphatics

Simultaneous cannulation of afferent and efferent vessels of single lymph nodes has previously been reported by only a few workers. Drinker *et al.* [7] and Widdicombe *et al.* [8] used the method for studying the barrier function of isolated lymph nodes in dogs and rabbits, respectively, against bacteria and erythrocytes. Sabiston *et al.* [9] cannulated afferent and efferent lymph vessels in investigations of the lymphatico-venous pathways in isolated lymph nodes in the neck of dogs. Hall and Morris [10] used a similar technique in sheep to observe changes in the cell population of efferent lymph after injection of antigen into afferent vessels. Fisher and Fisher [11] applied the procedure in studies

of the barrier function of normal popliteal lymph nodes in rabbits against transplantable tumour cells.

Our investigation of the barrier function of popliteal lymph nodes against cancer cells in connection with lymphography was performed on full-grown domestic rabbits of both sexes. The rabbits were anaesthetized *via* a polythene catheter (PE 50, Clay-Adams Co. Inc.) inserted into a marginal ear vein. Veterinary Nembutal (Abbott 60 mg/ml) diluted 1:5 in 0·9 % NaCl was used. By leaving the catheter in position throughout the experiment, maintenance doses could be given as required.

The lateral aspect of the thigh was shaved and cleaned and the popliteal fossa was explored through an incision into skin and muscle. The rabbit has a single lymph node in the popliteal fossa. This node has four to six afferent lymph vessels and as a rule a single efferent vessel, which is easily identifiable under a dissection microscope. If two efferent vessels were seen the experiment was discontinued.

The efferent lymph vessel was carefully freed from its surrounding tissues. A polythene catheter (PE 50), having at its tip a small cuff, was inserted into this vessel and was fixed with a ligature. The cuff was formed by heating the catheter tip over a flame [12]. The afferent lymphatics were visualized by injection of about 1ml of Patent Blue Violet (Gurr & Co.) into the paw. The most prominent of the afferent vessels was then threaded with a metal cannula or a polythene catheter (PE 10) introduced in proximal direction close to the node and fixed with a ligature (Fig. 1). To permit insertion of the catheter, its tip had to be thinned out by pulling it over a flame as recommended by Fisch [13].

The cannulations were done under a Zeiss dissection microscope and at 6–16 × magnification. In order to test the system for leakage, 1 ml of 0·9 % NaCl was injected into the afferent lymphatic and the fluid from the efferent vessel was collected in a test tube.

Cannulation of afferent and efferent lymphatics was successfully performed in 16 experiments. After a little practice, we found no difficulty in cannulating efferent lymphatics, but the greater difficuties presented by the finer afferent vessels constituted the main reason for lack of success in 6 experiments.

Preparation of cell suspensions for infusion

A prerequisite for studying the barrier function of lymph nodes is that the cells which may penctrate the barrier should be readily identifiable. Tjernberg and Zajicek [14] found unmistakable malignant cells in efferent lymph after digital massage of lymph nodes with metastatic Vx2 carcinoma. A search of the literature, however, has revealed no investigation on whether or not individual cells of Vx2 carcinoma can always be confidently distinguished on morphologic grounds (cell size, cytoplasmic: nuclear ratio, etc.) from the cells normally present in the lymph.

We therefore performed morphologic studies on Vx2 carcinoma cells of two types. These cells were obtained either directly by aspiration biopsy of transplantable tumours growing in domestic rabbits (transplantable tumour cells, Fig. 2) or were taken from tissue cultures (cultured tumour cells, Fig. 3).

Tissue cultures of Vx2 carcinoma cells were prepared by one of us (C. Rubio) as follows. A biopsy specimen measuring approximately 2 cm^3 was taken from a 1-month-old transplanted Vx2 tumour in a rabbit and was immersed in sterile Early medium complemented with L-glutamine, antibiotics and 20% bovine serum. Using sterile technique, the specimen was transferred to a Petri dish and areas of suspected necrosis were removed. The specimen was now washed in various changes of the mentioned medium and was chopped until a creamy material was obtained. After addition of 100 ml trypsin (1 % in trypsin buffer SBL), the specimen was incubated under constant magnetic stirring for ten minutes. The supernatant was discarded. The sediment was resuspended in 100 ml trypsin solution and the suspension was incubated for an additional 40 min. In order to remove clumps, the suspension was sieved through four layers of surgical gauze.

The percolated material was centrifuged at room temperature (1000 rev/min for 3 minutes) and the resulting sediment was resuspended in fresh medium. From this suspension 12×10^6 trypan-blue negative cells were transferred in 15 ml of the medium to stoppered milk flasks. After a lag period of three weeks, the cultures entered into a phase of growth. Sixteen passages were then made before the cells were ready for use in our experiments.

These cultured Vx2 carcinoma cells were suspended in rabbit lymph which had been rendered acellular by centrifugation. The transplantable Vx2 cells obtained directly by aspiration biopsy were suspended in the same way.

The tumour cells were sedimented by centrifugation of the suspension. They were then washed in 0·9% NaCl and fixed with methanol-acetic acid mixture [14]. To a drop of fixed

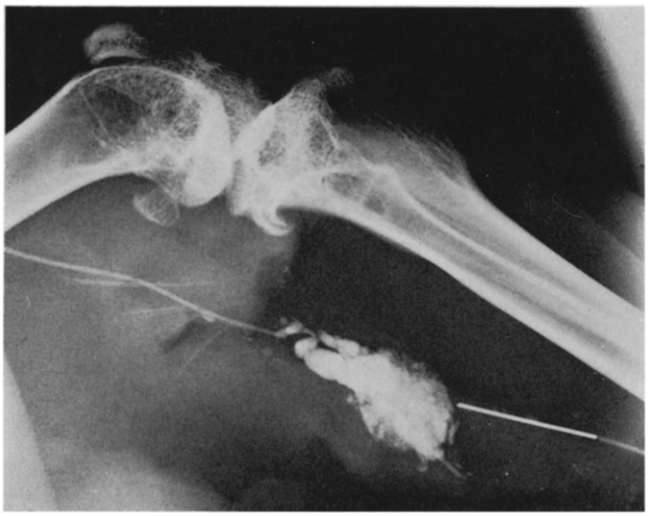


Fig. 1. *Lymphogram showing the popliteal node in a rabbit. A polythene catheter has been inserted into the efferent vessel and a metal cannula into an afferent lymphatic.*

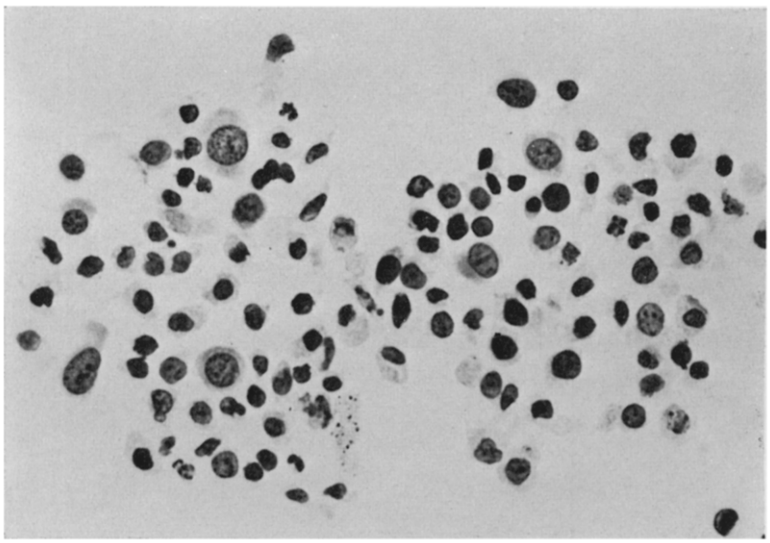


Fig. 2. *Slide prepared from Vx2 carcinoma cells aspirated from a tumour growing in the hind paw of a rabbit. Harris' haematoxylin stain* × 480.

(to face p. 306)



Fig. 3. *Cultured Vx2 carcinoma cells spread on a slide after trypsinization and washing. Harris' haematoxylin stain* ×480.

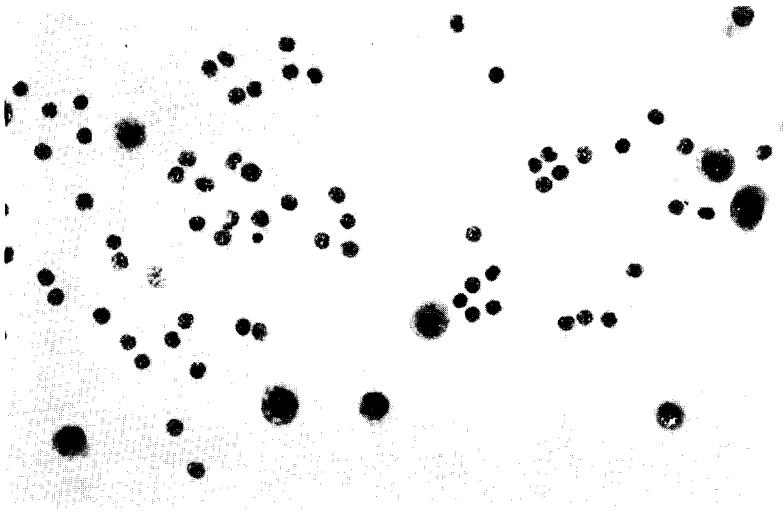


Fig. 6. *Cultured Vx2 carcinoma cells collected from the efferent popliteal lymphatic. The cells had been injected into the afferent lymphatic of the same node after lymphography with Lipiodol Ultrafluid. Harris' haematoxylin stain* ×480.

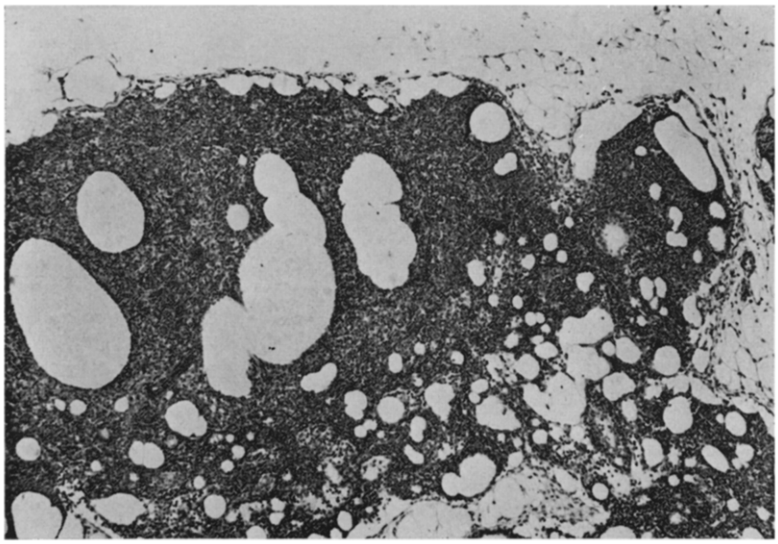


Fig. 7. *Section from a popliteal lymph node after lymphography with oily contrast medium (Lipiodol Ultrafluid). Sinuses are greatly distended by the medium. Haematoxylin-eosin stain* $\times$ 75,6.

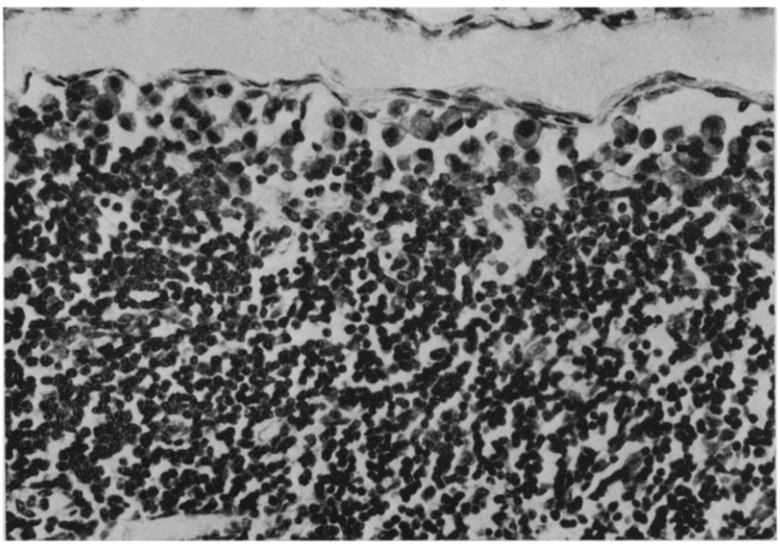


Fig. 8. *Section from a popliteal lymph node after lymphography with water-soluble contrast medium (Urografin) and subsequent infusion of cultured Vx2 carcinoma cells into the afferent lymphatic. The carcinoma cells have accumulated in subcapsular sinuses. Haematoxylin-eosin* $\times$ 270.

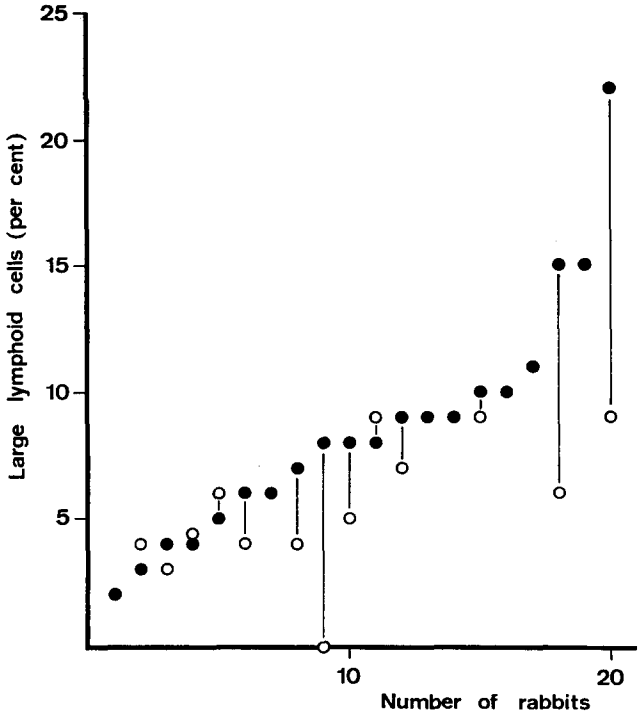


Fig. 4. *Percentages of large lymphoid cells (dia.* $\geqslant 8$ μ) *in efferent lymph from the popliteal node in* 20 *rabbits (filled circles) and in fluid leaving the node after perfusion with* 1 *ml* 0·9% *NaCl in* 13 *rabbits (open circles).*
The rabbits are arranged in order of frequency of large cells in the efferent lymph.

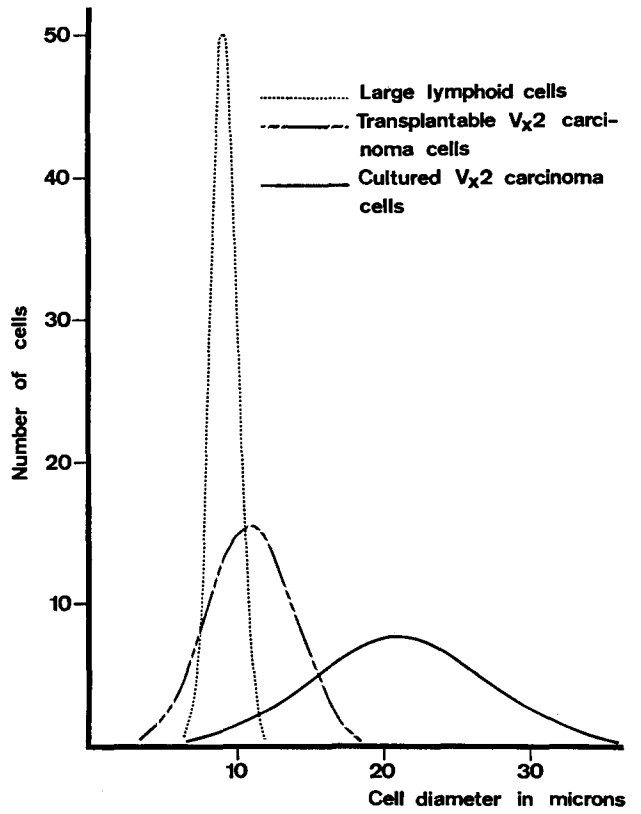


Fig. 5. *Size distribution curves for large lymphoid cells (diameter* $\geqslant$ 8 μ), *aspirated Vx2 carcinoma cells and cultured Vx2 carcinoma cells.*

carcinoma cells on a glass slide a drop of 1% pectin adhesive solution [15] was added and mixed well. The slides were dried in air and then stained with haematoxylin-eosin. Slides were prepared in the same way from cells sedimented from efferent lymph.

Comparisons of the slides showed that small lymphoid cells (diameter up to 8μ) could readily be distinguished from both aspirated and cultured Vx2 carcinoma cells by virtue of *morphologic details in the cytoplasm or nuclei.* Possible exceptions were lymphocytes with pyknotic nuclei, which were occasionally found in the efferent lymph and closely resembled pyknotic, small Vx2 carcinoma cells in aspirate.

When cell diameter was 8μ or more, however, differentiation on the same morphologic basis between lymphoid cells and aspirated Vx2 carcinoma cells was difficult. The frequency of such large lymphoid cells among 200 consecutive cells in the lymph leaving a popliteal node was determined in 20 healthy rabbits. In 13 of them the fluid leaving the node after perfusion with 1 ml 0·9% NaCl was also analyzed. The results are shown in Fig. 4. Lymphoid cells whose diameter was at least 8μ formed up to 22% of the total cell population in the undiluted lymph (filled circles) and up to 9% in the efferent fluid after NaCl perfusion (open circles).

Since individual cells of transplantable Vx2 carcinoma were not reliably distinguishable from the larger cells commonly occurring in normal efferent lymph, the possibilities offered by the cultured Vx2 cells were next explored. These cells appeared generally to be larger than the Vx2 cells aspirated from transplantable tumours, and therefore the feasibility of basing the differentiation on *cell size* was investigated.

For this purpose, micrometric determinations were made on consecutively observed cells—100 Vx2 cells from aspirates and 100 from cultures and also 100 lymphoid cells with a diameter of at least 8μ. Figure 5 presents the size distribution curves with 95% tolerance limits at 95% confidence levels. The limits for the curve representing large lymphoid cells are wholly within those of the curve for aspirated Vx2 carcinoma cells. The tolerance limits were calculated from the formula $\bar{x} \pm 2 \cdot 233\, s$, where $\bar{x}$ is the mean and s the standard deviation.

	Lower limit	Upper limit
Large lymphoid cells	6·99	11·07
Aspirated Vx2 carcinoma cells	4·45	17·39
Tissue-cultured Vx2 cells	7·97	34·40

The numerical calculations of the tolerance limits were as follows.

From these studies it was concluded that individual Vx2 cells aspirated from transplantable tumours cannot always be distinguished from large lymphoid cells on morphologic grounds, including cell size. Cultured Vx2 carcinoma cells, however, are larger than the cells in efferent lymph and thus are more easily identified. Consequently, cultured Vx2 carcinoma cells were used in the present investigation on the barrier function of lymph nodes in connection with lymphography.

Intralymphatic infusion of cultured Vx2 carcinoma cells

The cells were taken from culture flasks and after trypsinization were washed twice in Hanks' balanced salt solution. Concentrations of $1 \cdot 5\text{–}2 \times 10^6$ cancer cells in 1 ml of Hanks' solution were used for infusion. One ml of the cell suspension was injected manually, using an ordinary plastic syringe, into a cannulated afferent vessel over a period of 20 to 30 minutes. In this way 50,000 – 100,000 cancer cells per min. entered the lymph node. During the infusion the syringe was repeatedly shaken in order to minimize sedimentation of the cells.

The efferent lymph was collected in siliconized tubes which contained 2 drops of heparin. The tubes were immersed in an icebath (+4°C).

Infusion of 0·9% NaCl

After infusion of the Vx2 cancer cell suspension, 1 ml of 0·9% NaCl solution was injected into the same vessel and the efferent fluid was collected as before for cytologic analysis.

Lymphography

After infusion of 1 ml of 0·9% NaCl the contrast medium was injected into the afferent lymphatic over a period of 15 to 25 minutes. When a few drops of the contrast medium were recovered from the efferent vessel, the injection was discontinued. The volume of injected contrast medium was about 0·2–0·3 ml. Lipiodol Ultrafluid (Laboratories André Guerbet, France) was used in 6 cases, Urografin 60% (Schering A. G., Berlin) in 3 cases and Emulsion Leo 372 (Leo A. B., Sweden) in 5 cases.

Post-lymphography infusion of 0·9% NaCl and of cultured Vx2 carcinoma cells

When contrast medium appeared in the efferent lymph, 1 ml of an 0·9% NaCl solution was injected over 10 to 15 minutes into the afferent vessel in 12 cases. Efferent fluid was collected for cytologic analysis as previously

Table 1. Presence of cultured Vx2 carcinoma cells in efferent lymph before, during and after lymphography. Experimental study on the popliteal node in 16 rabbits

Injection into an afferent lymphatic	No. of rabbits	Cytologic findings in efferent lymph: No cancer cells (no. of rabbits)	Cancer cells (no. of rabbits)
Cancer cells in Hanks' solution	16	15	1
0·9% NaCl solution	14	14	0
Contrast medium	14	13	1 of 6 injected with Lipiodol 0 ,, 5 ,, ,, Emulsion Leo 372 0 ,, 3 ,, ,, Urografin 60%
0·9% NaCl solution	12	10	1 ,, 4 ,, ,, Lipiodol 0 ,, 5 ,, ,, Emulsion Leo 372 1 ,, 3 ,, ,, Urografin 60%
Cancer cells in Hanks' solution	11	5	3 " 3 ,, ,, Lipiodol 2 ,, 5 ,, ,, Emulsion Leo 372 1 ,, 3 ,, ,, Urografin 60%

described. Inspection of this fluid indicated that some of the contrast medium was removed from the lymph node during the NaCl injection.

As a final step in the experimental procedure, a suspension of $1 \cdot 5–2 \times 10^6$ Vx2 cancer cells in 1 ml of Hanks' solution was injected in 11 cases and efferent lymph was again collected for cytologic analysis.

Cytologic processing

From the samples collected from efferent lymph vessels the cells were isolated as follows.

When Lipiodol had been injected, 1 ml of 0·9 % NaCl solution was added to the sample. Because of its higher specific gravity as compared with lymph or lymph-NaCl, the Lipiodol settled to the bottom of the tube. The supernatant was pipetted off and the cells were isolated by centrifugation.

When Emulsion Leo, Urografin or NaCl solution had been injected, the sample was first centrifuged at 1500 rev/min for 10 minutes. The supernatant was pipetted off and the sediment was washed twice in NaCl solution by centrifugation.

The sedimented cells were immediately fixed with methanol-acetic acid and allowed to stand overnight. The fixed cells were centrifuged at 1500 rev/min for 7 minutes and the sediment was transferred to precleaned slides. The bottom of the emptied centrifuge tube was rinsed with a drop of 1% pectin solution, which was then added to the slides to increase adhesion of the cells to the glass. The slides were stained with Harris haematoxylin for one minute and mounted with Eukitt synthetic resin.

RESULTS

Table 1 summarizes the experiments and the results of the cytologic analyses.

It is seen that following injection of cultured Vx2 carcinoma cells into an afferent lymphatic of the popliteal node, cancer cells were detected in the efferent lymph in only 1 of the 16 rabbits.

When NaCl solution was thereafter injected into the afferent lymphatic in 14 of these 16 rabbits, none of them was found to have cancer cells in the efferent lymph from this node. The reason for withdrawal of 2 rabbits at this stage of the experiments was difficulty in keeping the cannula system running. One of the 2 rabbits was that mentioned as having cancer cells in efferent lymph.

In the remaining 14 rabbits the experiments were continued by injecting contrast medium into the afferent lymph vessel. Only one of the samples collected after passage of contrast medium through the lymph node contained cancer cells (Table 1). The medium in this case was Lipiodol Ultrafluid. In the same rabbit, severe bleeding could be seen in the lymph node and the efferent lymph also was haemorrhagic.

Perfusion of the lymph node with contrast medium was followed by injection of 1 ml NaCl in 12 of the 14 rabbits. (The 2 other rabbits were withdrawn because of leakage in the system. Both had been injected with Lipiodol). The fluid collected from the efferent lymphatic after this NaCl injection was found to contain contrast medium. The fluid contained cancer cells in the rabbit in which such cells had already been detected after passage of Lipiodol, and also in 1 of the 3 rabbits which had been injected with Urografin. No Vx2 carcinoma

cells were found in the efferent lymphatic fluid collected from the other 10 rabbits (Table 1).

A suspension of cancer cells was finally injected into the afferent lymph vessel in 11 of the 12 rabbits. (One rabbit was withdrawn because of leakage in the system.) In 5 cases, no malignant cells appeared in the efferent fluid. Numerous cancer cells were found in this fluid in all 3 rabbits which had been injected with Lipiodol (Fig. 6). A few cancer cells were detected in 2 of the 5 rabbits in which Emulsion Leo had been used and in 1 of the 3 injected with Urografin.

DISCUSSION

The filtering action of lymph nodes on tumour cells was recognized more than a century ago by Virchow [16], who discussed this action in connection with lymph-borne metastases of malignant mammary tumours. The lymph node barrier against malignant cells has previously been studied along two lines. Some writers noted the occurrence of distant metastases after intralymphatic tumour-cell injection [17–19]. Others attempted a more direct approach by recording the presence of tumour cells in efferent lymphatic vessels [11, 20].

Pressman *et al.* [20] performed intranodal injection of a highly concentrated suspension of HeLa cells and then found such cells in the efferent lymph. But this technique may be traumatic and therefore may create abnormal communications in the injected node [9]. Intralymphatic and subcutaneous injection of Vx2, Brown-Pearce and Walker carcinoma cells into the paw in rabbits was reported by Fisher and Fisher [11] to be followed within 60 minutes of the injection by appearance of tumour cells in the efferent lymph from popliteal nodes. When we tried to reproduce their experiments in 6 rabbits by subcutaneous injection of Vx2 carcinoma cells into the paw, no significant change occurred in the cell population of the efferent lymph as compared with control rabbits.

One of the prerequisities for investigating the lymph node barrier function by recording the presence of tumour cells in efferent lymph is the possibility of differentiating tumour cells from the cells that are ordinarily present in the lymph. We found difficulty in making reliable distinction between individual Vx2 carcinoma cells obtained by aspiration biopsy and large lymphoid cells. But cultured Vx2 carcinoma cells were more easily distinguishable from normal constituents of the lymph, particularly because of their larger size, and therefore were better suited for such experimental work.

The results of these experiments showed that the lymph node barrier, as a rule, is effective against cultured Vx2 carcinoma cells, free or forming clusters, injected into an afferent lymphatic. The carcinoma cells thus were detected in efferent lymph in only 1 of the 16 tested rabbits. Whether in this rabbit the barrier was damaged during the injection or whether the cells escaped through some pre-existing direct communication between the afferent and efferent lymphatics is not known, however.

The main purpose of our study was to evolve an experimental procedure by which the possibility of tumour-cell dissemination in lymphography using oily or water-soluble contrast medium could be assessed. By introducing freely suspended clustered or individual carcinoma cells into a lymph node *via* an afferent lymphatic, we created presumably optimal conditions for spreading these cells during or after subsequent lymphography. Two aspects of the problem could thus be studied, viz., the possible transportation of cancer cells by contrast medium in its passage through a lymph node, and the barrier function of that node against cancer cells before and after lymphography.

The observations hitherto made indicate that transportation of tumour cells by contrast medium is not a common phenomenon in lymphography. When injection of cancer cells (free or in cluster) into an afferent lymphatic was followed by injection of contrast medium into the same vessel, cancer cells were found in the efferent contrast-containing lymph in only 1 of 14 rabbits. During its passage through the lymph node the contrast medium apparently did not attract malignant cells previously deposited in the node. It is pertinent that in suspensions which contained, in addition to lymphocytes or Vx2 carcinoma cells, droplets of Lipiodol Ultrafluid or Emulsion Leo 372, there was no discernible mutual adhesion between the cells and the oily contrast medium.

Does lymphography damage the structure of lymph nodes in such a way that their barrier function against passage of tumour cells is impaired?

Lymphography with Lipiodol Ultrafluid seemed to cause complete breakdown of the lymph node barrier, since subsequently injected Vx2 cells were found in large numbers in the efferent lymph in all of the tested rabbits. Emulsion Leo 372, which contains microscopic droplets of oily medium, possibly was less

traumatic: The efferent lymph contained a few carcinoma cells in only two of the five rabbits studied in the same way. The water-soluble Urografin likewise seemed to be relatively nontraumatic [Figs. 7 and 8].

The observations concerning Lipiodol Ultrafluid may, however, be inapplicable to clinical lymphography in man. The contrast medium then remaining in the lymph nodes is slowly absorbed over a period of weeks or months. The only conclusion permitted by our experiments is that it is inadvisable to remove contrast medium immediately after lymphography by perfusion of the nodes, for example with chemotherapeutic agents, if there is any suspicion that the area which drains towards the nodes still contains cancer cells. Post-lymphography perfusion of lymph nodes may conceivably open up communications blocked by oily contrast medium and cause dissemination of the tumour from a primary site through the perfused nodes.

RESUME

L'effet de la lymphographie sur la fonction des ganglions lymphatiques, comme barrière vis-à-vis des cellules carcinomateuses, a été étudié, par la cathétérisation des lymphatiques afférents et efferents du ganglion poplité, chez le lapin. Des cellules carcinomateuses Vx2 cultivées étaient injectées dans le vaisseau afférent, avant et après lymphographie par le Lipiodol Ultrafluide, l'Emulsion Leo 372, ou l'Urographine. Ces cellules étaient facilement reconnaissables parmi les cellules du ganglion. Les modifications de la fonction de barrière du ganglion étaient estimées par l'analyse cytologique du liquide recueilli au niveau du vaisseau lymphatique efférent.

Les résultats préliminaires indiquent:
(1) *que le ganglion est une barrière efficace vis-à-vis des cellules carcinomateuses Vx2 cultivées, injectées dans le vaisseau afférent;*
(2) *que les cellules Vx2, introduites dans un ganglion, avant la lymphographie, ne sont pas emportées par le produit de contraste lors de son passage à travers le ganglion;*
(3) *que la fonction de barrière du ganglion est diminuée après lymphographie, particulièrement avec le Lipiodol Ultrafluide: des cellules carcinomateuses Vx2, injectées après lymphographie, se retrouvaient dans le liquide lymphatique efférent.*

SUMMARY

In order to study the effect of lymphography on the function of lymph nodes as a barrier to carcinoma cells, cannulation of afferent and efferent lymphatics of the popliteal node was performed in domestic rabbits. Cultured Vx2 carcinoma cells were injected into an afferent vessel before and after lymphography with Lipiodol Ultrafluid, Emulsion Leo 372 or Urografin. These cells were readily recognizable among lymph cell populations. The changes in the barrier function of the node were judged by cytologic analysis of fluid collected from the cannulated efferent lymphatic.

The preliminary results indicated:
(1) *that the lymph node is an effective barrier against cultured Vx2 carcinoma cells injected into an afferent lymphatic;*
(2) *that Vx2 carcinoma cells introduced into a node prior to lymphography are not transported by the contrast medium in its passage through the node, and*
(3) *that the barrier function of lymph nodes is impaired after lymphography, especially with Lipiodol Ultrafluid: Vx2 carcinoma cells injected after lymphography were found in the efferent lymph.*

ZUSAMMENFASSUNG

Um die Auswirkung der Lymphographie auf die Funktion der Lymphknoten als Barriere für Karzinomzellen zu untersuchen, wurden die afferenten und efferenten Lymphgefäße von Lymphknoten in der Poplitea bei Kaninchen kanüliert. Kulturen von Vx2-Karzinomzellen wurden in die afferenten Gefäße injiziert und zwar vor und nach Lymphographie mit Lipiodol Ultrafluid, Emulsion Leo 372 oder Urografin. Diese Zellen waren leicht erkennbar innerhalb der Lymphknotenzellpopulation. Veränderungen in der Barrieren-

funktion der Knoten wurden beurteilt durch eine zytologische Analyse der Flüssigkeit, die aus der kannulierten efferenten Bahn gesammelt wurde.
Die vorläufigen Ergebnisse zeigen, daß erstens der Lymphknoten eine wirksame Barriere gegen kultivierte Vx2-Karzinomzellen ist, die in das afferente Lymphgefäß injiziert werden. Zweitens, daß Vx2-Karzinomzellen, die in einen Knoten vor der Lymphographie injiziert werden, nicht durch das Kontrastmedium bei seiner Passage durch den Lymphknoten mitgeschleppt werden, und drittens, daß die Barrierefunktion von Lymphknoten nach der Lymphographie verschlechtert ist, speziell bei Verwendung von Lipiodol Ultrafluid. Vx2-Karzinomzellen, die nach der Lymphographie injiziert wurden, konnten in den efferenten Lymphgefäßen gefunden wereden.

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Phosphorylation of Histones in Mouse Mammary Tumors

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PHOSPHORYLATED histones have been detected in nuclei of rat thymus and liver [1, 2] calf thymus lymphocytes [3] and human lymphocytes [4]. Kleinsmith *et al.* have presented evidence that phosphoproteins play a role in the modulation of DNA template activity, even though they did not exclude alternative explanations such as phosphate storage [4]. In view of the proposed role of phosphorylated histones in gene activation the present work was performed to study the phosphorylation of acid extractable proteins in the nuclear fruction of malignant tumors of the mouse mammary glands.

METHODS

C3H/HeSy mice bearing mammary tumors were used throughout this work. The animals were anesthetized with ether and the tumors were removed and placed in chilled 0·25 M sucrose–1·8 mM $CaCl_2$ solution. The nuclei were isolated as described by Hogeboom [5]. *In vitro* incubations of the isolated nuclei with ^{32}P-phosphate were performed essentially according to the method of Kleinsmith *et al.* [4]: nuclei from 10 g wet liver were suspended in 8·0 ml 0·25 M sucrose and incubated for ½ hr at 37°C with 4·0 ml 0·1 M tris-HCl buffer pH 7·1, 3·2 ml glucose containing 12 mg NaCl and 13·5 mg $MgCl_2 \cdot 4H_2O$ and 0·4 ml H_2O containing 400 μC ^{32}P-orthophosphate (purchased from Amersham, Bucks, England). For *in vivo* experiments, groups of 5 mice were administered intraperitoneally a daily dose of 100 μC ^{32}P-phosphate for five consecutive days and the tumors were excised 1 hr following the last isotope administration. The isolated nuclei from both the *in vivo* and the *in vitro* experiments were further washed with 3 mM $MgCl_2$ in 0·01 M tris-HCl buffer pH 7·1 and with 80% ethanol–0·01 M HCl as suggested by Kleinsmith *et al.* (4). The histones were extracted from the nuclei with 0·15 0·3 ml 0·2 N HCl/g-w. of initial tissue. For the gel filtration experiments the acid extracts were used as such, while for the CM-cellulose fractionation experiments, the histones were precipitated with 10 vol. of acetone and further dehydrated by acetone and ether.

Histones were hydrolyzed in sealed tubes with 2N HCl at 120° for 12 hr. Phosphoserine and phosphothreonine were separated from the remaining amino acids by paper chromatography on Whatman No. 1 paper (solvent: ethanol, water, conc. ammonium hydroxide 100 : 20 : 20). The radioactive spots were located by passing the paper chromatogram through a Selo chromatoscan.

Protein was determined by the method of Lowry *et al.* [6] while radioactivity of emerging fractions was measured in a Selo automatic G-M counter.

RESULTS

In vivo phosphorylation of histones. Figure 1 is the filtration profile on Sephadex G-75 of nuclear histones from mammary tumors of ^{32}P-treated mice. Radioactivity in histones is associated mainly with the first protein peak. The second radioactive peak is not due to

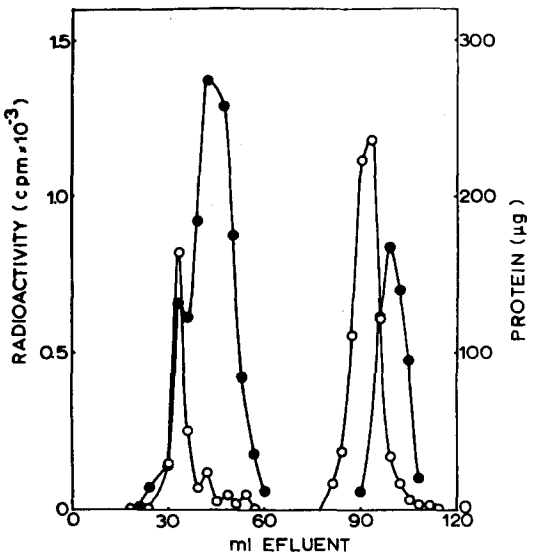


Fig. 1. *Filtration profile through Sephadex G-75 of a* 0·2 N *HCl extract of mammary tumor nuclei from mice treated for* 5 *consecutive days with* ^{32}*P-phosphate. Column size* 53*X* (1·3) *cm. Fraction volume* 3 *ml. Filtration medium* 0·02 N *HCl* ●——● *Protein* o——o *radioactivity.*

histones (negative Lowry test). When the protein-associated radioactive peak was hydrolyzed with 2 N HCl and the hydrolysate was chromatographed on paper and passed through the chromatoscan, Fig. 2 was obtained. Radioactivity was associated with inorganic phosphate, which remains at the origin in the system used, as well as with spots moving the same distance as phosphoserine and phosphothreonine. A third radioactive spot also appeared, but no attempts were made to identify it.

Interaction of histones with phosphate at pH 7·1. Since Kleinsmith *et al.* have shown that

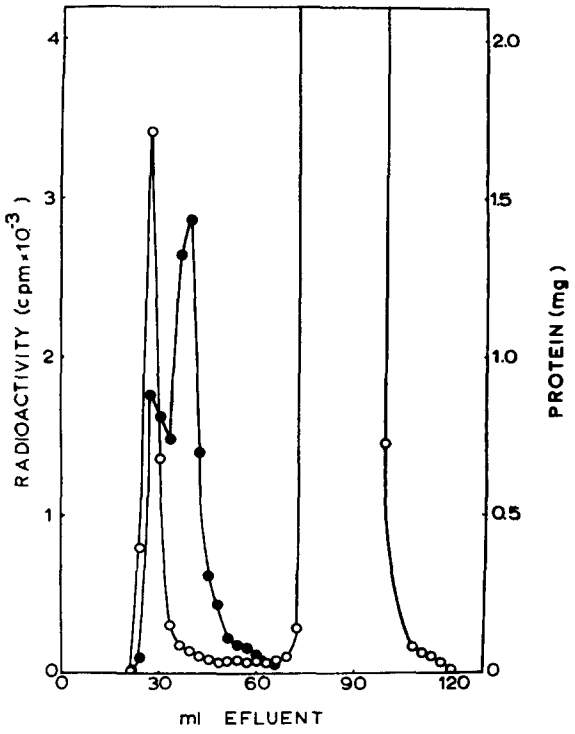


Fig. 3. *Filtration profile through Sephadex G-75 of nuclear histones incubated* in vitro *with* ^{32}*P-phosphate. Column size* 53*X* (1·3) *cm. Fraction volume* 3 *ml. Filtration medium* 0·02 N *HCl* ●——● *Protein* o——o *radioactivity.*

histone phosphorylation is an energy-dependent process [3], it was rather surprising to find inorganic phosphate attached to histones in the *in vivo* experiments. To clarify this point, 1 µC of ^{32}P-phosphate was added to 14 mg acetone-dried histones suspended in 1·5 ml 0·01 M tris-HCl pH 7·1 30 min later, the mixture was rendered 0·2N with respect to HCl and filtered through Sephadex G-75 in

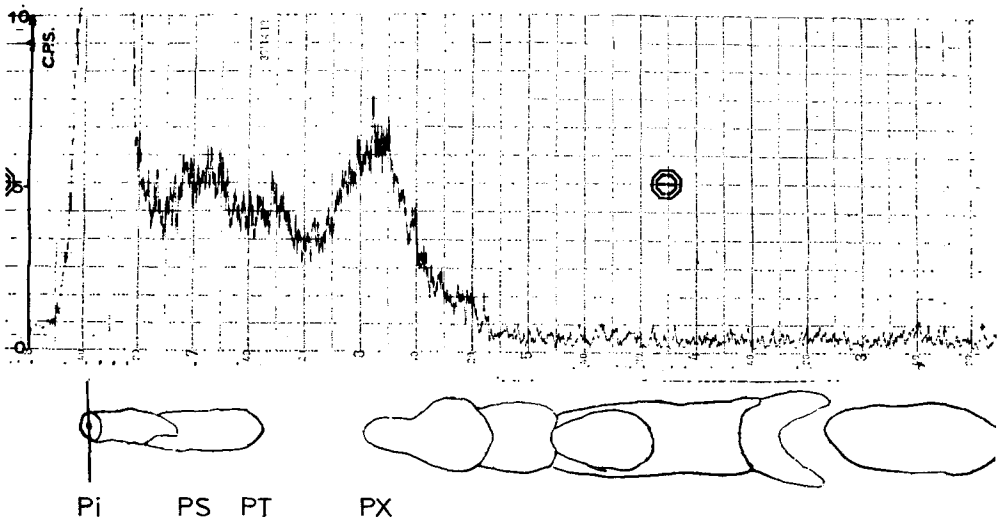


Fig. 2. *Paper chromatogram superimposed by its scanning profile of an acid hydrolysate of nuclear histones phosphorylated* in vivo (*radioactive peak in Fig.* 1). *Pi, inorganic phosphate; PS, phosphoserine; PT, phosphothreonine; PX, unidentified radioactive area.*

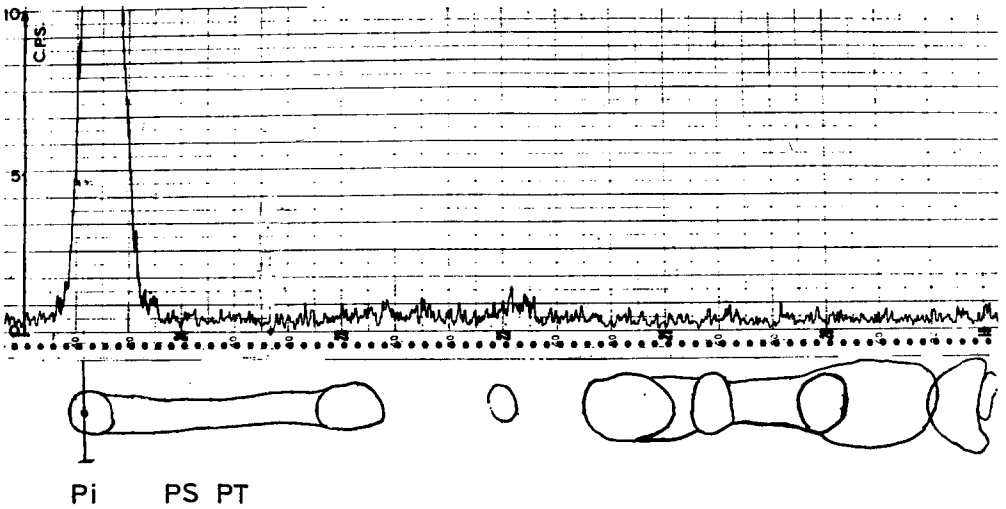


Fig. 4. Paper chromatogram superimposed by its scanning profile of an acid hydrolysate of nuclear histones "phosphorylated" in vitro, *(radioactive peak in Fig. 2). Pi, inorganic phosphate; PS, phosphoserine; PT, phosphothreonine.*

0·01 N HCl. As can be seen in Fig. 3 a portion of the radioactivity accompanies the histones. When, however, these radioactive histones were hydrolyzed with 2 N HCl and chromatographed only inorganic phosphate was detected on the chromatograms, phosphorylated amino acids being totally absent (Fig. 4). This indicates that there is a non-energy dependent binding of phosphate to histones, probably ionic in nature.

In vitro *phosphorylation of histones.* In order to ascertain whether histones could be phosphorylated in isolated nuclei of mammary tumors, the procedure of Kleinsmith *et al.* was followed [4]. Following incubation of the nuclei with ^{32}P-phosphate at pH 7·1, the acid extractable proteins were precipitated with acetone and chromatographed on CM-cellulose. Figure 5 is the chromatographic profile of one such experiment. Labeled f_1 histones emerged from the column but no f_3 histones, labeled or otherwise appeared with 0·2 N HCl. Similar

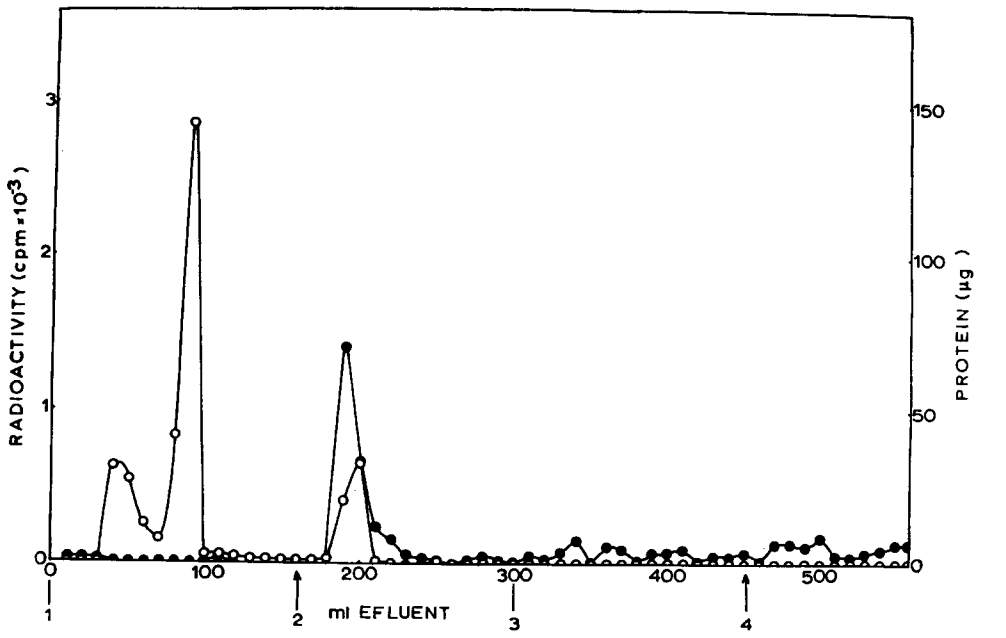


Fig. 5. CM. cellulose chromatography of a 0·2N *HCl extract of mouse mammary tumor nuclei incubated* in vitro *with* ^{32} *P-phosphate. Column size* 22×1 *cm. Fraction volume* 5 *ml. Arrow* 1*:* 0·1M *sodium acetate PH* (4·2) *in* 4 M *Urea; arrow* 2, 0·17 M *sodium acetate and* 0·42 M *NaCl pH* 4·2 *in* 4 M *urea; arrow* 3, 0·01 N *HCl in* 4 M *urea; arrow* 4, 0·2 N *HCl in* 4 M *urea.* ●——● *Protein,* o——o *radioactivity.*

results were obtained when the column was eluted with three times the volumes of buffers as in the work of Kleinsmith *et al* [4].

DISCUSSION

The present experiments clearly show that histone phosphorylation takes place in nuclei of malignant tumors of the mouse mammary gland. As in the case of rat thymus and liver as well as human lymphocytes, this phosphorylation occurs by esterification of the hydroxy amino acids serine and threonine. Not all histones are phosphorylated. The *in vivo* experiments have shown that only a portion of the basic proteins is phosphorylated while the *in vitro* experiments showed that portion to be histone f_1. No histone f_3 could be isolated from the tumor nuclei on a CM-cellulose column (Fig. 5). It has already been shown that phosphorylation of serine and threonine residues in histones is an energy-linked process [4]. The present work, however, reveals that inorganic phosphate may easily attach to histones in the absence of enzymic catalysis or any other energy requiring process (Fig. 3). It is apparent that phosphate attaches to histones by at least two types of bonds: an ester bond between hydroxy-amino acids and phosphate stable to acid hydrolysis, and an acid-labile bond, presumably ionic in nature. If the ester linked phosphate plays a role in the modulation of DNA template activity, as suggested by Kleinsmith *et al.* (4) then the ionically bound phosphate may well serve for storage purposes.

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RESUME

Des histones phosphorylées furent isolées d'un extrait acide des noyaux de tumeurs mammaires de la souris, par la filtration sous gel, et par la chromatographie avec échangeurs de cations. La phosphorylation des histones apparaît aussi bien après l'administration de phosphate ^{32}P à des souris C3H in vivo *qu'après l'incubation de noyeaux isolés avec le précurseur radioactif,* in vitro. *Le phosphate se lie aux histones de deux façons différentes: une liaison, stable aux acides, entre les acides hydroxy-aminés (serine et thréonine) et le phosphate, et une liaison, labile aux acides, probablement de nature ionique. Cette liaison, non spécifique, du phosphate aux histones, apparaît même sans la participation de processus énergétiques.*

SUMMARY

Phosphorylated histones were isolated from acid extracts of nuclei of mouse mammary tumours both by gel filtration and cation exchange chromatography. Histone phosphorylation was shown to take place both after administration of ^{32}P-phosphate to C3H mice in vivo, *as well as after* in vitro *incubation of isolated nuclei with the radioactive precursor. Phosphate attaches to histones by two types of bonds: an acid-stable bond between the hydroxy amino acids, serine and threonine, and phosphate, and a non-specific acid-labile bond, presumably ionic in nature. This non-specific attachment of phosphate to histones occurs even without the participation of energy-dependent processes.*

ZUSAMMENFASSUNG

Phosphorylierte Histone wurden durch Gelfiltration und Kationenaustauschchromatographie aus Säureextrakten von Zellkernen muriner Mammatumoren isoliert. Histonphosphorylierung fand sowohl in vivo *nach Verabfolgung von P-32 an C3H-Mäusen als auch* in vitro *nach Inkubation isolierter Zellkerne mit radioaktivem Phosphat statt. Phosphat bindet sich auf zwei Arten an Histone: eine säure-stabile Bindung zwischen den Hydroxyaminosäuren Serin und Threonin und dem Phosphat, und eine unspezifische säure-labile Bindung wahrscheinlich elektrostatischer Natur. Dieses unspezifische Anhaften von Phosphat an die Histone geschieht sogar ohne Beteiligung ernergieabhängiger Prozesse.*

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Preferential Sites of Involvement and Spread in Malignant Lymphomas

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IN THE past decade the long-term survival rates achieved with adequate radiation therapy in localized malignant lymphomas and particularly in Hodgkin's disease mainly by Peters [1, 2], Easson [3] and Kaplan [4] have been gradually changing the general attitude of pessimism based on the idea that these diseases were almost invariably incurable. These data and the widespread use of lymphography have yielded a large number of useful information about the natural history as well as new lines concerning a standardized clinical staging classification [5] and a meticulous diagnostic evaluation for Hodgkin's disease. Concomitant investigations on the correlation between clinical stages and histologic expressions [6] and on the significance of the immunologic status [7] widened considerably both clinical and biological interest for malignant lymphomas.

Although localized malignant lymphomas are now considered potentially curable [8], the technique and the extent of the intensive radiotherapy are still somewhat controversial. More detailed studies on the natural history of these diseases are desirable: they should provide further bases for a rational therapeutic approach.

The present investigation has been undertaken in a group of 500 unselected malignant lymphomas with the intention to further contribute to the knowledge of their preferential sites of involvement and mode of spread. This report is on the line of previous studies performed by Scheer [9], Rosenberg and Kaplan [10] and Hant and Stutzman [11].

MATERIAL AND METHODS

The study was performed on 500 consecutive untreated patients (200 Hodgkin's disease, 150 lymphosarcomas and 150 reticulum cell sarcomas).

All patients had a detailed clinical history and a complete physical examination as well as one or two excisional biopsies. The histologic diagnosis was confirmed by two pathologists. Radiological examination including a postero-anterior and lateral chest film with mediastinal tomograms and skeletal survey (skull, spine, pelvis) were obtained in all cases. Bilateral lymophography was performed in 100 patients with Hodgkin's disease, in 50 patients with lymphosarcomas and in 50 patients with reticulum cell sarcomas. Urography and pneumoretroperitoneum with tomography were performed mostly in those patients studied before the advent of lymphography. Laboratory studies have included complete blood count, platelet count, erytrosedimentation rate, bone marrow aspiration, alkaline phosphatase, bromsulphalein excretion at 45 min serum acid, calcium and protein electrophoresis.

In patients with hepatomegaly, a liver scan with ^{198}Au and a liver biopsy through peritoneoscopy were performed.

Table 1. *International staging classification for Hodgkin's disease proposed at Rye* (1965)

Stage I	Disease limited to one anatomic region (Stage I_1) or two contiguous anatomic regions (Stage I_2) on the same side of the diaphragm
Stage II	Disease in more than two anatomic regions or in two non contiguous regions in the same side of the diaphragm
Stage III	Disease on both sides of the diaphragm, but not extending beyond the involvement of lymph nodes, spleen, and/or Waldeyer's ring
Stage IV*	Involvement of the bone marrow, lung parenchyma, pleura, liver, bone, skin, kidneys, gastrointestinal tract, or any tissue or organ in addiction to lymph nodes, spleen or Waldeyer's ring

All groups are subclassified as A or B to indicate the absence or presence, respectively, of one or more of following systemic symptoms: fever, night sweats, generalized pruritus

*Patients with involvement of viscera or tissue, such as stomach, bowel or skin without apparent disease in the lymph nodes or spleen should not be included in any of the groups described, but listed separately in clinical reports.

After diagnostic evaluation the patients were staged according to what appears to be the new standardized clinical classification proposed at Rye for Hodgkin's disease (See Table 1 and reference 5). This classification has also been adopted for lymphosarcoma and reticulum cell sarcoma with primary involvement of lymph nodes and spleen since an agreement on an international basis has not yet been achieved for these diseases. The anatomical regions for the above proposed clinical classification were also adopted [4].

When treatment was completed patients were followed at frequent intervals (every 1–3 months according to the different clinical situations) in the out-patient department by at at least one radiotherapist and one chemotherapist. Complete blood count, chest X-ray were obtained almost always on each visit.

Skeletal survey was repeated in case of suspicious bone pain as well as flat of abdomen to check on the previously opacified retroperitoneal nodes as long as the contrast material was present.

RESULTS

Figure 1 shows the preferential sites of involvement in untreated Hodgkin's disease, lymphosarcoma and reticulum cell sarcoma. From this diagram it appears that, in 200 cases of Hodgkin's disease, the involvement was confined to lymph nodes and spleen in 199 patients (99·5%) and that in only one case (0·5%) the disease arose in the Waldeyer's ring. On the contrary, the per cent of involvement of Waldeyer's ring is considerably increasing from lymphosarcoma (20%) to reticulum cell sarcoma (53%) while the other sites (bone, liver, lung parenchyma, skin, gastro-intestinal tract, marrow, etc.) show no significant difference between these two diseases (12–11%).

Figure 2 shows a comparative study of the distribution among the different lymph node-bearing areas including spleen. The cervical area (cervical, supraclavicular, occipital and preauricular nodes) is by far the highest involved region in all three diseases but mostly in Hodgkin's disease (45%) and lesser in

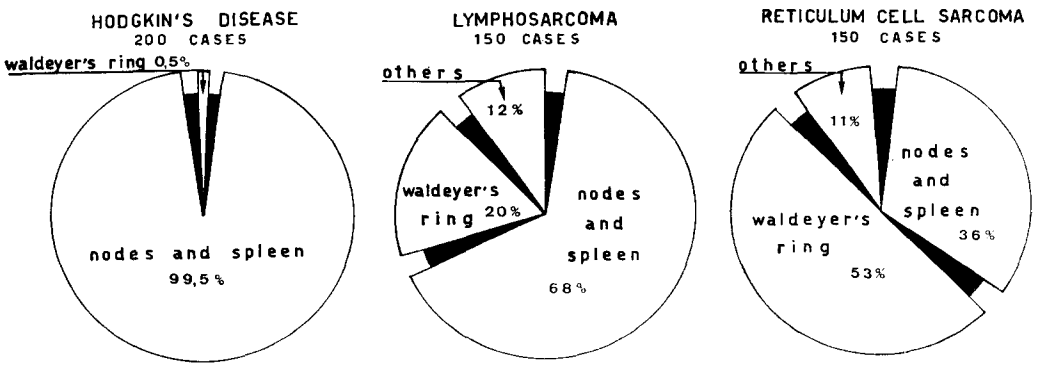


Fig. 1. *Diagrams showing the distribution of case material* (500 *consecutive untreated malignant lymphomas*) *according to the sites of involvement.*

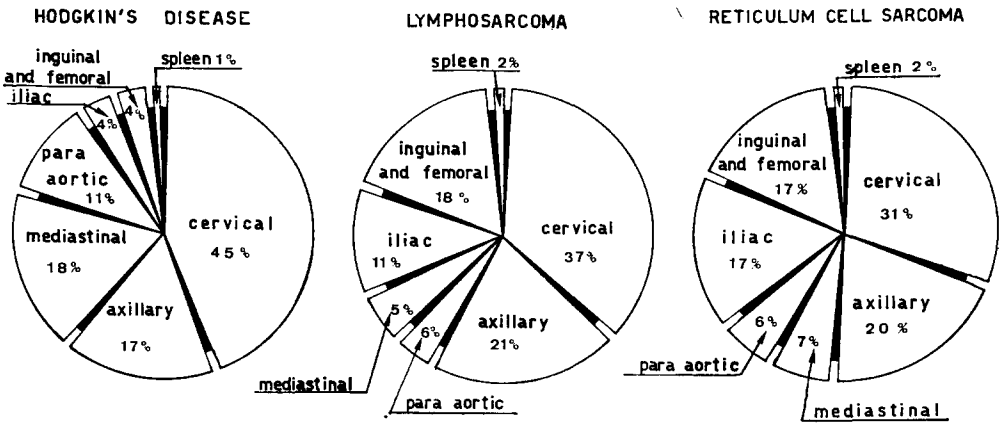


Fig. 2. Diagrams showing the distribution of the different localizations in untreated patients with primary involvement of lymph nodes and spleen (Hodgkin's disease: 199 cases, lymphosarcoma 102 cases, reticulum cell sarcoma 53 cases).

reticulum cell sarcoma (31%). While the axilla appears to be equally involved, mediastinal (18%) and para-aortic (17%) adenopathies are more frequently found in Hodgkin's disease than in lymphosarcoma and in reticulum cell sarcoma where, on the contrary, there is a higher involvement of iliac (11–17%), inguinal and femoral (18–17%) nodes. The splenic involvement seems, from this study, to be practically identical in all three types of lymphomas.

The preferential "central" involvement of Hodgkin's disease rather than the "peripheral" one of lymphoreticulum sarcomas is more accurately described in Fig. 3. The anatomical sketch shows a comparative study carried out only in patients (always with nodal and splenic extent) in whom bilateral lymphography has been performed. The more detailed investigation of the retroperitoneal space accounts here for the higher percentage of para-aortic adenopathies in Hodgkin's disease (20%) and respectively of iliac adenopathies in lymphoreticular sarcomas (23%).

The mode of spread has been studies by counting the number of untreated patients with contiguous and non contiguous adenopathies as well as the next site of involvement after initial radiation therapy. This study does not include patients with involvement of Waldeyer's ring or other organs or tissues besides lymph nodes and spleen.

Table 2 shows that in about 2/3 (66%) of patients with Hodgkin's disease the adenopathies are found to be distributed in adjacent lymphoid areas while this finding occurred only in 35% of lymphoreticular sarcomas. In the group of patients with Hodgkin's disease in whom the work-up included lymphography, the percentage of contiguous involvement is lower (59%) than in those without the lymphographic study (74%). This is due to the fact that

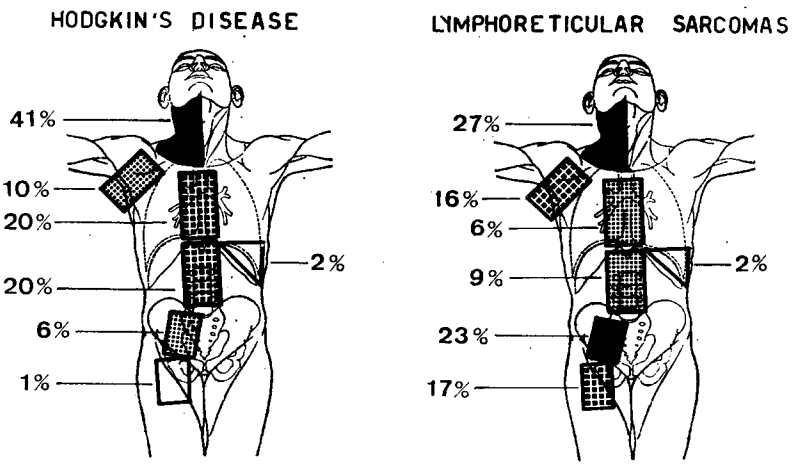


Fig. 3. Anatomical sketch showing the percentage of involvement of the different lymph nodes-bearing areas in patients with primary involvement of lymph nodes and spleen (Hodgkin's disease: 99 patients, lymphoreticular sarcoma: 67 patients) studied with lymphography.

Table 2. Hodgkin's disease and lymphoreticular sarcomas; primary lymphoid onset—number of contiguous involvement in untreated patients

Disease		Number of cases	Contiguous involvement	
Hodgkin's disease				
	without lymphography	73	54	(74%)
	with lymphography	80	47	(59%)
	total	153	101	(66%)
Lymphoreticular sarcomas				
	without lymphography	53	19	(36%)
	with lymphography	54	18	(33%)
	total	107	37	(35%)

lymphography showed para-aortic adenopathies in a nymber of patients with neck or axillary nodes without concomitant involvement of mediastinum. Hodgkin's disease may have arised in the paraaortic area and via the thoracic duct spread to the cervical and/or axillary region without involving the mediastinum.

Table 3 reports the sites of extension in new areas after local radiation therapy. Only patients classified after lymphography as stage I_1 (cervical onset) had precautionary or prophylactic irradiation to mediastinum. In 72% of cases with Hodgkin's disease, the next site of involvement after initial treatment was found in adjacent lymonode-bearing areas. This finding occurred only in 45% of lymphoreticular sarcomas. Furthermore, in Hodgkin's disease, the first site of extention after localized radiation therapy occurred practically always in lymph node regions; on the contrary, in lympho-reticular sarcomas, this was found also in viscera or bones.

DISCUSSION

In clinical oncology, the detailed knowledge of the natural history of a given tumor is of fundamental importance mainly for deciding whether a radical or a palliative treatment should be started after histologic diagnosis and proper clinical evaluation. As far as malignant lymphomas are concerned, a growing number of data have been recently provided by different investigators showing that Hodgkin's disease behaves somewhat differently from lymphoreticular sarcomas.

From our study performed on 500 consecutive cases of untreated malignant lymphomas emerges as first observation that in Hodgkin's disease the primary extra-nodal involvement is at least rare. This does not occur in lymphoreticular sarcomas where for instance the Waldeyer's ring is frequently involved and in much higher percentage than usually reported [12, 13]. Furthermore in the sarcoma group the primary involvement of other extranodal sites is not unusual.

The comparative study of the lymph nodes extention has revealed, at first diagnostic evaluation, that the involvement in Hodgkin's disease is besides the cervical regions predominantly "central" (mediastinal and paraaortic) while in lymphoreticular sarcomas it is mainly "peripheral" (axillary, iliac and inguinal). This diverse nodal distribution has been better described after the advent of lymphography. Bilateral foot lymphography, although not exempt from occasional doubtful interpretations, remains today the most appropriate diagnostic tool to explore the retroperitoneal nodes. Therefore it should be performed as a routine in all patients with a

Table 3. Hodgkin's disease and lymphoreticular sarcomas with primary lymph nodes onset: comparative study of the mode of spread (next site of involvement after initial irradiation) in patients classified at stage I_1 and I_2 and at stage II with contiguous involvement

Disease	Number of cases	Spread			
		contiguous		discontiguous	
Hodgkin's disease	79	57	(72%)	22	(28%)
Lymphoreticular sarcomas	44	20	(45%)	24	(55%)

biopsy-proved diagnosis of malignant lymphoma, no matter what is the initial extent of involvement. In case of equivocal evidence, the exploration of the retroperitoneal space should be completed with pneumoretroperitoneum or with urography and cavography, and even through an exploratory laparotomy.

As far as the mode of spread of malignant lymphomas is concerned our study performed on untreated patients with more than one lymphoid region involved (Stage I_1, II, and III) reveals:

(1) In Hodgkin's disease the number of cases with contiguous involvement was 66% while in lymphoreticular sarcoma it was only 35%. The number of contiguous involvement is definitely higher in both diseases if one considers that the primary onset can occur also in the para-aortic area and in the gastrointestinal tract, and spread via the thoracic duct to the neck and to the axillae.

(2) The next site of involvement in patients at stage I_1 I_2 and II occurred in contiguous areas in 72% for Hodgkin's disease and in 45% for lymphoreticular sarcomas. The sarcomas had also tendency to spread early to viscera or bones.

Cumulatively all these data provide sufficient evidence that Hodgkin's disease and lymphoreticular sarcomas, although probably unicentric in origin, have a different pattern as far as the preferential sites of involvement and the node of spread are concerned. In particular our results confirm the observation of Rosenberg and Kaplan [10] and of Han and Stutzman [11] and, at the same time, are in favour of the therapeutic approach adopted by Peters [1, 2], Rubin [13] and Kaplan [4]. These Authors in fact administer prophylactic irradiation only to localize Hodgkin's disease and not to lymphoreticular sarcomas.

On a theoretical basis, the results of our study support the hypothesis, not shared by all investigators [14], of the unicentric onset of both diseases and of the initially slow and orderly lymphatic spread of Hodgkin's disease, while lymphoreticular sarcomas seem to disseminate predominantly through the blood stream.

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RESUME

Les auteurs ont étudié le siège initial et les modalités de diffusion de la maladie sur 500 *sujets atteints de lymphomes malins* (200 *cas de maladie de Hodgkin,* 150 *lymphosarcomes et* 150 *réticulosarcomes*). *Ils ont constaté que, dans* 99, 5% *des cas, la maladie de Hodgkin est localisée initialement dans les ganglions et la rate. Chez les malades atteints de réticulosarcome, la maladie envahit d'abord, dans la plupart des cas, l'anneau de Waldeyer, et d'autres sièges extra-ganglionnaires. Dans la maladie de Hodgkin, l'envahissement ganglionnaire prédomine dans le médiastin, dans les régions para-aortiques, ainsi que dans les régions cervicales, alors que, dans les lympho-réticulosarcomes, l'affection siège principalement au niveau des aisselles, des régions iliaques et des creux inguinaux.*

L'étude des modalités de diffusion a montré que, pour la maladie de Hodgkin non traitée, le nombre des cas avec envahissement des régions avoisinantes est de l'ordre de 66%, *alors que pour les lympho-réticulosarcomes, ces régions ne sont envahies que dans* 35% *des cas. De plus, après traitement radiologique, la tendance à récidiver la maladie de Hodgkin, dans les régions ganglionnaires adjacentes, est plus fréquente* (72%) *que celle des lympho-réticulosarcomes* (45%).

Les auteurs discutent les indications thérapeutiques qui dérivent de cette étude.

SUMMARY

The authors studied the preferential sites of involvement as well as the mode of spread in 500 *consecutive untreated patients with malignant lymphomas* (200 *Hodgkin's disease,* 150 *lymphosarcomas and* 150 *reticulum cell sarcomas*). *The primary involvement in Hodgkin's disease was confined in* 99.5% *to lymph nodes and spleen. In lymphoreticular sarcomas, there was a high primary involvement of the Waldeyer's ring and of other extranodal sites. Besides the cervical regions the distribution of lymph nodes involvement occurred in Hodgkin's disease predominantly in the mediastinum and in the para-aortic area, while in lymphoreticular sarcomas it can be observed mainly in axillary, iliac and inguinal regions. The study of the mode of spread showed that in untreated Hodgkin's disease the*

number of cases with contiguous involvement was 66% *while in lymphoreticular sarcomas it was only* 35%. *Furthermore, after localized radiation therapy, Hodgkin's disease has higher* (72%) *tendency to recur in adjacent lymphoid regions than lymphoreticular sarcomas* (45%). *The therapeutic implications of this study are discussed.*

ZUSAMMENFASSUNG

Die Autoren studierten die bevorzugten Stellen des Befalls sowie den Ausbreitungsmechanismus bei 500 *aufeinanderfolgenden unbehandelten Patienten mit bösartigen Lymphomen* (200 *Hodgkin'sche Krankheit,* 150 *Lymphosarkome und* 150 *Reticulumzell-Sarkomen*). *Hauptächlich* (*mit* 99, 5%) *ist die Hodgkin'sche Krankheit auf Lymphknoten und Milz beschänkt. Bei Lymphoreticulum-Sarkom gibt es eine hohe Primärbeteiligung des Waldeyer'schen-Ringes und anderer Stellen auβerhalb der Lymphknoten. Neben den Zervikalregionen betrifft die Lymphknoten-beiteiligung bei der Hodgkin'schen Krankheit hauptsächlich das Mediastinum und die para-aortische Region, während bei Lymphoreticulum-Sarkomen es hauptsächlich Achsel-, Darmbein- und Leistengegenden sind.*

Die Untersuchung der Ausbreitung zeigte bei unbehandelter Hodgkin'scher Krankheit mehrere Fälle mit angrenzender Beteiligung, und zwar 66%, *während es bei Lymphoreticulum-Sarkom nur* 35% *waren. Weiterhin gab es nach lokaler Bestrahlungstherapie der Hodgkin'schen Krankheit eine höhere Tendenz* (72%) *zur Ausbreitung in anliegende Lymphregionen als bei Lymphoreticulum-Sarkom* (45%).

Die therapeutische Bedeutung dieser Untersuchung wird diskutiert.

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A Modification of the Sensitivity of Mammalian Cells Surviving Treatment with Methyl Methanesulphonate

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INTRODUCTION

CONSIDERABLE attention has been focused recently on the study of the growth kinetics of mammalian cells surviving X-irradiation *in vitro*. Sinclair [1] demonstrated heritable, non-lethal damage in cells surviving X-irradiation, which resulted in the formation of slowly growing "small clones". These "small clones" were also shown to be more radiosensitive. An analogous situation has recently been described for P388 lymphoma cells growing *in vivo* in DBA/2JN mice by Berry [2]. A significant increase in the generation time of L1210 leukaemia cells growing *in vivo* in CDF_1 mice and surviving cyclophosphamide treatment has also been described by Johnson *et al.* [3]. In this case, although the surviving fraction was low, the whole population was affected, and in addition to an increase in doubling time it also showed decreased virulence. Return to control population doubling times and virulence did, however, occur after 4–5 transplant generations. Similar findings have also been reported by Johnson *et al.* [4] for L1210 cells surviving amethopterin treatment *in vivo*. In view of the importance of these findings in relation to cancer chemotherapy, it seemed important to determine whether this phenomenon occurred with other drugs of potential clinical application. Two *in vitro* tumour systems were chosen for the present study with a view to detailed characterization of surviving populations and clones.

Methyl methanesulphonate (MMS), the drug chosen for this study, has been shown to cause specific lymphopenia in monkeys and rabbits [5], and considerable information exists regarding its effects on cellular kinetics and macromolecular biosynthesis in the P388F lymphoma *in vitro* [6, 7].

MATERIALS AND METHODS

The techniques for *in vitro* culture of mouse lymphoma cells have been described previously by Fischer and Sartorelli [8], and the origin of the P388F clone has been described by Fox and Gilbert [9]. Cells do not attach to glass and grow in static suspension cultures. They were maintained in logarithmic growth by appropriate dilutions every 2–3 days. Under these conditions the population doubling time of the cultures was 12–14 hr.

HeLa cells, cloned in this laboratory, were grown in monolayer culture in 4 oz medical flat bottles using techniques previously des-

cribed by Nias *et al.* [10]. The doubling time under these conditions was 25 hr.

Methyl methanesulphonate (MMS) was dissolved in water and sterilized by passage through a membrane filter (pore size 0·22 μ Millipore Filter Corp.). The concentrations of stock solutions of the drug were adjusted so that the required dose was given to 5 ml cultures (P388F) or 10 ml cultures (HeLa cells) by the addition of 0·025–0·2 ml. Cultures were exposed to the drug for varying periods of time during the different experimental procedures. In the case of the lymphoma cultures, the drug containing medium was decanted after centrifugation (800 rev/min for 10 min) and replaced by fresh drug-free medium. Drug-containing medium was removed from HeLa cell cultures by aspiration and replaced by fresh drug-free medium.

Cell survival after treatment of P388F cells was determined by the extrapolated growth curve technique described by Alexander and Mikulski [11]. Survival of HeLa cells was determined by the colony-forming techniques described by Puck and Marcus [12].

For determination of cellular multiplicity, 5ml aliquots of a 2000 cell/ml suspension of HeLa cells were plated in Falcon plastic bottles. The monolayer was fixed in formol-saline and subsequently stained in haematoxylin at appropriate times after plating. Counts were made of single and paired cells over a minimum of 100 cells in several separate experiments.

For labelling experiments, replicate samples of 5 ml, 2000 cells/ml were plated in Falcon plastic bottles. Half the cultures were treated with MMS 100 μg/ml for 15 minutes at 37°C. At the end of this period, the drug containing medium was removed and replaced by medium containing ^{3}H thymidine 0·1 μc/ml. Similarly, medium was removed from the control cultures and replaced by medium containing ^{3}H thymidine at the same concentration. All bottles were subsequently incubated at 37°C and samples, control and treated, were fixed acetic alcohol at hourly intervals. After staining with acetic-orcein, the bottoms of the plastic bottles were removed and coated with Kodak AR 10 stripping film. Exposure time was 10 days.

All irradiations were carried out at room temperature on a Resomax machine operating at 300 kVp 2·0 mm Cu h.v.l. with a dose rate of 100 rad/minute. The cells were irraditated in 4 oz medical flat bottles after attachment to the glass.

RESULTS

Effect of single doses of methyl methanesulphonate on P388*F lymphoma cells*

The half-time of hydrolysis of methyl methanesulphonate in aqueous solution is 9·1 hr at 37°C [13]. The survival of P388F cells after treatment with MMS has previously been shown to fall with increasing time of exposure to the drug [6]. For the present studies, a three-hour exposure period was found to be most convenient. Figure 1 shows the survival curve for lymphoma cells in exponential growth treated with various dose levels of MMS for 3 hours, together with the survival curve for a second treatment. After a first treatment of 40 μg/ml for 3 hours, the surviving fraction was reduced to ~ 3%. Eleven to 18 days after the initial treatment, when the population was again growing exponentially with a population doubling time equal to that of the controls, cells were given a second (3 hr) treatment with the same range of MMS doses. The dose response curve for these cells (given an initial treatment of 40 μg/ml 3 hr and subsequently treated a second time) can be compared with that of cells treated once only (Fig. 1). Both survival curves show a similar form with a shoulder and an exponential portion, but the slopes of the exponential part differ by a factor of ~ 2:1.

The results in Fig. 1 represent a mean of 4 experiments, cells receiving the second treatment between 11 and 18 days after the initial treatment. It was of interest therefore to determine how long this increased sensitivity persisted.

A series of replicate cultures of P388F cells were treated with MMS (40 μg/ml 3 hr). Three days later, one of these cultures was given a second similar dose and survival of cells to the second dose was determined. Other replicate cultures were given a second dose at day 6, day 9 and up to 24 days after the initial treatment. The mean survival to the first treatment was 2·8±0·3%. The survival of cells given a second treatment between 3 and 24 days after the first is shown in Fig. 2. From 3–18 days survival was less than that for the first dose. The mean survival for all treatments during this period was 0·37±0·02%. Treatment at 21 and 24 days showed a return of sensitivity to normal levels, i.e. ≈ 3%.

The effect of a single dose of 50 μg/ml and 80 μg/ml is included in Fig. 2 for comparison. It would appear therefore that although a large proportion of damage is in fact repaired, there is some residual damage which cannot

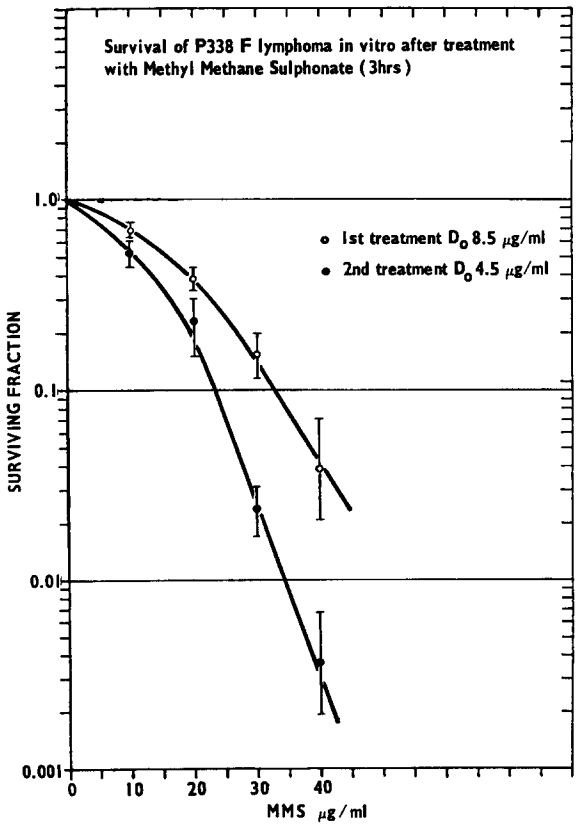


Fig. 1. *Sensitivity of populations of P388F lymphoma* in vitro *to an initial treatment with methyl methanesulphonate compared with the sensitivity of cells surviving a treatment of* 40 μg/ml *MMS* (3 *hr*) *given* 11–18 *days earlier. Mean of* 4 *experiments.*

be repaired. The prolonged period of time before normal sensitivity returns suggests that the majority of the surviving populations is affected, and hence a long period of time elapses before the few remaining normal cells overgrow the damaged ones.

Characterization of HeLa cell clones surviving treatment with methyl methanesulphonate

HeLa cultures were exposed to various doses of MMS during the whole of a ten-day culture period. At the end of this time several clones which had survived 2 μg/ml were isolated. These showed changes which were related to the clone size at the time of isolation. Small clones (i.e. those with less than 50 cells) were not viable at all. Medium clones (with less than 200 cells) showed reduced viability and other characteristics described below. Large clones (i.e. control sized with around 1000 cells) also showed these changes to a lesser extent.

Clones M_3 and M_4 are examples of a large and a medium clone respectively. The cells from these clones were repeatedly sub-cultured over the following 4–5 months. The sensitivity of the two clones to second doses of MMS was tested at intervals during this period. The survival curves obtained for clones M_3 and M_4 after a second continuous treatment with MMS are shown in Fig. 3 together with that of a control clone isolated at a similar time. The Do for the control is 3μg/ml; those for clones M_3 and M_4 are 1·3 and 1 μg/ml respectively. All three cell survival curves extrapolate to the same point. Both clones M_3 and M_4 are significantly more sensitive to MMS treatment than control cultures. However, they do not differ significantly from each other with regard to sensitivity. Both clones M_3 and M_4 showed reduced growth rates and plating efficiencies as well as greater sensitivity to further MMS treatment, Table 1. The clone size distribution (Fig. 4) illustrates the slower growth and increased spread of cell cycle times of these clones; M_3 completed a maximum of 6–7 divisions in 10 days and M_4, 4–6 divisions during the same period. The control clone completed 9 10 divisions during the same period. There is little overlap between these distributions. The characteristics of reduced growth rate, and plating efficiency, and increased sensitivity have been retained over a period of 4–5 months of continuous subculture.

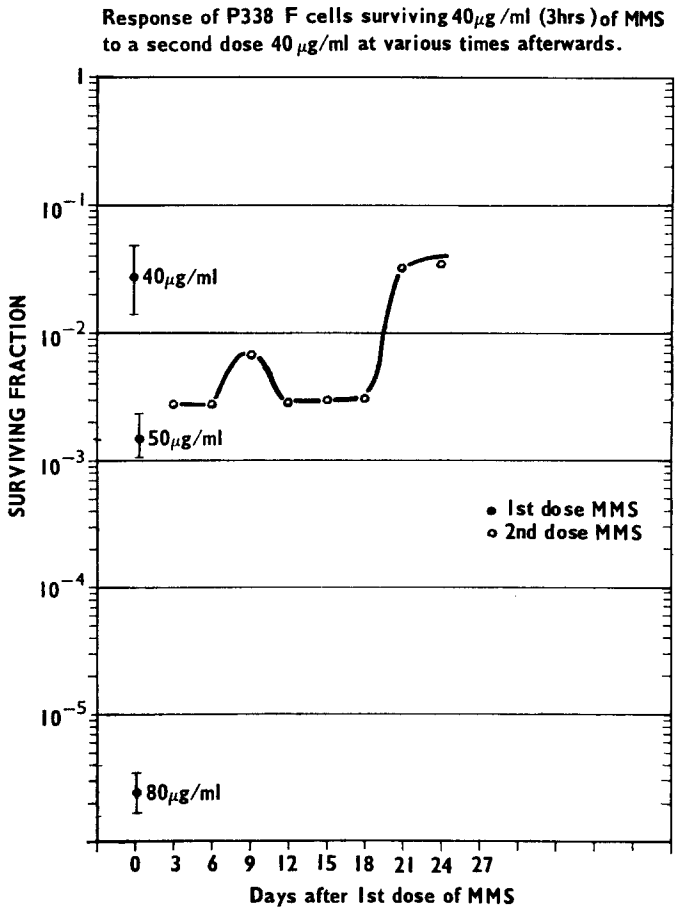


Fig. 2. Time course of return to normal sensitivity of a population of P388F cells surviving an initial dose of 40 μg/ml (3 *hr*). *Survival determined by the extrapolated growth curve technique.*

In P388F populations, decrease in exposure time to the drug resulted in decreased toxicity. A similar relationship was demonstrated for HeLa cells. The Do value for 10-day exposure of control populations was 3·0 μg/ml. that for a 15 minute exposure at 37°C was 30 μg/ml (Fig. 5). Cells were treated with MMS for 15 min either 4 hr or 17 hr after plating. The survival curves obtained had similar Do values but the extrapolation numbers differed considerably (Fig. 5). For 17 hr cells the extrapolation number was 9·0 and for 4 hr cells 5·0. Correction for a cellular multiplicity of $1 \cdot 51 \pm 0 \cdot 03$ at 17 hr from that of $1 \cdot 09 \pm 0 \cdot 15$ at 4 hr could account for these differences. One must therefore conclude that there is no major difference in sensitivity of cells treated at these two times after plating. For convenience therefore the experiments described below were commenced on cells 17 hours after plating.

Effects of divided doses of methyl methanesulphonate on HeLa cells

Two 15-minute treatments of 50 μg/ml of MMS at 37°C separated by periods of up to 8 hours were given to a series of replicate HeLa cell cultures starting 17 hours after plating. The surviving fraction was determined and compared with survival after a single dose of 100 μg/ml which was taken as unity. The mean results from four experiments are shown in Fig. 6. Separation of the two 50 μg/ml doses by one hour results in a slight reduction in survival, separation of the two doses by greater time intervals results in a further reduction.

Changes in the survival pattern of cells given two doses of 150 rads X-rays separated by various periods of time, relative to cells given a single dose of 300 rads, are shown for comparison (Fig. 6). A two-fold increase in survival occurred during the first two hours. This is a similar finding to that of Lockhart, Elkind and Moses [14]. Elkind and Sutton [15] have interpreted this increased survival to indicate repair of sub-lethal damage.

The single doses of MMS and X-rays were chosen to give similar surviving fractions. HeLa cells in this laboratory have previously

been shown to have a Do in air of 150 rads. with an extrapolation number of 3 [16].

Treatment of HeLa cell cultures with divided doses of MMS required that the medium be changed twice. Control and X-irradiated cultures were therefore subjected to the same procedure. This handling alone did not affect the total number of colonies which developed.

It is possible that the initial dose of MMS (100 μg/ml 15 min) induces some synchrony in the population and that cells are in their most sensitive stage when the second dose is given. Continous labelling of the culture with ^3H thymidine has shown, however, that the rate of passage of cells from G_1 and G_2 into S is the same in control and MMS treated cultures (Fig. 7). There cannot therefore be any appreciable induction of synchrony by MMS.

DISCUSSION

The importance of the modification of growth kinetics of cells surviving drug treatment *in vivo* has been stressed by Johnson *et al.* [4]. At that time, however, the additional important factor of increased sensitivity to subsequent drug treatment had not been described. Increased radiosensitivity of small slowly growing clones surviving radiation is now, however, well established by the work of Sinclair with Chinese hamster cells [1] and has been confirmed for HeLa cells used in the present study. The present results indicate an increase in sensitivity of cells of two mammalian tumour lines *in vitro* surviving methyl methanesulphonate treatment.

The increased drug sensitivity of HeLa cell cultures is clearly associated with an increased population doubling time and a reduced plating efficiency. This is apparently not the case in P388F cultures. However, the populations of this latter cell line were not cloned, and it is quite possible, therefore, that a slightly reduced growth rate of sensitive cells could be masked by the presence of normal cells with normal growth rates. That normal cells are present in the surviving populations is indicated by the eventual return of the total population to normal sensitivity (Fig. 2). The stability of the change to increased sensitivity in HeLa cell clones suggests that it is genetic in origin.

Stable changes to drug resistance by somatic cells in culture have been demonstrated for many drugs in many cell lines, e.g., to analogues of folic acid [17], pyrimidines [18, 19] purines [20, 21]. In most cases [17, 22, 23] clones were

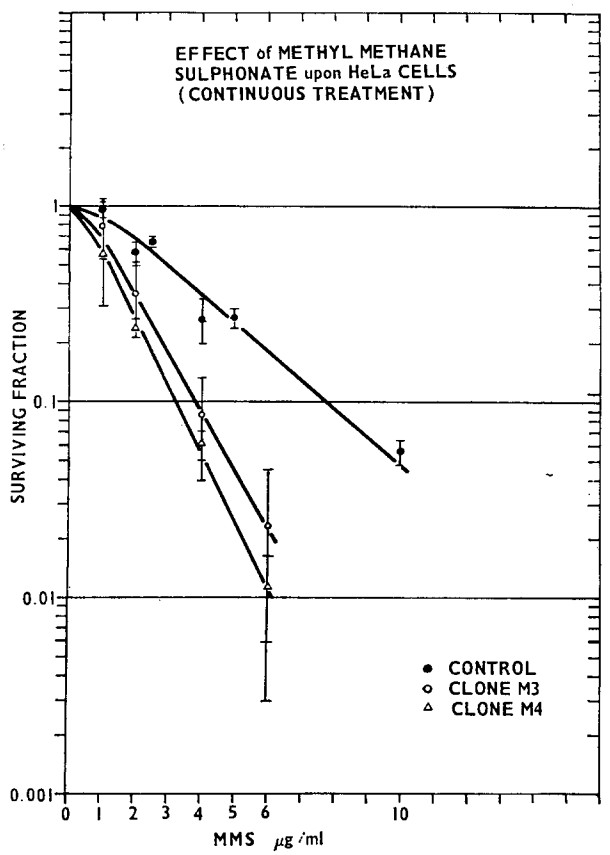


Fig. 3. Survival of HeLa cell clones as determined by the "Puck" colony technique after growth in methyl methanesulphonate for a 10 day period. Clones M_3 and M_4 were slow growing survivors of an initial treatment with the drug.

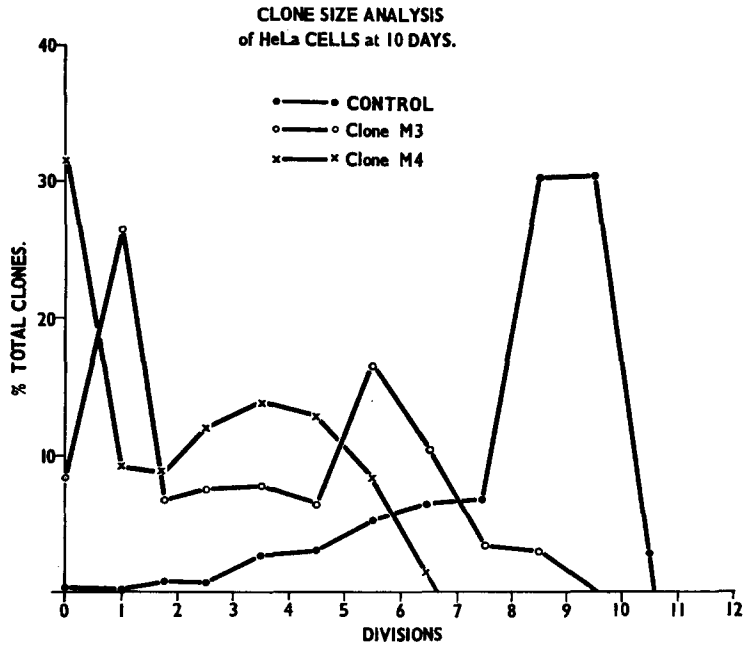


Fig. 4. *Clone size analysis of HeLa cells after* 10*-days incubation. All clones were cultured in the absence of the drug, clones* M_3 *and* M_4 *show a slower growth rate than the control culture.*

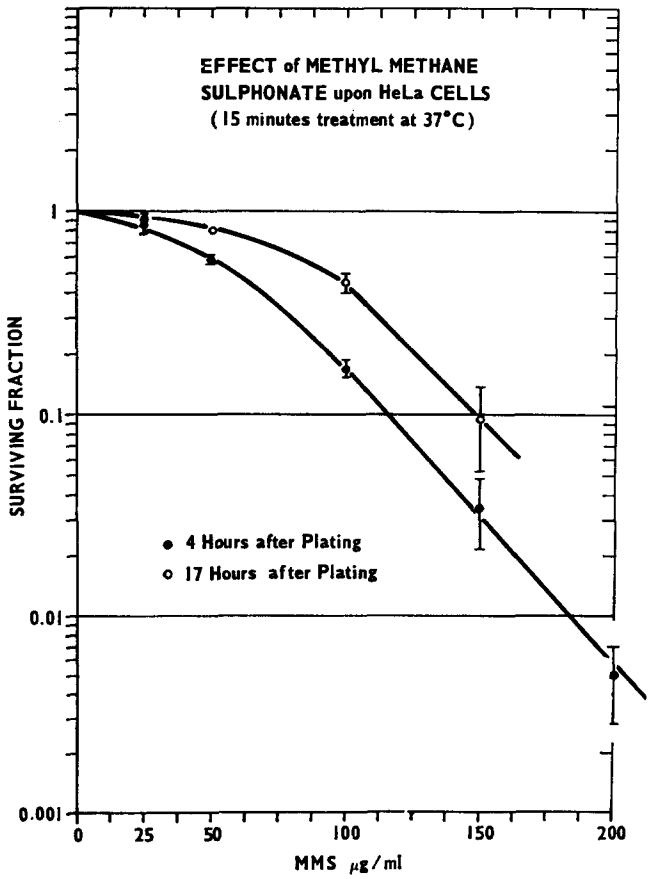


Fig. 5. *The effect of* 15*-minute treatment of methyl methanesulphonate on the survival of HeLa cells. Treatments were given either* 4 *hours or* 17 *hours after plating* ●—● 4 *hrs,* o—o 17 *hours.*

Table 1

Clone	Population doubling time	Plating efficiency
Control	25 hr	70%
M_3	38 hr	14%
M_4	53 hr	7%

isolated which remained stable over long periods of time. Cases of decline of resistance or spontaneous reversion in cloned populations [19] and overgrowth of resistant by sensitive cells in uncloned populations [24] have also been reported. In most of these cases, relatively high degrees of resistance were obtained, e.g., 20,000 fold to amethopterin was reported by Fischer [17]. Mutation to resistance has however been shown to be an event of relatively low frequency. Lieberman and Ove [20] gave a figure of $3{\cdot}6\times10^{-6}$ mutation per cell per generation in monkey kidney cells and Szybalski and Smith [25] gave a rate of 5×10^{-4} mutations per cell per generation in Detroit D98 cells. Mutation to resistance to alkylating agents has less frequently been studied, few reports are available and the degrees of resistance obtained are very low [26, 27], usually two to threefold. They are thus of the same order of magnitude as the increased sensitivity observed in the present study.

Obviously, therefore, the frequency of occurrence of resistant mutants is low and perhaps a more frequent occurrence is a mixture of surviving normal cells and surviving damaged cells. The damaged cells may not always show greater sensitivity and inheritance of the change, as this possibly depends on the initial lesion and mode of action of the drug.

The results of the divided dose experiments (Fig. 6) are open to a number of interpretations. Firstly, it is possible that the initial dose of MMS induces some synchrony and cells are therefore in a sensitive phase of the cell cycle when the second dose is given. Preliminary

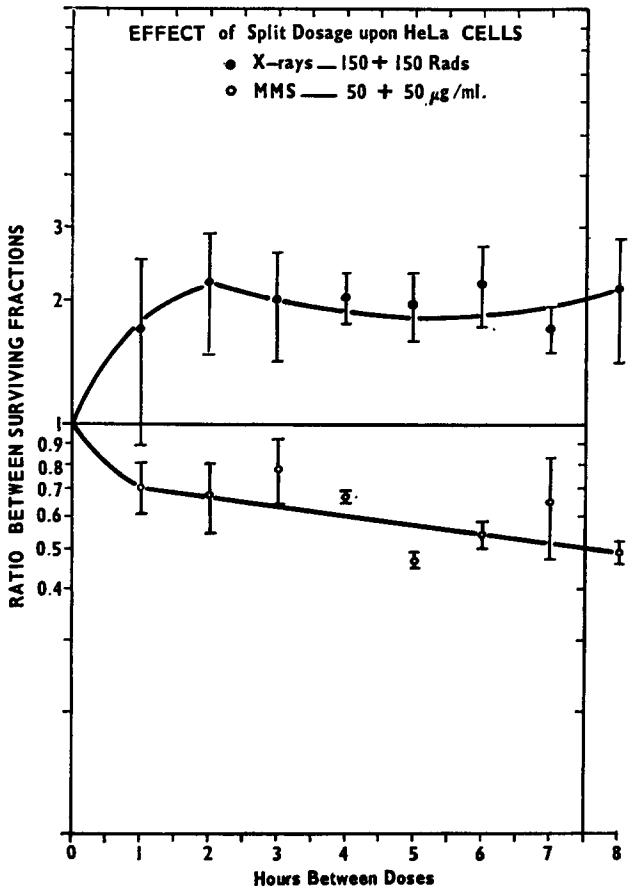


Fig. 6. Effect of divided doses of X-rays and methyl methanesulphonate on survival of HeLa cells. Treatments were started 17 hours after plating the cells. The survival after 300 rad. X-rays and 100 μg/ml MMS is taken as unity.

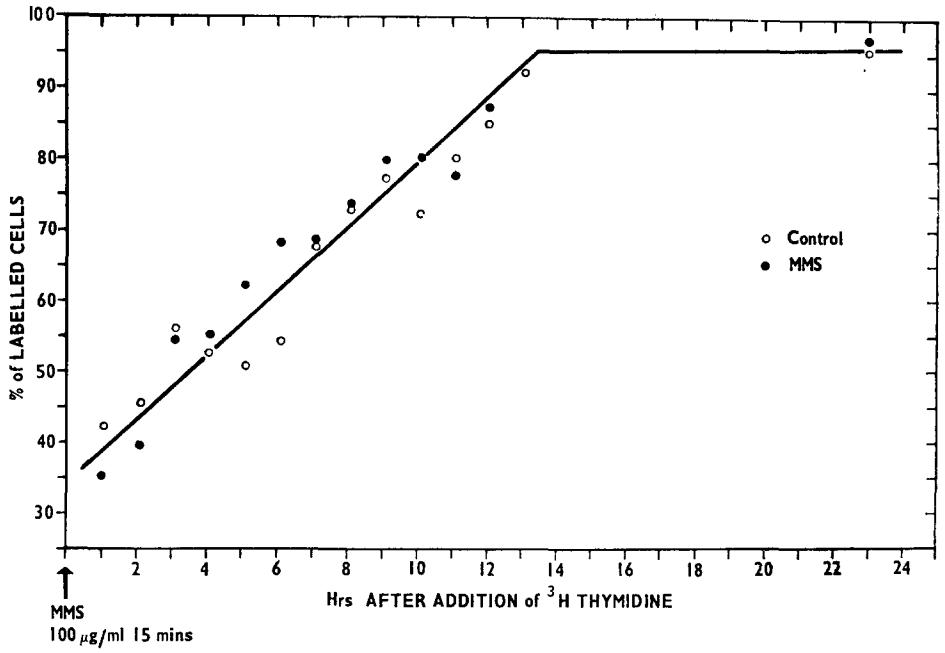


Fig. 7. Rate of increase of labelled cells during the continuous presence of ^{3}H-thymidine in control populations of HeLa cells and in cells exposed to MMS (100 µg/ml 15 mins) 17 hr after plating and immediately prior to addition of the ^{3}H-thymidine.

experiments in this laboratory using the synchronisation technique described by Terasima and Tolmach [28] have shown that cells in G_1 show the greatest sensitivity to MMS [29]. The continuous labelling experiments (Fig. 7) indicated however that the rate of progress of cells into S is unaffected by MMS treatment. Induced synchrony would therefore not appear to be responsible for the decline in survival observed in the divided dose experiments.

The two alternative interpretations of this observation are as follows: the fall in survival observed (Fig. 6) is possibly an indication of the early development of increased sensitivity as shown in the surviving HeLa cell clones (Fig. 3) and P388F populations (Fig. 1 and 2). However, in these divided dose experiments, the number of survivors is much higher ~ 50 % (Fig. 4) than in experiments from which sensitive clones were isolated (Fig. 3). The implication in this case would be that all cells surviving an initial treatment would show greater sensitivity. This possibility has not been examined in detail in HeLa cells, although nearly all the (3%) survivors of 40 µg/ml to P388F cells did show increased sensitivity (Fig. 2).

These results also suggest that there is no recovery from alkylation by MMS, but one must consider in this case, and in the previous situation, the possibility that unreacted drug could still be present in the cells after the 15 min pulse treatment. The effect of this in split dose experiments would essentially be to extend the treatment period, which has been shown to increase the effect of the drug (cf Figs. 3 and 5). However, a thirty-minute treatment of HeLa cells did prove approximately twice as effective as a fifteen-minute treatment at the same dose level implying that the reaction of the drug can be effectively stopped by removing it from the medium bathing the cells.

The implications are therefore that in the lymphoma cells some, though not all the damage sustained by cells after treatment with MMS can be repaired, e.g. two doses of MMS 40 µg/ml (3 hr) separated by 3 days (Fig. 2) resulted in a survival level equivalent to that produced by a single dose of 50 µg/ml (3 hr). Thus, although the two doses are not strictly additive, indicating some repair, some residual damage must remain in the population. In HeLa cells, heritable damage was demonstrable as increased sensitivity and reduced growth rate and plating efficiency of clones M_3 and M_4. The divided dose experiments suggest that the increase in sensitivity develops within one hour and increases progressively with time, and also indicate that HeLa cells are unable to repair MMS damage in the same way as X-ray damage is repaired.

Acknowledgements—The authors are grateful for the technical assistance of Mrs. Elisabeth Moore, Mrs. Dorothy Robinson and Mr. J. B. Harrison.

RESUME

Des cellules du lymphome P388F et des cellules HeLa furent traitées in vitro *par des doses uniques d'un agent alkylant monofonctionnel, le méthyl méthane sulfonate. La survie dépendait du temps d'exposition du produit et de la dose. Des cellules P388F survivantes, et des clones de cellules HeLa, reçurent un second traitement, ce qui mit en évidence une augmentation de la sensibilité. Les clones de cellules HeLa, qui présentaient une telle sensibilité augmentée, avaient aussi un temps de doublement allongé et une possibilité d'étalement diminuée. Ces clones, isolés en sous-culture continue, conservaient leur sensibilité augmentée, leur croissance retardée, et leur efficacité d'étalement réduite, pendant* 4 *à* 5 *mois. Les cellules P388F retrouvaient leur sensibilité normale après* 21 *jours.*

La division de la dose en deux fractions égales, administrées à un intervalle de 1 *à* 8 *heures, produisait, pour les cellules HeLa, une diminution de la survie, par rapport au traitement par dose unique. On compare ces résultats à ceux obtenus avec des doses fractionnées de rayons X qui montrent que la survie est doublée lorsque le traitement est fractionné en deux doses égales, administrées à* 2 *heures d'intervalle.*

SUMMARY

P388F lymphoma cells and HeLa cells in vitro *were treated with single doses of the monofunctional alkylating agent methyl methanesulphonate. Survival was found to depend on duration of exposure as well as dose of the drug. Surviving populations of P388F cells and surviving clones of HeLa cells were given second treatments with the drug and found to show an increased sensitivity. HeLa cell clones showing increased sensitivity also showed increased population doubling time and a reduced plating efficiency. The HeLa cell clones isolated, maintained the characteristics of increased sensitivity and reduced growth rate and plating efficiency over* 4–5 *months of continuous sub-culture. P388F polulations returned to normal sensitivity after* 21 *days growth.*

Divided dose experiments with HeLa cells showed a decrease in survival, compared with that to a single treatment, when the dose was split into two equal fractions separated by 1–8 *hr. These results are compared with those obtained after divided doses of X-rays when survival shows a two-fold increase if two equal fractions are given two hours apart.*

ZUSAMMENFASSUNG

P388F Lymphom- und HeLa-Zellen wurden in vitro *mit Einzeldosen des monofunktionellen alkylierenden Agens Methansulfonsäuremethylester behandelt. Die Uberlebenszeit der Zellen hing sowohl von der Dauer der Einwirkung als von der verwendeten Dosis ab. Die überlebenden Populationen von P388F Zellen und überlebende Klone von HeLa-Zellen wurden einer zweiten Behandlung mit der Verbindung unterworfen, wobei sie erhöhte Empfindlichkeit zeigten. Diesen HeLa-Zellen zeigten auch vergrösste Vermehrungsgeschwindigkeit und verminderte Explantierbarkeit als Einzelzellen. Diese Charakteristiken behielten die Zellen während einer Weiterzüchtung über* 4–5 *Monate. P388F Zellen hingegen wiesen nach* 21 *tägigem Wachstum wieder normale Empfindlichkeit auf.*

Im Vergleich zur Einzelbehandlung zeigten Versuche mit HeLa-Zellen, bei denen die Dosis in zwei gleichen Fraktionen in zeitlichem Abstand von 1–8 *Stunden gegeben wurde, eine Verminderung an überlebenden Zellen. Im Vergleich hierzu ergibt sich bei einer fraktionierten Röntgenbestrahlung (zwei gleiche Dosen in zweistüngdigem Abtsand) eine Verdopplung der Uberlebenswahrscheinlichkeit.*

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Announcements

THE EUROPEAN Association for Cancer Research was founded in Amsterdam, The Netherlands, on January 9th 1968, by a committee appointed for that purpose in Vienna during the European Cancer Meeting in July 1967.

It is the intention of this Association to hold a scientific meeting every two years. The first will be in Switzerland in October 1969.

Membership is open to those who possess an academic degree or the equivalent and have held an appointment or worked actively in cancer research for at least three years or in research for five years, two of which were in cancer research.

The annual subscription, which should be paid to the Treasurer, will be about 20 Guilders per annum.

Applications for membership and further information can be obtained from the Secretary of the Association, Dr. G. J. V. Swaen, Academisch Ziekenhuis Wilhelmina Gasthuis, Pathologisch-Anatomisch Laboratorium, Amsterdam-Oud West, Eerste Helmersstraat 104, The Netherlands. Applicants will be considered for election by the acting executive committee and council.

Membership of the European Association for Cancer Research is open to all suitably qualified cancer research workers. It is distinct from the "European Organization for Research on Treatment of Cancer (G.E.C.A.)" which is a multidisciplinary group of workers studying definite problems as a team.

L'Association Européenne pour la Recherche du Cancer fut fondée à Amsterdam, Pays-Bas, le 9 janvier 1968, par un comité réuni dans ce but à Vienne, lors du "European Cancer Meeting", en juillet 1967.

L'intention de cette association est de tenir un congrès scientifique tous les deux ans. Le premier se tiendra en Suisse en octobre 1969.

Peuvent être membres les personnes porteuses d'un grade académique ou équivalent, travaillant activement en recherche cancérologique, depuis au moins trois ans, ou faisant de la recherche depuis cinq ans, dont au moins deux en cancérologie.

L'inscription annuelle, qui sera payée au trésorier, s'élève à environ 20 florins.

Les formulaires et des renseignements complémentaires peuvent être obtenus auprès du secrétaire de l'association, le Docteur G. J. V. Swaen, Academisch Ziekenhuis Wilhelmina Gasthuis, Pathologisch-Anatomisch Laboratorium, Amsterdam-Oud West, Eerste Helmersstraat 140, Pays-Bas. Tous les membres peuvent poser leur candidature lors de l'élection du comité exécutif et du conseil.

Tout chercheur qualifié dans le domaine du cancer peut devenir membre de l'Association Européenne pour la Recherche sur le Cancer. Il s'agit d'un groupement distinct de l'"Organisation Européenne de Recherche sur le Traitement du Cancer (G.E.C.A.)" qui est constituée par un groupe multidisciplinaire de chercheurs étudiant en collaboration des problèmes bien définis.

Human Carcinogenesis: Working Group

THE WORKING group on Human Carcinogenesis under the Co-ordinating Committee for Human Tumour Investigations met in København on October 5th and 6th 1967 with participation of the following research workers:

Dr. C. van den Berg, *Leiden*
Dr. Johannes Clemmesen, *København* (Convenor)
Professor L. A. Elson, *London*
Professor Åke Espmark, *Stockholm*
Dr. P. Holsti, *Helsinki*
Dr. Eva Klein, *Stockholm*
Professor P. Koller, *London*
Professor C. Maltoni, *Bologna*
Professor R. D. Passey, *London*
Professor D. Schmähl, *Heidelberg*
Dr. R. I. G. Willighagen, *Leiden*

The group discussed the need for the collection of material and data for further characterization of human carcinogenesis and made the following observations.

Genetical aspects. It was considered advisable to study the chromosome pattern of normal cells from persons in families showing an excess of cases of malignant disease among close relatives (parents and sibs), particularly when such cases occur at an unusually early age. Also a followup of children with chromosome anomalies will be desirable.

Chemical aspects. To increase knowledge on occupational and other environmental exposure it is urgently necessary to collect regularly individual histories, especially on high risk groups, and to exchange information on suggestive experience among workers in occupations where carcinogenesis from chemicals might constitute a hazard.

As an example, it appeared that asbestos calls for more attention than dedicated to this factor up to now. It has been found that not only cases of mesothelioma, but also other malignant neoplasms may be associated with exposure to asbestos dust which might occur both occupationally and in other ways. For a list of some of the many and varied sources of exposure, see Hueper: Occupational and Environmental Cancers of the Respiratory System, p. 40, Springer, Berlin, New York, 1966 (Recent results in Cancer Research, Vol. 3).

Cigarette smoking. It has been suggested that part of the international differences in incidence of bronchial carcinoma may perhaps be attributed to differences in the use of flue-cured tobacco which has been heated in the drying process in contradistinction to tobaccos, in which slow drying has allowed enzymes to reduce the sugar content.

Immunological aspects (lymphosarcomas). In the study of the nature of carcinogenesis, the juvenile African lymphosarcoma (Burkitt) has offered particularly useful lines of approach and the rare fulminant cases of other lymph node neoplasia following acute fever or vaccination may offer important opportunities for study.

From cases of Burkitt's lymphosarcoma, as well as from lymphosarcoma cases of the classical type, and of the said fulminant form it will be desirable for specialized laboratories to obtain material of the following kind.

(1) Biopsy tissue, at least about pea-size dispatched in any tissue culture medium containing *foetal* calf serum (e.g. Hank's or Parker medium 199), packed in ice (NOT FROZEN).

(2) Serum, 10 ml packed in dry ice (CO_2).

This material should be sent to Dr. Eva Klein, Institute of Tumour Biology, Stockholm 60, Sweden, from whom in return cell lines established from "cured" cases of Burkitt's tumour, kept under observation, are available for experimental studies.

Organisation Européenne de Recherche sur le Traitement du Cancer
G.E.C.A.
Groupe Coopérateur des Cancers Thyroïdiens

Registre du Cancer Thyroïdien

Secrétariat: Docteur Pierre DOR
Institut Jules Bordet
1, rue Héger-Bordet
Bruxelles 1.

L'enregistrement des cas de cancer thyroïdien a débuté officiellement le 1er janvier 1967. Cent-douze cas ont été enregistrés jusqu'au 31 décembre 1967. Pour tous ces cas, le résumé de l'observation, sous forme de réponse à un questionnaire, et les lames histologiques caractéristiques, ont été adressées au Secrétariat.

Les médecins désireux de collaborer au travail du Groupe sont invités à se mettre en rapport avec le secrétaire.

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Cell Proliferation in Cancer*†

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1. INTRODUCTION

THE SYNTHESIS of tritiated thymidine (^{3}H-TdR) was carried out independently at Brookhaven [1] and in Belgium [2] about 10 years ago. This made available a marker specific for deoxyribonucleic acid (DNA) and highly suitable for cell autoradiography; since then research on cell proliferation kinetics has expanded rapidly. The new facility of identifying cells in DNA synthesis in the tissue and of following their fate for several generations, the ingenious use of the labeled mitoses index [3], of double labeling of DNA in combination with ^{14}C-thymidine [4, 5], as well as other methods combining autoradiography with DNA labeling, enormously increased the power of investigation in the field of kinetics of cell proliferation. After this technological advance, a large amount of data was collected on cell proliferation in cancer. Recently, Baserga [6] extensively reviewed most of the available data in relationship to the cell cycle and control of cell division. Mendelsohn [7–9] has repeatedly discussed the kinetic of tumor growth. At the last Symposium of the Leukemia Society Inc., held in December 1966 in New Orleans, Cronkite [10] brought up-to-date the results and ideas about kinetics of leukemic cell proliferation. And at the Second Symposium on Biological Characterization of Human Tumors in Rome, the Cell Proliferation Study Group held a special session on growth patterns of human tumors under the chairmanship of L. F. Lamerton [11]. It is difficult to add to what these distinguished scientists in the field have already said. The only meaningful thing to do is to refer to their work.

There is one topic, however, in the immense field of cell proliferation in cancer to which, in my opinion, some further contribution can be given at this time. This is the comparison between kinetics of proliferation of tumors and normal tissue, and in this paper I shall be primarily concerned with this problem.

Comparison of normal and neoplastic proliferation may be viewed from different angles. For instance, the comparison may be made from the point of view of setting a biological basis for therapy. In this case, one should make a general comparison of all normal tissues of the body with the type of cancer under consideration. In this paper, however, the comparison will be aimed especially at a basic biological question; that is, identifying changes of proliferation parameters ensuing the transformation of a normal cell into a cancerous one, and investigating the relevance of these changes to the kinetics of unlimited growth. To fulfill the above stated purpose best, one should restrict the comparison to the neoplastic populations which are closest to the initial transformed cells and the normal populations from which they originated. For this reason, the comparison is limited in this paper to primary, autochthonous tumors with normal counterpart.

The general outline of the paper will be as follows: after discussion of the structure of a cell system with proliferative activity, all available relevant data will be critically

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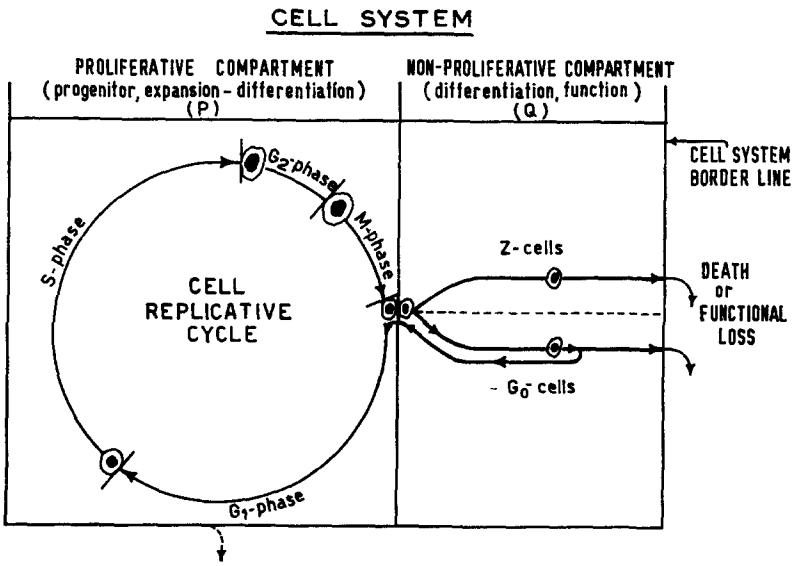


Fig. 1. General structure of a cell system with proliferative activity. See text for detailed explanation.

reviewed; a theoretical analysis of transition from steady-state proliferation to the condition of unlimited growth will follow; and finally, general conclusions will be drawn.

2. THE GENERAL STRUCTURE OF A CELL SYSTEM WITH PROLIFERATIVE ACTIVITY

It has been well established that the process of cell replication is subdivided into four phases, G_2, S, G_1 and M, during which an orderly progression of biochemical events takes place, culminating in the birth of two new cells (Fig. 1). The new-DNA synthesis occurs during the S phase, while during the phases G_2 and G_1 only ribonucleic acid (RNA) and protein syntheses go on. During mitosis protein synthesis is minimal and RNA synthesis is limited to early prophase and late telophase. Many reviews are available on biochemical events during the cell cycle and I refer to Baserga [6] for a full treatment.

The process of cell replication is the cell replicative cycle. After mitosis the new cells may undergo a new replication process or go into a condition of non-proliferation which is generally associated with differentiation and function. This transition to the non-proliferative condition can be irreversible, and in this case I shall refer so these cells as z-cells; or after a variable period of proliferative rest, the cell may replicate again. These cells which have retained the ability to divide are called G_0-cells [12]. Under normal conditions, cells die or are removed for functional reasons after a given sojourn in the non-proliferating condition, but cell death during replication should be kept in mind especially in pathological cases. Removals of cells from the system in a normal condition generally occurs by senescence but, as in the cases of functional removal it can also occur as a random process. In both cases an average transit time can be measured, but one should be aware of the vast difference in the form of the distribution of transit times in the two cases.

In a cell system with proliferative activity, one can distinguish a proliferative compartment (p-compartment) and a non-proliferative compartment (q-compartment). The p-compartment can be further subdivided according to the particular phase of cells in proliferation, i.e., into G_1-, G_2-, S- and M-compartments. The q-compartment may be qualified as z- of G_0-compartment. The p-compartment may consist of an expanding-maturing compartment (e-m-compartment) in addition to a progenitor cell compartment (pr-compartment). The pr-compartment is self-sustaining in that a fraction of the newborn cells will remain in the compartment and continue to replicate. On the contrary, the e-m-compartment is one in which the cell will progress through one or more successive divisions following an irreversible pattern of maturation. A clear instance of an e-m-compartment is given in the haemopoietic series. The e-m- compartment requires feeding from a pr-compartment. If the progenitor cell source is abolished, the e-m-compartment decreases and disappears within a time corresponding to the duration of the e-m-process. One thus distinguishes compartments in a cell population not only on a functional basis but also by morphology as well as by anatomical location. Actually, these criteria often coincide or are combined in delimiting a compartment,

as in the case of identification of the *e-m*-compartment in the bone marrow by cell morphology or in the case of location of the progenitor compartment of intestinal epithelium in the crypts. In tumors, distinction of the population in a *p*- and a *q*- compartment presents the research worker with a difficult problem because morphological or locational criteria are generally lacking. This is also the reason why only relatively recently it has been clearly realized that in tumors, as in normal tissues, not all cells are proliferating. The measurements of the *p*-fraction of cells in a population is of basic importance when duration of the cell replicative cycle is calculated indirectly from the relationship [12]:

$$\text{cell replication time} = \text{DNA replication time} \times \times \frac{\text{No. of proliferating cells}}{\text{No. of cells in DNA synthesis}} \qquad (1)$$

Relationship (1) applies to an asynchronously replicating cell population with uniform age distribution of cells in the *p*-compartment. A correction factor should be introduced in (1) for the case of a growing population. Detailed treatment of this problem is already given by Johnson [13] and others. Biased estimates of cell replication time due to the assumption that all cells in the tissue are proliferating are abundant in the literature.

Definition of terminology used

As stated before, the cell replicative cycle or cell generative cycle (C) consists of the process of cell replication, i.e., the orderly sequence of specific biochemical events which are required and culminate with actual cell division. The definition "interval between midpoint of mitosis in the parent cell and midpoint of the subsequent mitosis in the daughter cell" corresponds to the process of cell replication only in the case that cells divide without interruption. For instance, in the case of cells which go into the G_0 condition and eventually re-enter the cell replicative cycle, the interval between the midpoints of two successive mitoses also covers a period of proliferative rest.

The size of a compartment is indicated by n (number of cells) with the letter in lower index indicating the compartment: n_p, n_{pr}, n_{em}, n_q, n_s, n_m, etc., with N indicating the total number of cells in the system. When the compartment is expressed as a fraction of the total number of cells in the population, the corresponding index is obtained. Thus $n_p/N = I_p$, proliferative index, or fraction of the population which is in proliferation; $n_s/N = I_s$, DNA synthesis index or fraction of the population which is in the process of synthesizing DNA, etc. I_s corresponds to the labeling index of the cell population after flash labeling with ^{3}H-TdR. The average transit time of cells through a given compartment is indicated by t with the lower index letter indicating the specific compartment. Thus transit through the cell replicative cycle is indicated by t_c, which is given by the sum of $t_{G1}+t_s+t_{G2}+t_M$; t_q is the transit time through the q-compartment and so on.

Birth rate and removal rate are defined as the number of cells produced or removed from the tissue per unit time and per cell in the total population. Removal can occur by function, loss (for instance metastasis) or death. I shall also consider a "distribution parameter", i.e. the cell distribution ratio (d-ratio), which for the *pr*-compartment is the ratio between the fraction of newborn cells (just passed through mitosis) which remain in the compartment and continue to replicate ($\bar{\eta}$) and the fraction which leaves the compartment progressing into differentiation ($\bar{\eta}$).

Clearly, if the $\bar{\eta}$ fraction from the *pr*-compartment feeds directly into the q-compartment it will no longer divide, while if it feeds into an *e-m*-compartment it will continue to replicate. For the *e-m*-compartment the definition of the distribution parameter is apparently more complicated because of the possibility of distinguishing serial replicative cycles due to progressive differentiation. The d-ratio in this case can be defined as the ratio between the fraction of cells which does not progress further but remains in the same stage of maturation and continues to divide, and the fraction which progresses into the next stage of differentiation. In the normal *e-m*-compartment, the cells can only progress along the differentiation pathway ($\bar{\eta}=0$; $\bar{\eta}=1$) and therefore to speak of a d-ratio in this case is not very proper; but in pathological cases a recycling cell pool could form in the *e-m*-compartment and $\bar{\eta}$ become a positive number. Doubling time (t_d) is the time necessary for a growing population to double its size. It corresponds to t_c only when the population grows with $I_p=1$ and without cell loss.

It is outside the scope of this paper to go into a discussion of statistical distribution of parameters of a cell population. This has already been discussed in detail by Quastler [12] and others. I will consider here only average parameters, for this is sufficient for the following discussion. Methods available for measurement of proliferation parameters have already been described in detail [14]. The age

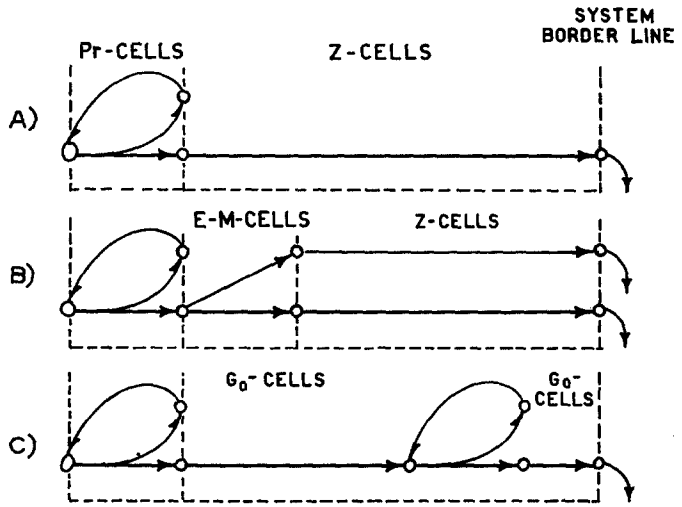


Fig. 2. Wire diagrams of basic cell systems with proliferative activity as found in the adult animal. See text for detailed explanation.

distribution of cells in a growing population has also been dealt with [13].

Basic types of proliferation systems in the adult animal

These are shown as wire diagrams in Fig. 2. System type A consists of a *pr*-compartment in which part of the cells recycle and part after irreversible maturation, stop dividing and pass into a *q*-compartment from which, after an orderly transit, the cells are lost. System type A schematically represents cell populations mainly covering external and internal surfaces of the body. In these cases the transit in the *q*-compartment actually corresponds to physical movement of the cells toward the boundary of the body surface, with loss by desquamation. In the intestinal epithelium the *pr*-compartment is located in the crypts, and the villi are the *q*-compartment. After the $p \rightarrow q$ transformation at about the neck of the crypts, the cells progress from the bottom to the end of the villi, from which pont they are lost into the intestinal lumen. In the epidermis, the *pr*-compartment is the stratum germinativum from which cells progress toward the surface with final desquamation. Other cell systems like skin appendages the crystalin lens, etc., also fall into this category.

System B differs from the former in that an *e-m*-compartment is interpolated between the progenitor and *q*-compartments. System type B represent mainly haemopoyesis, the *e-m*-compartment representing the irreversible process of maturation into specific cell types during which the cells continue to divide. The mature, non-dividing cells sojourn in the marrow and the blood before being removed from the blood mostly by function.

System type C is different from type A in that cells, after being produced, may either replicate again—although with limitations—or go into a G_0-period from which they may again go into replication. System C schematically represents tissues like liver and kidney. In these cases the kinetic data available suggest that cells are lost at random in the tissue and are replaced by division of nearby cells. Cells, therefore, intercalate long periods of proliferative rest with short periods of multiplication. Under conditions in which a larger part of the parenchyma is lost by hepatectomy or intoxication, all surviving cells start proliferating in order to replace destroyed cells. This shows that all liver cells retain the ability to divide.

In the adult animal, cell systems with proliferative activity are in a condition of steady-state, which indicates that the total number of cells in the whole system (N) as well as the number of cells in the single compartments (n) do not change with time when observations are sufficiently far apart in time to avoid cyclic fluctuations connected with biological rhythms. Steady-state is to say that cell birth rate and removal rate in the tissue are equivalent. In the growing tissue, cell birth rate prevails over cell death rate and n and N increase with time.

3. COMPARISON OF NEOPLASTIC GROWTHS WITH NORMAL COUNTERPART

Normal counterpart is here intended to mean the normal tissue from which the cancer originated, possibly in the same animal or at least in animals of about the same age as those carrying the neoplastic lesions. In this section,

all available instances of comparison of primary autochthonous neoplastic growth to normal counterpart, with respect to cell proliferation kinetics, will be reviewed. Comparison of primary tumors to normal counterpart is clearly the choice. The profound transformation that serially transplanted tumors undergo makes these tumors peculair biological entities which may bear little relationship to the primary tumor and tissue from which they originated. The changes due to serial transplantation are likely to influence cell kinetics aspecifically, and this may obscure changes in proliferation relevant to neoplastic transformation. Comparison with normal counterpart, furthermore, prevents kinetic differences between different normal cell types from interfering with recognition of changes due to neoplastic transformation.

Blast population in acute leukemia and myeloid cell population in man

To date 14 cases of acute leukemia in man have been studied with injection *in vivo* of ^{3}H-TdR and subsequent sampling of cells and autoradiography. The investigations were made by Killmann *et al.* [15, 16], Mauer and Fisher [17–19], Gavosto *et al.* [20], Clarkson *et al.* [21] and Greenberg *et al.* [22–23]. The results are summarized in Table 1, with the addition of some data on fraction of cells in DNA synthesis in bone marrow and blood, which were obtained by incubation *in vitro* of cells with ^{3}H-TdR by Pileri *et al.* [24]. These results on leukemia can be compared with a summary of data on the myeloid series, as obtained essentially from papers of the Brookhaven group of hematologists [10, 25]. Even in recent literature [6, 26], it is generally reported the leukemic blast cells have a much longer t_c than normal myeloid precursor cells. This statement, which is frequently quoted in support of the more general idea that cancer cells often proliferate slower than their normal counterpart, is based on pioneer research in this field [15, 27]. Due to difficulty in applying to humans more precise methods of estimating t_c these research workers used the method of dilution of the label [14]. As it is also reported in Table 1, they obtained a grain count halving time of about 24 hr for normal myeloblasts and of 50–100 hr for leukemic blasts. From this they concluded that the leukemic blasts replicate much slower than their normal counterpart. Unfortunately, they were not aware at that time that a larger fraction, on the average about 80% of the leukemic blast population, is not dividing [16–22]. As pointed out by Mendelsohn [7], in such conditions the ditulion of the label with time in the cell population does not reflect, or only very distortedly, the actual t_c. This is due to cells which incorporate the label at the time of injection but then stop dividing. More recent data of Mauer and Fisher [19] on dilution of label of replicating blasts, selectively indicate a grain count halving time of about 20 hr. On the basis of the increase of the number of labeled blasts, Cronkite [10] has also recently estimated, from data of Clarkson *et al.* [21], a t_c of about 24 hr for leukemic blasts, and Greenberg *et al.* [22,23] also put the t_c of these cells at 20–24 hr. In conclusion, leukemic blasts have a t_c of about 20 hr, with possible oscillations between a minimum of 15·3 hr, as calculated by this author using equation (1) and a probable maximum of about 24 hr. These values are of about the same order of t_c as normal myeloblasts, which is about 24 hr, with minimum and maximum estimates of 15·3 hr and 48 hr. More precise measurements are necessary for finer conclusions.

Contrary to a previous idea pointed out above that all leukemic blasts are engaged in proliferation, solid evidence, both *in vivo* and *in vitro*, has been obtained by a number of research workers [17–24] to show that the leukemic blast population can be differentiated into a dividing blast population consisting of larger blasts and a non-dividing population of smaller blasts. This is based on the grouping of blast cells into different size classes and then comparing the ^{3}H-TdR labeling index of the different classes; and on the observation that only larger cells are labeled after a flash of ^{3}H-Tdr. With time, the small cells become labeled as the intensity of the label in the larger blasts decreases. This last result is evidence that the large blasts eventually become smaller cells. As Table 2 shows, the I_s of the larger blasts is on the average about 0·5–0·7, that is, it approaches that of myelocytes, which is 0·65. In contrast, the I_s of the total population, including large and small blasts, is much lower and may show very large variations from patient to patient. In a recent paper, Pileri *et al.* [24] studied in detail the I_s of bone marrow in 42 patients with acute leukemia, by *in vitro* incubation of cells. Their results are also shown in Table 2. They find that in the marrow of patients with clinical diagnosis of fast-expanding leukemia, I_s is, on the average, twice as large as in patients with leukemia in stationary condition, with a large variability present in both groups

Table 1. Cell proliferation parameters in normal as well as acute leukemic bone-marrow and blood in man [based on data from refs. 10, 15–25]

	Myeloid normal bone-marrow ($I_p \times 10^2 \sim 30$)						Normal Blood
Kinetic parameter		Dividing (~0·3 of total myeloid population)			Non-dividing	Total myeloid marrow averages	
	stem cells	myeloblast	promyelocyte	myelocyte	neutrophilic compartment		neutrophiles
$I_m \times 10^2$		2·5	1·5	1·1	0	0·4	0
$I_s \times 10^2$		85	65	33(60–15)¶	0	~14	0
Grain count halving		~24 hr	~24 hr	54–60 hr			
t_m		0·6 hr	0–6 hr	0·6 hr			
t_s		13hr (6–24 hr)	13–24 hr	13–24 hr			
t_c	⩽20–25 hr	24 hr (15·3–48 hr)					
Compartment transit time		23·3 hr	26–78 hr	17–126 hr	33–72 hr†	99·3–⩽299 hr	9·6 hr‡
Emergence time marrow→blood							96–144 hr
Half life							6·6 hr

Table 1. *cont.*

	Bone marrow in acute leukemia ($I_p \times 10^2 \sim 3$ to 60)			Blood in acute leukemia
	Dividing blasts (larger) (from 0·03 to 0·60 of total blast population)	Non-dividing blasts (smaller)	Total marrow blast population averages	
$I_m \times 10^2$			0·65‖ (0·2–0·95)	0–0·02
$I_s \times 10^2$	50–70	~0 expanding	slow: 7·6‖ fast: 14·7‖	slow: 2·2‖ fast: 8·1‖
Grain count halving	50–100 hr			
t_s	13 hr (8–20 hr)			
t_c	20 hr (15·3*–24 hr)			
Compartment transit time Emergence time marrow→blood			≧192–240 hr	33–38 hr < 1 hr

Numbers in parenthesis represent maximal oscillations.
*Calculated by this author on the basis of equation (1) of text and from $t_s = 13$ hr, $I_s = 0{\cdot}85$ and $I_p = 1$.
†Release of band and segmented neutrophiles does not follow a strict pipeline; the values given in the table represent a lower limit.
‡Random loss truncated by senescence.
‖Average value. In bone marrow, maximal oscillations in slow expanding conditions are 1·5–13·7 and in fast expanding conditions 4·7–30.
¶Average values for small and large myelocytes, respectively, were put in parentheses.

Table 2. Cell proliferation parameters in rat liver and 3'-Me-DAB induced hepatoma (based on data from refs. 28–31)

		Age of rats (weeks)	$I_s \times 10^2$	$I_m \times 10^2$	t_s	t_{G2}	t_m	t_{G1}	t_c	$I_p \times 10^2$
Liver	body and liver wt. growing	3	4·2	1·1	9 hr†	1·8 hr†	1·7 hr†	9 hr†	21–25 hr†	10*
		8	1·7	0·4						4*
		24	0·2	0·0						0·5*
Primary hepatoma		24	8·1	2·8		2 hr			14–16 hr‡	14–15§*

*Calculated by this author from $I_p = (I_s \times t_c)/t_s$.
†Values for diploid cells only; the diploid population is the major replicating group, but tetraploids play a minor role.
*Calculated by this author from Post and Hoffman data [28] interpreting skewness of 1st wave of labeled mitoses according to later interpretation by the same authors [31] of corresponding normal liver in 8-week old rats; values is valid for diploid cells only; see also Fig. 3.
§Calculated by this author assuming $t_s = 7$–9 hr.

of patients. In general, the I_s may vary from as low as 0·015 to 0·30, while the myeloid series of normal bone marrow, including mature leukocites, shows an I_s of about 0·14. On the basis of t_s and of the data which show that t_c is about twice the former in both normal and leukemic cells, one can calculate I_p to be about 0·30 for the whole normal myeloid bone marrow. In leukemic bone marrow, the I_p is highly variable, from 0·03 to 0·60, in relation to the state of the patient, i.e., in general being lower in the static and higher in the fast expanding condition.

Another difference of interest in normal and leukemic cell kinetics is in the "emergence" time, i.e., the time elapsing between the initiation of maturation of a progenitor-cell and its passage into the blood. In normal marrow the emergence time is 4–6 days, which is the time required for maturation from myeloblasts to granulocytes, while in acute leukemia it is only 1 hr or less. That is, leukemic blasts may pass into the blood even in the very early stage of marrow life, although there is a higher probability for smaller than for larger cells to emerge into the blood. This last result, at least in part, explains the generally much lower labeling index of blood, which in leukemia is, relatively speaking, an almost sterile compartment [10]. Because of the difficult kinetic nature of the "emergence", it is difficult to compare normal and leukemic bone marrow with regard to transit time of cells through the non-proliferative compartment of marrow. A rough comparison of main transit values shows that the average t_a of leukemic blasts in marrow is higher than normal. The transit time of leukemic blasts in blood is certainly much higher than that of normal leukocytes: about 3–4 times higher at least. These data thus suggest that, on the whole, the life span of non-dividing leukemic blast cells is longer than that of the mature cells of the normal myeloid cell line.

Primary 3'-Me-DAB induced hepatoma and normal liver in rats

In 1964 Post and Hoffman [28] studied the cell replication pattern of 3'-Me-DAB induced hepatoma in 5-month old rats by the method of the waves of labeled mitoses after pulse labeling with ^{3}H-TdR. Their data on prophase labeling are presented in Fig. 3 and are connected by this author with a broken line. The same group of research workers [29, 30] also investigated cell proliferation in the normal

Table 3. Cell proliferation parameters of basal epithelium and 7,12-DMBA induced hyperplasia and tumors of the hamster pouch (based on data from ref. 32)

	$I_s \times 10^2$	$I_m \times 10^2$	t_s (hr)	t_{G2} (hr)	t_m (hr)	t_{G1} (hr)	t_c (hr)
Basal epithelium	7·2	1·7	10·2	1·6	2·6	128	142
Hyperplastic epithelium	24·6	1·5	10·2		0·5		42
Epidermoid carcinoma†	29·5	2·0	6·1	2·2	0·4	11·9	20·7
				2·3	0·6	8·6	17·6

†Upper transit values were estimated assuming linear growth; lower values assuming exponential growth; $I_p = 1$ was assumed for all tissues.

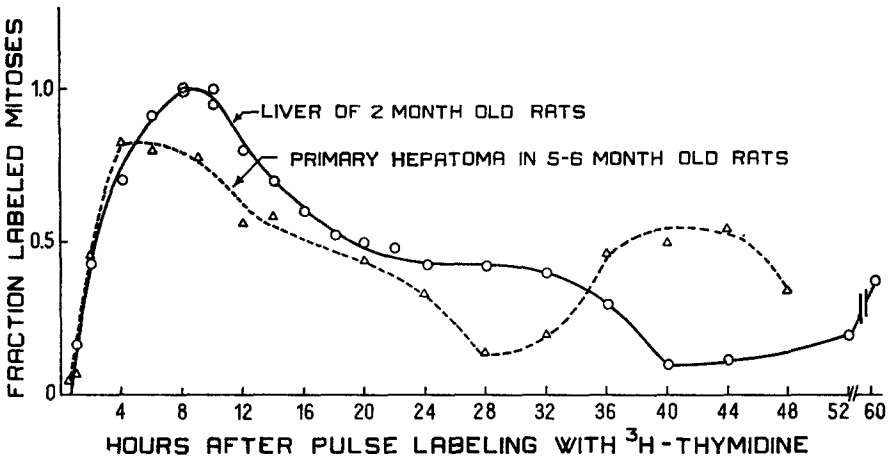


Fig. 3. Labeled mitoses curves of 3'-Me-DAB induced hepatoma and normal liver cells after pulse labeling with ^{3}H-thymidine in vivo. *See text for detailed explanation.*

liver of rats from 1 day to 24 weeks of age. At 8 weeks of age, when the rat is still growing, there is still sufficient proliferation in the liver to make feasible a study of cell replication with the waves of labeled mitoses method. Although to be strictly correct, hepatoma cells should be compared with normal liver cells of rats of the same age as those carrying the hepatoma, to date liver of 8-week old rats is the closest normal counterpart available. The data on normal liver are also plotted in Fig. 3 and connected by this author with a continous line. Clearly, the first wave of normal liver has a marked skewness on the trailing edge. This very obvious anomaly caused further studies by Post and Hoffman [31] who re-interpreted the first wave of labeled mitoses as actually containing two waves of labeled mitoses of diploid cells, which do not appear as two distinct waves because of contribution to proliferative activity of tetraploid cells with a longer DNA replication time. This interpretation is based on correlation between the curve of labeled mitoses and per cent contribution of diploids, tetraploids and octaploids to proliferation of labeled cells; and on comparison with the labeled mitoses pattern in liver of 1 day old rats, at which age the only proliferating cells are diploid.

The same anomaly of the first wave of labeled mitoses is, however, also clearly to be seen in the case of the hepatoma. Since the primary hepatoma consists of aneuploid-polyploid cells, if the interpretation given by Post and Hoffman for normal liver is correct, it should also be valid for hepatoma. With a t_s of 7–9 hr and applying the usual graphic technique [12] to graphs of Fig. 3, one obtains a t_c of 14–16 hr for hepatoma. Therefore t_c of hepatoma is shorter than that of normal liver in rats of about the same age, while I_p is larger. A summary of data is presented in Table 2.

Normal basal epithelium, hyperplasia and epidermoid carcinoma of the hamster pouch induced by 7,12-*DMBA*

Reiskin and Mendelsohn [32] carried out these measurements with precise methods and the specific aim of strictly comparing proliferation kinetics of cancer cells to their normal counterpart. A summary of their data was compiled and is shown in Table 3. They estimate t_s by the indirect method based on equation (1) from t_s as measured by the wave of labeled mitoses method, and from I_s measured as ^{3}H-TdR labeling index. They assume $I_p=1$ for both the basal epithelium and the tumors. This assumption is reasonable for the stratum germinativum, but uncertain for the deriving carcinoma. If the I_p of tumors is smaller than 1, however, the t_c of tumors would be even shorter than calculated and the difference with normal epithelium larger. While the question of possible difference of I_p in normal and cancerous tissue is to be left open in this case, the decrease of t_c to about 1/3 of the normal in hyperplasia and to 1/10–1/15 in cancer is dramatic and undeniable. Another conclusion from the data of Reiskin and Mendelsohn is that all phases of the cell replicative cycle, with the possible exception of mitosis, contribute to the decrease of t_c in cancer cells.

Normal epidermis hyperplasia and epidermoid carcinoma by 3-*MC in the mouse*

Dörmer *et al.* [33] compared proliferation kinetics of the whole normal epidermis with 3-methylcolanthrane induced hyperplasia and

carcinoma in the mouse. Their data support the conclusion that in hyperplasia and in cancer of the epidermis t_c is much shorter than in normal counterpart. They find a replication time of 150 hr for normal epidermis against 56 hr for hyperplasia and only 32 for carcinoma. Thus t_c of carcinoma cells is reduced to about 1/5 of its normal counterpart.

Finally, I should mention the extensive work by Iversen and collaborators on epidermal cell population kinetics in hyperplasia and carcinogenesis. However, these data have already been excellently summarized and interpreted by the same author [34, 35] and it would be redundant to review them again in this paper.

Normal, hyperplastic and neoplastic mammary epithelium of the C3H mouse

An accurate comparison of cancer with its normal counterpart is best obtained when cancer and corresponding normal tissue are compared in the same animal. This possibility is offered by the C3H mouse which shows spontaneous mammary tumors as well as preneoplastic lesions, the so-called hyperplastic nodules, while still a large part of the mammary tissue is exempt from these changes.

Measurements of proliferation parameters in normal, hyperplastic and neoplastic mammary tissue in this mouse strain were carried out by this author [36–38]. From the same C3H mouse carrying one or more spontaneous primary mammary tumors groups of alveoli from normal tissue as well as hyperplastic nodules were micro-dissected. Small bits of tissue were also obtained from tumors showing an alveolar structure. These tissues were then "squashed" on slides in order to obtain cytological preparations with cells spread well apart. This is a necessary prerequisite in order to obtain good results with the double-labeling method with ^{3}H- and ^{14}C-TdR, because cells labeled with one or the other radioactive isotope can easily be identified. By applying the double-labeling method, t_s as well as I_s and I_m were measured simultaneously in the different mammary tissues in the same animal [36].

In further work I_p of normal alveoli and hyperplastic nodules was also estimated, as well as re-estimated in mammary tumors [37, 38]. I_p in C3H mammary tumors was found to be on the average about 0·40, which confirms earlier experimental measurements of Mendelsohn [39]. I_p of normal alveoli and hyperplastic nodules were estimated at about 0·14 and 0·26 respectively. Thus, in both these tissues the fraction of cells engaged in proliferation is definitely smaller than in cancer. Once these data are obtained, cell replication time and mitotic time can also be computed from equation (1). The C3H mammary tumors studied by us were generally at least about 1 cm^3 in volume and most often much larger. Mendelsohn [8] has shown that the C3H mammary tumors have a striking tendency to cube-root growth, which agrees with the finding that the tumors used in our experiment showed a quasi linear mode of growth. For this reason t_c and t_m may be calculated with good approximation by equation (1) as such even in the case of tumors. The complete set of values for the cell replicative cycle and proliferating fractions for normal alveoli, hyperplastic nodules and mammary cancer in the C3H mouse are summarized in Table 4. One can see that while t_c decreases from normal to neoplastic cells by a factor of 2, I_p increases by a factor of about 2·5. The decrease of t_c is a consequence of reduction of cell transit through all phases of the cycle.*

*In a paper published after the presentation of this one, Hoffman and Post (*Cancer Res.*, **27**, 898; 1967) carried out studies *in vivo* on DNA synthesis in normal and cancer cells in human beings. They injected ^{3}H-thymidine in seven elderly subjects about to undergo surgery for carcinoma, and then obtained specimens of tumors and adjacent normal tissues. In 3 out of the 4 cases of gastric and intestinal carcinoma where they obtained samples of normal gastric and intestinal tissues, they measured a decrease of the average grain count on labeled cells and of the percentage of labeled interphase cells in cancer tissues compared to normal. In the fourth case, they did not find notable differences between cancer and corresponding normal tissue. They interpret the decrease of grain count to indicate a decrease of DNA synthesis rate, i.e. a longer t_s in cancer cells; and the decrease of per cent labeling to indicate a decrease of the fraction of cells engaged in DNA synthesis. From this, they further speculate that normal cells have a shorter replication time compared to cancer cells.

There are many pitfalls, most of which are extremely difficult to cope with, in extrapolating from grain count to rate of DNA synthesis; they are technical, as for instance the error involved in efficiency of tritium counting by a photographic method, and the geometry of nuclei in a tissue section. These factors, together with true variations of DNA synthesis rate over the *S*-period, induce an extreme variance in grain counts, which span at least over more than one order of magnitude. Furthermore, most important in the case of a comparison of grain counts in a normal and pathological tissue is the error connected with differences in blood flow and in the size of the cold thymidine pool of the tissue. In particular this last factor is of basic importance in a comparison of normal and cancer tissue, becauses it is well known that the thymidine pool of cancer tissues is generally much larger than that of normal tissues. This is not surprising considering the large cell distruction within tumors, even in the abscence of visible necrosis, as

Table 4. Cell proliferation parameters of alveolar cells of normal mammary gland, hyperplastic nodules and cancer in the same C3H mouse
(based on data from refs. 36–38)

	$I_s \times 10^2$	$I_m \times 10^2$	t_s	t_{G2}	t_m	t_{G1}	t_c	$I_p \times 10^2$
Normal alveoli	4·9	0·4	21·7	3	1·6	37·7	64	14·3
Hyperplastic nodules	9·7	0·5	15·9	2	1·3	21·8	41	25·8
Mammary tumors	14·1	1·1	11·6	1·4	0·9	19·1	33	40·0

Average values of six mice bearing 12 mammary tumors with alveolar structure

Conclusions

From the available instances of tissue- and age-specific comparison of neoplastic growth to normal counterpart presented in this section, the following conclusions may be drawn:

(1) Replication time of cancer cells is generally shorter than that of normal counterpart in steady-state. Cells of hyperplastic, pre-neoplastic lesions generally show a t_c intermediate between normal and neoplastic values. Leukemic blasts, contrary to an opinion held until recently, show about the same t_c as myeloid precursor cells in the normal bone marrow. Because very precise estimates are still lacking at this time, the possibility that leukemic blasts actually replicate faster than normal counterpart cannot be excluded.

(2) Decrease of all phases of the cell replicative cycle contributes to the decrease of t_c. This, while dismissing the idea of an immutable t_s, indicates a speed-up of all biochemical events connected with cell replication. Non-proportional decrease of cell transit times of different phases of the replicative cycle points to the fragility of estimation of proliferation changes on the basis of I_s and/or I_m alone.

(3) Correctly, one should consider the whole normal tissue in the calculation of I_p. In the cases in which it was measured or calculated, I_p of solid cancer and hyperplasia was found to be larger than I_p of normal counterpart as a whole. Leukemic bone marrow is a notable exception. I_p of blast population in acute leukemic bone marrow is in most cases much lower than I_p calculated for the whole myeloid series of normal marrow. However, in instances of fast expanding leukemic conditions the I_p of leukemic bone marrow may be larger than I_p of normal counterpart.

4. OVERALL COMPARISON OF NEOPLASTIC AND NORMAL CELL PROLIFERATION

Experimental animals

In the previous section tissue- and age-specific comparison of neoplastic and normal proliferation was carried out. However, one should also explicitely consider that (1) cells in embryos or in new-born fast-growing animals are usually replicating faster than neoplastic cells of the same type in the adult animal, and (2) normal cells of some fast proliferating renewal tissue like intestinal epithelium in the adult animal, replicate faster than many cancer cells. Furthermore, it has been shown that normal cells—specifically the mammary alveolar cells of C3H mouse—which show a longer t_c than corresponding cancer cells under normal hormonal conditions, replicate faster than cancer cells under artificially high doses of

calculated by Steel (*Europ. J. Cancer*, **3/4**, 381, 1967) and Bresciani (1st Symp. Ass. Ital. Radiobiol. Med., Casaccia del C.N.E.N., 1967; in press). Thus the injected ^{3}H-thymidine will have a lower specific activity within tumors compared to normal counterpart, with resulting lower grain count even in the case of a higher rate of DNA synthesis. In our own studies with C3H mammary tumors and normal mammary gland, attempts failed to correlate grain counts with duration of the *S*-phase as measured with more reliable methods. The ratio between grain counts in normal tissues and t_s, as measured by the double labeling method, was different from the same ratio in tumors—an effect most likely connected with differences in thymidine pools in the two tissues, although contribution by other factors cannot be ruled out. Cases of grain counts lower in cancer than in corresponding normal cells were observed, while the double labeling method was giving opposite results. With regard to the percentage of labeled interphase cells, one should consider that lower specific activity thymidine, eliminating the lower part of the grain count cell spectrum as compared to higher specific activity thymidine, produces a lower labeling index. Another main criticism of the comparison of labeling indices by Hoffman and Post is that in the case of intestinal epithelium, due to the localization of cells in DNA synthesis in the crypts only, one should specify where the labeling index is being measured. Unfortunately, they do not give this information. From data of Lipkin *et al.* in Table 4 of this paper, the average labeling index for the whole epithelium of human intestine is 1·8–3·6%, a value lower than those for intestinal cancer reported by Hoffman and Post. As discussed in this paper, the labeling index of the whole epithelium should be considered in a comparison under correct kinetic premises of cancer of the intestines with normal counterpart.

Table 5. Overall comparison of neoplastic and normal proliferation parameters in mouse and rat

	t_c (hr.)	$I_p \times 10^2$	Refs.
Mouse			
Tail bud of embryo	11		14
Growing hair follicle	12		40
Duodenum: crypts	13·5†	65†‡	41
crypts + villi		9·8†‡	
Mammary gland			
normal alveoli:			
in intact mouse	64	14·3	36–38
in mouse ♂ and treated with E+P*	13	16·0	
cancer with alveolar structure	33	40·0	
Ear epidermis	30–100	8–25‡	42
Rat			
duodenum	9·4		43
Liver			
3-wk old rats	21·5	10‡	28–31
1-day old rats	13·8	32‡	
primary hepatoma	14–16	14–15‡	

*E=17β-estradiol; P=progesterone.
†Average values of animals of different ages.
‡Calculated by this author from $I_p = (I_s \times t_c)/t_s$; for duodenal crypts average values are $t_s = 9$ *hr*, $I_s = 0{\cdot}425$; for villi+crypts $I_s \simeq 0{\cdot}065$ [41]. For whole ear epidermis $I_s = 0{\cdot}045$, $t_s = 18$ *hr* [42].

ovarian hormones. Data from experimental animals supporting these statements are summarized in Table 5 [14, 28–31, 40–43].

Man

In several instances it has been attempted to estimate kinetics parameters of tumors in man. For obvious reasons, the measurements in the case of solid tumors were generally limited to estimation of I_s by pulse labelling the patient with ^{3}H-TdR a short time before surgery. Cell replication time was then indirectly calculated by equation (1) as such, or modified for growing populations assuming an arbitrary t_s and $I_p = 1$ [44–46]. As I_p may vary largely from tumor to tumor, and variation is the rule also in t_s, these indirect estimates, unfortunately, may bear very little relationship to actual values. Because of the possibility of serial sampling, only leukemia was studied in depth in humans, as reported in the previous section.

Table 6. Cell proliferation parameters of tumors and normal tissues in man as studied with ^{3}H-TdR in vivo

	$I_s \times 10^2$	t_s (hr)	t_c (hr)	$I_p \times 10^2$	Ref.
Tumors					
Pleural and/or peritoneal effusions					
carcinoma endometrium (S.C.)	11	60	110	20*	47
carcinoma ovary (M.C.)	18	(28)	(120)	(77*)	
carcinoma ovary (E.C.)	20	(34)	(120)	(70*)	
carcinoma stomach (M.P.)	28	20	(72)	(100*)	
carcinoma stomach (S.M.)	27	32	(110)	(93*)	
lymphosarcoma (B.H.)	4·5	17	(91)	(24*)	
Basal cell carcinoma I	5	19	(97)	(29)	48
Basal cell carcinoma II	12		(67)	(39)	
Normal tissues					
Epidermis			(1080)		45
Ileum: crypts	8·9	11	24	19*	49
crypts+villi	1·8			4*	
Colon and rectum: crypts	15·0	11–15	24	24–33*	50
Colon and rectum: crypts+villi	3·6				

*Calculated by this author from $I_p = (n_s \times t_c)/t_s$.

More recently, however, several cases of neoplastic effusions and solid tumors in man were investigated with improved methodology by Clarkson *et al.* [47] and Malaise *et al.* [48], respectively. These data are summarized in Table 6, together with available data for normal tissues in man [45, 49, 50]. Values which, in the opinion of this author, cannot be considered definitely certain, are put in parenthesis. The results shown in Table 6 are in agreement with data obtained with experimental animals. In the case of basal cell carcinomas compared to normal epidermis, the decrease of t_c appears to be very relevant. At the same time, as in experimental animals, overlapping of normal with neoplastic values is undeniable, with t_c of intestinal epitheliums being shorter than t_c of most tumors. Is it of interest to point out the generally longer t_c of normal as well as neoplastic cells in man in comparison with experimental animals.

Conclusions

The overlapping of spectrums of proliferation parameters when comparison is not tissue- and age-specific, as well as the faster replication of normal cells in comparison to cancer cells when stimulaton by hormones or other factors is applied, has already been repeatedly pointed out [6, 26, 32, 36]. These more recent observations obtained by methods employing ^{3}H-TdR and autoradiography are significant in that they have further contributed to disprove that tumors are all-out growths. However, they do not challenge the validity of the conclusions reached when a strict comparison of cancer with normal counterpart is carried out with the criteria already expressed in the previous section. The results shown in this section only further qualify the conclusions of the previous section by indicating that the parameters of the kinetic changes in the transformed, neoplastic cell do not necessarily reach the potential limit values of the specific cell type, or of mammalian cells in general. The wide spectrum of values of proliferation parameters of different primary tumors is in itself evidence that differences inherent to the normal cell type at least in part persist in the ensuing cancer cell.

5. AN ANALYSIS OF THE CHANGE FROM STEADY-STATE TO UNLIMITED GROWTH

In the first section of this paper the basic compartments, which may be found differently combined in proliferation cell systems, were defined and presented as wire diagrams in Fig. 2. The number of cells in a compartment, i.e., the size of the compartment, is however of relevance to the continuation of this discussion and should now be defined. The compartment will now be represented on a plane with the x-axis measuring time (cell age) as fractions of t_c, and the y-axis the number of cells in the age class (v). Thus the area delimited by these coordinates represent n, the total number of cells in the compartment. In the steady-state condition, in which n does not change with time, the *pr*- and the *q*-compartments correspond to rectangular parallelograms, because v does not change throughout the compartment. In the *e-m*-compartment, on the other hand, v increases with time. In the simplified assumption of this discussion of lack of intercellular and intracellular variability of proliferation parameters, the increase in size of the cell flux throughout the compartment will occur stepwise at each time fraction t/t_c. The *e-m*-compartment can thus be approximated by a trapezoid with the smaller side t measuring the influx from the progenitor compartment and the larger one the efflux into the *q*-compartment. The proliferative index of the *e-m*-compartment may be lower than 1.

The three compartments are combined in basic cell systems A and B of Figs. 4 and 5. System type A consists of a *pr*- and a *q*-compartment, while system type B has an *e-m*-compartment interpolated between the previous two. As discussed before, cell systems A and B cover most cases of steady-state populations in the adult animal. The self-refeeding ability of the *pr*-compartment is indicated by a backward pointing arrow, while its subdivision into G_2, S, G_1 and M compartments is not shown because it is irrevelant to the following discussion.

The effect of a change of t_c *on steady-state*

Let us consider the case of an irreversible decrease of t_c. Referring to basic system type A of Fig. 4, one may assume that, without modification of other parameters, t_c of all progenitor cells decreases to one half at once. The rate of cell production of the *pr*-compartment will thus double and, as a consequence, the flux of cells into the *q*-compartment will also double. From the time of the change the *q*-compartment will therefore start to increase linearly with time and for a time $t=t_q$, until the flux of cells has doubled throughout the *q*-compartment. At this time a condition of steady-state will again set in. The new system corresponds to A1 of Fig. 4.

An irreversible decrease of t_c will thus transform steady-state into growth for a

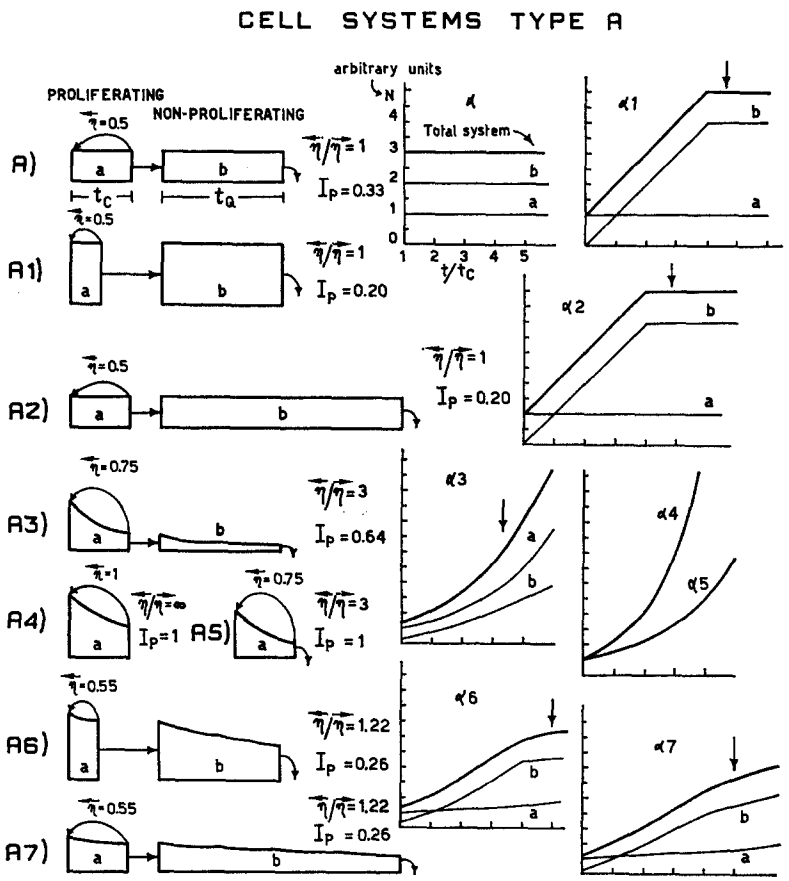


Fig. 4. An analysis of the change from steady-state to unlimited growth in the case of basic system type A. The growth curves α were generated assuming different changes or combination of changes of proliferation parameters (t_c, t_q, $\vec{\eta}/\vec{\eta}$) *with respect to basic system type A, occuring in an arbitrary unit of progenitor cells. Arrows on curves indicate phase of growth coincident with the two-dimensional representation of corresponding cell system (A). Concave trapezoids represent age distribution of cells in growing populations. See text for detailed explanation.*

limited time only. Clearly, the final size of the system will be:

$$N = n_{pr} + n_q \frac{(t_c)_a}{(t_c)_b}. \qquad (2)$$

where N is the number of cells in the whole system, and lower indexes a and b indicate values before and after the change. One can also easily calculate that I_p decreases almost proportionally to t_c.

In basic system type B of Fig. 5, a decrease of t_c of progenitor cells will bring about changes similar to those described for system A. The final size of the new system after the phase of growth will be:

$$N = n_{pr} + \frac{(t_c)_a}{(t_c)_b}(n_{e-m} + n_q). \qquad (3)$$

Also, in the case of irreversible decrease of t_c of cells in the *e-m*-compartment it is simple to see that the resulting growth is limited in time, and for conciseness this case will not be discussed here. Furthermore, it is clear that, on the contrary, an increase of t_c will bring about an opposite effect on the steady-state system, i.e., a decrease in size, but hypoplasia is outside the scope of this paper.

Cancer arises from a single or few transformed cells, while in the previous discussion it was assumed that t_c of all cells changes. Graph α 1 was generated starting from an arbitrary number of progenitor cells which were assumed to have undergone a decrease in t_c to one half compared to t_c of the population from which they originated, while remaining unchanged with respect to duration of non-proliferative life (t_q). Curve α1 thus describes in general the initial increase and subsequent steady-state in cell number for any fraction of cells of the original population which undergoes such a decrease of t_c.

One may calculate with the help of equations (2) and (3) the final size of the total cell system in which only a fraction of the progenitor cells undergoes a decrease in t_c; it will have increased proportionally to the fraction of changed cells. At least within the limits of our assumption, there is no evidence that the changed cells with

a shorter t_c will "take over" and replace the fraction of cells which have not undergone the change. A change in t_c alone does not confer the property of unlimited growth and after the phase of increase two sub-populations with different t_c will co-exist. The changed cells will however represent a larger portion of the population at the end of the phase of growth than before the change occurred. For instance, if in a system type A of Fig. 4 only 10% of the progenitor population undergoes a decrease of t_c to one half, it can be calculated from equation (2) that the whole system will reach a final size 6·6% larger than the initial one. In comparison to the initial 10%, when the final size is reached the new sub-population with decreased t_c will represent 15·5% of the total population.

In conclusion, an irreversible decrease of t_c in a fraction or the whole of a cell population in steady-state is followed by growth of the system for only a limited period of time after which the changed cells settle down to steady-state again. Thus, an irreversible decrease of t_c cannot generate the unlimited growth characteristic of cancer.

The effect of a change of t_q *on steady-state*

Referring to system A in Fig. 4 one may assume that, while all other parameters remain unchanged, t_q doubles at once. From this moment cells will no longer be eliminated after the old t_q; the size of the q-compartment will increase linearly in time and for a time equal to the increase of t_q, after which the steady-state sets in again. At this time the new system corresponds to A2 of Fig. 4. The final size of a system that undergoes a change of t_q can be derived to be:

$$N = n_{pr} + n_q \frac{(t_q)_b}{(t_q)_a} \cdot \qquad (4)$$

with lower indexes a and b indicating values before and after the change, respectively. An increase of t_q will have the same effect on a system type B of Fig. 5, the final size of which will be

$$N = n_{pr} + n_{e-m} + n_q \frac{(t_q)_b}{(t_q)_a} \cdot \qquad (5)$$

The proliferative index of the new population can easily be calculated to be lower than the original one. If t_q should increase in only a fraction of the population, likewise the case of an increase of t_c in only a fraction of cells, there will be a limited phase of growth, after which the population will consist of two sub-populations with different t_q in a fixed ratio. The final increase in size of the population can be calculated from equations (4) and (5), and will be a fraction of the increase occuring when the whole cell population shows a change of t_q. Graph α2 refers to the behaviour of the sub-population changed in t_q and it was generated starting from an arbitrary number of progenitor cells.

A decrease of t_q will bring about opposite changes as described above, which are of no interest to the present discussion.

In conclusion, as in the case of a decrease of t_c, an irreversible increase of t_q in a fraction or the whole of a cell population in steady-state will bring forth growth for a limited period of time only, after which the changed cells settle down to steady-state again. Again, therefore, an increase of t_q alone, even in association with a decrease of t_c, cannot explain the unlimited growth characteristic of cancer.

The effect of a change of t_{e-m} *on steady-state*

An increase of t_{e-m} in a cell system of type B will affect the whole system like the increase of t_q already described, with the complication, however that within a longer t_{e-m} more cell divisions occur and therefore the efflux from the *e-m*-compartment will increase. This means an increase in size of the q-compartment also. In the case in which t_{e-m} of basic system B is assumed to double without any change of other parameters, system BI of Fig. 5 is the resulting population after the period of growth following the change. In general, the size of the *e-m*-compartment at the end of the period of growth may be computed as follows:

$$\frac{m_{out} \cdot t_{e-m}}{2} \cdot \qquad (6)$$

where m_{out} represents the efflux from the compartment. The q-compartment will increase to the final size:

$$n_q = t_q \, (m_{out})_b \cdot \qquad (7)$$

and, because the *pr*-compartment does not change in size, final N of the whole system can be computed from (6) and (7). Graph β1 was generated assuming that the change had occurred in a fraction only of progenitor cells, and describes the behavior of such a fraction.

Decrease of t_{e-m} will bring forth opposite changes as described above and therefore this case is of no interest for this discussion.

In conclusion, as in the case of t_c and t_q, an irreversible increase of t_{e-m} transforms a steady-state condition into growth for only a limited period of time, after which the changed cells

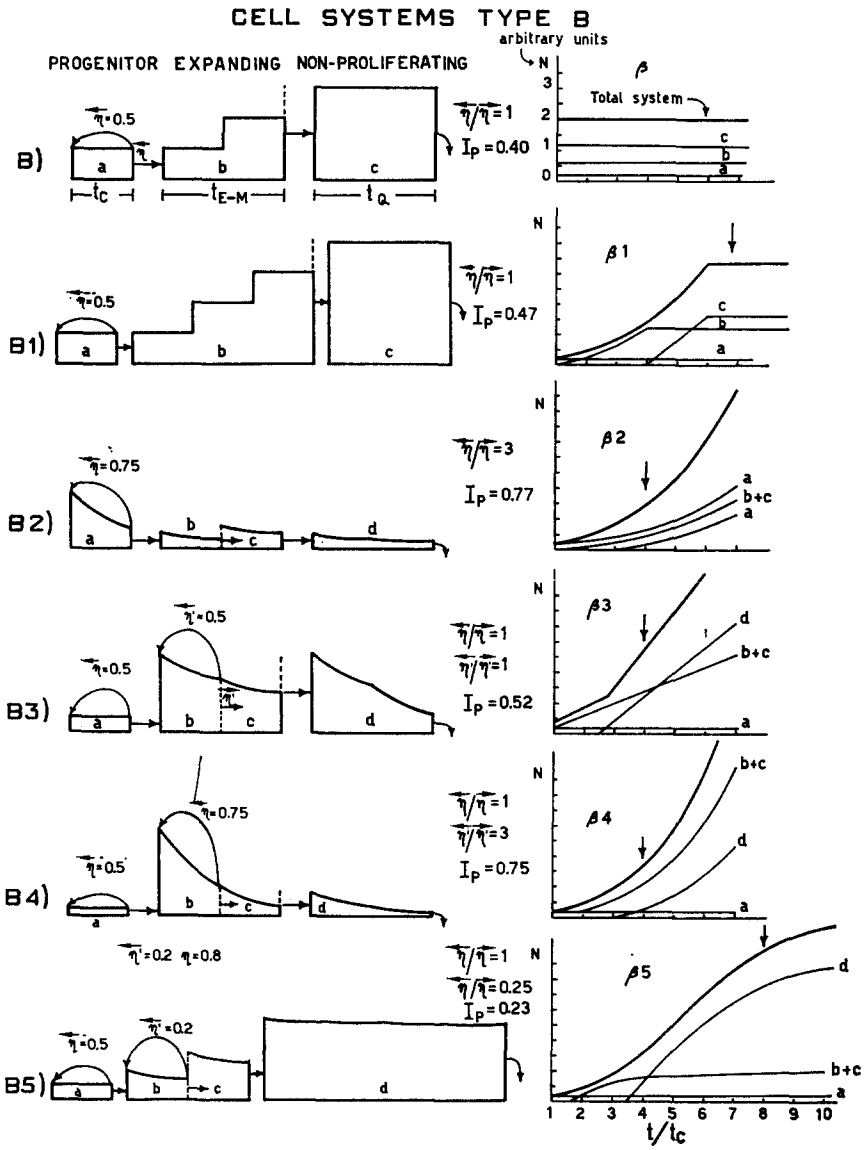


Fig. 5. An analysis of the change from steady-state to unlimited growth in the case of basic system type B. Growth curves were generated as described in legend to Fig. 4; in addition, increase of t_{e-m} *was also taken into consideration (B1). Broken lines dividing* e-m-*compartments indicate assumption of two consecutive replication cycles during maturation. Appearance of a pathological recycling pool (B3, B4, B5) was assumed to occur during first stage of maturation only, corresponding to first replicative cycle. Arrows on curves and concave trapezoids have the same meaning as described in legend to Fig. 4. See text for detailed explanation.*

settle down to steady-state. Thus, unlimited growth cannot result from such a change.

The effect of a change in the "distribution ratio" $(\bar{\eta}/\vec{\eta})$ *on steady-state*

In the steady-state the number of cells n in the compartments does not change with time. Therefore cell efflux from the compartment is equivalent to influx and the ratio $\bar{\eta}/\vec{\eta}$ of the *pr*-compartment is 1 in the steady-state. All parameters remaining unchanged, if the $\bar{\eta}/\vec{\eta}$ ratio irreversibly increases to any value large than 1, i.e. $\bar{\eta}/\vec{\eta}$, in a fraction or the whole *pr*-compartment, then the initial number of changed progenitor cells will increase according to:

$$(n_{pr})_t = (n_{pr})_0 \cdot (2\bar{\eta})^{t/t_c} \cdot \qquad (8)$$

from which:

$$(n_{pr})_t = (n_{pr})_0 \cdot \exp\left[(\ln 2\bar{\eta}) \frac{t}{t_c} \right] \qquad (9)$$

and putting $\ln 2\bar{\eta}/t_c = \gamma$ (growth constant) one obtains the more manageable equation:

$$(n_{pr})_t = (n_{pr})_0 \cdot \exp(\gamma t) \cdot \qquad (10)$$

In a cell system type A efflux from the *pr*-compartment feeds directly into the *q*-com-

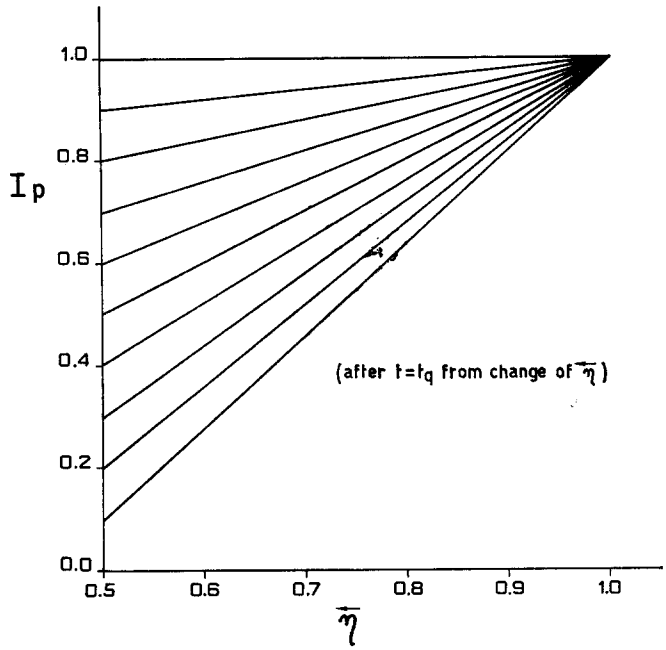


Fig. 6. Effect of changes of the cell distribution ratio ($\overleftarrow{\eta}/\overrightarrow{\eta}$) on the Proliferative Index (I_p) in a cell system. All other proliferation parameters of system are kept constant. See also text.

partment. Thus, the size of the q-compartment at time t will be:

$$(n_q)_t = \sum_{t-t_q}^{t} t_c \left[\frac{\vec{\eta}}{\overleftarrow{\eta}} (n_{pr})_0 \cdot \exp(\gamma t) \right] . \quad (11)$$

The growth of the whole system type A when the $\overleftarrow{\eta}/\vec{\eta}$ ratio is greater than 1 will thus be given by addition of (10) and (11).

System A3 corresponds to basic A in t_c and t_q but has an $\overleftarrow{\eta}/\vec{\eta}$ ratio of 3 instead of 1. Graph α3 was generated starting from an arbitrary number of cells with the same t_c and t_q as system A but with $\overleftarrow{\eta}/\vec{\eta}=3$, and it describes the growth of such a fraction of cells.

It should immediately be pointed out that systems like A3 which are in a condition of growth cannot be represented by rectangular parallelograms like systems in steady-state, because the age distribution of cells is not uniform like in the steady-state. Since the population increases with each t_c, there will be a larger fraction of newest cells than there is of oldest. This point has already been fully discussed by Johnson [13], Steel and Bensted [51] and others, and does not need to be treated in detail here. The non-uniformity of age distribution of cells was accounted for in the graphic presentation of growing compartments of Figs. 4 and 5 by means of calculated trapezoids with the concave side portraying the age-distribution of cells in the compartment. Only relative sizes of compartments were taken into consideration in the construction of the growing cell systems. Growth curves were drawn for each system, starting from an arbitrary number of progenitor cells. In every case the arrows on the curves point out where the growth curve coincides with the corresponding two-dimensional representation of the system.

Simpler cases than A3 are systems A4 and A5. Type A4 is a population growing without cell loss ($\overleftarrow{\eta}=1$) and A5 is also growing exponentially but with a cell loss of 25 per cent ($\overleftarrow{\eta}=0{\cdot}75$). Their growth pattern is described by equation (3) and is also drawn as curves α4 and α5 in the same figure. Other systems could be constructed in addition to A3–A5, but these three exhaust basic types. Further discussion of similar cell systems would therefore add nothing to the general principles which are of interest to this discussion.

The value of I_p in a system type A3 can be calculated by dividing equation (10) by equations (10)+(11), or approximated from areas of geometric models. For system A3 the calculation gives $I_p=0{\cdot}65$, a value higher than the $0{\cdot}33$ of the original system A in steady-state. For t_c and t_q constants, I_p was calculated for the whole range of $\overleftarrow{\eta}$, from $0{\cdot}5$ to 1 (i.e. for the ratio $\overleftarrow{\eta}/\vec{\eta}$ from 1 to ∞) and the results are given in the graphs of Fig. 6. These results show a direct linear relationship between the $\overleftarrow{\eta}/\vec{\eta}$ ratio and I_p, that is, any increase of the $\overleftarrow{\eta}/\vec{\eta}$ ratio over 1 ($\overleftarrow{\eta}>0{\cdot}5$; always generates an increase of I_p, together with the condition of growth. This is true for any value of t_c, t_s and initial I_q of the cell system. Obviously, an increase of I_p results in a relative decrease of n_q, and at the

limit condition $\bar{\eta}=1$ the q-compartment disappears. In other words, because $\bar{\eta}$ of an unlimitedly growing population is by definition larger than 0·5, which is the value of steady-state, it follows that the I_p of such a growing population is necessarily larger than the I_p of the corresponding cell system in steady-state.

When in a fraction of progenitor cells of basic system type B of Fig. 5 the $\bar{\eta}/\vec{\eta}$ ratio increases irreversibly to a value larger than 1, the changed cells star growing. Basically the growth of this fraction of cells is similar to that described in the case of system type A. The only difference is due to the magnifying ability of the *e-m*-compartment, but to go into detailed treatment of this compartment is outside the scope of this paper.

In Fig. 5, system B2 represents a system with the same t_c, t_{e-m} and t_q of basic B, but with a $\bar{\eta}/\vec{\eta}$ ratio of 3. I_p of system B3 is larger than I_p of the corresponding steady-state system, and in case B as well there exists a direct correlation between $\bar{\eta}$ and I_p. The statement already made for system A, i.e. that I_p of a population growing unlimitedly is necessarily larger than the I_p of the corresponding population in steady-state may be repeated for type B system.

A novel possibility with the system type B is that a recycling pool may appear in the *e-m*-compartment. In this case the *e-m*-compartment and tributary q-compartment will start to grow, indefinitely if the change is irreversible, while the *pr*-compartment will continue to be in steady-state. Clearly, with time, the *pr*-compartment will become an irrevelant fraction of the total population. This possibility is depicted as systems B3 and B4 and growth curves β3 and β4 of Fig. 5. Basically, regarding growth and behavior of I_p such systems B3 and B4 are similar to the previous one and do not require detailed treatment. They are reported here for their possible relevance to a hypothesis of Cronkite [10] regarding cell proliferation in acute leukemia.

In conclusion, when in a cell system in steady-rate the distribution ratio $\bar{\eta}/\vec{\eta}$ of a fraction of progenitor cells irreversibly changes from 1 to a value larger than 1, the changed cells will start to grow unlimitedly and the resulting population will show an I_p larger than that of the original population in steady-state. The appearance of a recycling pool of cells in the *e-m*-compartment will also result in unlimited growth, with the exclusion, however, of the progenitor compartment. The increase of I_p will in any case be directly proportional to the increase of the $\bar{\eta}/\vec{\eta}$ ratio.

The effect of changes of t_c *and/or* t_q *on the correlation between the* $\bar{\eta}/\vec{\eta}$ *ratio and* I_p

As Fig. 6 shows, for t_c and t_q constant a direct linear correlation exists between $\bar{\eta}$ and I_p in a cell system. However, experimental data show that most often t_c is decreased in tumors compared to normal counterpart, while at least in the case of leukemia, an increased t_q seems well supported by evidence. Moreover, in several cases the I_p of tumors is not much higher than that of corresponding normal counterpart in steady-state, and in acute leukemia the I_p of leukemic bone marrow is most often decidely lower than that of normal bone marrow.

Fig. 7 shows in a graphic display the influence of increasing t_q/t_c on I_p in systems with different $\bar{\eta}$. Clearly, the increase of t_q/t_c may result from an increase of t_q alone or from a decrease of t_c alone, or both. If any of these changes occur in cells together with an increase of the $\bar{\eta}/\vec{\eta}$ ratio, depending on the relative changes of the involved parameters, the positive correlation between $\bar{\eta}/\vec{\eta}$ and I_p may be reduced, offset, or even reversed. For instance, one can see from Fig. 7 that if $\bar{\eta}$ of a cell system with $t_q/t_c=2$ changes from 0·5 (steady-state) to 0·55 (slow growth) the I_p changes from 0·33 to 0·39 if t_c and t_q remain constant. But, if the t_q/t_c ratio increases to 10, for $\bar{\eta}=0{\cdot}55$ the I_p will actually decrease to 0·15.

Systems A6 and A7 of Fig. 4 were obtained assuming a $\bar{\eta}/\vec{\eta}$ ratio of 1·22 and a t_q/t_c ratio of 2, compared with $\bar{\eta}/\vec{\eta}=1$ and $t_q/t_c=1$ of basic system A. Corresponding growth curves α6 and α7 were obtained starting from an arbitrary number of progenitor cells. Arrows on the curves show where the growth curve coincides with corresponding two dimensional representation of the cell system, after the initial phase of faster growth due to building up of the q-compartment. Note that at this time I_p of the new growing population is smaller than I_p of basic cell system A in steady-state, in which the growth was assumed to originate. Thus, the increase of the ratio t_q/t_c occuring together with the increase of the $\bar{\eta}/\vec{\eta}$ ratio reversed in these cases the increase of I_p as expected on the basis of the $\bar{\eta}/\vec{\eta}$ change.

The same conclusions reported above are valid in the case of system type B. Therefore, Fig. 5 shows only the peculiar case of a growing population with a pathological recycling pool in the *e-m*-compartment, in which t_q had doubled compared to basic system in steady-state (B5). Graph β5 describes the growth of such a cell population starting from an initial arbitrary number of cells. The first

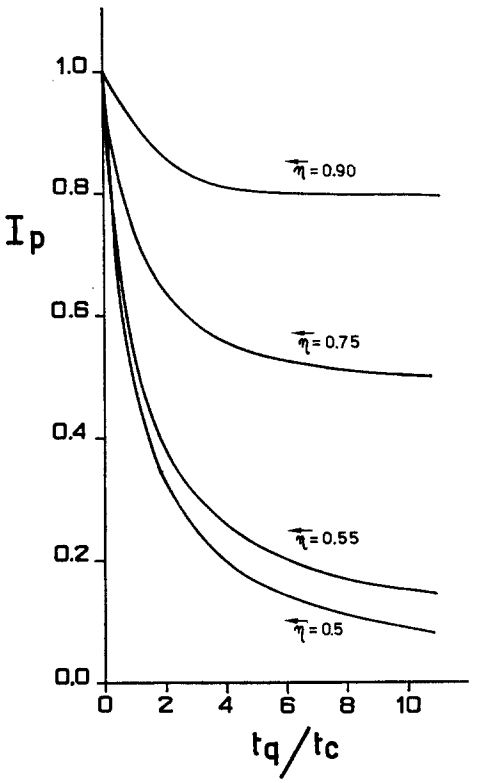


Fig. 7. The negative influence of increase of the ratio t_c/t_q *on the positive correlation between* $\bar{\eta}/\vec{\eta}$ *and* I_p. *Depending on the relative changes of involved parameters, the increase of* I_p *as a result of an increase of* $\bar{\eta}/\vec{\eta}$ *may be reduced, offset, or reversed by a simultaneous increase of* t_q *or decrease of* t_c. *Curves were calculated for a few values of* $\bar{\eta}/\vec{\eta}$. *See also text.*

phase of rapid growth is the result of building up of *e-m-* and *q*-compartments, while the subsequent phase of much slower growth represents the final rate of increase of the system, and coincides with the corresponding two-dimensional diagram of system B5. At this stage of slower growth the I_p of the B5 system is smaller than I_p of corresponding basic steady-state system B, from which the growing B5 was assumed to originate. Thus, also in this case the expected increase of I_p in the growing system in comparison to steady-state counterpart was reversed by the increase of t_q. It should be pointed out, however, that in the initial stages of growth the I_p of B5 is larger than that of normal steady-state counterpart. This is a similarity with at least some cases of acute leukemia.

In conclusion, the direct correlation between $\bar{\eta}/\vec{\eta}$ and I_p may be reduced, offset or even reversed by a decrease of t_c, and/or an increase of t_q, and/or an increase of t_{e-m}, depending on the relative change of involved parameters. This may explain instances of neoplastic growths not conforming to the rule of a larger I_p in comparison to normal counterpart.

6. GENERAL CONCLUSIONS

(1) From the available instances, one may conclude that replication time (t_c) of cancer cells is generally decreased compared to normal counterpart in steady-state, that is, the corresponding normal cells in the adult animal from which the cancer originated. Leukemic blasts, contrary to an opinion held until recently, have a t_c of the same order of corresponding normal proliferating bone marrow cells. One may go as far as to say that at this time it cannot be excluded that leukemic blasts actually replicate faster than corresponding normal cells.

(2) All four phases of the cell replicative cycle contribute to the decrease of t_c in the cancer cell compared to normal counterpart, thus indicating a general speed-up of the metabolic activities connected with cell replication.

(3) A theoretical analysis shows, however, that an irreversible decrease of t_c alone cannot generate the unlimited growth characteristic of cancer. Decrease of t_c in a fraction or the whole of a cell population in steady-state results in growth for only a limited time, after which the changed cells settle down to steady-state again. Thus, the decrease of t_c is devoid of primary significance in carcinogenesis. Furthermore one can show that slower than normal counterpart replication rate is not incompatible with cancer growth. Thus, although the primary change of t_c appears to be a decrease, it is possible that especially in older tumors, when nutritional and other factors may interfere, a slowing down of replication rate could occur.

(4) The overlapping of t_c values in a comparison at large of normal and cancer cells, as well as the shorter t_c of normal cells compared to corresponding cancer cells when stimulation by hormones is applied, indicate that cancer cells do not necessarily replicate at the potential full speed characteristic of the cell line, or of mammalian cells in general. This disproves that cancer is necessarily an all-out growth.

(5) The proliferative index (I_p), in the cases where it was measured, was found to be generally higher in tumors compared to normal counterpart in steady-state. In acute leukemia I_p is generally smaller than normal and is accompanied by an increased transit time through the non-proliferating cell compartment (*q*-compartment).

(6) A theoretical analysis shows that an irreversible increase of the *q*-compartment transit time (t_q) alone, or of the expanding maturing compartment transit time (t_{e-m}),

alone, or both, cannot in itself produce the unlimited growth characteristic of cancer. This is true unless the transit time becomes infinite. Increase of t_q and/or t_{e-m} in a fraction or the whole of a cell population in steady-state results in growth for a limited time only, after which the steady-state condition sets in again.

(7) Unlimited growth is generated only by an irreversible increase to a value larger than 1 of the cell distribution ratio ($\bar{\eta}/\vec{\eta}$ in a fraction or the whole of the progenitor cell compartment; $\bar{\eta}/\vec{\eta}$ is the ratio between those post-mitotic cells which remain in the *pr*-compartment and continue to divide ($\bar{\eta}$) and those which leave the comprtment progressing into differentiation ($\vec{\eta}$). In a cell population with an *e-m*-compartment this can also be accomplished by formation of a pathological recycling cell pool in this compartment, while the progenitor compartment may remain unchanged.

(8) It is also shown that in the case that transit parameters remain constant, there is a direct correlation between increase of the $\bar{\eta}/\vec{\eta}$ ratio and the proliferative index of the cell population. A growth should thus have a higher I_p compared to normal counterpart in steady-state. However, if t_c decreases and/or t_q and/or t_{e-m} increase, the correlation between $\bar{\eta}/\vec{\eta}$ and I_p is disturbed and, depending on the relative changes of the involved parameters, the increase of I_p as expected from the increase of the $\bar{\eta}/\vec{\eta}$ ratio may be reduced, offset or even reversed. This is an explanation of possible instances in which the I_p of neoplastic growth may be found to be smaller than the I_p of the steady-state counterpart.

RESUME

Dans tous les cas de tumeurs solides étudiés in vivo, *le temps de doublement cellulaire* (t_c) *est plus court que dans les tissus normaux correspondants, en état d'équilibre. Le temps de doublement des cellules leucémiques blastiques est approximativement le même que celui des cellules médullaires normales correspondantes, et, à l'heure actuelle, on ne peut pas exclure qu'il soit, en fait, plus court. Une analyse théorique montre cependant qu'un raccourcissement irréversible de* t_c *n'est pas suffisant pour expliquer la croissance illimitée, caractéristique des cancers. Ainsi, le raccourcissement de* t_c *est dépourvu de signification primaire dans la cancérogénèse. De plus, on peut montrer qu'un* t_c *plus long que le temps normal est compatible avec une croissance cancéreuse. Comme d'autres chercheurs l'ont montré, la comparaison, en général, des cellules normales et cancéreuses met en évidence un chevauchement des valeurs de* t_c*; il ressort en outre de ce travail que le raccourcissement de* t_c *des cellules mammaires normales, après injection d'une grosse dose d'hormones ovariennes, a une valeur plus basse que celui des cellules cancéreuses correspondantes. Ceci montre, de manière non équivoque, que les cellules cancéreuses ne doublent pas nécessairement à la vitesse potentielle maximum de la souche cellulaire correspondante, ou des cellules de mammifères en général. L'analyse théorique a encore montré qu'il n'existe pas de modification irréversible de l'un ou l'autre des temps de transit, à travers les autres compartiments d'un système cellulaire, qui puisse expliquer la croissance illimitée, caractéristique de cancer. La croissance illimitée ne peut se produire que si la valeur du "rapport de distribution" dépasse l'unité. Ce rapport de distribution* (d-ratio) *est le rapport entre les cellules post-mitotiques qui restent dans le compartiment générateur primitif et les cellules qui se différencient. En état d'équilibre, le "d-ratio" correspond à l'unité. On peut montrer qu'il existe une corrélation directe entre le "d-ratio" et la fraction de cellules engagées dans la prolifération* (I_p). *Donc,* I_p *doit être plus grand dans le cancer que dans le tissu correspondant en état d'équilibre, puisque le "d-ratio" est nécessairement plus élevé dans la croissance que dans l'état d'équilibre. C'est d'ailleurs ce que démontre l'expérience, la plupart du temps. Il existe cependant une exception notable: la moelle leucémique a, le plus souvent, un* I_c *nettement plus bas que la moelle normale. Cette anomalie peut être expliquée. L'analyse théorique montre qu'une diminution de* t_c*, ou une augmentation du temps de transit à travers le compartiment non prolifératif, coïncidant avec une augmentation du "d-ratio", modifie la corrélation entre l'augmentation du "d-ratio" et de* I_p. *Il en résulte que l'augmentation de* I_p *peut être réduite, supprimée, ou même inversée, selon les changements relatifs des paramètres concernés. Cette explication a une valeur générale et peut être valide pour d'autres cas possibles de croissance cancéreuse, dans lesquels* I_p *est plus petit que dans le tissu normal correspondant.*

SUMMARY

In all cases of in vivo *solid primary tumors investigated up to date, the cell replication time* (t_c) *is shorter than in the normal corresponding tissues in steady-state. Replication time of leukemic blasts is of the same order of magnitude as that of normal marrow cells, and at the present time one cannot exclude that it might actually be shorter. A theoretical analysis shows, however, that an irreversible decrease of* t_c *alone cannot generate the unlimited growth characteristic of cancer, and furthermore that a* t_c *longer than that of normal counterpart would be compatible with cancer growth. Thus, the decrease of* t_c *is devoid of primary significance in carcinogenesis. As other research workers have pointed out already, a comparison at large of normal and cancer cells shows overlapping of* t_c *values; moreover, the decrease of* t_c *of normal mammary cells after injection of large doses of ovarian hormones to a value lower than that of corresponding cancer cells is also pointed out in this paper. This definitively shows that cancer cells do not necessarily replicate at the potential full speed characteristic of the cell line, or of mammalian cells in general. With regard to transit times through other compartments of a cell system, one can show that in general no irreversible change of any transit time, alone or in combination, is sufficient to explain the unlimited growth characteristic of cancer. Unlimited growth can be generated only by an irreversible increase to a value larger than one of the cell "distribution ratio"* (d-*ratio*), *which is the ration between the post-mitotic cells which remain in the progenitor compartment and those which progress towards differentiation. In the steady-state condition the* d-*ratio is one. One can show that there is a direct correlation between the* d-*ratio and the fraction of cells engaged in proliferation in the tissue* (I_p). *Thus, because in a growth the* d-*ratio is necessarily higher than in the steady-rate, also* I_p *should be higher in cancer compared to normal counterpart in steady-state. This is indeed generally found to be true. There is, however, at least one notable exception, namely leukemic marrow which most often shows an* I_p *definitely lower than that of normal marrow. This anomaly may have an explanation. One can show that a decrease of* t_c *and/or an increase of transit time through non-proliferative compartments, if occurring in coincidence with the increase of the* d-*ratio, disturbs the correlation between increase of the* d-*ratio and of* I_p*; and, depending on the relative changes of the involved parameters, the expected increase of* I_p *may be reduced, offset or even reversed. This explanation is of general significance and may be valid for other possible instances of neoplastic growth in which the* I_p *may be smaller than that of normal counterpart in steady-state.*

ZUSAMMENFASSUNG

Bei primären soliden Tumoren in vivo *ist die Zellverdoppelungszeit* (t_c) *kürzer als die der Normalzellen des entsprechenden Gewebes. Die* t_c *leukämischer Blasten entspricht grössenordnungsmässig der von normalen Knochenmarkzellen; ob sie tatsächlich etwas geringer ist, lässt sich anhand der verfügbaren Daten gegenwärtig nicht entscheiden. Eine theoretische Analyse zeigt, dass eine irreversibele Reduktion der* t_c *nicht die Ursache eines unbergrenzten Zellwachstums sein kann und daher für die Krebsentstehung primär nicht bedeutungsvoll ist; theoretisch ist Tumorwachstum auch mit einer Erhöhung der* t_c *vereinbar. Wie schon andere Autoren betont haben, überschneiden sich die Zellverdoppelungszeiten verschiedenartiger normaler und maligner Zellen; in dieser Arbeit wird ferner auf die kürzere* t_c *normaler Brustdrüsenzellen unter dem Einfluss hoher Dosen von Ovarial-Hormonen verglichen mit Tumorzellen gleichartigen Gewebes hingewiesen. Daraus folgt, dass wachsende Krebszellen nicht notwendigerweise die maximale Zellverdoppelungsgeschwindigkeit realisiert haben müssen. Es lässt sich allgemein zeigen, dass keine irreversibelen Änderungen der Durchgangszeiten von Anteilen eines Zellzyklus das unbegrenzte Wachstum erklären könnten. Unbegrenztes Wachstum kann nur stattfinden, wenn der Quotient von nachmitotischen Zellen, die im "Generativen Anteil" bleiben, zu Zellen, die in den "Differenzierungsanteil" übergehen* (d-*ratio*), *irreversibel grösser als eins ist. In einem "steady-state"-System ist die* d-*ratio gleich eins. Die Analyse zeigt ferner, dass zwischen der* d-*ratio und dem Proliferationsindex* I_p (*Zahl der proliferierenden Zellen bezogen auf die Gesamtzahl der Gewebezellen*) *eine positive Korrelation besteht. Aus diesem Grunde sollte der* I_p-*Wert eines Tumorgewebes grösser als der von entsprechendem "steady-state"-System Normalgewebe sein. Bei einem*

Vergleich unter korrekten Voraussetzungen wird dies in der Tat auch in der Regel experimentell gefunden. Eine bemerkenswerte Ausnahme bildet leukämisches Khochenmark: sein I_p*-Wert ist meistens niedriger als der von normalem Knochenmark. Diese Anomalie könnte folgendermass erklärt werden: erfolgt eine Abnahme der* t_c *oder eine Zunahme der Durchgangszeit im "nichtproliferierenden Anteil" zusammen mit der Zunahme der* d*-ratio, so wird das Verhältniss zwischen* d*-ratio und* I_p *verändert; entsprechend der relativen Veränderung der betroffenen Parameter kann die normalweise zu erwartende Zunahme von* I_p *reduziert, annuliert oder in ihr Gegenteil verkehrt werden. Diese Erklärung ist von allgemeiner Bedeutung und könnte auf andere theoretisch möglich Fälle von neoplastischem Wachstum anwendbar sein, in denen der* I_p*-Wert kleiner als beim entsprechenden Normalgewebe gefunden wird.*

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Tumour Cure Rate and Cell Survival of a Transplantable Rat Rhabdomyosarcoma Following X-Irradiation

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INTRODUCTION

THE CURE rate of tumours after irradiation in experimental systems is usually evaluated in terms of 50% Tumour Cure Dose or TCD_{50} [1]. Most work concerning tumour cure rate of experimental tumour systems has been performed with the C_3H mammary tumour [2–4]. The probability of tumour recurrences after irradiation probably depends to a great extent upon the proportion of tumour cells which has escaped from the radiation-induced sterilization, and consequently on the X-ray dose applied. The dose-effect relationship between the X-ray dose and the percentage of surviving (i.e. non-sterilized) cells is currently termed the "cell survival curve". Such cell survival curves have been determined for a great variety of *in vitro* and *in vivo* systems, not only for tumour cells but also for cells of normal tissues [5–8]. Some excellent reviews, covering the whole subject of cell survival curves have recently been published [9–11].

The shape of many survival curves of mammalian cells for X- or γ-rays may be characterized by an initial shoulder followed by a straight part on a semi-log plot. A characterization of the shoulder may be found in the extrapolation number (N); this is the point of interception obtained by the extrapolation of the exponential part of the curve to the ordinate. The exponential portion is usually only that part of the curve related to surviving fractions below 10^{-1} and its slope is generally described by the D_{37}, being the *increment* of dose required to decrease the surviving fraction to 1/e or 37% (where e=base natural logarithm).

By means of a quantitative transplantation technique following irradiation, survival curves for malignant cells of a number of experimental solid tumour systems have been determined [12–18].

The D_{37} values for irradiation under anoxic conditions obtained by various authors varied from 260 to 380 rads; the mean value being in the order of 350 rads. In all the solid tumour systems investigated so far by means of quantitative transplantation a certain proportion of anoxic cells was found*. The percentages of anoxic cells ranged from 100% for a round-celled sarcoma [13] to 1% for a lymphosarcoma [15]. Values of 20, 19, 14 and 15% have also been found for respectively Adenocarcinoma MTG-B [20], a squamous carcinoma [14], Sarcoma KHT [17] and Rhab-

*The only experimental solid tumour system known to the authors in which no anoxic cells have been demonstrated is a methyl-cholanthrene-induced rat carcinoma. This tumour showed no increase in radiosensitivity when irradiated under high pressure oxygen. The response of this tumour to irradiation was investigated by means of the growth-delay of the tumour [19].

domyosarcoma BA 1112 [18]. When the assays are made by means of quantitative transplantation it seems hardly possible, however, to obtain information below a surviving fraction of 10^{-5} because of the limited yield of the cell dispersion procedures. Consequently if information is required with respect to very low surviving fractions of malignant cells in small solid tumours it seems that the only possible approach is to leave the tumour *in situ* after irradiation and use the cure rate of the tumour as the endpoint. For the evaluation of the effects of extended irradiation courses of the type employed in clinical radiotherapy, measurement of the tumour cure rate also seems the only possible method. Following such fractionated irradiations, the proportion of viable cells in the tumour is subject to changes during the interval between irradiations as a result of removal of dead cells and of proliferation of the surviving tumour cells [21, 22] so that an estimate of the surviving fraction following a course of irradiation seems to have little sense. Before such investigations could be initiated it was essential to establish whether the tumour cell survival data obtained with the quantitative transplantation technique (cell titration) following a single dose of X-rays would agree with the results obtained from cure rate experiments. These comparative experiments will be reported in the present paper.

The probability of the recurrence of a tumour after X-ray treatment will not only depend upon the number of cells which survive the irradiation, but also upon the minimum number of malignant cells which are required to initiate such a recurrence—the so-called "critical cell number" [2].

In the following pages it has been assumed that the number of viable cells required to cause "tumour takes" after inoculation of non-irradiated tumour cells is equal to the number of surviving cells required to cause tumour recurrences after irradiation. Two coextensive groups of experiments were performed. These consisted of a group of cure rate experiments and an equal number of tumour cell titrations with unirradiated tumour cells; the latter allowing the calculation of the "critical cell number".

MATERIAL AND METHODS

Tumour system

The tumour system employed was the transplantable Rhabdomyosarcoma BA 1112, growing in the inbred strain of WAG/Rij rats. The method of tumour cell titrations used for the determination of the cell survival curves of this tumour has been reported in earlier publications [18, 23].

The animals to be used for the cure rate experiments were inoculated approximately fourteen days before the irradiation with a suspension of 10,000 cells in 0·5 ml Simm's solution in the subcutaneous tissues of the head between the ears. In the first experiments some animals showed a multifocal tumour growth, manifesting itself in the appearance of an elongated tumour mass. According to Hewitt [24] this could be attributed to a deposition of tumour cells along the needle track and it could largely be avoided by fitting a collar around the tip of the needle, leaving approximately 1 mm for subcutaneous penetration. A total number of 804 animals were used for these experiments; 410 for the tumour cell titrations and 394 for the cure rate experiments. The age of the animals at the time of irradiation was between 7 and 8 weeks. The size of every individual tumour was measured at the day of irradiation by means of a plastic calibre, fitted with holes varying between 4 and 16 mm dia. [25]. These measurements obviously included skin etc. and in a separate analysis, the corresponding net tumour weight was determined by sacrificing a number of animals and weighing the tumour after its removal. The relationship found between the actual tumour weight (mg) and the measured diameter (mm) was: log tumour weight= 3·0708 log dia.−0·888.

In the irradiation experiments as well as in the tumour cell titrations 30–40 animals were used per dose of X-rays.

Irradiation

The irradiation set-up is depicted in Fig. 1. The animal is anaesthetized by means of an intraperitoneal injection of sodium pentothal (5 mg/100g) and is positioned unrestrained on an adjustable jack, which can be lifted in such a way that only the tumour and the upper parts of the skull will be exposed to the X-ray beam. Geometrical adjustment is facilitated by using a sight. By a lead shield the extension of the X-ray beam is confined to the left upper quadrant of the application field and the position of the tumour and the skull of the animal can be checked continuously during the irradiation by observation of a fluorescent screen which is put in place just before the X-ray generator is started. An ionization chamber of the Baldwin "Ionex" dosimeter is located directly over the tumour and thus

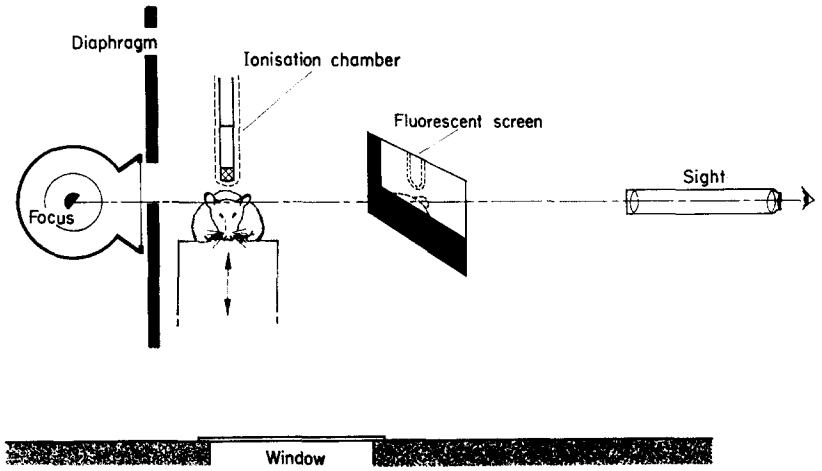


Fig. 1. *Irradiation set-up used for cure rate experiments. Only the tissues above the centre of the beam are being irradiated.*

receives an identical dose of X-rays. A Philips Müller MG 300 Industrial X-ray unit (300 kVp, 10 mA) was employed. In the first experiments the filter consisted of 1 mm Cu; later this was changed to 0·6 mm Cu+1 mm Al, the HVL being respectively 2·7 mm Cu and 2·0 mm Cu. The dose distribution in the tumour was estimated by sandwiching a second ionization chamber between pieces of "masonite" imitating the thickness of the tumour. The maximum deviation from that ionization chamber to the readings of the uncovered ionization chamber was—in this situation—found to be less than 5%. The focus-skin distance was 18 cm, focus–diaphragm distance 15 cm; the dose rate varied between 384 and 624 rads/min.

The size of most of the irradiated tumours varied from 4 to 15 mm dia., the mean diameter being 6·9 mm. In addition, a number of much smaller tumours was irradiated when just palpable as subcutaneous strands or "sand grains". It seemed interesting to investigate whether the "anoxic" response found with the larger tumours bears any relation to the size of the tumour.

Follow-up of the tumour bearing animals

An animal was considered cured if—after a follow-up period of 100 days—no trace of tumour tissue was detectable. The "clinical course" was as follows. During the first weeks after irradiation with doses exceeding 3500 rads the tumour mass was gradually absorbed and simultaneously a moist desquamation of the skin in the irradiated field developed. In the subsequent months the skin healed; permanent epilation remained however.

Recurrence of a tumour was noted first by the appearance of a tiny nodule, located centrally in the epilated area. The size of that tumour nodule increased slowly in the next few days and due to the damage of the overlying skin, an ulcerating tumour mass developed. This tumourous ulcer progressed slowly until it reached the border of the irradiated (epilated) area and from that time on a fast growing tumour mass invaded the non-irradiated subcutaneous tissues. There was a remarkable difference in aspect between the "cures" and "recurrences" leaving little uncertainty about the final score. The pooled data were submitted to probit-analysis for the determination of the ED_{50} and TCD_{50} values.

RESULTS

The results of the irradiation experiments are shown in Fig. 2 and Fig. 3. The TCD_{50} found for the first group of tumours (mean dia. 6·9 mm) is 5466 rads, that of the smaller tumours (sand grains) is 5208 rads.

The pooled results of all tumour cell titrations are presented in Fig. 4. The ED_{50} is 51 cells; this means that inoculation of 51 cells will—on the average—result in 50% tumour "takes" at the site of inoculation within 100 days.

The surviving fraction (s.fr.) of the tumour cells after irradiation—for example after TCD_{50}—can be calculated as follows:

$$\text{s.fr.}_{(TCD_{50})} = \frac{\text{mean no. of cells, resulting in 50\% tumour "takes"}}{\text{mean initial no. of cells in irradiated tumours}}$$

Of this fraction, the numerator equals the ED_{50}. The divisor has been estimated as follows: The mean diameter of the 199 tumours irradiated with doses ranging from 4500 rads to 7000 rads was 6·9 mm and from the weight–

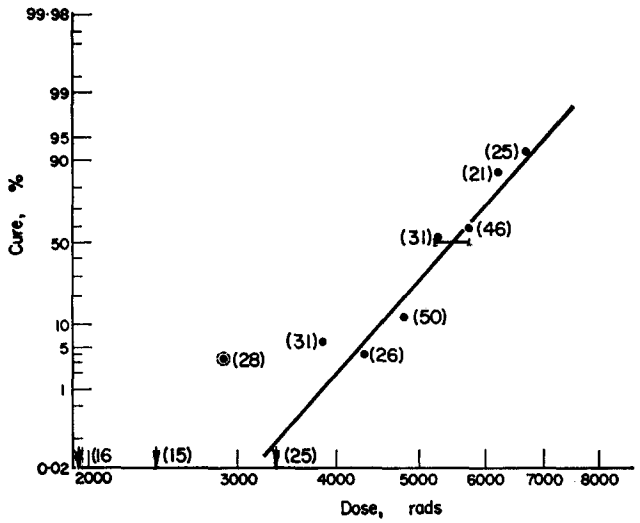


Fig. 2. Relationship between the X-ray dose and the cure rate of tumours with a mean diameter of 6·9 mm. The values between brackets indicate the number of animals per point. The TCD50 *was 5466 rads, the 95% confidence limits of the* TCD50 *are indicated by the horizontal bars. The encircled point (1/28) was removed from the data; this did not influence the results of the probit-analysis.*

diameter relationship a mean tumour weight of about 50 mg was assessed. Assuming a cellularity of 5×18^8 cell/g [23], a tumour of 6·9 mm dia. will contain ~ $2{\cdot}5\times10^7$ cells. Using this value, the surviving fraction of cells following the TCD50 could be calculated and was found to be $\sim2\times10^{-6}$. This value is plotted in Fig. 5.

On the assumption that the same relationship will hold true for the other doses employed—and thus for the other surviving fractions—additional points were derived (Fig. 5). The surviving numbers of the cells for the calculation of these points have been taken from the probit line of Fig. 4, assuming that similar percentages of tumour takes and recurrences will be evoked by equal numbers of inoculated and surviving cells.

The weight of the tiny "sand grain" sized tumours was estimated by careful excision and weighing of thirteen of such tumours. The average weight was 6 mg, and thus the number of cells in these tumours will be ~ 3×10^6. From this it follows that the surviving fraction of cells corresponding with the TCD50 (5208 rads) will be in the order of $1{\cdot}7\times10^{-5}$. This value is also plotted in Fig. 5.

DISCUSSION

The aim of these investigations was to verify

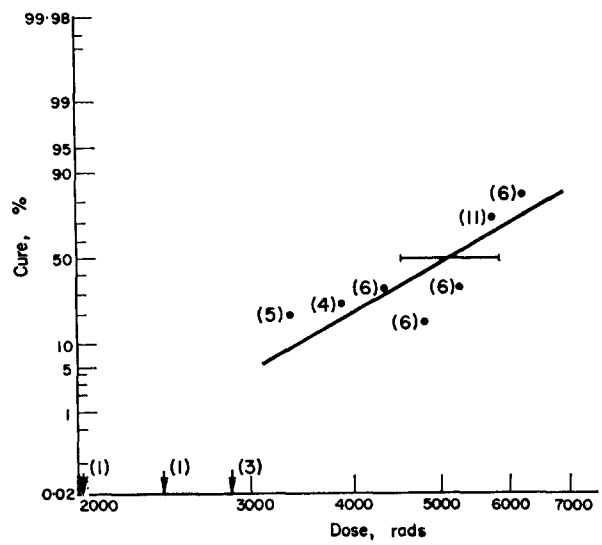


Fig. 3. Relationship between the X-ray dose and the cure rate of the "sand grain" sized tumours. The values between brackets indicate the number of animals per point. The TCD50 *was 5208 rads, the 95% confidence limits of the* TCD50 *are indicated in the horizonatl bars.*

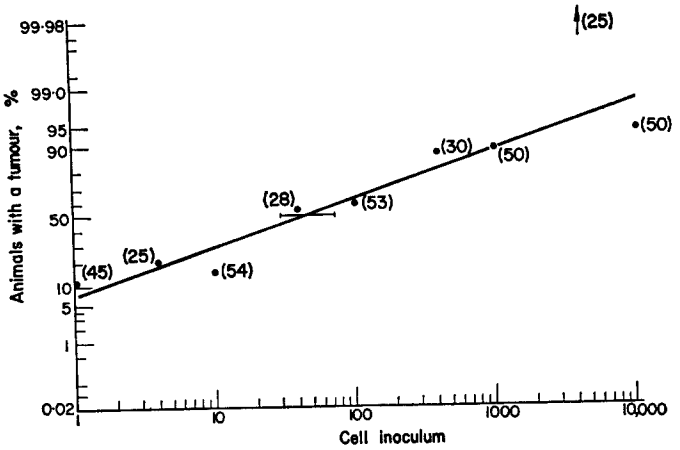


Fig. 4. Results of the tumour cell titrations.
Indicating the relationship between the number of tumour cells inoculated and the percentage of animals showing a tumour at the site of inoculation within 100 days. The values between brackets indicate the number of animals per point. The 95% confidence limits of the ED50 *are indicated by the horizontal bars.*

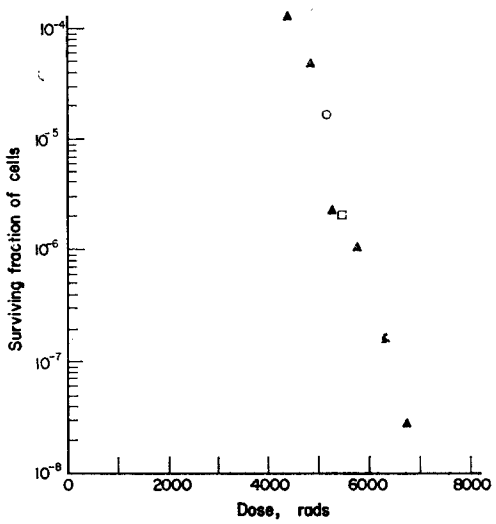


Fig. 5. Relationship between the therapeutic X-ray dose and the resulting surviving fraction of tumour cells. The method of calculation is described in the text.
▲ *Points obtained by calculating the cure rates of tumours treated with different doses of X-rays (Fig. 2).*
O *Surviving fraction of cells in "sand grain" sized tumours (mean weight 6 mg) derived from* ED50 *value of Fig. 3.*
□ *The mean surviving fraction of the 6·9 mm dia. tumours; also calculated at the 50% level* (TCD50 *of Fig. 2).*

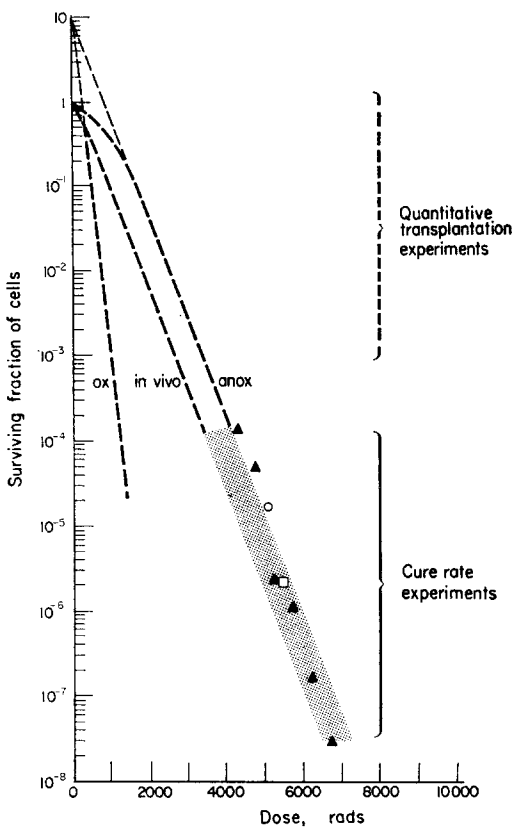


Fig. 6. Combined plot of the cure rate results and the cell survival curves as determined by tumour cell titrations. The broken lines indicate the "in vivo", *the "well-oxygenated" and the "anoxic* in situ" *cell survival curves* (18). *The shaded area lies between the extrapolation of the "anoxic* in situ" *and the* "in vivo" *cell survival curves. The "cure rate" points of Fig. 5 are also plotted in this diagram. Symbols as in Fig. 5.*

whether the cure rate of a tumour could actually be derived from the survival curve of its cells as determined by the Hewitt-type of assay. In order to be able to compare the cure rate results and the "cell survival" curves, the results of both types of assays have been combined in one diagram (Fig. 6).

The upper part of this diagram is derived from the cell survival curves, as determined by quantitative transplantation [18]. The shaded area lies between the extrapolation of the survival curve of the "anoxic *in situ*" irradiation and the "*in vivo*" irradiation. Evidently the "cure rate" points are located in or very near the shaded area. The conclusion seems to be

justified that the cure rate of a tumour after a single dose of X-rays can indeed be derived by extrapolation from transplantation–assay cell survival curves. Now that the results obtained with both systems have been adequately compared, future studies concerning the radiosensitivity of this tumour can be carried out with the system of choice depending on the problems to be studied.

This type of combined experiments has obvious limitations: the set of data, presented in Fig. 5 and Fig. 6 is subjected to experimental errors in horizontal as well as in the vertical direction. The "horizontal" experimental errors are—if we consider the dosimetry sufficiently reliable—mainly due to "geographic missers". Despite careful alignment of the tumours in the X-ray beam some 5 per cent of the irradiated animals showed an early tumour growth in the rear of the (epilated) treatment area and had to be discarded. Very likely these tumour extensions were caused by a hidden "needle-track" strand of tumour tissue which was not palpable at the time of irradiation. The possible "vertical" experimental errors are manifold. For the calculation of the surviving fraction the use of a number of different parameters was required, e.g. tumour diameter, the relation tumour diameter–tumour weight and the number of cells per gram of tumour. Each of these parameters is subject to one or more experimental errors. In addition, the cells required for the tumour cell titrations have been isolated by trypsinization. Their metabolic condition may thus differ from that of the cells remaining in situ in the tumours after irradiation.

The "cure rate" results still leave a considerable lack of information about the exact shape of the shoulder of the "*in vivo*" cell survival curve. From the cell survival curves, determined with the Hewitt-type of assay an extrapolation number of about 10 was estimated [18]. The data from the present investigation suggest that the extrapolation number for the cells of this tumour is similarly rather high.

The transplantation–assay survival curves also indicated the presence of about 15% hypoxic cells in small tumours of the Rhabdomyosarcoma BA 1112 [18]. The results of the cure rate experiments, as depicted in Fig. 6, are in accordance with this assumption.

A matter of special interest is formed by the results obtained with the sand grain sized tumours. These small tumours have been included in these investigations because of the idea that such tiny tumours might be without anoxic foci and this problem could be investigated best by using the cure rate technique. The results (Fig. 6) seem to indicate that the radiosensitivity of the "sand grain" sized tumours does not differ essentially from that of the larger tumours, so that it has to be assumed that the existence of anoxic foci is not confined to a specific minimum size of tumour.

This finding agrees with results obtained by Suit and Maeda [26], whose data also support the view that anoxic foci exists in very small C_3H mammary tumour isotransplants.

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RESUME

Nous avons effectué des expériences dans le but de vérifier si les résultats obtenus par titrage des cellules de la tumeur Rhabdomyosarcome BA 1112 *pouvaient correspondre au taux de guérison de la tumeur, après irradiation. Ces deux méthodes ont donné sensiblement les mêmes résultats. Puisque la radiosensibilité des petites tumeurs de l'ordre de* 6 *mg est très semblable à celle des tumeurs plus grandes, nous concluons à l'existence de régions dépourvues d'oxygène dans ces petites tumeurs.*

SUMMARY

Cure rate experiments with the Rhabdomyosarcoma BA 1112 *were performed to verify whether the results, obtained by tumour cell titrations agree with tumour cure rates after irradiation. Satisfactory agreement between the results, obtained with the two systems was found. It was also found that very tiny tumours of about* 6 *mg are likely to contain anoxic areas, because their radiosensitivity was very similar to that of larger tumours.*

ZUSAMMENFASSUNG

Das transplantierbare Rhabdomyosarcom BA 1112 *wurde einer Serie kurativer Experimente unterworfen.*

Es ergab sich, dass die Resultate gut übereinstimmten mit was auf Grund von schon früher bestimmten Zellüberlebungskurven erwartet werden konnte.

Es wurden auch Anweisungen gefunden, dass sich in sehr kleinen Tumoren von etwa 6 *mg schon anoxische Zonen befinden, die sich in einer relativ hohen Strahlungsresistenz aussern.*

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Search for Correlation between Tissue Cultures of Human Tumors and the Clinical Course of Cancer in Man*

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TUMOR tissue samples were obtained for cultures from 61 patients, 22 of whom had benign tumors and 39 had malignant growth. In addition, unaffected tissue samples of the tumor-bearing organs were cultured from 4 patients of the former group and from 10 patients with malignant tumors (Table 1). More than half of these patients had breast tumors, while the rest originated from the stomach, peritoneum, intestines, parotid gland, thyroid, ovary, skin and liver. The methods employed throughout were modified from those described by Ambrose *et al.* [1]. Outgrowth was observed in 36 out of the 39 cultures of malignant tumors, in 20 out of the 22 cultures of benign tumors and in 13 out of the 14 normal tissue samples explanted. Thus, successful growth was achieved in 69 of the total of 75 cultures (92%), (Table 2). The growth pattern seen in the cultures was independent of the source from which the tissue sample was obtained. In almost half of the successfully grown tissues, additional passage could be achieved. As the largest single group investigated was breast tumor, we shall restrict our remarks to some of the observations made in this group only.

The classification of the 21 mammary cancers according to the criteria suggested by the WHO—which were explanted into tissue cultures—are summarized in Table 3. These criteria are based upon the type of proliferation, the amount of fibrosis, and the degree of malignancy of the individual cancer cells. The question arose, whether the classification represented in this Table would find an expression in the pattern of growth of these cells in tissue culture. Figure 1 illustrates 3 parameters characterizing cell growth. The first parameter was the latent period or the time which elapses from the moment of explantation until the microscopic appearance of out growth. It can be seen that there is a wide variation in the latent periods from culture to culture and there was no correlation whatsoever between this parameter and the type of the tumor as established histologically. The second parameter studied was the period required from the time of the initial outgrowth until the production of the complete monolayer. Looking at these results, it is immediately obvious that here too a marked variation was the rule from culture to culture, without any regard to the source of the sample obtained. The length of survival of the cultured cells was the third parameter examined. It can be seen that one successful passage was achieved in 6 cases, two passages in 2 cases, and three passages in 1 case only. As with the other two parameters, there was no correlation here either between the type of the tumor explanted and the ability of the cells to proliferate after successive transfers. The relative ease with which normal tissue could be kept alive for many successive passages under identical conditions is well demonstrated in Fig. 1. Of the four cultures shown in this Figure, two have gone through more than ten passages. The

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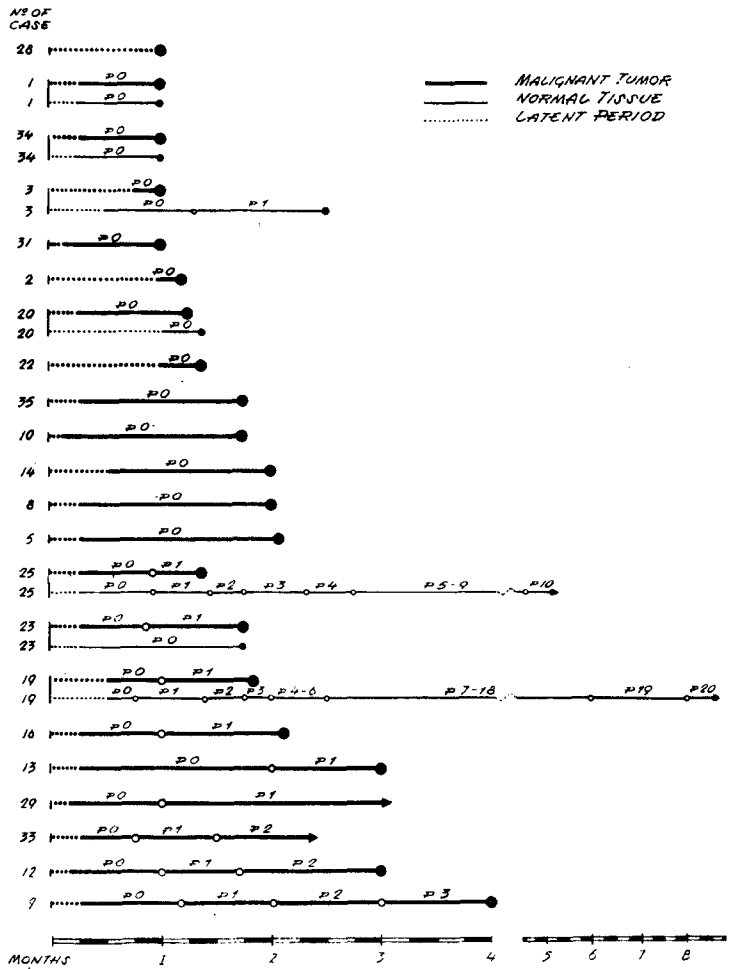


Fig. 1. *Development of outgrowth and passages in tissue culture. Malignant mammary tumors and their adequate normal tissue.*

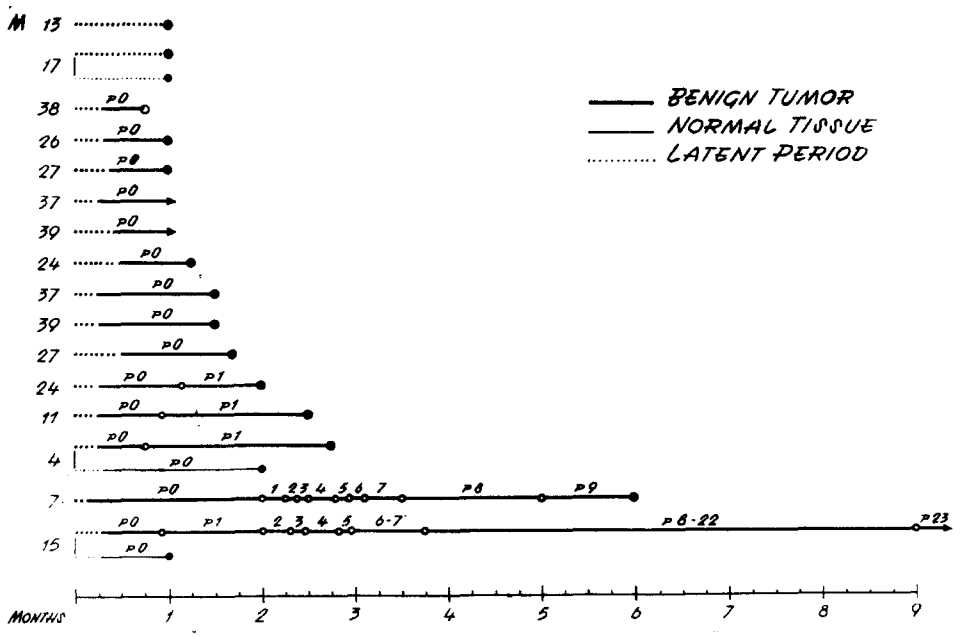


Fig 2. *Development of outgrowth and passages in tissue cultvre. Benign mammary tumors and their adequate normal tissue.*

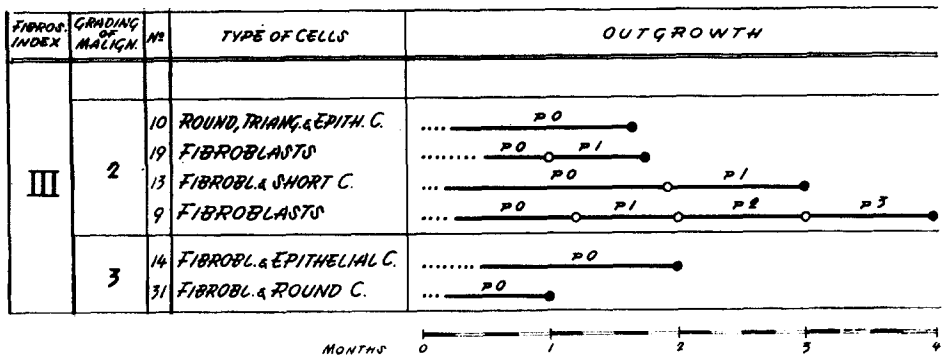


Fig. 3. Infiltrating duct carcinoma. Note the lack of correlation between the cell type explanted and the growth pattern observed in tissue culture.

same variability was observed in the 14 successfully cultured benign breast tumors (Fig. 2). Two of these, however, could be carried through a large number of passages. A cystic mastopathy for 6 months in 9, and a sclerosing adenosis in 23 passages for over 9 months, the latter having established a cell line.

It seemed to us of particular interest to study the morphological characteristics of the cells grown in these cultures with the aim to compare these properties, with those of the tissues originally explanted. It can be seen from Table 4 that pure fibroblast-like cells grew in 25 cultures and that a mixture of the above cells and epithelioid cells was found in 13 cultures, while small round cells were found associated

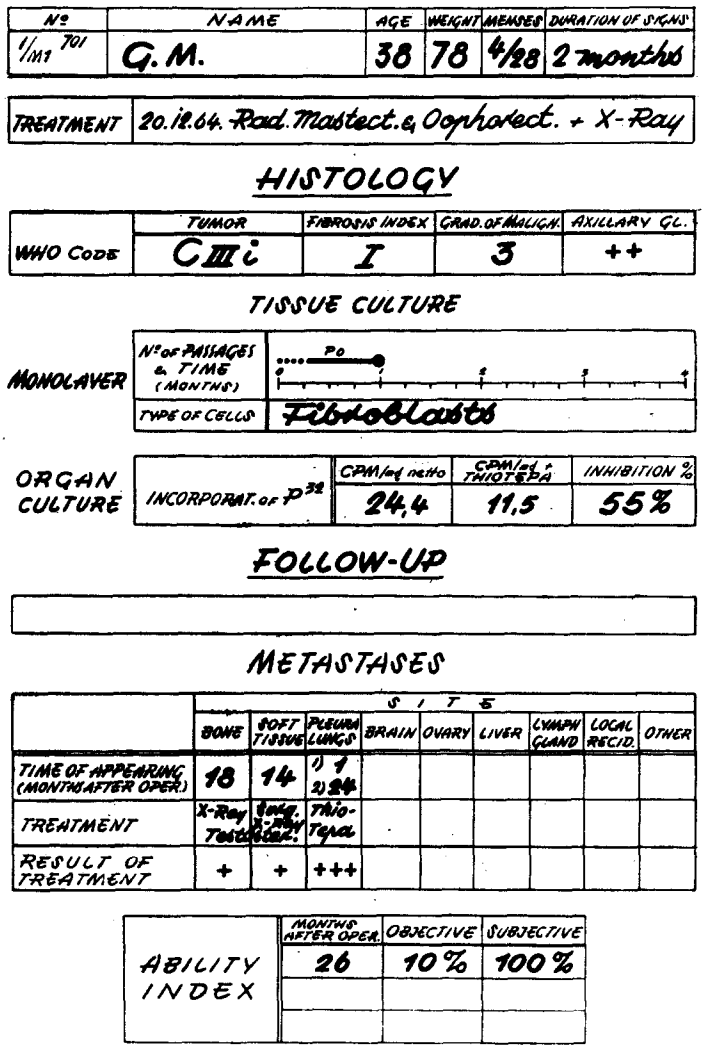


Fig. 4. Patient's chart employed in the course of the present investigation.

Table 2. Development of outgrowth and number of passages

No. of passages carried	Histology	Organ: Breast	Stomach	Peritoneum	Intestine	Parotis	Thyroid	Ovary	Skin	Liver	Cervical lymph node	Total
First outgrowth	B	$9_{(2^*)}$		1			1				$_{(1^*)}$	$11_{(3^*)}$
	M	$12_{(4^*)}$	$3_{(1^*)}$		1	1		1	$1_{(1^*)}$			$19_{(6^*)}$
1	B	3				1						4
	M	$6_{(1^*)}$	2		1				$1_{(1^*)}$			$10_{(2^*)}$
2	B											
	M	2		1								3
3	B											
	M	1	1									2
4–6	B			1_{p4}			1_{p4}					2
	M			$_{p6}1$						$_{p4}1$		2
8	B	1										1
	M											
10	B											
	M	$_{(1^*)}$										$_{(1^*)}$
16	B										1	1
	M											
20	B											
	M	$_{(1^*)}$										$_{(1^*)}$
23	B	1										1
	M											
Without outgrowth	B	$2_{(1^*)}$										$2_{(1^*)}$
	M	1			1					1		3
Total		$16_{(3^*)}$ $22_{(7^*)}$	$6_{(1^*)}$	2 2	3	1 1	2	1	$2_{(1^*)}$	2	$1_{(1^*)}$ 1	$22_{(4^*)}$ $39_{(10^*)}$
		$38_{(10^*)}$	$6_{(1^*)}$	4	3	2	2	1	$2_{(2^*)}$	2	$1_{(1^*)}$	$61_{(14^*)}$

B=Benign M=Malignant *=Normal tissue

Table 1. Source of culture specimens according to organ and histology

Histology	Organ										
	Breast	Stomach	Perito-neum	Intestine	Parotis	Thyroid	Ovary	Skin	Liver	Cervical lymph node	Total
Benign	$16_{(3)}$*		2		1	2				1†$_{(1)}$	$22_{(4)}$
Malignant	$22_{(7)}$	$6_{(1)}$	2	3	1		1	$2_{(2)}$	2		$39_{(10)}$
Total	$38_{(10)}$	$6_{(1)}$	4	3	2	2	1	$2_{(2)}$	2	$1_{(1)}$	$61_{(14)}$

*Numbers in parentheses indicate specimens of normal tissue taken from the adequate organ.
†Sarcoidosis.

with the above combination in six additional cultures. It was noted that the fibro-blast-like cells in cultures derived from normal tissue formed parallel bands, while the so-called criss-cross phenomenon was mainly encountered in cultures taken from malignant tumors. It is further noteworthy that the cultures containing small round cells originated also mainly from malignant tumors.

All the above observations were found to be true not only regarding the tumors of the breast, but also for all the 69 cultures studied.

The above observations allow us to draw two important conclusions. Firstly, there is no correlation between the type of the tumor established on the basis of histological criteria on the one hand, and the pattern of growth in the tissue culture on the other hand (Fig. 3). Secondly, tumors belonging to the same category as defined by their morphological characteristics, behave entirely differently in their growth pattern in the tissue culture.

The question arises, whether there is any correlation between the growth pattern of a tumor in tissue culture and the clinical course of the disease? This question cannot be answered at present. An attempt in this direction is represented by the project illustrated here, which was initiated recently in our institution. The follow-up sheet depicted in Fig. 4 included the histological type of the tumor, its growth pattern in monolayer culture, the results of the organ culture, which, according to Ambrose [2], is most useful as a sensitivity test for chemotherapy, and the results of treatment.

Of particular importance in relation to the clinical follow-up are the growth pattern of the cultures, i.e. loss of contact inhibition as shown by failure to show alignment of cells and the appearance of rounded cells, because these characteristics may be related to the invasive and metastasising capacity of the tumor. In such studies the dynamic biological behaviour of human tumors can be studied in a way, not possible by histology. Evidence accumulating rapidly indicates that each human tumor possesses individual biological characteristics which are not revealed by histology. Furthermore, the testing of the sensitivity of cultured tumor tissue to hormones and/or chemotherapeutic agents may serve as guide-

Table 3. Distribution according to histopathology, fibrosis and grade of malignancy

WHO Code	Tumor	Fibrosis index									
		I			II			III			
		Grading of malignancy									
		1	2	3	1	2	3	1	2	3	Total
CIIa	Medullary Ca	–	–	3	–	–	1	–	1	–	5
CIIb	Papillary Ca	–	–	–	–	1	–	–	–	–	1
CIIe	Lobular Ca	–	–	2	–	–	2	–	–	1	5
CIIi	Undifferentiated Ca	–	–	3	–	1	–	–	–	–	4
CIIj	Infiltrating duct Ca	–	–	–	–	–	–		4	2	6
	Total	–	–	8	–	2	3	–	5	3	21

Table 4. Type of cells in tissue culture

Type of cells	Histology	Organ: Breast	Stomach	Peritoneum	Intestine	Parotis	Thyroid	Ovary	Skin	Liver	Cervical lymph node	Total
Fibroblasts-like cells	B	6(2*)		1		1	1				1(1)	10(3)
	M	13(4)	3(1)	1	2		1		1(1)			21(6)
Fibroblasts-like and Epitheloid cells	B	6		1								7
	M	4(3)	1	1					1			7(3)
Fibroblasts-like, Epitheloid and little round cells	B	2										2
	M	4	2			1		1	(1)	1		9(1)
Total	B	14(2)		2		1	1				1(1)	19(3)
	M	21(7)	6(1)	2	2	1	1	1	2(2)	1		37(10)
Grand total		35(9)	6(1)	4	2	2	2	1	2(2)	1	1(1)	56(13)

B=Benign
M=Malignant
*Numbers in parentheses indicate specimen of normal tissue taken from the adequate organ.

lines in the therapeutic procedures to be employed. By a systematic and long-term survey of the type described here significant and useful criteria, hitherto unavailable, can be obtained.

Acknowledgement—We should like to use this opportunity to thank Professor Ambrose for his kind help and guidance.

RESUME

Des tissus cancéreux humains, et des tissus normaux de mêmes organes, furent cultivés en couches mono-cellulaires et en culture d'organe. On obtient une croissance satisfaisante dans la plupart des cas.

Les paramètres étudiés, c'est-à-dire la période de latence, le temps nécessaire pour la formation de la couche mono-cellulaire, le nombre de passage réalisés, variaient, dans une large mesure, indépendamment de la nature et du type du tissu explanté. Une variation semblable fut observée dans la morphologie des cellules cultivées; le tissu nouvellement formé était constitué soit par une majorité de fibroblastes, soit par une majorité de cellules épithéliales et de petites cellules rondes, soit par une association de ces divers types de cellules. Le phénomène désigné par le terme d'entrecroisement, associé aux petites cellules rondes, était surtout observé dans les cas de cultures malignes.

Des études, ayant pour but de rechercher une corrélation entre ces observations et l'évolution clinique de la maladie, sont actuellement en cours. Les résultats en seront publiés ultérieurement.

SUMMARY

Cancerous and normal tissues from the tumorbearing organs of human origin were cultured by the monolayer and organ culture methods. Successful growth could be achieved in the vast majority of experiments.

There was a wide variation in the parameters studied, i.e. in the latent period, in the time required for the completion of the monolayer and in the number of passages achieved, irrespective of the nature or the type of the tissue explanted. A similar variation was noted in the morphology of the cultured cells and the newly formed tissue consisted of, either predominantly fibroblasts, or mainly epithelial cells and small round cells, or a mixture of all the above cell types. The so-called criss-cross phenomenon associated with small round cells was mostly observed in cultures of malignant tumors.

A study aimed to correlate these findings with the clinical course of the disease is in progress at present, and its results will be reported separately.

ZUSAMMENFASSUNG

Malignes und normales Gewebe der vom Tumor befallen menschlichen Organe wurde in Monolayer oder Organkultur gezüchtet. Bei der Mehrzahl dieser Versuche konnte gutes Wachstum erzielt werden.

Die Kriterien, nach denen diese Kulturen beurteilt wurden, die Latenzperiode, die erforderliche Zeit zur Monolayer-Formierung und die Anzahl der erreichten Passagen zeigten eine erhebliche Variationsbreite, unabhängig vom Typ des explantierten Gewebes. Eine ähnliche Vielfältigkeit trat auf in der Morphologie der kultivierten Zellen und des aus ihnen neugebildeten Gewebes, das entweder vorwiegend aus Fibroblasten, oder Epithelzellen und kleinen Rundzellen oder einer Mischung aller dieser Zellen bestand. Das sogenannte "criss-cross" Phänomen in Verbindung mit kleinen Rundzellen wurde meist in Kulturen maligner Tumoren beobachtet.

Studien mit dem Ziel, einen Zusammenhang zwischen diesen Ergebnissen und dem klinischen Verlauf der Erkrankung zu finden, sind im Gang. Über sie wird an anderer Stelle berichtet werden.

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Burkitt's Tumour in Norway
A survey of possible cases occurring in the period 1953-1962

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INTRODUCTION

THE HISTOPATHOLOGICAL and clinical features of Burkitt's tumour ('African lymphoma') have been extensively discussed [1–4]. Histologically the tumour is characterized by tightly packed areas of primitive lymphoblastic cells with lipide containing vacuoles and scattered large histiocytes with signs of pronounced phagocytic activity, giving the tumour the so called 'starry sky' appearance. Clinically the disease is characterized by a peculiar age distribution, occurring most commonly in children, with only occasional adult cases [4]. There is a frequent involvement of bones, especially of the jaws, and of the abdominal viscera including the ovaries and the retroperitoneal lymph nodes, with relative sparing of the peripheral nodes.

It was first thought that this was an African disease. The distribution of Burkitt's tumour in Africa seems to correspond closely to areas that have a mean annual minimum temperature of not less than 60°F and a rainfall of more than 20 in/year [5]. Later studies have, however, shown (see various review articles and case reports in Vol. 2, No 6, Int. J. Cancer 1967) that similar cases can be found all over the world.

As a consequence of the geographical distribution in Africa, the theory has been put forward that the tumour may be induced by an arthropod-borne virus [5, 6]. More recently different viruses have been isolated from cases of Burkitt's tumour [7], but if they have any causal relationship to the tumour is as yet disputable. The fact that the tumour occur in widely differing climatic areas seems, however, to render the original 'insect borne virus'—hypothesis less acceptable.

In a previous publication one of us [8] reported a lymphosarcoma resembling Burkitt's tumour from the northern part of Norway. This encouraged us to start a study of other possible cases of this tumour in Norway. The purpose of the present paper is to give a brief survey of this study, and to discuss some aspects of the disease on the basis of our observations.

MATERIALS AND METHODS

By courtesy of Dr. Einar Pedersen, head of the Norwegian Cancer Registry, we received reports of all malignant lymphomas occurring in Norway during the years 1953 to 1962 inclusive, and in the age groups from birth up to 20 years. Cases registered as Hodgin's lymphoma and Brill Symmers 'giant follicular lymphomas' were excluded from our study. Only cases confirmed by histological diagnosis were considered. Evidence of leukaemia at the initial presentation led to rejection of the case, whereas cases that initially were non-leukaemic, but with subsequent development of leukaemia were included.

The Norwegian Cancer Registry receives reports from all cases of malignant disease in Norway. It is thus probable that we have been able to include well-nigh all cases of malignant lymphoma in patients below the age of 20 years during this period.

Clinical records were obtained from hospitals all over the country, and from doctors working in general practice. We are grateful to the Pathological Anatomical Departments at Radiumhospitalet, Gades Institutt in Bergen, Sentralsykehuset in Trondheim and Ullevål sykehus in Oslo for giving us access to microscopical sections from all the cases. Most of these were biopsies, but there were also several post mortem examinations.

The histological specimens were studied and classified as a 'blind test' without knowledge of the clinical information. Only tumours built up of lymphoblastic cells with definite large areas of 'starry sky' appearance were accepted as 'Burkitt positive' cases.

The criteria used for the clinical classification of the cases were more difficult to establish. Primary location to bones, especially to the jaws and facial bones, or dominating abdominal tumours including the ovaries with spread of tumour preferentially to the retroperitoneal glands, sparing the peripheral and mediastinal lymph nodes, were accepted as clinical criteria. It is, however, evident that such a classification will be somewhat arbitrary. The clinical grouping was also performed as a 'blind test', without knowledge of the histological diagnosis of the cases.

RESULTS

We received 112 reported cases of lymphosarcomas and suspected lymphosarcomas from the Cancer Registry during the actual 10 years period in patients below 20 years of age. Of these 80 fulfilled our criteria of being non-leukemic lymphosarcomas. 58 of these belonged to the age groups below 15 years.

Classification based on clinical criteria. Table 1 shows the type of initial clinical presentation of tumour. The clinical picture was in no case pathognomonic for Burkitt's tumour. However, 13 cases revealed a pattern similar to many of the Burkitt's lymphoma published from Africa and from non-African countries. Table 2 shows the main clinical findings in these cases.

Cases 1–7 are characterized by initial tumour location to the jaw and facial bones. In cases 8 and 9 the primary site of tumour was in osseous tissue elsewhere and the remaining 4 cases (Nos. 10–13) had all one or more abdominal tumours as the dominating feature.

Out of these 13 cases that clinically were in conformity with Burkitt's lymphoma, merely 3 cases (Nos. 11–13) had a histological picture in accordance with the accepted criteria for Burkitt's tumour.

Classification based on histological criteria. Table 3 shows the results. It is seen that 8 cases could be histologically classified as resembling Burkitt's tumour. Only 3 of these were accepted as clinically in accordance with Burkitt's tumour (case Nos. 6–8), thus being present also in Table 2 (case Nos. 11–13).

Table 1. *Classification of* 80 *cases of non-leukaemic childhood lymphosarcoma**

Type		No. of patients	Percentage
Extranodal tumour		34	42·5
Abdominal	9		
Jaw/facial bones	8		
Ovarian	0		
Other	17		
Peripheral lymphadenopathy		30	37·5
Widespread		16	20·0

*Based on initial clinical presentation.

DISCUSSION

Burkitt's lymphoma cannot, as first supposed, be regarded as an 'African disease'. This is evident from the many reports of lymphomata of this type occurring in different parts of the world. However, it is still a fact that the tumour is seen much more frequent in tropical Africa than elsewhere. Tables 4 and 5 illustrate this.

In most non-African countries the malignant lymphomas constitutes less than 10% of the total number of childhood cancer [9, 1, 10]. A frequence of about 5% in our series is sharply contrasted by the 45% in the Ugandan

Table 2. Summary of major findings in 13 cases clinically resembling Burkitt's tumour

Case	Age (years)	Sex	Survival Onset to death (months)	Presenting clinical feature	Subsequent course	Histology
1. H. M. H.	5	M	1½	Swelling of right maxilla	Spread of tumour to maxillary and zygomatic bone, with X-ray destruction. Lung metastasis. No peripheral lymphadenopathy	Lymphosarcoma
2. A. M.	7	M	14	Flat, massive tumour in left cheek, fixed to maxilla	Spread to pelvic bone. No p.l.a.	Lymphosarcoma
3. M. M.	14	M	4	Swelling of right maxilla	Spread of tumour to the orbit and lower vertebrae. No p.l.a.	Lymphosarcoma
4. S. A. L.	18	M	22	Swelling of right mandible	Paresis of both legs, intra-abdominal tumour. No p.l.a.	Lymphosarcoma
5. K. J.	3	F	2½	Propthosis and destruction of orbital bone. Epipharyngeal tumour with spread to adjacent bone	Spread to abdominal viscera and cervical lymph nodes.	Lymphosarcoma
6. A. L.	10	F	13	Retronasal tumour with destruction of palate, orbital and adjacent cranial bones	Unknown	Lymphosarcoma
7. B. E.	2	F	3	Tumour of frontal bone	Spread to liver and sceleton No p.l.a.	Lymphosarcoma
8. R. H.	6	F	7	Tumour of first rib	Metastasis to other bones. No p.l.a.	Lymphosarcoma
9. E. H.	9	F	3	Vomitus, pain in left hip	Rapid deterioration. Lymphadenopathy left axilla	Lymphosarcoma
10. U. M.	6	F	1½	Multiple abdominal tumours	Rapid deterioration.' Autopsia revealed spread of tumour to most abdominal organs and retroperitoneal tissue. Both ovaries enlarged. No p.l.a.	Lymphosarcoma
11. E. T.	5	M	5	Abdominal tumours, mainly located to mesocolon	Terminally ascites, hydrothorax and slight generalized adenopathy	Burkitt's tumour
12. S. L.	17	M	8	Gastric tumour	Spread to retroperitoneal tissue and abdominal viscera. No p.l.a.	Burkitt's tumour
13. G.G.S.	15	F	6	Gastric tumour	Huge pelvic tumour mass. No p.l.a.	Burkitt's tumour

Table 3. Summary of major clinical findings in 8 cases histologically classified as Burkitt's tumour

Case	Age (years)	Sex	Survival: Onset to lst cons (months)	Survival: Onset to death (months)	Presenting clinical feature	Subsequent course
1. T. H. G.	7	M	2	8	Cervical lymphadenopathy	Mediastinal mass, later generalized adenopathy
2. V. S.	4	M	5	10	Abdominal pain, movable abdominal tumour (intestinal) Generalized adenopathy	Unknown
3. W. F. L.	17	M	1	4	Cervical tumour, generalized adenopathy	Multiple abdominal tumours, central amaurosis, orbital oedema
4. I. A. B.	4	M	Alive after 6 years		Preauricular tumour	No recidivism
5. E. T.	18	F	Alive after 9 years		Axillary adenopathy	Unknown
6. E. T.	Identical with case No. 11 in Table 2					
7. S. L.	Identical with case No. 12 in Table 2					
8. G. G. S.	Identical with case No. 13 in Table 2					

Table 6. Clinical features of histologically diagnosed Burkitt's tumour in various series

	Main presenting location: Jaw or facial bones (%)	Abdomen/retroperitoneum (%)	Periph. or mediast. lymph nodes (%)	Other locations (%)	Leukemic transf. (%)
Uganda [1] (106 cases)	55*	30	1	14	0
Canada [10] (12 cases)	17	83	0	0	0
Columbia [13] (10 cases)	20	70	10	0	0
U.S.A. [12] (9 cases)†	33	44	22	0	?
England [14] (9 cases)	11	22	33·5	33·5	22(?)
U.S.A. [20] (33 cases)	3	51	40	6	18
Norway (present series) (8 cases)	0	33	50	17	0

*According to the author [1] probably an overestimate.
†Including only lymphoblastic lymphoma with 'starry sky'.

and 70% in the Nigerian series. Estimates of the total incidence of cancer is of course only approximate in those African regions here involved, but a difference of this magnitude obviously cannot be ascribed merely to difficulties concerning cancer registration in Africa. In fact, according to figures from Ibadan [11] the malignant lymphomas in childhood is about 12 times more common in those regions than in Norway.

Quantitatively, therefore, the 'African lymphoma' seems to differ from lymphomas elsewhere. But if the Burkitt's tumours also differ from other malignant lymphomas of childhood in qualitative respect, thus being a well defined and separate pathological, clinical and etiological entity, is a matter of dispute. The tumour is regarded merely as a variant of lymphosarcoma by some authors [2, 3, 12, 13] whereas Wright [4, 14] and others in Africa favour the opinion that the Burkitt's tumour is a unique disease entity, having a distinct histological picture, according to which it may be defined. The latter concept has been given some additional support by immunological studies [15] and the behaviour of the tumour cells in tissue culture has further strengthened this view [16, 17].

Based on the histological criteria, how uniform is the syndrome from a clinical point of view?

Case reports are mostly concerned with the 'typical' features of the syndrome and thus are apt to blur the variability in the development of the disease. During the last years, however, some reviews of non-African lymphosarcoma similar to Burkitt's lymphoma have been reported. Together with figures from the Ugandan series of Burkitt and O'Conor [1] the major presenting clinical features are extracted from the different series and listed in Table 6.

Most of the series include only a few cases, but it appears from the table that the histologically diagnosed Burkitt's lymphoma may differ significantly in the clinical behaviour. Thus, the tumours of the jaws and facial bones, the dominating location of the Ugandan cases, is a rather rare manifestation in the non-African series. It constitutes 11 per cent in an American series (O'Conor) and 3 per cent in an English series (Wright). In the present series we found no case of jaw tumour in the histologically Burkitt positive group.

On the other hand, the tumour was manifested by peripheral lymphandenopathy in many non-African patients. This is only exceptionally met with among the Africans.

We feel that the clinical characterization hitherto used is not adequate. If this disease is defined according to the traditional histological criteria, we have to accept a rather heterogeneous clinical group. If we then remember that some of the African cases has a 'non-typical' histological appearance [2] and that many 'conventional' lymphosarcomas demonstrate the same clinical behaviour as the Burkitt's tumour, it seems reasonable to

Table 4. *Occurrence of malignant lymphomas in children* 0–14 *years relative to total cancer*

	Total cancer	Malignant lymphoma %
Uganda/Kempala (Burkitt)	81	44·5
Kenya/Nairobi (Dalldorf)	300	43·0
Nigeria/Ibadan (Edington)	230	70·0
Norway (present series)*	1089	5·5

*Including intracerebral tumours of all kinds.

Table 5. *Rates of malignant lymphoma per* 100,000 *children/year*

	Ibadan	Norway
All cancer	12·6*	12·1†
Malignant lymphomas	8·9	0·7

*Calculated from Edington and Maclean [11].
†Including intracerebral tumours of all kinds.

"question the concept that Burkitt's tumour is a distinct disease entity, separable from the so-called conventional lymphosarcoma" [12].

There is broad agreement that the histiocytes in Burkitt's tumour which give it the 'starry sky' appearance, is a non-specific phenomenon, seen in many other malignant and non-malignant conditions. According to O'Conor [3] this finding merely characterizes lymphosarcomas in children generally and occasionally are seen in tumours of adults as well.

The idea put forward by O'Conor [2] that the signs of phagocytosis in the tumour indicates a high proliferation rate and a high rate of cell loss, has been later confirmed by others [18–20]. This property of the tumour cells may perhaps account for the characteristic behaviour of the cells in tissue culture, and for their immunological peculiarity, as compared with other lymphomas. If these latter criteria should qualify the tumour as a unique disease entity, is a rather semantic question, partially depending upon whether the tumour displays a distinct clinical behaviour or not.

Our study has shown that this is not the fact in the Norwegian material. Out of 8 cases histologically defined as Burkitt's tumour only 3 could be clinically accepted according to the classical criteria. Out of 13 cases clinically resembling Burkitt's tumour, only 3 had the histological criteria present. We feel that the following statement is in accordance with all observations hitherto reported: lymphosarcoma in childhood is a disease with many different clinical manifestations. Some of these tumours grow rapidly, and respond initially well to treatment with cytostatics because of the rapid proliferation. These tumours have also a rapid cell loss. Macrophages are taking care of the material from the dead cells, giving these tumours the 'starry sky' appearance. The rapid proliferation and the short life span of the tumour cells make them recognisable in tissue culture and give them their immunological characteristics. Something is wrong with the energy metabolism in the cells leading to the characteristic degeneration called 'fatty change', characterized by accumulation of lipid droplets in the cells.

The carcinogenic agent may in some cases be a virus. This may especially be true in tropical Africa where a virus and other environmental conditions may contribute to an unusually high prevalence of the disease with a tendency to location to the jaws, and abdominal lymph nodes and viscera.

RESUME

On a examiné, du point de vue anatomo-pathologique, tous les cas de lymphomes malins diagnostiqués en Norvège, pendant les années s'étendant de 1953 *à* 1962, *chez les sujets âgés de* 20 *ans ou moins. Le but de cette étude était de trouver si quelques-uns des cas de cette catégorie d'âge pouvaient être considérés comme des tumeurs de Burkitt. On a trouvé* 80 *cas dont* 58 *avaient moins de* 15 *ans. Dans* 8 *cas, histologiquement définis comme tumeurs de Burkitt,* 3 *seulement ont présenté les caractéristiques cliniques de la même maladie. Dans* 13 *cas, cliniquement pareils à la tumeur de Burkitt,* 3 *seulement présentaient une histologie caractéristique. On se demande, sur les bases de nos observations et d'autres rapports publiés, si la tumeur de Burkitt est une maladie bien caractéristique en soi, ou non. Selon notre opinion, la tumeur de Burkitt ne représente probablement qu'une catégorie de lymphosarcome, à prolifération rapide, de l'enfance et de la jeunesse.*

SUMMARY

All malignant lymphomas occurring in Norway during the years 1953–1962 *in the age groups from birth up to* 20 *years have been studied clinically and pathological anatomically. The aim of the study was to find possible cases of Burkitt's tumour. The material consisted of* 80 *cases of which* 58 *belonged to the age group below* 15 *years. Out of* 8 *cases histologically defined as Burkitt's tumour only three could be clinically accepted according to the classical criteria. Out of* 13 *cases clinically resembling Burkitt's tumour, only three had the histological criteria present. The concept of Burkitt's tumour as a unique disease entity is discussed on the basis of our observations and other reports given in the literature. It is the authors' opinion that Burkitt's tumour is probably a fast growing variety of lymphosarcoma in childhood, and not a separate disease.*

ZUSAMMENFASSUNG

Alle malignen Lymphome, die in den Jahren von 1953–1962 *bei der Altersgruppe bis* 20 *Jahre und darunter, in Norwegen aufgetreten sind, wurden unter anatomisch-patholo-*

gischen Kriterien erfaßt. Das ziel dieser Studie war, herauszufinden ob einzelne Fälle in dieser Alterskategorie als Burkitt-Tumoren betrachtet werden könnten. Achtzig Fälle wurden festgestellt, 58 *davon waren weniger als* 15 *Jahre alt. Von* 8 *histologisch als Burkitt-Tumoren diagnostizierten Fällen, zeigten nur* 3 *die klinischen Charakteristiken dieser Krankheit. Von* 13 *klinisch typischen Fällen wiesen nur* 3 *die histologischen Befunden des Burkitt-Tumors auf. Auf der Basis dieser eigenen Beobachtungen und anderer Veröffentlichungen erhebt sich die Frage, ob der Burkitt-Tumor überhaupt eine Krankheit per se ist. Die Möglichkeit wird diskutiert, ob der Burkitt-Tumor nur die rapide wachsende Form eines in der Kindheit und in der Jugend auftretenden Lymphosarkoms darstellt.*

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Transplantation of Canine Lymphosarcoma

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INTRODUCTION

APART from the naturally occurring canine venereal tumour [1] only three neoplasms of dogs have been homologously and serially transplanted. Allam *et al.* [2] succeeded in transplanting an anaplastic carcinoma of the thyroid following pre-treatment of recipient dogs with cortisone and total body X-irradiation. A canine mast-cell leukaemia has been successfully transplanted on two separate occasions [3, 4]. No pre-treatment was required and the tumour could, after a number of passages, be transmitted by cell-free material. Finally, a lymphosarcoma was transplanted into X-irradiated new-born puppies by Moldovanu *et al.* [5]

The dog, because of its larger size, is better suited for research on surgical, irradiation and perfusion techniques in cancer therapy than the smaller laboratory animals where many transplantable tumours are readily available. The relative infrequency of success of canine tumour transplantation and the need for transplantable tumour in a large species prompted us to pursue a new approach.

Description of donor dog

The original tumour-bearing dog was a male Cairn aged 8 years with a clinical history of lymphosarcoma of 7 months. Widespread lymphosarcomas were present in many internal lymph nodes and organs. Especially large tumour masses up to 4 cm dia. were located in the lungs and in the liver but marked enlargement of the peripheral lymph nodes, as is a frequent occurrence in canine lymphosarcomas, was not a feature. Tumour masses were soft and white and without the gross appearance of central necrosis. The histological appearance was that of a lymphoblastic tumour with scattered vacuolated histiocytes giving a "starry sky" appearance identical to that seen in the Burkitt lymphoma of children and in several canine lymphomas [6–8].

METHOD

A two-year old cross-bred bitch (Bitch A) 46 days pregnant, was pre-medicated with acetylpromazine 1 mg and atropine sulphate 0·65 mg. Anaesthesia was induced with thiopentone sodium and maintained with halothane. Following a laparotomy foetuses were palpated through the uterine wall and held at the pelvis by the index finger and thumb. A 26-gauge needle was inserted through the uterine wall into the abdomen of the foetuses and a mixture of approximately equal numbers of spleen and neoplastic splenic lymph node cells from the donor dog was injected. Seven out of 8 foetuses present each received $6{\cdot}5 \times 10^8$ cells contained in 0·2 ml of suspension in Hanks' solution.

RESULTS

Bitch A gave birth to 8 live puppies but one of these died on the same day and in this animal no lymphosarcoma was found. Between 17 and 38 days post-partum (34–55 days post injection) 5 more puppies died or were killed and all these had advanced lymphosarcoma (Table 1, Figs. 1 and 2). The large tumour masses which occurred in the lungs in 3 cases were seen radiologically 2 or 3 days before the

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Table 1. *Bitch A*

Pup	Death	Age (days)	Main lesions						Other sites
			Lungs	Spleen	Thymus	Lymph nodes Thoracic	Abdominal	Peripheral	
1	Died	1							
2	Died	17	+++	+		+	+		Pleura ++
3	Killed	20	+++	+	+	++	+	+	
4	Killed	25		+		+	+		Pancreas +
5*	Killed	32	+++	+		+			Pleura+
6†	Killed	38				+		++	
7	Alive	95							
8	Alive	95							

*Used as donor for transplantation into Bitch B.
†Used as donor for transplantation into Bitch C.
Key: + to +++ denotes increasing size of lymphoid tumours (approx. 2 mm–3 cm).

puppies became clinically ill. Two puppies in the litter are still alive and appear normal 95 days after birth.

A cell suspension of bronchial lymph node and spleen from Pup 5 killed 32 days post-partum was injected intraperitoneally into 6 of 8 foetuses of a miniature poodle (Bitch B) on the 42nd day of pregnancy. Each foetus received $4{\cdot}7 \times 10^7$ cells. Three puppies died the day after birth. All were found to have lymphosarcoma and a fourth animal with lymphosarcoma was killed when 3 days old (Table 2). In a third experiment 4 out of 5 foetuses in a Toy poodle (Bitch C) 48 days pregnant were injected with 4×10^7 cells of spleen and lymph node from Pup 6 (Bitch A). Eighteen days post-partum one puppy was noted to have developed an enlarged abdomen. On post-mortem examination lymphosarcoma was found in the spleen, peripheral lymph nodes, lungs, thymus and surrounding both ovaries. A second puppy was killed when 37 days old. Lymphosarcoma tumours up to 2 cm dia. were present bilaterally in the mandibular lymph nodes and lymphosarcoma was also present in the left kidney. Two puppies died within 5 days of birth in this litter without evidence of tumour.

The histological picture of both first and second tumour passages closely mimicked that of the original donor except that the frequency of vacuolated histiocytes had increased thus accentuating the "starry sky" pattern (Fig. 3).

Preliminary chromosome studies on cell cultures from the tumour in a female first passage puppy (Pup 5, Bitch A) indicated that cellular transplantation had occurred rather than viral transmission. Studies on cell free transmission using the same techniques are in progress.

DISCUSSION

It seems likely that the canine foetus is immunologically incompetent at the time of cell injection and that tolerance has occurred.

Table 2. *Bitch B*

Pup	Death	Age (days)	Main lesions						Other sites
			Lungs	Spleen	Thymus	Lymph nodes Thoracic	Adbominal	Peripheral	
1	Killed	1							
2	Killed	1							
3	Died	1						++	R. scapula region +++
4	Died	1					+++	+++	Liver ++
5	Died	1	++	+		+	+	++	Subcapsular L. Kidney ++
6	Killed	2							
7	Killed	3			+			++	R. scapula region +++
8	Alive	36							

Key: + to +++ denotes increasing size of lymphoid tumours (approx. 2 mm–1·5 cm).

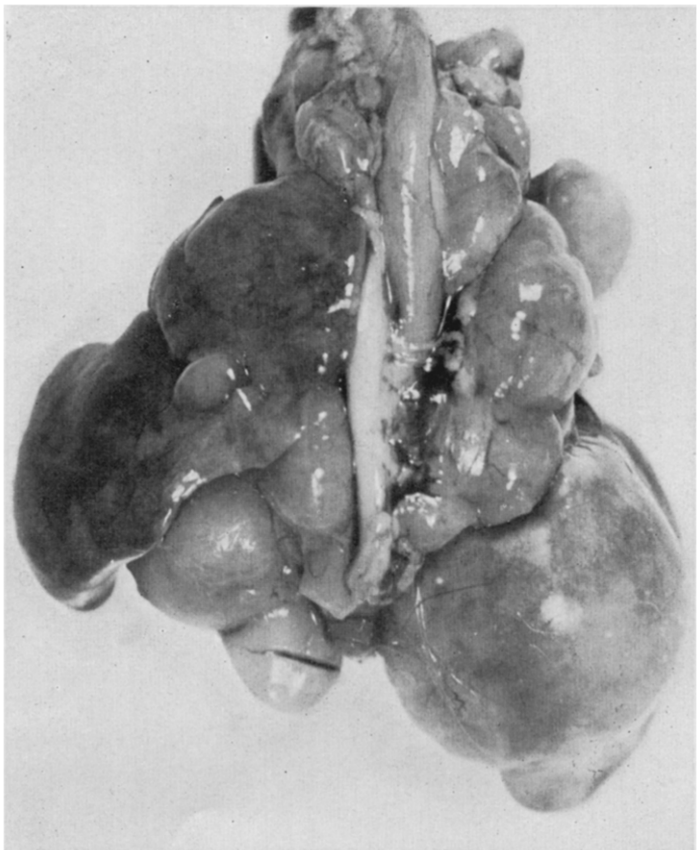


Fig. 1. Gross lesions in pup 3 of Bitch A showing several large lymphoid tumours of mediastinum and lung parenchyma.

(to face p. 392)

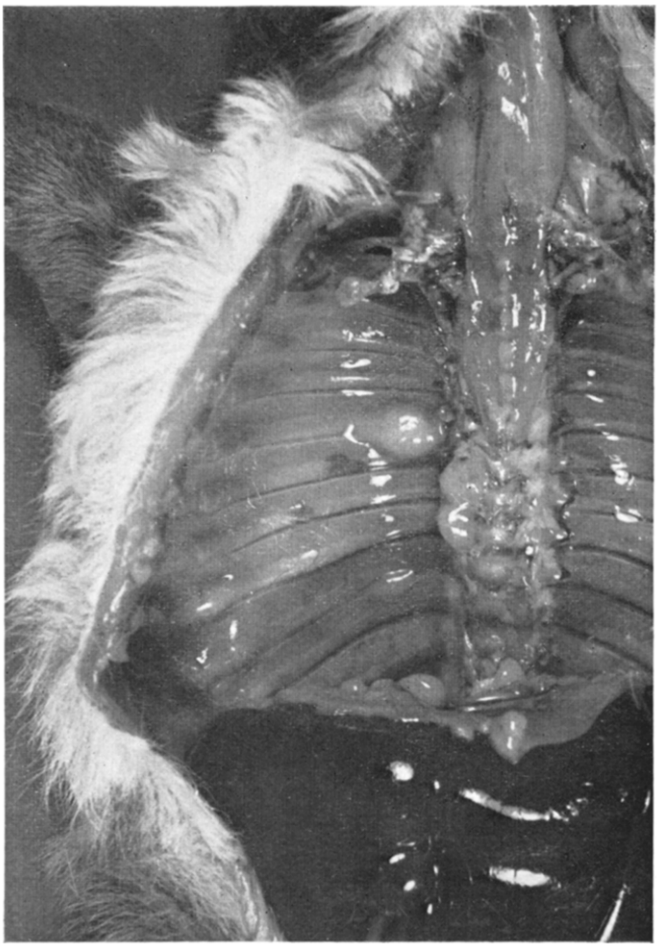


Fig. 2. Gross lesions of pup 2 of Bitch A showing several nodules in parietal pleura.

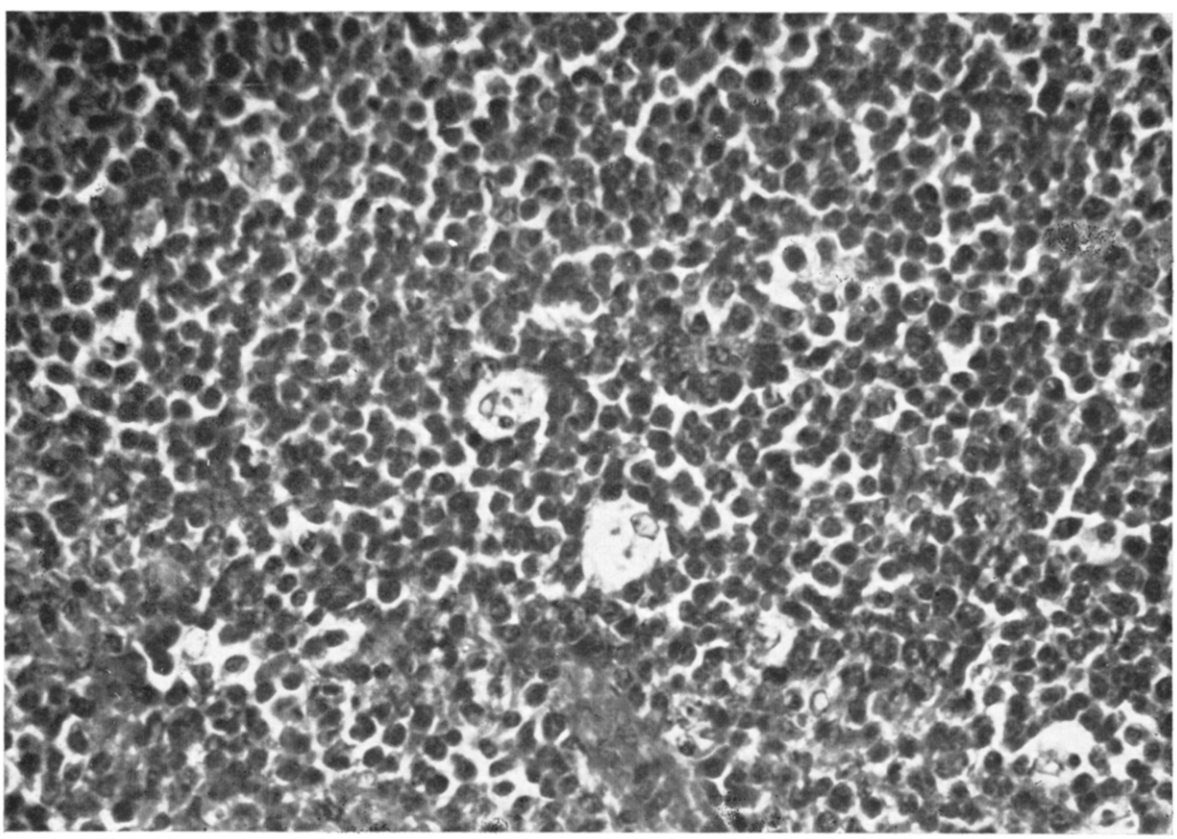


Fig. 3. Photomicrograph of first passage lung tumour of pup 5 from Bitch A showing a uniformly lymphoblastic cell population with 2 vacuolated histiocytes in centre.

In some strains of mice and rats tolerance can be induced at birth [9, 10]. Using intravenous injections of spleen cells into rabbits at birth, followed by skin grafting, only a very weak tolerance could be obtained in a small percentage of animals [9]. Attempts at heterografting rabbit skin in rats following the intraperitoneal injection of rat foetuses *in utero* with donor spleen cells produced a partial tolerance [11]. Tolerance to skin homografts was produced in pigs by injection of foetuses with bone marrow or lymphoid cells at 60 days but not at 80 days gestation [12].

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RESUME

L'inoculation in utero *de cellules fraîches de lymphosarcomes, provenant d'un chien non apparenté, dans des foetus canins, a produit des lymphosarcomes disséminés dans 5/7 des chiens, au cours des 38 jours suivant leur naissance (55 jours après l'inoculation). Un second passage des cellules, dans deux autres portées, a également réussi.*

SUMMARY

Canine foetuses-in-utero *were inoculated with fresh lymphosarcoma cells from an unrelated dog and widespread lymphosarcoma was produced in 5 out of 7 dogs within 38 days of birth (55 days post inoculation). A second passage into two more litters was also successful.*

ZUSAMMENFASSUNG

Hundefetuse wurden in Utero *mit frischen Lymphosarkomzellen von einem unverwandten Hund inokuliert. Ein generalisierter Lymphosarkom wurde in 5 von insgesamt 7 Hunden innerhalb 38 postpartaler Tage (55 Tage nach Inokulation) festgestellt. Ein zweiter Durchgang in zwei weiteren Würfen war ebenfalls erfolgreich.*

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The Uptake of [6,7-^{3}H] Oestradiol by Oestrogen Dependent and Independent Hamster Kidney Tumours

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INTRODUCTION

THE OESTROGEN-induced renal tumour of hamsters is of great interest as it is a hormone-induced tumour in an organ which is not normally regarded as belonging to the endocrine system [1]; also the hormone concerned with the induction of the tumour is necessary for continued growth of the tumour.

On repeated transplantation the tumour becomes oestrogen independent, but the mechanism by which this occurs is unknown. Other studies on hormone dependent and independent tumours—[6,7-^{3}H] oestradiol-17β uptake in dimethylbenzanthracene-induced rat mammary tumours [2]; [2,3,4,5-^{3}H] hexoestrol and human breast tumours [3]; [1,2-^{3}H] cortisol and mouse P1798 lymphosarcoma [4]—indicated that, in each case, the dependent tumours contained more tritium than the comparable independent tumours. These studies did not show if the decreased uptake of hormone by the independent tumour was due to a change in the binding mechanism or to altered tumour catabolism of the hormone resulting in a lowered cellular concentration of hormone. It was thought that a comparison of oestrogen binding in dependent and independent hamster tumours might furnish information on possible changes in the oestrogen receptor with the loss of oestrogen dependency.

The RCA_1 renal adenocarcinoma [5] was included in the study as a hormone independent control tumour.

MATERIALS AND METHODS

Animals and tumours

Unless otherwise stated, male hamsters were 6–8 weeks old when tumour transplantation or hormone treatment began. The oestrogen-independent tumour was transplanted subcutaneously, and the interval between successive transplantations was about 21 days. In 4 animals, a concomitant kidney capsule transplant was also done. The RCA_1 renal adenocarcinoma was transplanted subcutaneously every 7–21 days. Animals with subcutaneous tumours were killed when the tumour dia. was about 2 cm. Pellets of oestradiol-17β (20 mg) or stilboestrol (Burroughs Wellcome & Co. Ltd., 15 mg Stilboestrol implants) were implanted subpanicularly at 3 monthly intervals for the duration of the experiment. Most animals were used with the pellet *in situ*, in others the pellets were removed 48 hr prior to injection of [6,7-^{3}H] oestradiol-17β. In one experiment, stilboestrol pellets were implanted 19 days before subcutaneous transplantation of the independent tumour.

Animals survived better on stilboestrol, consequently the use of oestradiol was discontinued.

Operations were carried out under light ether anaesthetic, except for the transplantation under the kidney capsule, and for removal of oestrogen pellets. In these instances Avertin (Winthrop Laboratories, New York) was used.

Purification of [6,7-^3H] *oestradiol-17β and measurement of radioactivity*

The preparation and purification of [6,7-^{3}H] oestradiol-17β has been described [6]. Three batches have been used with specific activities of 180, 216 and 232 μc/μg and all results on *in vivo* uptake have been calculated as moles of oestradiol-17β incorporated. A single subcutaneous injection of about 1·0μg [6,7-^{3}H] oestradiol-17β/100 g body wt. was given into the left groin of the hamster. The exact amount injected was found by counting an aliquot of the injection solution. All results have been corrected to an injection dose of 1·0 μg [6,7-^{3}H] oestradiol-17β/100 g body wt. The actual amount injected was usually between 0·7–1·3 μg/100 g body wt. All samples were counted in a Packard liquid scintillation spectrometer model 3003. The phosphor consisted of naphthalene (100 g), diphenyl oxazole (3·5 g), dissolved in dioxane (385 ml), xylene (385 ml) and ethanol (230 ml). Quenching was assessed by the method of Baillie [7]. Paper chromatograms were scanned in a Nuclear Chicago windowless chromatogram scanner, model 1036.

The following systems were used for paper chromatography:-

(1) light petroleum (b.p. 80–100°): toluene: methanol: water (50: 50: 70: 30);

(2) Ligroin: toluene: methanol: water (100: 50: 70: 30:); Whatman's No. 2 paper was used throughout. In some instances the chromatograms were cut into 1 cm sections, and the radioactivity measured in each section by liquid scintillation counting.

Preparation and purification of tissue extracts

Animals were killed with CO_2 gas either 0·5 or 4 hr after injection of [6,7-^{3}H] oestradiol-17β. In the initial experiments the tritium in kidney, liver and tumour was separated into "bound", "free steroid", "glucosuronide", "sulphate" and "polar" fractions [8]. Samples of all fractions were counted in duplicate. Subsequently, weighed pieces of tissue (15–45 mg) were digested in 0·3 ml 1N NaOH prior to counting.

The method of characterization of metabolites has previously been described [8].

In vitro *incubations*

The tissues were finely minced with scissors and duplicate 100 mg. portions incubated at 37° for 0·5 hr in 5 ml Krebs ringer phosphate solution pH 7·4 containing 0·4 μc [6,7-^{3}H] oestradiol-17β/ml. Stilboestrol-treated kidney tissue was taken from kidneys that had visible primary tumours and which had been excised.

RESULTS

The distribution of tritium in dependent and independent tumour 0·5 and 4 hr after injection of [6,7-^{3}H] oestradiol-17β is given in Table 1. In both tumours, free oestradiol-17β was the major steroid recovered after 0·5 hr, whereas at 4 hr "glucosuronide" fractions contained most of the radioactivity. Good recoveries of radioactivity were obtained at 0·5 hr; the varied recoveries at 4 hr may explain the difference between the dependent and independent tumour "free steroid" fractions. Negligible amounts of radioactivity were associated with the "bound" and "hexane soluble" fractions.

The free oestradiol-17β from the 0·5 hr dependent tumour was characterized by mixing with 50 mg of carrier oestradiol-17β. Successive crystallizations from absolute ethanol gave

Table 1. *Distribution of* [6,7-^{3}H] *oestradiol-17β and its metabolites in oestrogen dependent and independent tumours*

Tumour	Time (hr)	Fractions: Free steroid A*	Free steroid E_2†	Free steroid E_1	Glucosuronide A	Glucosuronide E_2	Glucosuronide E_1	Sulphate A	Sulphate E_2	Sulphate E_1	Hexane	Bound	Polar	Total tumour tritium recovered in the fraction%
Dependent with stilboestrol implants	0·5	91	78	20	6	16	16	1	40	46	2	1	2	102
	4	9	29	28	32	15	12	14	36	25	2	5	27	89
Indpendent	0·5	65	83	1	9	35	55	11	18	21	11	1	7	103
	4	42	70	2	55	70	13	8	54	7	1	1	13	118

*A is the percentage of total tissue radioactivity present in the fraction.

†E_2 and E_1 are the amounts of oestradiol-17β and oestrone respectively expressed as a percentage of A.

Table 2. Uptake of radioactivity by hamster tissues 0·5 and 4 hr after the subcutaneous injection of 1·0 μg [6,7-^{3}H] oestradiol-17β/100 g body wt. expressed as M oestradiol (x 10^{-17})/mg wet tissue ±S.E.

Group No.	Treatment of animals	Time of injection prior to death	Dependent tumour	Independent tumour		RCA_1 Tumour	Kidney	Liver	Pituitary	Muscle	Hypothalamus
				Subcutaneous	Kidney capsule						
1	None	0·5	—	328±340 (15)*	—	182± 98 (4)	1706±139 (31)	4350±669 (16)	1204±217 (14)	409±132 (6)	319± 49 (13)
2		4·0	—	253± 41 (6)	—	—	737± 78 (5)	3355±411 (6)	645 (2)	105 (2)	119± 15 (6)
3	Oestradiol	0·5	5746±3171 (6)	—	—	—	3739±3031 (16)	4033±2291 (13)	1713±937 (4)	—	958 (2)
4	Stilboestrol	0·5	3140±2006 (11)	—	—	—	2825±2132 (15)	4237±1425 (7)	1523±957 (6)	632±356 (6)	729±485 (6)
5		4·0	295± 47 (7)	—	—	—	569± 52 (8)	773±135 (4)	218± 83 (4)	144± 37 (4)	101± 39 (4)
6	Stilboestrol implants removed 48 hr prior to injection of	0·5	3112±438 (7)	—	—	—	2089±159 (8)	2120±733 (4)	2121±534 (4)	685±332 (3)	948±261 (4)
7	[6, 7-^{3}H] oestradiol	4·0	1001±141 (14)	—	—	—	1489±171 (8)	951±214 (4)	343± 57 (4)	119± 14 (4)	107± 21 (4)
8	Stilboestrol for 9 days prior to tumour transplant	0·5	—	410±287 (4)	—	—	2241±817 (6)	3495±247 (3)	793±250 (3)	219± 68 (3)	418±221 (3)
9	None	0·5	—	784±632 (9)	1142±683 (4)	—	2681±1374 (12)	4788±1526 (7)	1369±661 (7)	308±101 (6)	473±221 (8)

*No. in parentheses refers to the number of tissues in sample.

Table 3. Level of significance in the comparison of uptake of tritium between tumours

Groups being compared	Significance (P value)
0·5 hr dependent and independent tumours with stilboestrol implants (4 and 8)*	$<0·001$
0·5 hr dependent and independent tumours without stilboestrol implants (6 and 1)	$<0·001$
0·5 hr dependent tumours with and without stilboestrol implants (4 and 6)	$>0·1$
0·5 hr dependent tumours with either stilboestrol or oestradiol implants (4 and 3)	$0·02>P>0·01$
0·5 hr independent tumours with and without stilboestrol implants (8 and 1)	$>0·1$
4·0 hr dependent and independent tumours without stilboestrol implants (7 and 2)	$0·01>P>0·001$
0·5 hr independent tumour without implants transplanted either subcutaneous or under kidney capsule (9 and 9)	$>0·1$

*Numbers in brackets refer to group No. in Table 2.

crystals with specific activities of 412, 435 and 428 dis/min/μmole respectively. The mother liquors had activities of 738, 767 and 445 dis/min/μmole respectively. The final crop of crystals was acetylated and recrystallized from n-hexane to give crystals of specific activities 407 and 471 dis/min/μmole respectively, with mother liquors of activities 457 and 460 dis/min/μmole respectively.

The distribution of tritium in kidney and liver of tumour bearing animals did not differ from that previously reported for untreated animals [8].

The uptake of radioactivity by various hamster tissues is given in Table 2. The difference in tritium content of dependent and independent tumours at 0·5 hr was highly significant (Table 3); this was true both in the comparison of tumours from animals which had stilboestrol implants, and in those which did not. There was no difference between dependent tumours in animals with or without stilboestrol implants, or in similar independent tumours. The difference between dependent tumours in animals with stilboestrol or oestradiol implants was significant ($0·02<P<0·01$).

Table 4. In vitro *metabolism of* [6,7-^{3}H] *oestradiol-17β by hamster kidney tissue*

Tissue	Fractions												
	Free steroid			Glucosuronide			Sulphate			Hexane	Bound	Polar	Tritium recovered (%)
	A	E_2†	E_1	A	E_2	E_1	A	E_2	E_1				
None	90	95	2	—						2	<1	1	93
Dependent tumour	89	95	1	—			—			2	<1	4	95
Independent tumour	90	95	2	—			—			2	<1	2	94
RCA_1	93	93	5	—			—			<1	<1	2	95
Kidney	68	71	22	2	29	30	21	39	56	3	1	4	99
Stilboestrol-treated kidney	74	82	13	2	56	14	11	75	21	2	<1	2	91

*A is the percentage of total tissue radioactivity present in the fraction.
†E_2 and E_1 are the amounts of oestradiol-17β and oestrone respectively expressed as a percentage of A.

The tritium content of RCA_1 tumours was lower than that of independent tumours.

The difference at 4 hr between dependent and independent tumours in animals without stilboestrol implants was significant, but not if the dependent tumour was from an animal with an implant. There was no significant difference between independent tumours transplanted subcutaneously or under the kidney capsule.

There was no significant difference at 0·5 hr in the tritium content of other tissues in untreated and stilboestrol treated animals. This was also true at 4 hr except for liver. In this instance the differences between untreated (group 2) and stilboestrol treated animals (group 5) was highly significant ($P<0·001$).

In vitro *metabolism of* [6,7-^3H] *oestradiol*-17β

Table 4 gives the results obtained from incubating [6,7-^{3}H] oestradiol-17β with various kidney preparations. Oestradiol-17β was the only steroid isolated from the tumour incubations. The metabolic activity of the kidney was unrelated to hormone treatment.

DISCUSSION

The significant difference in uptake of [6,7-^{3}H] oestradiol-17β between oestrogen dependent and independent renal tumours is consistent with studies on other hormone dependent and independent tumours [2–4]. This difference is unlikely to be due to the different blood supply of the two types of tumours, since subcutaneous and kidney capsule transplants of the independent tumour retain tritium to a comparable extent. The slightly higher uptake by the kidney capsule transplant may have been due to its haemorrhagic nature. Another possible explanation of the higher uptake by the dependent tumour was that it metabolised and lost oestradiol-17β at a slower rate than the independent tumour. This does not appear to be the case, since *in vitro* experiments demonstrated the inability of both types of tumour to metabolise oestradiol. Thus the significant difference in the uptake of oestradiol by dependent and independent tumours at 0·5 hr is related to either a change in the nature of the oestrogen–receptor complex, or in the number of oestrogen receptor sites available in these tumours [9].

Only dependent tumours in animals without stilboestrol implants retained tritium after 4 hr. In animals with implants the uptake of tritium was suppressed at 4 hr suggesting that endogeneous stilboestrol was competing for the available oestrogen binding sites. The reason for the decreased ability of the stilboestrol treated liver to retain tritium at 4 hr. is unknown. Both tumours had little free oestradiol at 4 hr, and this could be indicative of a low affinity for the oestrogen–receptor complex, especially when compared to other target tissues [10].

The independent tumour had a higher tritium content than the RCA_1 independent renal adenocarcinoma suggesting that the so-called independent tumour retains some oestrogen dependency. This supports the evidencee of Bloom *et al.* [11] that the anti-oestrogen UIII00A (1-{2-[p-(3,4-dihydro-6-methoxy-2-phenyl-1-naphthyl)phenoxy]-ehtyl} pyrrolidine, HCl) inhibited the growth of such tumours, and that concomitant injections of oestradiol benzoate prevented this inhibition.

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RESUME

Le métabolisme du (6,7-^3H) *oestradiol*-17β *a été étudié, chez des hamsters mâles, dans des tumeurs rénales, dont la croissance était soit dépendante, soit indépendante des oestrogènes. L'étude était faite* 1/2 *heure et* 4 *heures après l'injection sous-cutanée de* (6,7-^3H) *oestradiol*-17β. *Après* 1/2 *heure, la fraction "stéroïde libre", dans les* 2 *types de tumeurs, ne contenait que de l'oestradiol*-17β, *et, dans les fractions conjuguées, on ne trouavit, à ce moment, que des traces de tritium. Après* 4 *heures, la plus grande partie du tritium se trouvait dans les fractions conjuguées. Divers métabolites furent mis en évidence dans le foie et dans le rein, après* 1/2 *heure et après* 4 *heures.*

La tumeur primitive, oestrogéno-dépendante, captait jusqu'à 10 *fois plus de* (6,7-^3H) *oestradiol*-17β *que la tumeur indépendante transplantée. Cette constatation ne pouvait pas s'expliquer par le site de la tumeur. Ni l'une ni l'autre des tumeurs ne métabolisaient le* (6,7-^3H) *oestradiol*-17β in vitro; *on en conclut que la captation différente, observée* in vivo, *était due à une perte ou à modification de la structure du récepteur d'oestrogènes, dans la tumeur indépendante. Ceci indiquerait une différence fondamentale de la capacité*

d'utilisation de l'oestradiol-17β par la tumeur dépendante et par la teumur indépendante. La tumeur rénale RCA_1, qui ne répond pas aux oestrogènes, captait moins de tritium que la tumeur indépendante.

SUMMARY

The fate of [6,7-^3H] *oestradiol-17β has been studied in oestrogen-dependent and independent kidney tumours in male hamsters 0·5 and 4 hr after the subcutaneous injection of* [6,7-^3H] *oestradiol-17β. After 0·5 hr the "free steroid" fraction from both tumours contained only oestradiol-17β, and only trace amounts of tritium were present in the conjugated fractions. At 4 hr most of the tritium was in the conjugated fractions. In kidney and liver several metabolites were detected at 0·5 and 4 hr.*

The primary, oestrogen-dependent tumour took up about 10 times more [6,7-^3H] *oestradiol-17β than the transplanted independent tumour. This was not due to the site of the tumour. Neither tumour metabolised* [6,7-^3H] *oestradiol-17β* in vitro, *and it was concluded that the different* in vivo *uptake was due to a loss or change in structure of the oestrogen receptor in the independent tumour. This would incidate a fundamental difference in the ability to handle oestradiol-17β by dependent and independent tumours. The oestrogen unresponsive RCA_1 kidney tumour took up less tritium than the independent tumour.*

ZUSAMMENFASSUNG

Das Schicksal von 6,7-^3H Oestradiol-17β in Oestrogenabhängigen und -unabhängigen Nierentumoren männlicher Hamster wurde 0, 5 und 4 Stunden nach subcutaner Injektion von 6,7-^3H Oestradiol-17β untersucht.

Nach 0,5 Stunden enthielt die Fraktion "freier Steroide" beider Tumortypen nur Oestradiol-17β und es waren nur Spuren von Tritium in den konjugierten Fraktionen vorhanden. Nacht 4 Stunden befand sich der größte Teil des Tritiums in den konjugierten Fraktionen. Sowohlin der Niere, als auch in der Leber konnten nach 0,5 und 4 Stunden mehrere Metabolite nachgewiesen werden. Der ursprüngliche oestrogenabhängige Tumor nahm etwa 10 mal mehr 6,7-^3H Oestradiol-17β auf, als der transplantierte oestrogenunabhängige Tumor.

Dies lag nicht an der Lokalisation des Tumors, denn keiner der beiden Tumoren metabolisierte 6,7-^3H Oestradiol-17β in vitro *und es wurde daraus geschlossen, daß die unterschiedliche Aufnahme* in vivo *durch einen Verlust oder eine Strukturänderung des Oestrogen-Receptors im oestrogenunabhängigen Tumor bedingt war. Dies würde einen grundlegenden Unterschied zwischen oestradiolabhängigen und -unabhängigen Tumoren bezüglich der Behandlung von Oestradiol-17β bedeuten.*

Der auf Oestrogen nicht ausprechende Nierentumor RCA_1 nahm noch weniger Tritium auf als der oestrogenunabhängige Tumor.

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Sur les Interactions entre une Tumeur Maligne et un Tératome Expérimental, chez la Souris et le Rat

I. Influence Réciproque des Cellules Malignes et des Cellules Embryonnaires Réunies sur un même Hôte

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1. HISTORIQUE

DANS certains tératomes spontanés coexistent des cellules indifférenciées, à multiplication intense, et des cellules adultes d'aspect normal. Il s'agit de tératomes malins, siégeant essentiellement dans les gonades, le caractère malin est lié à la présence des cellules indifférenciées. De tels tératomes spontanés ont été observés et décrits en particulier chez l'Homme par Cabanne [1] et J. Gaillard [2–4], chez le Chien et le Cheval par Peyron [5], chez la Souris par Stevens et Little [6] Stevens et Hummel [7], Stevens [8–11].

L'existence de telles tumeurs nous a donné l'idée de mélanger des cellules cancéreuses à de la pulpe d'embryons, et d'injecter le tout à un hôte adulte. Nous nous proposions de suivre l'évolution des deux types de cellules ainsi réunis. Nous nous sommes trouvé alors devant le difficile, mais intéressant problème de l'influence d'un tissu normal sur le développement d'une tumeur cancéreuse.

L'étude de cette influence a été entreprise depuis longtemps. Ainsi, dès 1932, Baroni et Comsia [12], greffant des organes adultes à des Rats porteurs d'une tumeur maligne, constatent la diminution de la croissance de cette tumeur, et même, dans quelques cas, sa régression totale.

En 1934, Murphy et Sturm [13] préparent des extraits de tissus normaux variés et observent que les extraits de placenta et de peau d'embryons inhibent la croissance d'un cancer transplanté sur la Souris. Murphy [14] remarque qu'un facteur inhibiteur existe également dans in sarcome d'Oiseau. Ce facteur retarde l'évolution de plusieurs cancers.

Depuis, les expériences se sont multipliées. De nombreux auteurs ont essayé l'action des greffes d'organes, ou l'injection d'extraits de tissus, sur l'évolution de différentes tumeurs malignes. Ils ont obtenu des résultats contradictoires. Il y a, dans certains cas, une action stimulatrice des extraits de tissus ou d'organes sains sur la tumeur maligne, et, dans d'autres cas, une action inhibitrice de ces mêmes extraits. Citons, en particulier, les travaux de Schneyer [15], Vasiliev [16], Wynn, Stein, Werblowsky et Amoroso [17], Solomon [18]. Schneyer prépare des suspensions de tissus embryonnaires, d'une part, de tissus adultes, d'autre part. Ces suspensions, ajoutées aux cellules cancéreuses, puis injectées aux Souris hôtes, ont une action stimulatrice sur la

croissance de la tumeur maligne. Cet effet favorable des tissus embryonnaires est supérieur à celui des tissus adultes. L'auteur conclut à la présence de substances stimulatrices de la croissance dans les deux types de tissus. C'est également un effet favorable des tissus sains qu'ont observé Vasiliev et Olshevskaya [19] en 1958, et Vasiliev [16] en 1962, après l'injection de suspensions de différents tissus normaux à des Souris recevant en même temps des cellules cancéreuses. Ces suspensions favorisent la croissance de la tumeur maligne cancéreuse; ce sont surtout les suspensions de tissus embryonnaires isologues qui sont très actives. De même, Solomon [18] constate que la présence de trophoblastes de Souris implantés sur des Souris adultes, en même temps que des fragments du sarcome S180, favorise le développement de ce sarcome.

En revanche, Wynn et collaborateurs [17] n'observent aucun effet des suspensions de placenta de Rats, de 7 à 20 jours de gestation, sur le développement de la tumeur humaine HS1 transplantable chez le Rat.

On peut expliquer ces résultats contradictoires par l'hypothèse de Szent-Györgyi, Hegyeli et McLanghlin [20], selon laquelle il y aurait, dans certains tissus, deux substances antagonistes, la promine et la rétine ayant, l'une un effet stimulant, l'autre un effet inhibiteur sur la prolifération des cancers et sur la croissance en général. Lorsqu'elles sont réunies dans un extrait, c'est la proportion entre les deux substances qui détermine l'action de cet extrait. La rétine serait plus instable que la promine et se décomposerait facilement. Ceci expliquerait que le même extrait ne possède pas toujours le même pouvoir. En effet, Szent-Györgyi et collaborateurs ont observé qu'un extrait, d'abord inhibiteur, peut, après 2 jours, devenir favorable. Dans ce cas, c'est la rétine que s'est d'abord manifestée, mais, après 2 jours, cette substance instable a disparu, et la promine peut alors agir.

Cette hypothèse a été reprise en 1964 par Rubnitz, McKee et Miller [21]. Ces auteurs éprouvent l'action de tissus embryonnaires secs et frais, et constatent que les extraits secs ont un effet inhibiteur et les extraits frais un effet favorable. Dans les extraits frais, le facteur favorable neutraliserait l'action du facteur inhibiteur; dans les extraits secs, au contraire, la dessication détruisant le facteur favorable permettrait au facteur inhibiteur de se manifester. Signalons à ce propos que, dès 1907, Reinke [22] observait que certains tissus, traités préalablement à l'éther, se multipliaient activement, et pensait qu'il y aurait dans ces tissus une substance inhibitrice de la division mitotique. Cette substance, soluble dans l'éther, disparaîtrait des tissus traités, ce qui provoquerait leur croissance exagérée. En 1944, Cook et Walsch [23] ont testé l'activité de certains extraits de tissus normaux, adultes et embryonnaires, et de tissus cancéreux, sur la croissance de cultures de levure. Ils constatent que les extraits les plus actifs sur la croissance des levures sont les extraits de cerveau et de foie adultes. Les extraits embryonnaires sont également actifs, mais les extraits de tumeurs n'ont qu'une faible action. De même, en 1965, Parshley et Mandl [24] ont extrait, à partir de certains tissus adultes, des substances exerçant soit un effet inhibiteur, soit un effet favorable, sur la croissance de fibroblastes normaux, cultivés *in vitro*. L'effet dépend du mode d'extraction des substances. Ces extraits ont une forte action sur le sarcome de Rous et le sarcome HS1, cultivés *in vitro*. Les substances actives seraient des peptides. On peut rapprocher ces résultats du récent travail de Rubin [25]. Rubin cultive des cellules du sarcome de Rous en association avec des cellules embryonnaires. Le milieu est ensuite prélevé et centrifugé; le surnageant obtenu est ajouté à un milieu frais sur lequel on cultive des cellules embryonnaires normales. L'auteur constate que le surnageant d'un milieu ayant servi à cultiver, pendant 3 jours, les cellules cancéreuses, favorise la croissance des cellules embryonnaires. Le surnageant, extrait à partir d'un milieu utilisé pendant 4 à 5 jours à la culture des cellules cancéreuses, possède un pouvoir inhibiteur sur la croissance des cellules normales. Il y aurait donc deux substances dans les cellules cancéreuses, l'une stimulatrice de la croissance et agissant à faible concentration, l'autre inhibitrice de la croissance et agissant à forte concentration.

Mais pour de nombreux auteurs, l'influence des extraits d'un tissu sur l'évolution d'un greffon cancéreux est liée à un phénomène d'immunologie appelé "enhancement" ou "facilitation". La "facilitation" observée en particulier par Kaliss et Snell [26], Shear, Imagawa, Syverton et Bittner [27], Snell [28], Kaliss [29–31], Voisin [32] est un état paradoxal obtenu après le traitement de l'hôte par des extraits de tissus. La pré-injection de tels extraits permet une meilleure "prise" des greffons cancéreux. Cet effet favorable est obtenu après l'injection de tissus lyophilisés préparés à partir d'organes normaux: foie, rein, rate de Souris et de Rats adultes ou à partir de tumeurs malignes. Pour Casey et Casey [33] cette action serait due à un facteur,

le facteur XYZ, isolé par ces auteurs dans les extraits de cancers. Pour Green et Wilson [34, 35] "l'enhancement" fait intervenir des lipoprotéines. Ces lipoprotéines, extraites d'une tumeur, favorisent la croissance d'un greffon cancéreux; elles agissent comme antigènes et provoquent, chez l'hôte, la formation d'anticorps; mais, à forte dose, ces anticorps suppriment la réaction de l'hôte. L'importance de la quantité de tissus injectés avait été constatée par Kaliss en 1952 [30]; cet auteur avait remarqué qu'une pré-injection d'une petite quantité de tissus lyophilisés inhibait la croissance de la tumeur maligne, alors que les fortes quantités avaient un effet favorable. Snell [28] signale également cette contradiction dans l'action des extraits de tissus ou d'organes sains sur l'évolution d'un greffon cancéreux; dans certains cas, il observe une action inhibitrice, dans d'autres cas, une action stimulatrice.

Que conclure de ces travaux? L'influence d'extraits de tissus normaux sur la croissance d'un greffon cancéreux est-elle liée à un phénomène immunologique, ou est-elle sous la dépendance de deux substances antagonistes existant dans de nombreux tissus? Le problème n'est pas résolu; les deux théories continuent à coexister, soutenues chacune par de nombreux travaux. Il nous a paru intéressant d'associer, sur le même hôte, cellules embryonnaires et cellules cancéreuses, tumeur bénigne et tumeur maligne, afin d'étudier les influences réciproques éventuelles de chaque type de cellules et de tumeurs ainsi réunies.

Dans un premier type d'essais, nous avons injecté les cellules embryonnaires et cancéreuses, intimement mêlées aux animaux hôtes; dans une deuxième série d'expériences, nous avons greffé une tumeur maligne à un hôte porteur d'un tératome bien développé; dans la dernière série, enfin, nous avons greffé la tumeur maligne à un animal porteur d'un tératome en voie de croissance. Les premiers essais de Salaün [36] nous ont appris que

1. L'association réalisée sur le même hôte entre un tissu cancéreux et un tissu embryonnaire est toujours favorable au tissu cancéreux. Le tissu embryonnaire est utilisé par le tissu malin comme élément nutritif, lorsque ces deux tissus sont réunis et injectés ensemble.
2. La tumeur maligne nuit au développement du jeune tératome en voie de croissance.
3. Un tératome volumineux ne freine en rien l'évolution d'un greffon cancéreux. La tumeur bénigne et la tumeur maligne coexistent alors sur l'animal.

Ces premières expériences ont été confirmées par les résultats ultérieurs que nous exposons ci-dessous.

2. MATERIEL ET METHODES

Nous avons opéré sur la Souris RAP et surtout le Rat Wistar.

Nous préparons la pulpe à partir d'embryons de 13 à 15 jours, selon la technique décrite dans la première partie [37].

Nous avons utilisé le sarcome S 180 chez la Souris, l'épithélioma utérin T8 de Guérin chez le Rat.

Le sarcome est entretenu régulièrement dans notre laboratoire. Les repiquages se font tous les 13 à 18 jours. La Souris cancéreuse, endormie à l'éther, est fixée sur la table d'opération. On prélève un fragment sain de la tumeur; on le lave plusieurs fois dans du liquide de Tyrode stérile, et on le découpe en petits morceaux avec des fins ciseaux courbes. On injecte les morceaux de tumeur en suspension dans le Tyrode, dans le tissu sous-cutané de l'abdomen de nouvelles Souris mâles ou femelles. Chaque animal reçoit 0,25 à 0,35 cm^3 de tissu malin. Toutes ces opérations sont faites avec soin pour éviter les infections.

L'épithélioma T8 est fourni aimablement par le Docteur Rivière, de Villejuif, à qui nous exprimons ici tous nos remerciements. Le repiquage se fait par greffe d'un petit nodule cancéreux prélevé sur un Rat. On rase une partie du dos du futur porte-greffe, on incise la peau et on glisse le nodule de tumeur sous la peau, aussi loin que possible de l'incision. On referme ensuite avec quelques agrafes et on badigeonne la peau avec du curichrome pour empêcher toute infection. La tumeur grossit, des métastases apparaissent dans les ganglions lymphatiques des aines et des aisselles. Les animaux meurent après 30 à 40 jours.

3. RESULTATS EXPERIMENTAUX

Chez la Souris

Nous avons réalisé 26 cas d'association de la pulpe d'embryons de Souris et du sarcome S180. La pulpe est préparée à partir d'embryons de Souris de 13 jours, selon la méthode habituelle.

Le sarcome est prélevé stérilement sur une Souris. Un fragment de Sarcome est isolé et placé dans une boîte de Pétri, dans du liquide de Tyrode, maintenu à 38°C. Un petit morceau de ce sarcome est ajouté à la pulpe et un autre, de volume égal, est injecté aux témoins. Les proportions du mélange sont les suivantes: 0,50 cm^3 de sarcome finement hâché est intimement mêlé à 5 cm^3 de pulpe d'embryons. Il y a donc une disproportion importante entre les volumes des deux matériels mis en présence; chaque animal reçoit 1 cm^3 du mélange. Les témoins sont injectés avec le même volume

Tableau. 1. *Mortalité des Souris injectées avec le mélange pulpe d'embryons-sarcome S180 et des Souris témoins injectées avec le sarcome S180 seulement*.*

Date de la mort (jours)	Souris injectées avec le mélange pulpe—S180		Souris témoins (injection de S180 seulement)	
	Nombre de Souris	%	Nombre de Souris	%
17	3	≠ 13,5		
20	4	18		
25	3	13,5	0	0
26	3	13,5		
27			2	≠ 14,5
28	3	13,5		
29			2	14,5
30			4	28,5
31			2	14,5
32			1	7
35			1	7
40	2	9,5		
42	1	4,5		
44	1	4,5		
45	2	9,5		
48			1	7
55			1	7
	Total: 22		Total: 14 sur 25 traités	

*Vingt-deux Souris sont comptées dans ce tableau; les 4 autres ayant été sacrifiées pour le repiquage des tumeurs. Chez les témoins, 14 Souris sur 25 traitées meurent, les autres sont normales.

de sarcome en suspension dans du Tyrode; on inocule ainsi à chaque Souris une très faible quantité du tissu malin: 0,10 cm^3 environ.

Sur chacune des 26 Souris traitées par le mélange pulpe d'embryons-cancer, se développe rapidement une tumeur qui finit par devenir énorme. L'injection a été faite sur le flanc; mais, après 20 à 30 jours, la tumeur occupe non seulement le flanc, mais aussi le dos, et même une partie de l'abdomen. L'animal bien souvent ne peut se mouvoir que difficilement, la tumeur empêchant les membres de toucher le sol (Figs. 1, 2).

La mortalité survient dans un délai variant entre le 13e et le 45e jour. Chez les témoins, le sarcome évolue en 7 à 8 semaines. La tumeur, au bout de ce temps, est grosse, mais n'atteint cependant pas la taille des tumeurs issues de l'injection du mélange pulpe d'embryons-sarcome. Elle se creuse d'un cratère qui s'infecte; le centre est nécrosé. La mortalité se produit entre le 27e et le 55e jour.

De plus, à l'époque où nous avons entrepris ces essais, le sarcome S180 se développait avec difficulté au laboratoire. Dans plusieurs repiquages entrepris pour maintenir le sarcome, on a constaté une régression du nodule cancéreux. Dans nos propres témoins, le sarcome n'a pas pris dans 11 cas sur 25. Or, dans tous les cas où nous avons injecté à la fois le sarcome et la pulpe d'embryons, nous avons obtenu la formation d'une tumeur saine et très envahissante. Les Souris porteuses d'une telle tumeur meurent plus tôt que les Souris témoins (Tableau 1).

On voit sur le Tableau 2 que, 26 jours après l'injection, 40% seulement des Souris en expériences survivent, alors que toutes les Souris témoins sont en vie.

A l'autopsie, on constate que, malgré sa masse, la tumeur est saine. Elle a macroscopiquement l'aspect homogène du sarcome S180 (Fig. 3). Cependant, dans deux cas (Souris mortes le 25e et le 28e jour après

Tableau 2. Survie des Souris injectées avec le sarcome S180 ajouté à la pulpe d'embryon et le sarcome S180 seul

Souris survivant	Traitement			
	pulpe+S180		S180	
Jusqu'au 26e jour	9	40%	25	100%
Au-delà du 45e jour	0	0%	13	52%



PLANCHE I

Fig. 1 *et* 2. *Souris injectées avec* 1 cm^3 *du mélange pulpe d'embryons de Souris—sarcome S* 180, *photographiées* 30 *jours après l'injection.* T: *tumeur.* (*Gr.* × 1)

Fig. 3. *Autopsie d'une souris injectée avec le mélange pulpe d'embryons—sarcome S*180, 40 *jours après l'injection. Aspect homogène de la tumeur.* T: *tumeur.* (*Gr.* × 1).

Fig. 4. *Rat porteur d'une tumeur issue de l'injection du mélange pulpe d'embryons—épithélioma T*8, *sacrifié* 15 *jours après l'injection.* T: *tumeur.* (*Gr.* × 0,4)

(to face p. 406)

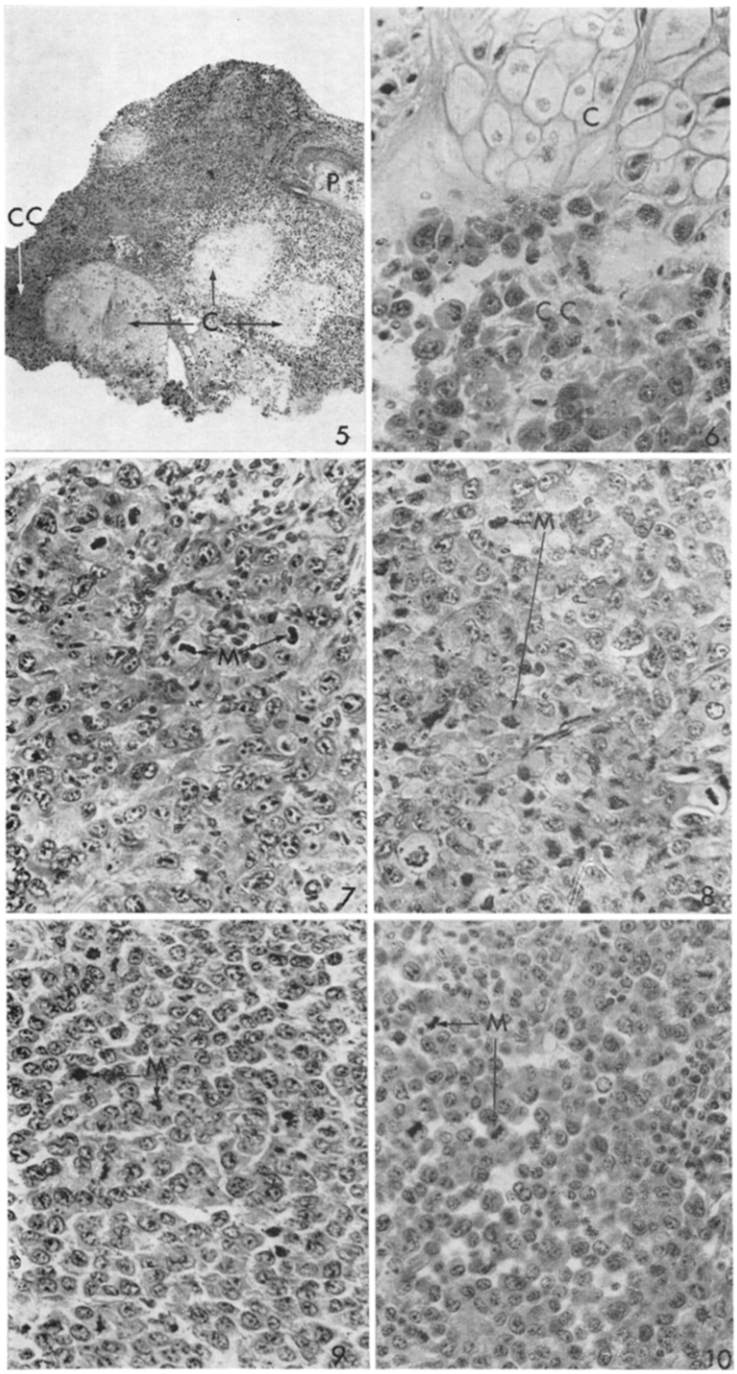


PLANCHE II

Fig. 5. *Aspect histologique d'une tumeur formée chez la Souris après l'injection du mélange pulpe d'embryons—sarcome* S180. *Cas où plusieurs structures normales persistent à côté de nodules de cellules cancéreuses.*
C: *cartilage* P: *peau.* CC: *cellules cancéreuses.* (*Gr.* × 18)

Fig. 6. *Détail de la Fig.* 1. C: *cartilage.* CC: *cellules cancéreuses.* (*Gr.* × 176)

Fig. 7. *Aspect histologique du sarcome* S180 *développé sur une Souris témoin.*
M: *mitose.* (*Gr.* × 176)

Fig. 8. *Repiquage d'une tumeur issue de l'injection, à une Souris, du mélange pulpe d'embryons—sarcome* S180. *Aspect de cette tumeur identique à celui de la tumeur témoin.*
M: *mitose.* (*Gr.* × 176)

Fig. 9. *Aspect histologique de la tumeur maligne* T 8 *témoin.* M: *mitose.* (*Gr.* × 176)

Fig. 10. *Tumeur issue de l'injection à un Rat du mélange pulpe-d'embryons—Tumeur* T 8. *Structure histologique identique à celle de la tumeur* T 8. M: *mitose.* (*Gr.* × 176)

l'injection), nous avons remarqué, au sein du sarcome, un petit amas blanchâtre, moins transparent que l'ensemble de la tumeur.

A l'histologie, nous avons observé, dans ces deux tumeurs, des cellules cancéreuses, mais aussi les tissus normaux décrits dans les tératomes: glandes sébacées, follicules pileux, cartilage (Fig. 5, 6).

Cet aspect rappelle celui des tératomes malins décrits par Stevens [8], mais ces deux cas sont restés isolés. Dans toutes les autres tumeurs, on ne retrouve aucun tissu embryonnaire, mais uniquement des cellules cancéreuses. Ces cellules cancéreuses montrent de nombreuses mitoses. Les tumeurs issues de l'injection du mélange pulpe d'embryons-cancer S180 sont histologiquement tout à fait semblables aux tumeurs S180 typiques. Nous avons isolé 4 Souris porteuses de telles tumeurs, pour en effectuer le repiquage. Nous procédons comme pour un repiquage classique. Les deux premières tumeurs transplantées ont 20 et 26 jours. Chacune est repiquée sur 2 nouvelles Souris. Une tumeur tout à fait semblable au sarcome témoin se développe sur 3 de ces 4 Souris. La 4e Souris est normale.

Ces Souris meurent entre le 28e et le 45e jour. Les deux autres tumeurs repiquées n'ont que 8 jours et ne forment qu'un nodule de faible volume.

Nous avons fixé un fragment de ce nodule et repiqué un autre fragment sur 4 Souris. Dans la fraction de ces nodules soumise à l'histologie, on reconnaît un peu de cartilage à côté de nombreuses cellules cancéreuses. Nous espérions retrouver, dans les tumeurs repiquées, quelques éléments embryonnaires. Mais ce sont des tumeurs composées uniquement de cellules malignes qui se développent sur les 4 Souris (Fig. 7, 8).

La mort survient entre le 32e et le 46e jour. Ces tumeurs, issues de la transplantation des tumeurs expérimentales, évoluent absolument comme les sarcomes témoins.

Ces résultats montrent que le tissu cancéreux, ajouté, même en très petite quantité, à de la pulpe d'embryons, subit une évolution accélérée. Les Souris sont porteuses d'énormes tumeurs et meurent précocement. Mais cette accélération de l'évolution de la tumeur maligne ne s'observe que sur la première tumeur et disparaît dès le repiquage. Il s'agit d'un effet favorable, sans aucun doute nutritif, du tissu embryonnaire sur le tissu cancéreux.

Chez le Rat

L'épithélioma T8 découpé en fins fragments,

Tableau 3.

Date de la mort (jours)	Rats injectés avec le mélange pulpe—T8			Rats témoins (Injection de T8 seulement)		
	Nombre de Rats		%	Nombre de Rats		%
17	1	≠	2			
18	3		7			
19	2		5			
20	3		7	0	=	0
21	3		7			
29	1		2			
30	7		16			
31	2		5	5	≠	18
32	1		2	1		4
33	2		5	1		4
34				3		11
35	4		9	3		11
36	2		5	1		4
38				1		4
39				2		7
40	1		2	4		14
41				1		4
42				2		7
43	5		12	1		4
45	3		7	1		4
46	2		5			
47	1		2			
56				1		4
	Total: 43			Total: 27 sur 36 traités		

Mortalité des Rats injectés avec le mélange pulpe d'embryons-épithélioma T8 et des Rats témoins injectés avec le tumeur T8 seulement.
Vingt-sept Rats témoins sur les 36 injectés portent un épithélioma; les autres sont normaux.

Tableau 4. Survie des Rats injectés avec le mélange pulpe—T8 et des Rats injectés avec la tumeur T8 seule

Rats survivants	Traitement			
	Pulpe+T8		T8	
Au 30e jour	23	53%	36	100%
Du 30 au 40e jour	11	25%	15	43%
Après le 47e jour	0	0%	10	27%

est intimement mêlé à la pulpe d'embryons de Rat de 14 jours. La proportion est de 0,50 cm³ de tumeur maligne pour 5 cm³ de pulpe d'embryons; chaque Rat reçoit 1 cm³ de ce mélange en injection sous-cutanée. On injecte aux témoins la même quantité (0,10 cm³ environ) d'épithélioma en suspension dans du liquide de Tyrode.

Nous avons injecté à 48 Rats le mélange pulpe cancer, et à 36 Rats l'épithélioma en suspension dans du Tyrode.

Sur chacun des 48 Rats apparaît une tumeur qui évolue rapidement:
— 5 animaux sont sacrifiés entre le 10e et le 22e jour, la tumeur est repiquée;
— 12 animaux meurent entre le 17e et le 21e jour après l'injection;
— 17 animaux entre le 29e et le 35e jour;
— 14 animaux entre le 36e et le 47e jour, aucun ne survit au-delà du 47e jour.

Au moment de la mort de l'hôte, la tumeur est très grosse et la surface est souvent à nu, les métastases sont très développées surtout dans les deux aines; elles sont présentes sous les aisselles, mais leur volume est moindre.

L'aspect de cette tumeur est absolument identique à celui de l'épithélioma témoin (Fig. 4). Sur les témoins, l'épithélioma se développe dans 27 cas sur 36; la mort survient chez ces derniers entre le 31e et le 56e jour. Ces expériences confirment les observations faites chez la Souris. Après l'injection de l'ensemble pulpe d'embryons-tumeur cancéreuse, une tumeur très envahissante apparait dans 100% des cas. Chez les témoins injectés avec la même quantité de tissu cancéreux, une tumeur ne se forme que dans 75% des cas. La tumeur expérimentale provoque la mort précoce des animaux (Tableau 3). Le Tableau 4 montre que, 30 jours après l'injection, tous les témoins sont en vie, alors que 53% seulement des expérimentés survivent.

L'aspect de la tumeur expérimentale est absolument identique à celui de la tumeur T8. Nous avons fait l'étude histologique de ces tumeurs. Nous constatons qu'elles ne contiennent que des cellules malignes. Nous n'y avons jamais observé de tissus embryonnaires (Fig. 9, 10).

Nous avons repiqué 5 tumeurs de 10, 17, 18, 20 et 22 jours. A partir de chaque tumeur, 2 petits nodules sont isolés et regreffés sur 2 autres Rats, selon la technique utilisée pour maintenir la tumeur T8; un fragment de la tumeur est isolé et placé dans une coupelle stérile. Le Rat hôte est endormi. Nous incisons la peau du flanc, préalablement rasée, et nous glissons le morceau de tumeur dans le tissu sous-cutané. Nous refermons l'incision avec des agrafes. Nous avons effectué, de la même manière, 8 repiquages de tumeur T8 témoins.

Les greffons provenant des tumeurs expérimentales se comportent comme les greffons des tumeurs malignes témoins. Une tumeur apparaît sur chacun des Rats opérés; des métastases se développent dans les aines et les aisselles. La mort se produit entre le 24e et le 38e jour après le repiquage; chez les témoins elle survient entre le 25e jour et le 40e jour. L'effet favorable de la pulpe d'embryons sur l'évolution du tissu cancéreux est donc de courte durée. Dès le premier repiquage, on n'observe plus de différence entre les 2 types de greffons.

4. CONCLUSION

Si on ajoute du tissu cancéreux à de la pulpe d'embryons, et qu'on injecte ce mélange à un hôte adulte, les tissus embryonnaires disparaissent. Malgré l'énorme inégalité des volumes de chaque matériel réunis au départ, inégalité en faveur du tissu embryonnaire, c'est toujours une tumeur cancéreuse qui se développe. Cette tumeur prolifère plus rapidement que la tumeur maligne témoin, mais cette amélioration de l'évolution de la tumeur maligne est liée à la présence des cellules embryonnaires et disparaît dès le premier repiquage.

La tumeur maligne se développe dans 100% des cas. Au contraire, chez les témoins injectés avec un volume égal de tissu malin, la tumeur ne prend pas dans 25% des cas chez le Rat,

et dans 44% des cas chez la Souris. Cette action favorable du tissu embryonnaire sur la croissance du tissu malin évoque le rôle des organes embryonnaires sur la culture *in vitro* des cellules cancéreuses. Dans la première partie de ce travail [37], nous avons déjà cité les nombreux travaux de Wolff et Schneider, et, Wolff et Wolff sur la culture organotypique des tumeurs malignes. Ces travaux ont montré que la tumeur maligne prolifère *in vitro* aux dépens de l'organe embryonnaire explanté sur le même milieu, soit directement, soit par dialyse à travers une membrane vitelline d'oeuf de Poule.

Le tissu embryonnaire sert à la nutrition du tissu malin. Dans nos expériences, les cellules embryonnaires sont utilisées par les cellules cancéreuses et ne peuvent former de tératome.

Peut-être ce rôle nutritif n'est-il pas le seul que joue le tissu embryonnaire. Nous avons déjà signalé les observations de Southam [38] sur "l'effet de masse" et la nécessité d'injecter un certain nombre de cellules pour provoquer la formation d'une tumeur [37]. Dans nos expériences, la "masse" représentée par les cellules embryonnaires favorise la prise et la prolifération du cancer. Chez les témoins, la "masse" injectée est faible, et, selon la résistance individuelle de l'hôte, parfois insuffisante pour permettre le développement de la tumeur cancéreuse.

En conclusion, dans nos expériences, la présence des cellules embryonnaires est toujours favorable à la croissance du tissu malin. Cette action se manifeste sur le maintien de la tumeur maligne d'une part, sur la rapidité de son évolution d'autre part.

RESUME

Nous avons introduit un petit fragment de tissu malin au sein d'une pulpe d'embryons. Les proportions du mélange sont de 0,5 *cm³ de tissu cancéreux pour* 5 *cm³ de pulpe d'embryons. Nous injectons* 1 *cm³ de ce mélange aux animaux. Une tumeur massive se développe dans* 100% *des cas. C'est une tumeur cancéreuse semblable à la tumeur maligne témoin, mais proliférant plus rapidement. La présence des cellules embryonnaires est favorable à l'évolution de la tumeur maligne. Il s'agit d'un effet nutritif du tissu embryonnaire sur le tissu cancéreux, effet déjà observé par Wolff et collaborateurs lors de la culture* in vitro *de tumeurs malignes.*

SUMMARY

A small fraction of malignant tissue was inserted into embryo pulp, the proportion being 0·5 *cm³ of malignant tissue for* 5 *cm³ of embryo pulp. One cm³ of this mixture was injected into the animals. A voluminous tumour developed in* 100% *of the cases. It was a malignant tumour, similar to the control malignant tumour, but proliferating more actively. Embryo cells appear to favour the growth of the malignant tumour. This is due to a nutritional effect of the embryonic tissue on the cancer tissue, and this effect has already been observed by Wolff* et al. *in the* in vitro *culture of malignant tumours.*

ZUSAMMENFASSUNG

Wir haben ein kleines Fragment von bösartigen Geschwulstgeweben in die Embryonenpulpe eingeführt. Die Verhältnisse in dieser Mischung ist 0,5 *ml Krebsgewebe für* 5 *ml Embryonenpulpe. Wir injizieren* 1 *ml dieser Mischung in die Tiere. In* 100% *der Fälle entwickelt sich ein massiver Tumor. Das ist ein Karzinom ähnlich dem bösartigen Kontrolltumor, aber mit einer schnellen Proliferation. Die Anwesenheit der embryonalen Zellen ist zugunste der Entwicklung dieses bösartigen Tumors. Es handelt sich um ein Ernährungseffekt der Embryonalgewebe auf dem Karzinomgewebe, welcher schon von Wolff und seinen Mitarbeitern in* in vitro *Erzeugung der bösartigen Tumoren beobachtet wurde.*

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Sur les Interactions entre une Tumeur Maligne et un Tératome Expérimental, chez la Souris et le Rat

II. Evolution sur le même Hôte d'une Tumeur Maligne et d'une Tumeur Bénigne

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1. INTRODUCTION

DANS la première partie de notre travail [1], nous avons montré que de la pulpe d'embryons de Rat et de Poulet, injectée à des animaux adultes, évolue en une tumeur appelée tératome. Le tératome, malgré un volume parfois très important, ne provoque pas la mort de l'hôte. C'est une tumeur bénigne, localisée à l'endroit de l'injection. Sa croissance, souvent considérable, n'est pas illimitée; après quelque temps, 1–2 mois, elle est définitivement terminée.

Nous avons utilisé le tératome pour étudier l'influence qu'exercent, l'une sur l'autre, deux tumeurs, l'une bénigne, l'autre maligne, lorsqu'elles sont réunies sur le même hôte.

Que se passe-t-il lorsqu'on injecte le tissu malin à un animal porteur d'un tératome?

Le cancer et la tumeur bénigne vont-ils coexister, ou, au contraire, entrer en compétition?

Une compétition a été observée, en particulier par Csaba, Acs et Horvath [2] entre le greffon intra-péritonéal et le greffon sous-cutané d'une même tumeur maligne, introduits sur le même hôte. Le greffon intra-péritonéal nuit au développement du greffon sous-cutané. Pour les auteurs, il s'agit d'une concurrence biologique entre les deux implants. La tumeur intra-péritonéale accapare certaines substances nutritives au détriment de la tumeur sous-cutanée.

2. RESULTATS EXPERIMENTAUX

1. *Greffe d'une tumeur maligne à un hôte porteur d'un tératome bien développé*

Nous avons greffe l'épithélioma T8 chez 29 Rats porteurs de tératomes volumineux. Ces tératomes étaient formés depuis 2 à 19 mois.

Nous avons introduit l'épithélioma sur le même flanc que celui sur lequel se développe le tératome, dans 10 cas, et sur le flance opposé, dans 16 cas. Dans les 3 derniers cas, nous avons greffé l'épithélioma chez des Rats porteurs de tératomes intrapéritonéaux.

Dix-huit Rats témoins ont reçu un nodule malin de volume semblable à celui du greffon introduit dans les animaux en expérience. Nous avons choisi ces témoins parmi des Rats adultes âgés de 4 à 20 mois, afin de comparer l'évolution de la tumeur maligne dans les 2 groupes d'animaux. L'épithélioma se développe plus lentement sur ces hôtes âgés que sur les Rats utilisés habituellement. Mais l'évolution de l'épithélioma est tout à fait identique chez les témoins et chez les Rats porteurs de tératomes.

L'épithélioma prolifère dans 24 cas sur 29

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Tableau 1. *Association sur le même Rat de la tumeur T8 et d'un tératome volumineux*

Age du tératome	Nombre de cas	Resultats	
		Tératome	T8
2 mois	3	3+	3+
3 mois	2	2+	1+1−
4 mois	6	6+	5+1−
6 mois	3	3+	3+
7 mois	2	2+	1+1−
9 mois	2	2+	2+
12 mois	3	3+	2+1−
18 mois	4	4+	3+1−
19 mois	4	4+	4+
Total	29	29+	24+5−

chez les Rats en expérience, et dans 15 cas sur 18 chez les témoins.

Dans les deux groupes d'animaux, les Rats meurent entre le 50e et le 70e jour après la greffe du cancer.

Le tératome ne subit aucune modification; les deux tumeurs coexistent et ne semblent pas exercer d'influence l'une sur l'autre (Tableau 1 et Figs. 1, 2).

Cependant, lorsque les deux tumeurs évoluent sur le même flanc, la tumeur maligne s'accroît et finit par rejoindre le tératome. Les deux tumeurs ne sont plus séparées que par un tissu lâche. Dans 6 cas, nous avons prélevé et greffé, à de nouveaux Rats, des fragment de ce tissu limitant les deux tumeurs. Une tumeur T8 se développe à partir de ces greffons.

Nous avons repiqué des fragments de 7 tératomes associés à l'épithélioma. Les greffons évoluent comme le feraient les greffons de tératomes normaux; ils se maintiennent, sans proliférer, sur ce nouvel hôte.

Nous avons enfin effectué le repiquage de 5 tumeurs malignes associées à un tératome. Un épithélioma absolument identique aux tumeurs malignes témoins se développe dans chaque cas.

L'étude histologique, effectuée sur 6 tératomes et 4 tumeurs cancéreuses, associés sur le même hôte, montre également que chaque type de tumeur a conservé sa structure habituelle.

Conclusion

La présence, sur un Rat, d'un tératome massif et étendu n'inhibe pas la prolifération de l'épithélioma T8 greffé ultérieurement à cet animal. Lorsque la greffe est faite controlatéralement, le Rat porte deux tumeurs indépendantes, l'une bénigne l'autre maligne. Lorsque les deux tumeurs se trouvent sur le même flanc, la tumeur maligne s'étend à côté de la tumeur bénigne et finit par la recouvrir en plusieurs endroits. Nous pensons qu'il ne s'agit pas d'une agression de la tumeur maligne contre le tératome, mais que la tumeur maligne, évoluant rapidement, tend, comme chez les témoins, à envahir le plus d'espace possible.

La mort des hôtes survient dans les mêmes délais que celle des témoins. Le développement de l'épithélioma ne subit aucune modification.

Expériences témoins

Nous avons réalisé plusieurs expériences d'associations témoins.

(1) Association de 2 greffons de la même tumeur maligne

(2) Association de 2 tératomes.

(1) Nous avons inoculé le sarcome S180 à 6 Souris préalablement injectées avec ce même sarcome. Le premier sarcome a 12 jours au moment de la seconde injection; il forme un petit nodule de la grosseur d'un pois sur le flanc gauche de l'animal. Nous avons fait la deuxième injection sur le flanc droit. Le deuxième sarcome se développe dans tous les cas. Les Souris meurent 20–30 jours après la seconde injection (soit pratiquement dans les mêmes délais que les Souris porteuses d'un seul sarcome).

Chaque flanc est envahi par un gros nodule cancéreux.

Nous avons également greffé un fragment de tumeur T8 à 10 Rats auxquels nous avions greffé cette tumeur. Les 2 greffes sont faites à 13 jours d'intervalle, l'une sur le flanc droit, l'autre sur le flanc gauche. La mort survient 20–30 jours après la 2e opération.

Nous constatons que, comme chez la Souris, une tumeur maligne s'est développée sur chacun des flancs.

Ainsi, dans ces expériences, nous n'avons observé aucune concurrence entre les 2 greffons sous-cutanés de la même tumeur maligne. Le premier greffon, quoique bien évolué au

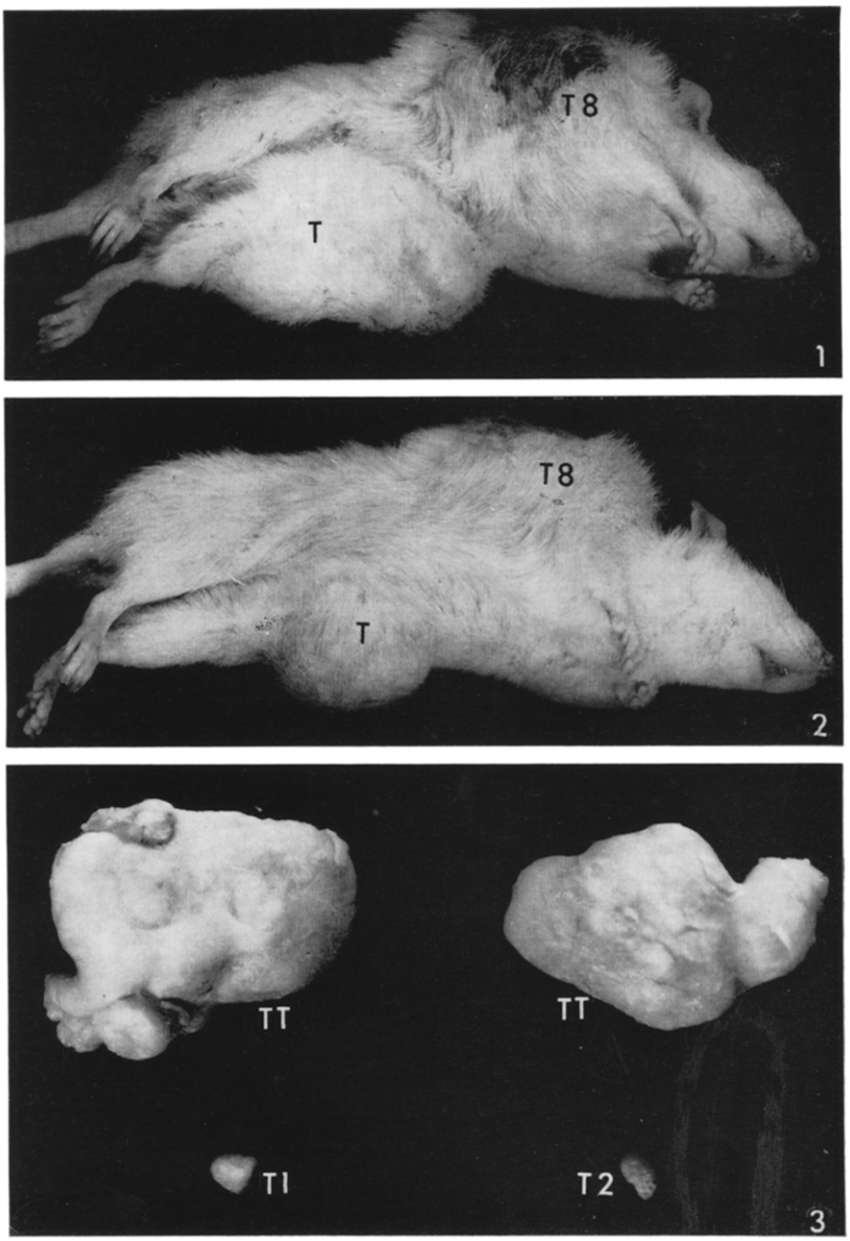


Fig. 1 *et* 2. *Associations entre un tératome volumineux de* 18 *mois et la tumeur maligne* T8. *Coexistence des deux tumeurs.* T*: tératome.* T8*: tumeur* T8. (*Gr.* × 0,5)

Fig. 3. *Résultats des injections de culot de sédimentation de l'extrait de l'épithélioma* T8, *à des Rats porteurs de jeunes tératomes agés de* 3 *à* 10 *jours: inhibition de la croissance des tératomes traités.* T.T.*: Tératomes témoins.* T_1–T_2*: tératomes traités, de la même série expérimentale que celle des témoins* T.T. (*Gr.* × 1)

(to face p. 414)

moment de la seconde intervention, n'inhibe pas le développement du 2e greffon.

(2) Nous avons injecté 1 cm³ de pulpe d'embryons à 8 Rats déjà porteurs d'un tératome sous-cutané. La 2e injection est faite sur le même flanc que la première dans 3 cas, et sur le flanc opposé dans 5 cas. L'âge du tératome au moment de la 2e opération varie entre 10 jours et 3 mois. Un 2e tératome se développe sur chacun de ces Rats, sans que l'évolution du 1er tératome semble modifiée (nous avons résumé ces expériences témoins dans le Tableau 2).

Dans ce type d'association, aucune concurrence ne paraît s'établir entre les deux tumeurs bénignes. La présence d'un tératome, même très volumineux, n'empêche pas la formation d'un deuxième tératome.

La mort survient dans les mêmes délais chez les Souris traitées et les Souris cancéreuses témoins (soit 30–40 jours après l'injection du sarcome).

Le tératome ne s'est développé que sur 10 Souris. Il ne forme qu'un petit nodule de la grosseur d'un pois, aplati contre le cancer.

On ne retrouve aucune trace le la pulpe d'embryons sur les 12 autres Souris (Tableau 3).

La 23e Souris, sur laquelle le sarcome n'a pas pris, porte un tératome de la taille d'un haricot.

Sur les Souris témoins, injectées avec la pulpe d'embryons seulement, un tératome s'est développé dans 7 cas sur 11.

Ce tératome est très volumineux dans 3 cas; il forme un nodule de 0,5 cm³ environ sur les 4 autres Souris. Bien que le nombre des

Tableau 2. *Associations témoins réalisées sur le même hôte entre* 2 *greffons d'une même tumeur maligne d'une part, et* 2 *tératomes d'autre part*

Type d'association	Nombre de cas	Resultat	
		1ère Tumeur	2e Tumeur
S180+S180	6	+	+
T8 +T8	10	+	+
Tératome + Tératome	8	+	+

2. Greffe d'un cancer à un hôte porteur d'un jeune tératome en voie de développement

Nous avons réalisé ce type d'association chez la Souris et chez le Rat.

La technique est la suivante: nous injectons, ou nous greffons, une tumeur maligne à des animaux récemment injectés avec de la pulpe d'embryons. Des animaux témoins reçoivent les uns la pulpe d'embryons seulement, les autres la tumeur maligne seulement.

Chez la Souris. Nous n'avons pu faire que 23 associations.

Dans 19 cas, nous avons injecté la pulpe d'embryons dans le tissu sous-cutané du flanc des Souris hôtes; puis, 4–5 heures après, et sur le même flanc, le sarcome S180.

Dans les 4 derniers cas, nous avons injecté le sarcome à des Souris porteuses de jeunes tératomes de 5 jours.

Témoins: 11 Souris témoins reçoivent 1 cm³ de pulpe d'embryons, 5 autres Souris reçoivent le sarcome S180.

Le sarcome se développe sur 22 des 23 animaux traités, et sur les 5 animaux témoins.

expériences soit restreint, on peut conclure que, dans ce type d'association, le sarcome évolue absolument comme sur les Souris témoins. En revanche, le tératome subit une nette inhibition. Il ne se développe pas dans 12 cas sur 22. Lorsqu'il apparaît, il reste de volume réduit, inférieur à celui des tératomes témoins.

Ces premiers résultats ont été confirmés par les expériences réalisées chez le Rat.

Chez le Rat. Nous avons fait 74 associations entre l'épithélioma T8 et le tératome âgé de 5 heures à 20 jours.

Dans 6 cas, l'épithélioma T8 n'a pas pris.

Sur les 68 cas restants, l'association a été réalisée sur le même flanc dans 38 cas, et sur le flanc opposé dans 30 cas.

Trente-neuf Rats témoins n'ont reçu que la pulpe d'embryons.

Vingt-cinq Rats n'ont reçu que la Tumeur T8.

On constate que sur les Rats en expérience, la Tumeur T8 évolue comme sur les Rats témoins.

Des métastases apparaissent dans le gan-

Tableau 3. *Injection du Sarcome S180 à des Souris récemment injectées avec de la pulpe d'embryons*

Age du tératome au moment de l'injection du sarcome	Nombre de cas	Résultat			
		S180		Tératome	
		Sarcome	Pas de sarcome	Tératome	Pas de tératome
4–5 H	19	19	0	9	10
5 jours	4	3	0	1	2
		0	1	1	0

glions lymphatiques. Les animaux meurent 30 à 45 jours après la greffe de la tumeur maligne. Le tératome, mesuré avec un palmer au moment de la greffe de l'épithélioma, est à nouveau mesuré au moment de la mort de l'hôte, et, dans certains cas, prélevé et fixé. Ces dimensions sont comparées aux premières dimensions et à celles des tératomes témoins.

Les résultats sont consignés dans le Tableau 4.

Le Tableau 5 montre l'évolution des tératomes témoins.

Les résultats sont particulièrement nets dans le cas des associations réalisées sur le même flanc entre l'épithélioma et le jeune tératome. La régression du tératome est alors importante (19 cas sur 38 soit 50%).

Dans 15 cas, le volume du tératome a diminué; le tératome n'est plus qu'un petit amas cartilagineux appliqué contre la tumeur maligne.

Dans 4 cas, le volume de la tumeur bénigne n'a pas subi de changement appréciable; le tératome s'est maintenu, et les deux tumeurs sont accolées l'une à l'autre. Un tissu lâche les sépare cependant.

Dans aucun cas, le tératome n'a progressé de façon sensible, alors que 18 tératomes sur 39 témoins, ont, dans le même temps, nettement proliféré, allant parfois jusqu'à doubler de volume.

Tableau 4. *Résultats de la greffe de l'épithélioma T8 à des Rats porteurs de jeunes tératomes*

Age du tératome au moment de la greffe de l'epithélioma T8	Nombre de cas	Evolution du tératome			
		Disparition	Réduction	Maintien	Croissance
Même flanc					
5–48 H	8	5	3	0	0
3–8 jours	9	4	4	1	0
10–20 jours	21	10	8	3	0
Total	38	19 (50%)	15 (39%) 89%	4 (11%)	0 (0%) 11%
Flanc opposé					
5–48 H	3	3	0	0	0
3–8 jours	24	4	14	6	0
10–20 jours	3	0	3	0	0
Total	30	7 (24%)	17 (56%) 80%	6 (20%)	0 (0%) 20%

Tableau 5. *Résultats de l'injection de la pulpe d'embryons à des Rats normaux*

Nombre de cas	Evolution des tératomes			
	Disparition	Réduction	Maintien	Croissance
39	8 20%	5 10% 30%	8 20%	18 50% 70%

Dans le cas d'association controlatérale, le tératome ne disparaît complètement que dans 7 cas. Son évolution est cependant inhibée, puisque ses dimensions diminuent dans 17 cas.

Aucune croissance notable ne se produit.

Nous avons examiné, au microscope, 11 tératomes de volume réduit, associés à l'épithélioma sur le même flanc et sur le flanc opposé. Ils se composent essentiellement de cartilage.

Nous avons repiqué, sur de nouveaux Rats, 5 petits tératomes associés à l'épithélioma sur le même flanc, et 5 tératomes associés sur le flanc opposé. Le tératome entier est repiqué; c'est un petit nodule de 0,5 cm de diamètre environ. Neuf de ces greffons évoluent comme les repiquages de tératomes normaux. Ils survivent sur le nouvel hôte, mais ne subissent aucune croissance. Le dernier tératome, au contraire, augmente rapidement de volume.

Il s'agit d'un tératome développé sur le flanc d'un Rat. L'épithélioma a été greffé, sur le flanc droit, 6 jours après l'injection de la pulpe d'embryons. Nous avons sacrifié ce Rat 43 jours après la greffe de la tumeur cancéreuse. L'animal est prostré, les métastases et la tumeur principale sont volumineuses. Le tératome, très petit et plat, est transplanté sur un nouveau Rat. A partir de ce tératome, se développe une tumeur importante. Nous avons sacrifié ce nouvel hôte après 55 jours, et prélevé des fragments de la tumeur. Certains sont fixés et soumis à l'histologie, les autres greffés à de nouveaux Rats. Nous constatons que cette tumeur est une tumeur cancéreuse, identique à l'épithélioma T8. Dans ce cas, la tumeur bénigne et la tumeur maligne, fixées chacune sur un flanc de l'hôte, ne sont pas entrées en contact. Le tératome était également éloigné des métastases. Cependant des cellules cancéreuses sont venues coloniser le tératome. Ce résultat bien qu'unique, nous paraît intéressant. On peut se demander pourquoi des cellules cancéreuses envahissent ainsi le tératome. Sont-elles spécialement attirées par la tumeur bénigne ou bien le hasard seul intervient-il dans ce phènomène? Il est difficile de répondre à cette question. Nous pensons que, au moment de la formation des métastases, des cellules cancéreuses ont migré vers les ganglions lymphatiques, et que certaines d'entre elles se sont fixées sur le tératome, sans qu'on puisse préciser pourquoi. Mais cette observation est restée unique. Dans tous les autres tératomes étudiées histologiquement ou repiqués sur de nouveaux hôtes, nous n'avons jamais noté la présence de cellules cancéreuses.

Nous avons complété ces expériences d'association entre l'épithélioma et un jeune tératome en injectant de la pulpe d'embryons à des Rats sur lesquels l'épithélioma se développe depuis plusieurs jours.

Douze Rats, porteurs de l'épithélioma depuis 12 à 15 jours, sont injectés avec 1 cm^3 de pulpe d'embryons. Nous faisons l'injection sur le même flanc dans 4 cas, et sur le flanc opposé dans 8 cas. Les animaux meurent 35–45 jours après la greffe du cancer.

On constate qu'aucun tératome ne se développe sur les 4 Rats de la 1ère série ni sur 3 Rats de la 2e série. Trois autres Rats de la 2e série portent un très petit kyste cartilagineux. Enfin, sur les 2 derniers Rats, un tératome d'à peine 0.5 cm^3 s'est développé; nous avons repiqué ces deux tératomes; les greffons ne montrent aucune croissance sensible.

Dans ce type d'association, le développement de l'épithélioma n'est pas modifié, alors que celui du tératome est réduit.

Conclusion

Lorsque nous greffons l'épithélioma T8 à un Rat, porteur d'un jeune tératome, formé depuis moins de 20 jours, l'épithélioma T8 prolifère. En revanche, le tératome subit une nette inhibition, particulièrement importante si les 2 tumeurs sont situées sur le même flanc. Le tératome peut disparaître complètement, il peut aussi ne former qu'un très petit kyste cartilagineux. Dans aucun cas il ne prolifère; il ne forme jamais d'amas volumineux comme le font certains tératomes témoins.

La tumeur maligne nuit au développement de la jeune tumeur bénigne. S'agit-il d'une concurrence nutritive ou d'une action toxique de la tumeur maligne. Il serait en effet possible que s'établisse une compétition entre les deux tumeurs, à évolution si différente. La tumeur maligne, proliférant très rapidement, utiliserait les réserves nutritives du milieu sur lequel elle évolue, au détriment de la tumeur bénigne. On peut penser que l'hôte, trop épuisé par la tumeur cancéreuse, ne peut nourrir le tératome. Mais rappelons que 2 greffons cancéreux d'âge différent se développent sur le même hôte et forment 2 tumeurs volumineuses. Aucune limite à la prolifération des 2 tumeurs ne semble intervenir dans ce cas.

Dans l'historique de ce travail, nous avons cité les conclusions de nombreux auteurs sur l'existence, dans certaines tumeurs cancéreuses, de substances soit stimulatrices, soit inhibitrices, de la croissance.

L'inhibition du tératome serait-elle provoquée par une telle substance émise par la tumeur maligne? Nous nous sommes proposés d'injecter des extraits de tumeur T8 à des Rats

porteurs de jeunes tératomes, et de suivre l'évolution de la tumeur bénigne. C'est ce que nous traitons dans le chapitre suivant.

3. *Action d'extraits de tumeur T8 sur un jeune tératome*

(*a*) *Préparation de l'extrait.* Nous utilisons un broyeur électrique, tournant à la vitesse de 8000 tours/min. La pulpe obtenue est centrifugée pendant ½ heure, à la vitesse de 10.000 tours/min. Nous recueillons le surnageant d'une part, le culot d'autre part, et nous les conservons dans des tubes placés dans un congélateur à −20°. Nous avons vérifié, au microscope, que les cellules étaient détruites.

(*b*) *Expériences témoins.* Nous avons injecté le surnageant, et le culot de l'extrait cancéreux, à des Rats normaux, afin de savoir si les animaux pourront supporter plusieurs injections successives d'extraits.

Douze Rats reçoivent ainsi 1 cm³ de surnageant par jour, pendant 10 jours, dans le tissu sous cutané du flanc gauche.

Après la 5e ou 6e injection, les Rats présentent un gonflement à l'endroit des piqûres. Mais ce gonflement finit par se résorber. Les animaux sont sacrifiés 3 mois après la dernière injection. Ils sont absolument normaux.

Sept Rats sont injectés, pendant 6 jours, avec le culot de l'extrait dilué par du Tyrode, à raison de 1 cm³ par jour.

Des petits nodules sont sensibles à l'emplacement des piqûres, mais ils se résorbent peu à peu. Nous avons sacrifié ces animaux deux mois après la dernière injection.

Aucune tumeur ne se développe à partir des injections répétées de culot ou de surnageant de l'extrait. Les animaux résistent parfaitement aux inoculations successives.

(*c*) *Injections du surnageant à des Rats porteurs de jeunes tértomes.* Nous avons injecté 1 cm³ du surnageant de l'extrait, congelé, puis réchauffé à la température du laboratoire, dans le tissu sous-cutané du flanc gauche de 15 Rats. Ces animaux portent, sur ce même flanc, des tératomes âgés de 3 à 12 jours.

Sept témoins sont injectés avec la pulpe d'embryons seulement. Les Rats en expérience subissent 10 à 16 injections d'extrait cancéreux, à raison de 1 injection par jour. Nous les sacrifions 30–40 jours après la dernière injection. Les tératomes sont prélevés et comparés aux tératomes témoins. Le tératome persiste dans 11 cas sur 15 chez les Rats en expérience, et dans 5 cas sur 7 chez les témoins. Dans 1 cas, le tératome traité est très petit, mais dans 8 cas, il forme un amas de la taille d'une noisette. Dans les deux derniers cas, enfin, c'est une tumeur importante, plus volumineuse que celle des témoins correspondant aux tératomes.

Aucune inhibition du développement de la tumeur bénigne ne se manifeste dans ces essais. Nous avons pensé que, peut-être, le séjour au congélateur altérait l'extrait cancéreux. Nous avons alors entrepris une nouvelle série d'expériences en utilisant un extrait frais pour chaque injection.

De plus, nous avons fait plusieurs injections d'extrait avant d'introduire la pulpe d'embryons.

Onze Rats sont injectés, pendant 5 jours, avec 1 cm³ d'extrait frais. Le 5e jour, chaque animal reçoit 1 cm³ de pulpe d'embryons sur le flanc traité préalablement par l'extrait.

Six Rats témoins sont inoculés avec la pulpe d'embryons.

Nous avons fait 1 injection quotidienne d'extrait frais aux 11 animaux, pendant à nouveau 14 et 15 jours. Les Rats sont sacrifiés 35 jours après la dernière opération. Un tératome s'est développé sur 9 de ces animaux. Cinq tératomes sont de volume réduit, inférieur à 1 cm³, mais les 4 autres forment des tumeurs massives.

Conclusion

Le surnageant de l'extrait frais, comme le surnageant de l'extrait congelé, ne provoque aucune modification de l'évolution des tératomes. Les injections, répétées pendant plusieurs jours, ne nuisent en aucune façon à la "prise" et au développement de la tumeur bénigne. Nous avons pourtant injecté l'extrait cancéreux le plus près possible du tératome. Mais les tératomes traités sont tout à fait comparables aux tératomes témoins.

Si une substance est responsable de l'inhibition de la croissance du tératome, observée lorsque l'épithélioma et le tératome se développent sur le même hôte, cette substance n'est pas présente dans le surnageant de l'extrait, ou bien sa concentration n'est pas suffisante pour qu'elle puisse exercer une action appréciable.

Nous avons alors essayé l'action du culot de l'extrait cancéreux

(*d*) *Injection du culot à des Rats porteurs de jeunes tératomes.* Dans un premier temps, nous avons voulu savoir si l'injection du culot de l'extrait cancéreux empêchait la "prise" du tératome. Nous avons injecté simultanément le culot et la pulpe d'embryons dans le flanc des Rats. Les 2 injections se font à 4 cm environ l'une de l'autre. Puis, nous avons inoculé aux animaux 1 cm³ de culot chaque jour, pendant 8–10 jours.

Dans certains cas, l'injection du culot précède

de plusieurs jours celle de la pulpe. Dans un second temps, nous avons étudié l'action du culot de l'extrait sur de jeunes tératomes en voie de développement.

Premier groupe d'expériences

C'est toujours le culot congelé, puis réchauffé progressivement à la température ambiante, et enfin dilué de moitié par du liquide de Tyrode, que nous avons utilisé. Nous avons fait 48 associations.

Dans 33 cas, nous injectons le culot de l'extrait; puis, 4 à 5 heures après, la pulpe d'embryons. Nous faisons ensuite 1 injection quotidienne de culot, pendant 9 jours.

Dans 15 cas, les animaux subissent 5 injections préalables de culot à raison de 1 injection par jour, puis reçoivent la pulpe d'embryons.

Après l'injection de la pulpe, les Rats sont traités, pendant 5 jours encore, avec le culot de l'extrait cancéreux.

Trente-sept Rats témoins reçoivent uniquement la pulpe d'embryons, et 10 autres Rats, 10 injections de culot cancéreux.

Nous sacrifions les animaux 2 à 6 mois après l'injection de la pulpe d'embryons.

Sur 48 Rats traités, 3 seulement portent un tératome de la taille d'un pois.

Sur les 45 autres, aucune tumeur ne s'est développée.

Les Rats témoins, injectés avec le culot de l'extrait, ne portent pas de tumeur; ils semblent parfaitement normaux.

Les tératomes témoins se sont développés dans 30 cas sur 37. Neuf ont un volume réduit, 8 sont de la grosseur d'une noisette, et 13 forment des tumeurs massives et étendues (Tableau 6).

Conclusion

L'injection répétée de culot de sédimentation de l'extrait cancéreux empêche la formation du tératome dans la grande majorité des cas (45 sur 48 essais). Les résultats sont identiques dans le cas où l'injection de la pulpe et celle du culot sont faites le même jour, et dans le cas où plusieurs injections du culot sont faites avant les injections de pulpe d'embryons. L'injection du culot ne provoque pas la formation d'une tumeur cancéreuse.

Il se forme, après plusieurs injections du culot, des petites boursouflures qui disparaissent totalement après une dizaine de jours. Les animaux, à l'autopsie, ne présentent plus aucune anomalie.

Deuxième groupe d'expériences

Dans cette série, des Rats porteurs de tératomes de 3 à 10 jours sont traités par le culot de l'extrait cancéreux. Les injections d'extrait sont faites dans le tissu sous-cutané, au voisinage du tératome.

Dans 13 cas, nous injectons le culot de sédimentation du broyat cancéreux T8 à des Rats porteurs de tératomes de 10 jours, et, dans 6 cas, à des Rats porteurs de tératomes de 3 jours.

Quatorze Rats porteurs de tératomes sont gardés comme témoins. Les tumeurs bénignes ont sensiblement la taille d'une noisette.

Nous continuons les injections pendant 9 jours consécutifs.

Quarante-cinq jours après la dernière injection de l'extrait cancéreux, nous sacrifions les animaux. La tératome a disparu sur 5 des 13 premiers Rats traités; dans 6 cas, il forme un très petit kyste cartilagineux, dans 2 cas enfin, c'est une tumeur de la taille d'une noisette, c'est-à-dire de 1 cm de diamètre environ (Fig. 3).

Sur les 6 Rats de la deuxième série, un petit tératome s'est maintenu dans 1 seul cas.

Dans les 5 autres cas, le tumeur bénigne a complètement régressé.

Tableau 6. *Evolution de la pulpe d'embryons, injectée à des Rats traités par le culot de l'extrait cancéreux d'une part, et à des Rats témoins d'autre part*

Injection de pulpe d'embryons Nombre de cas	Nombre d'injection de culot cancéreux	Evolution du tératome			
		Pas de tératome	Petit tératome de la taille d'un pois	Tératome de la taille d'une noisette	Tératome volumineux
			(diamètre ≠0,5 cm)	(diamètre ≠1 cm)	
33	10	31	2 (≠6%)	0	0
15	5+5	14	1 (≠6%)	0	0
37	0	7	9	8	13
				(81%)	

Chez les 14 témoins, le tératome a disparu dans 2 cas; il forme un petit amas ne dépassant pas 0,5 cm de diamètre dans 4 cas; dans 3 cas, il a sensiblement 1 diamètre de 1 cm; mais, dans 5 cas, c'est une tumeur très volumineuse (Fig. 3).

Nous avons repiqué, sur deux nouveaux Rats, deux petits tératomes traités. Les greffons ne s'accroissent pas.

Nous avons examiné au microscope 4 autres tératomes traités par le culot de l'extrait cancéreux.

Ce sont de petits kystes de cartilage.

Conclusion

Les résultats sont condensés dans le Tableau 7. Malgré le nombre réduit d'expériences, nous pouvons conclure que le jeune teratome subit une nette inhibition en présence du culot de centrifugation du broyat de l'épithélioma T8.

Il disparaît complètement dans 53% des cas (au lieu de 14% chez les témoins). Dans les autres cas, il ne s'accroît pas de façon appréciable; il forme tout au plus un petit nodule de la grosseur d'une noisette, alors que 36% des tératomes témoins sont des tumeurs volumineuses.

Les injections du culot de l'extrait cancéreux ne provoquent pas la formation de tumeur maligne. Les nodules, observés à la suite des injections successives, finissent par disparaître complètement.

Il semble bien qu'une substance toxique, présente dans le culot de l'extrait utilisé, soit responsable de l'inhibition du développement des tumeurs.

Nous nous sommes demandé si cette inhibition pouvait être provoquée par des extraits d'organes de Rats adultes normaux. La tumeur T8 étant un épithélioma utérin, il nous a paru intéressant d'essayer l'action des broyats d'utérus d'animaux sains. Nous avons également utilisé des broyats de foie de Rats normaux.

Nous broyons les utérus et nous les centrifugeons ½ heure à la vitesse de 10.000 tours par minute; le culot, dilué par du liquide de Tyrode, est seul conservé.

Le foie est simplement haché aux ciseaux fins et aux ciseaux de Pascheff. On obtient une pulpe facilement injectable.

Nous injectons 1 cm^3 de pulpe d'embryons à 10 Rats; 5 de ces animaux reçoivent ensuite 1 cm^3 d'extrait utérin, les 5 autres 1 cm^3 de pulpe de foie. Nous continuons le traitement pendant 10 jours. Comme dans le cas des injections d'extrait cancéreux, il se forme de petites nodules, à l'endroit des piqûres, mais ces nodules disparaissent après quelque temps.

Nous sacrifions les animaux 40 jours après la dernière injection.

Les 5 Rats traités par le broyat d'utérus portent un tératome dont 2 sont volumineux, et 3 sont de la grosseur d'une noisette.

Sur les 5 Rats injectés avec la pulpe de foie, 2 portent un tératome de la taille d'un haricot, 2 un petit nodule de la grosseur d'un pois. Sur le dernier, aucune tumeur ne s'est développée. Il ne se forme pas de tumeur chez des Rats témoins, traités uniquement par le broyat d'utérus, ou de la pulpe de foie. Ainsi, les extraits de tissus normaux n'exercent aucune action sur l'évolution des jeunes tératomes. Les tératomes évoluent sur les Rats traités par ces broyats, comme ils le feraient sur des Rats normaux.

Dans nos conditions expérimentales, seuls les broyats de l'épithélioma T8 manifestent une action sur l'évolution des tumeurs bénignes.

3. CONCLUSIONS

Dans tous les cas où nous avons réuni, sur le même hôte, le tissu embryonnaire et le tissu cancéreux (S180 et T8), la tumeur bénigne et la tumeur maligne, nous avons observé le maintien et la prolifération de la tumeur maligne, d'une part, l'inhibition de la croissance du tératome, d'autre part.

Tableau 7. Résultats de l'injection du culot de centrifugation de broyat de tumeur T8 à des Rats porteurs de tératomes de 10 et 3 jours. Evolution de ces tératomes et des tératomes témoins

Tératome Nombre de cas	Tératome Age	Nombre d'injections du culot cancéreux	Evolution du tératome Disparition	Réduction	Maintien	Forte croissance
13	10 jours	10	5	6	2	0
6	3 jours	10	5	1	0	0
Total 19			10 53%	7 37%	2 10%	
			90%			10%
14		0	2 14%	4 28%	3 22%	5 36%
			42%			58%

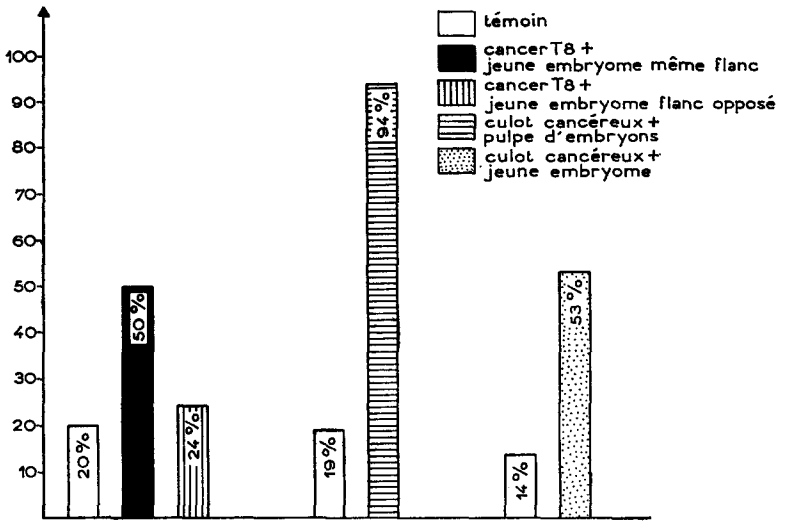


Fig. 4 Inhibition de la formation du tératome chez les Rats témoins et chez les Rats cancéreux ou injectés avec le culot de sédimentation de l'extrait cancéreux.

Si nous greffons la tumeur T8 à un Rat porteur d'un tératome volumineux, la tumeur T8 se développe. Elle n'est pas inhibée par la présence de la tumeur bénigne qui ne modifie ni la "prise", ni la croissance de la tumeur maligne. La tumeur maligne ne semble pas non plus gêner le tératome massif.

Comme nous l'avons montré dans la première partie de ce travail, les tissus conjonctif, cartilagineux, osseux sont abondants dans les tératomes âgés. Ces tissus résistants permettent sans doute au tératome de se maintenir, malgré le présence de la tumeur maligne. Les deux tumeurs coexistent alors sur le même animal.

En revanche, lorsque l'épithélioma T8 se développe sur un Rat porteur d'un jeune tératome, nous constatons un effet inhibiteur de l'épithélioma sur le maintien et la croissance du tératome. Le tératome disparaît dans un grand nombre de cas (50% si l'épithélioma et le tératome se développent sur le même flanc, 24% lorsqu'ils se développent sur les flancs opposés). Lorsqu'il persiste, il forme de petits kystes atteignant rarement le volume d'une noisette. Jamais il ne prolifère de façon sensible.

Il ne semble pas que cet effet soit lié à une mauvaise nutrition de la tumeur bénigne, mais à une action toxique de la tumeur cancéreuse. Depuis plusieurs années, de nombreux auteurs ont pensé qu'il existait, dans les tumeurs malignes, une ou des substances toxiques. Dès 1948, Nakahara et Fukuoka [3] isolent une substance, à partir de différentes tumeurs cancéreuses; cette substance inhibe l'activité enzymatique de la catalase du foie de Rats normaux. Ils l'appellent la "Toxohormone". Greenstein [4, 5] observe également cet effet sur la catalase du foie, en utilisant des extraits de plusieurs tumeurs malignes. En associant en parabiose deux Rats, l'un cancéreux, l'autre sain, Lucke et collaborateurs [6] constatent que des substances toxiques passent du Rat cancéreux vers le Rat sain. Ces toxines réduisent l'activité de la catalase du foie du Rat normal.

Tableau 8. Influence réciproque exercée par l'épithélioma T8 et les tératomes associés in vivo

Type d'expérience	Evolution du tératome	Evolution du cancer T8
Tissu cancéreux mélangé à la pulpe d'embryons	Inhibition	Favorisée
Tumeur cancéreuse + tératome bien développé	Aucune modification	Aucune modification
Tumeur cancéreuse + jeune tératome	Inhibition	Aucune modification
Culot de l'extrait cancéreux + jeune tératome	Inhibition	

Depuis, la "Toxohormone" a donné lieu à de nombreuses recherches. Les auteurs se sont efforcés de purifier la substance. On trouvera, dans l'ouvrage de Busch [7], l'historique de la question. Nous nous contenterons de préciser ici qu'il s'agirait d'un polypeptide de faible poids moléculaire. Il ne semble pas que cette substance soit spécifique des tumeurs malignes. Elle existerait également, en petite quantité, dans certains tissus normaux.

Cependant, selon Yunoki et Griffn [8], une fraction de la Toxohormone, la fraction TH2, ne se trouverait que dans les tissus malins. Le problème reste encore complexe, mais il semble bien que des substances toxiques soient élaborées par les tumeurs cancéreuses.

Nous avons préparé des broyats de l'épithélioma T8. Le culot de centrifugation de ces broyats, injecté pendant plusieurs jours à des animaux recevant une injection de pulpe d'embryons, manifeste une forte action inhibitrice sur le développement de la tumeur bénigne. Si l'injection de la pulpe d'embryons est faite le jour de la ère inoculation de l'extrait cancéreux, aucun tératome ne se développe dans 94% des cas. Si le tératome est déjà formé lorsque débute le traitement par l'extrait cancéreux, le tératome disparaît dans 53% des cas; dans 37%, il forme un petit amas cartilagineux de dimensions réduites. Son volume est toujours inférieur à celui des tératomes témoins (les résultats sont réunis dans la Fig. 4).

Ainsi, les culots de centrifugation des broyats de la tumeur T8, empêchent la croissance de la tumeur bénigne.

Les injections du surnageant de l'extrait cancéreux, d'une part, de broyats de foie et d'utérus normaux, d'autre part, sont sans action sur l'évolution du tératome.

Le traumatisme, produit par les piqûres répétées, et la présence de nodules étrangers au tératome, ne sont donc pas responsables de l'involution de la tumeur bénigne.

Ces expériences suggèrent que l'action manifestée par le culot de sédimentation des broyats de la tumeur maligne T8 pourrait être due à un facteur toxique présent dans l'extrait utilisé, que nous tenterons d'analyser dans un travail ultérieur.

Nous pouvons conclure, dès à présent, que:

(1) l'évolution du Sarcome S180 et de l'épithélioma utérin T8 est favorisée par les cellules embryonnaires lorsque les 2 types de cellules sont mélangées avant d'être injectées à l'hôte;

(2) la tumeur maligne (S180 et T8) exerce une action inhibitrice sur le développement des jeunes tératomes;

(3) cette action inhibitrice ne dépend pas de la présence de cellules cancéreuses intactes. Elle se manifeste également lorsque nous injectons le culot de sédimentation du broyat de la tumeur maligne T8.

Ces résultats sont consignés dans le Tableau 8.

Signalons que le jeune tératome, comme le tératome bien développé, n'exerce aucune influence sur la tumeur maligne qui se développe comme elle le ferait sur des Rats normaux.

RESUME

Nous avons réuni, sur le même hôte, un tératome et une tumeur maligne, et étudié l'influence qu'exercent l'une sur l'autre ces deux tumeurs. Quelques essais sont faits chez la Souris avec le sarcome S180, mais le plus grand nombre d'expériences sont faites chez le Rat avec l'épithélioma utérin T8.

1. *Lorsqu'on greffe la tumeur T8 à un Rat porteur d'un tératome massif, développé depuis plusieurs mois, la tumeur maligne et le tératome coexistent sur l'animal et ne semblent pas exercer d'influence l'un sur l'autre. En particulier, le tératome, même très volumineux, n'empêche nullement la prolifération de l'épithélioma qui se développe comme le font les tumeurs T8 témoins.*

2. *Lorsqu'on greffe la tumeur T8 à un Rat récemment injecté avec la pulpe d'embryons, on constate une inhibition du développement du tératome. Le développement de la tumeur maligne, lui, ne subit aucune modification. L'inhibition du tératome est particulièrement nette lorsque les deux tumeurs évoluent sur le même flanc. Elle se manifeste par une augmentation des pourcentages de la disparition du tératome ou de la réduction de son volume, par rapport aux pourcentages témoins* (50% *au lieu de* 20% *pour la disparition,* 39% *au lieu de* 10% *pour la réduction du volume*). *La croissance du tératome est arrêtée. Les tératomes inhibés sont constitués de fibroblastes et de cartilage. Nous n'y avons jamais observé de cellules malignes.*

3. *Nous nous sommes demandé si cette action de la tumeur T8 sur la croissance du tératome était due à une compétition nutritive entre les deux tumeurs ou à une action toxique du cancer. Nous avons préparé des extraits de tumeur T8 et constaté que le culot de*

centrifugation de l'extrait, injecté pendant plusieurs jours à des Rats recevant une injection de pulpes d'embryons au début du traitement, empêchait la formation du tératome dans 94% *des cas. Les injections de culot exercent également une action défavorable au développement d'un tératome âgé de* 3 *à* 10 *jours. La régression des tératomes est augmentée, leur croissance est bloquée* (53% *des tératomes disparaissent au lieu de* 14% *chez les témoins*).

4. *Des broyats de tissus normaux -foie et utérus- de Rats adultes n'ont aucun effet sur l'évolution des tératomes.*

5. *En conclusion, l'action inhibitrice de l'épithélioma utérin T*8 *sur le maintien et la croissance du tératome serait due à des facteurs toxiques produits par la tumeur maligne et présents dans le culot de centrifugation de l'extrait de cette tumeur.*

SUMMARY

*A teratoma and a malignant tumour were grafted on the same host and the way they are acting upon each other was studied. A few experiments were carried out on mice with sarcoma S*180, *but most of the experiences were performed on rats with the uterine epithelioma T*8.

1. *When a T*8 *tumour was grafted on a rat bearing a big several months old teratoma, the malignant tumour and the teratoma just co-existed on the animal and did not seem to influence each other. Particularly, even if very voluminous, the teratoma did not prevent the proliferation of the epithelioma, which developed as the T*8 *control tumours.*

2. *When a T*8 *tumour was grafted on a rat recently injected with embryo pulp, there was inhibition in the development of the teratoma. The development of the malignant tumour did not undergo any modification. The inhibition of teratoma was particularly apparent when both tumours developed on the same side of the body. This could be distinguished by a faster disappearing of the teratoma or by a reduction of its volume in comparison with the controls* (50% *instead of* 20% *for the disappearing and* 39% *instead of* 10% *for the reduction of volume*). *The growing of teratoma was stopped. The inhibited teratomas consisted of fibroblasts and cartilage. Malignant cells were never observed.*

3. *We were wondering whether this action of the T*8 *tumour on the growing of the teratoma was due to a nutrition competition between both tumours or to a basic action of the cancer. Extracts of T*8 *tumour were prepared and we noticed that the centrifugation precipitate of the extract, injected for several days to rats receiving an injection of embryo pulp at the beginning of the treatment, prevented the formation of the teratoma in* 94% *of the cases. The precipitate also had an unfavourable effect on the development of a teratoma aged* 3 *to* 10 *days. The regression of teratomas was increased, their growth was stopped* (53% *of the teratomas disappear instead of* 14% *of the controls*).

4. *Extracts from normal tissues of liver and uterus of adult rats had no effect on the evolution of the teratomas.*

5. *In conclusion, the inhibitory action of the uterine T*8 *epithelioma on the maintenance and growth of the teratoma could be due to toxic factors induced by the malignant tumour and present in the centrifugation precipitate of this tumour extract.*

ZUSAMMENFASSUNG

*Wir haben bei einem und demselben Tier ein Teratom und einen bösartigen Tumor gepflanzt und den Einfluss von einem auf den anderen untersucht. Einige Versuche sind an Mäusen mit Sarkom S*180 *durchgeführt, aber die grösste Zahl von Experimenten wurde bei den Ratten mit Uterusepitheliom T*8 *untersucht.*

1. *Wenn man eine Ratte impft, die seit mehreren Monaten einen massiven Teratom entwickelt hat stellt man fest, dass der bösartige Tumor und das Teratom koegsistieren und sie keinen Einfluss aufeinander ausüben. Das Teratom, auch dann, wenn es sehr gross ist, verhindert nicht die Entwicklung des Epithelioms, welches das gleiche ist wie bei Kontrolltieren mit einem T*8*-Tumor.*

2. *Einer Ratte, der man vor kurzem die Embryonenpulpe injiziert hat und anschliessend einen T*8*-Tumor geimpft hat, wurde eine Hemmung des Teratoms festgestellt. Die Entwicklung des bösartigen Tumors ist besonders ausgeprägt, wenn beide Tumoren sich*

auf derselben Flanke entwickelt. Diese Hemmung drückt sich aus in einer Prozenterhöhung der verschwundenen Teratomen oder in Verminderung ihrer Volumen in Vergleich zu den Kontrolltieren (50% *anstatt* 20%, *für das Verschwinden von Teratomen,* 39% *anstatt* 10% *für die Volumenveränderungen*). *Das Wachstum der Teratome ist unterbrochen. Die gehemmten Teratome bestehen aus Fibroplasten und Knorpel. Die bösartigen Zellen haben wir nie beobachtet.*

3. *Wir haben uns gefragt, ob diese Wirkung des T8-Tumors auf das Wachstum des Teratoms einer Ernährungskonkurrenz zuzuschreiben ist, oder einer toxischen Wirkung des Karzinoms. Wir haben zwei Extrakte vom T8-Tumor vorbereitet und festgestellt, dass das Extraktzentrifugat, das den Ratten—die mehrere Tage mit Embryonenpulpe vorbehandelt wurden—injiziert wurde, die Entwicklung des Teratoms in* 94% *der Fälle dadurch verhinderte. Die Verabreichungen des Zentrifugats beseitigen die Entwicklung eines* 3–10 *Tage alten Teratoms. Die Rückbildung der Teratomen ist erhöht und ihr Wachstum blockiert* (53% *der Teratomen bilden sich zurück gegenüber* 14% *bei Kontrolltieren*).

4. *Das homogenisierte normale Gewebe* (*Leber und Uterus von der erwachsenen Ratte*) *hat keinen Effekt auf die Entwicklung der Teratomen.*

5. *In Zusammenfassung: Die hemmende Wirkung von T8-Uterusepitheliom auf Erhaltung und Wachstum des Teratoms ist auf toxische Faktoren zurückzuführen, die durch einen bösartigen Tumor entstanden sind. Diese Faktoren sind in Zentrifugat des Tumorextraktes zu finden.*

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The Possible Mechanism of the Effect of the Pituitary–Adrenal System on Growth of a Transplanted Tumor

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THE ACTION of hypophysectomy and adrenalectomy on tumor growth has been studied by various authors. Hypophysectomy, like adrenalectomy, exerts an inhibitory effect on spontaneous tumorigenesis [1] and on growth of transplanted tumors [2, 3].

Talalay, Takano and Huggins [4] found that despite the ease with which hypophysectomized rats gained in body weight in response to tube-feeding their tumors grew at a much slower pace than in control animals. The authors believe that the inhibition of tumor growth may be dependent upon indirect factors, such as vascular supply and connective tissue reaction. However, no very convincing arguments have been offered to support this hypothesis.

The purpose of the present communication is to provide further information on the mechanism by which the pituitary–adrenal system affects tumor growth. Part of the results given here has previously been reported in an abstract [5].

MATERIAL AND METHODS

In recent publications we have attempted to study the relationship between tumor and host by means of diffusion chambers (1) and of transplantation tests (2).

Tumor

Rhabdomyoblastoma CRMI originated in 1961 in a rat treated by implantation of cellophane film between interacting components of muscle [6]. The tumor grows and kills 100 per cent of the animals.

1. *Diffusion chambers*

Small pieces not exceeding 0·5 mm^3 of tumor tissue or of the anterior pituitary gland were removed and freed from the surrounding tissue under sterile conditions. They were placed in Millipore diffusion chambers introduced intraperitoneally into rats. The chambers were 10 mm outer diameter and 6 mm inner diameter. The membranes had an average pore diameter of 0·1–0·3 μ.

The data from experiments of this type together with appropriate controls are presented in Table 1. The diffusion chambers were extracted from the peritoneum on the second third, fifth and tenth days.

2. *Transplantation tests*

In these experiments we studied the transplantability of intramuscular CRMI tumor implants. Three groups of animals were used: (1) intact rats (2) hypophysectomized rats and (3) rats treated with metopirone. (Metopirone—2-methyl-1,2-di-3′-pridylpropane-1-one—selectively blocks enzymatic II β-hydroxylation, thereby inhibiting the biosynthesis of cortisol, aldosterone and corticosterone).

Group (1). The intact rats were given one intramuscular injection of tumor in the following doses: 40 mg, 50 mg, 70 mg, 80 mg, 110 mg. 150 mg, 180 mg, 230 mg and 300 mg. One hundred animals were used.

Table 1. *Growth of tumor cells in presence and absence of pituitary tissue in recipient animals*

Experiment No.	Recipient animal	Type of tissues transplantated in diffusion chamber	Mitotic index at 5-day cultivation %
1.	Intact animal	Tumor CRMI	2–3
2.	Intact animal	Tumor CRMI plus pituitary tissue	30–35 40
3.	Intact animal	Pituitary tissue	2–2·5
4.	Animal with tumor CRMI	Pituitary tissue	25–30

Group (2). In this group of rats various doses of tumor (110 mg, 150 mg, 210 mg, 315 mg, 400 mg) were transplanted 3–4 weeks after hypophysectomy. Twenty-five animals were used.

Group (3). Each animal was injected intraperitoneally with a single dose of 10 mg of metopirone per day which was administered for 2 weeks. The various tumors (110 mg, 150 mg, 230 mg, 300 mg) were transplanted on day 4 after the first injection of metopirone.

RESULTS

1. *Tissue explants*

The tumor explants. In two days the cultures of tumor cells from the original piece grew in all series. These cells were growing in monolayer sheets. The shape of tumor cells in control experiments (Table 1, No. 1) varied from round to oval, polygonal, spindle-shaped and rod-shaped.

While in experiment No. 1 variability of cell shape of the tumor predominated, in No. 2 the cellular picture was homogeneous. Cells in this series were cubic or polygonal in shape (Fig. 1). The preparations revealed that tumor cells in contact with pituitary cells tended to align themselves on these pituitary cells.

The control preparations (experiment No. 1) showed less mitotic activity than when pituitary tissue was present.

The pituitary explants. In experiment No. 3 (Table 1) the cells had finely granular cytoplasm, the nuclei were round or somewhat ovoid and contained one or two nucleoli. The tendency of epithelial cells to adhere to each other and to divide resulted in the formation of cell aggregates containing some reconstructed histological patterns at the fifth day. Sometimes epithelial-cell-clones were seen to be separated from the primary transplant, but usually they were attached to pituitary fragments.

In experiments number 2 and 4 (Table 1) the outgrowth was more intense. The mitotic index characteristically reached levels much greater than in the control experiments (Table 1). One of the most remarkable features of the histological picture was the

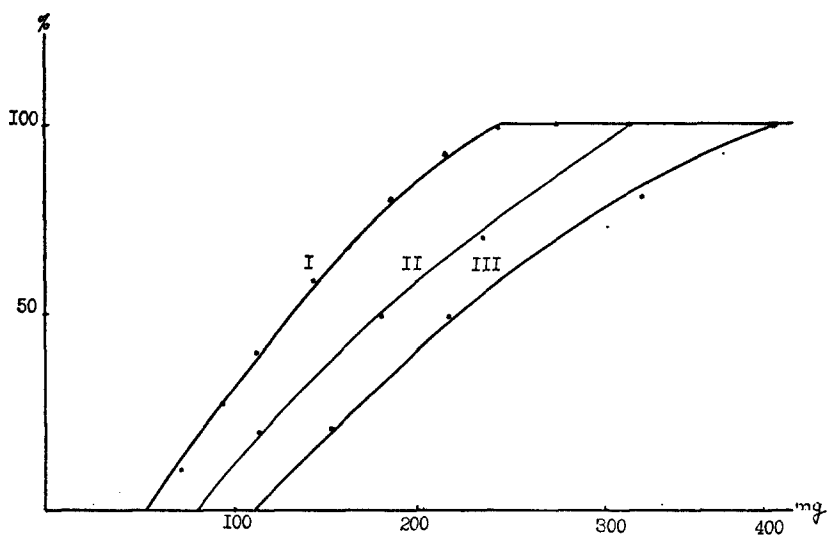


Fig. 4. *Growth of transplanted tumor tissue as a function of the amount of tissue transplanted.*
Curve I: *control*
Curve II: *in animals treated with metopirone*
Curve III: *in hyposectomized animals*

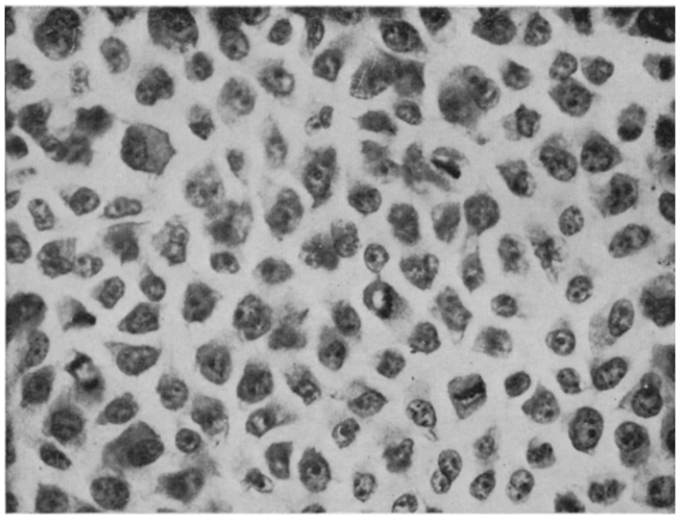


Fig. 1. *Microscopic aspect observed in experiment No.* 2 (*Table* 1).

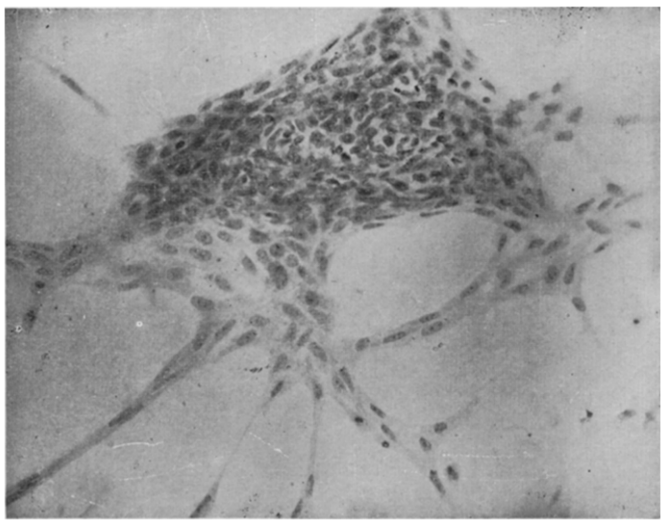


Fig. 2. *Cell colonies.*

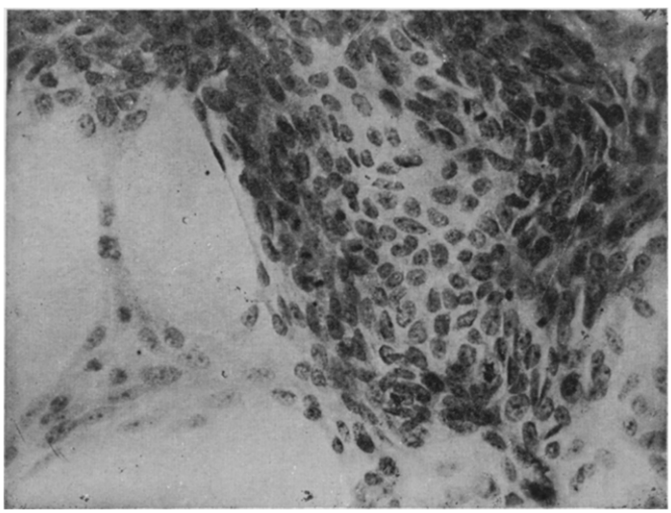


Fig. 3. *Cell colonies and mitoses.*

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formation (after 3 days) of cell colonies which we call epitheliades [7]. These structures increased in size during the following days and were observed at a considerable distance from the original piece (Fig 2). The cells were either round (outer region) or spindle-shaped (inner region). Mitoses were found in both the outer and inner regions (Fig. 3).

Cells of fibroblastic type were very rare. Their distribution on the filter was erratic.

2. The effect of metopirone injections and the effect of hypophysectomy are summarized in Fig. 4. Figure 4 shows the relationship between doses of tumor/abscissa and growth after transplantation/ordinate. It will be seen from this chart that both the effect of hypophysectomy and inhibition of adrenal cortex on the growth of transplanted tissue takes place at once. The curves are displaced in hypophysectomized rats (III) and in netopirone treated rats (II) to the right of that in control animals (I). In both experiments retardation of tumor growth occurred and was accompanied by a definite decrease in the rate of mortality of animals.

Tumor cells enclosed in cell-impermeable Millipore chambers and implanted in the peritoneum of treated animals remained viable for at least two weeks.

DISCUSSION

The present work shows that this particular tumor in culture had a significant effect on the number and size of organized pituitary structures. In addition there was a statistically highly significant difference in mitotic activity between the mixed samples and the controls. Experiment No. 4 gave the same mitotic index as experiment No. 2, indicating perhaps that the tumor exerted its effect on the pituitary even when the 2 tissues were widely separated.

In addition, the present studies showed that the pituitary had a significant effect on the differentiation and mitotic activity of tumor cells. Therefore there appears to be a reciprocal relation between adenohypophysis and the tumor.

In recent years, there has been increasing interest in cortisol as immunizing agents [8]. The data presented here clearly demonstrate that hypophysectomy markedly altered the rate of growth of a transplanted tumor. The administration of metopirone had a similar effect.

Little is known at present about the regulation of immunological reactions. The observed reciprocal influence of tumor and hypophysis in tissue culture should lead to more fundamental questions concerning regulation of tumor growth. The problem is undoubtedly very involved and conclusions must be only tentative.

Acknowledgments—I wish to thank Prof. A. N. Studitsky for advice and encouragement, as well as Miss E. A. Ivanova for carrying out the hypophysectomy.

RESUME

1. *Par rapport aux valeurs de contrôle, on a constaté que la culture de fragments du lobe antérieur d'un mélange de l'hypophyse et de tissu tumoral stimulait davantage la prolifération et la différentiation des tissus.*
2. *L'administration de métopirone et l'hypophysectomie provoquaient une diminution de croissance des tumeurs transplantées.*

SUMMARY

1. *In mixed cultures of fragments of the anterior lobe of the hypophysis with tumor tissue, the proliferation and the differentiation of tissues were found to be stimulated as compared to control values.*
2. *The administration of metopirone and hypophysectomy produced a decrease of growth of transplanted tumor.*

ZUSAMMENFASSUNG

1. *Im Vergleich mit den Kontrollwerten wurden die Proliferation sowie die Differezierung von Geweben bei einer gemischten Kultur von Fragmenten des Hypophysenvorderlappens mit dem Gewebe des Tumors stimuliert.*
2. *Zugabe von Metopiron und Hypophysektomie verringern das Wachstum des transplantierten Tumors.*

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Ultrastructure du *Mycoplasme Salivarium* et du *Mycoplasme Orale* en Culture de Tissus

Le Problème de leur Reproduction à Partir de la Surface Cellulaire*

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La microscopie électronique est de grande importance dans les recherches sur les mycoplasmes, et c'est grâce à des études ultrastructurales que l'on put mettre en évidence la ressemblance morphologique entre les unités de petite taille des mycoplasmes et certaines particules virales. Ces observations remirent en cause plusieurs publications qui semblaient impliquer la présence d'un virus dans les affections malignes humaines.

Toute une série de travaux viennent d'être publiés sur le sujet [1–11].

L'intérêt des recherches ultrastructurales sur les mycoplasmes est ainsi très évident, et les nouvelles observations rapportées ici ont un double but:

—insister encore sur certains aspects morphologiques des mycoplasmes qui ont été révélés en culture de tissus, aspects qui, de ce point de vue, les rapprochent de certains myxovirus et virus oncogènes à ARN,

—décrire ensuite deux souches dont l'une, le *mycoplasme orale*, n'a pas encore été examinée en coupe ultrafine sur culture de tissus, malgré son importance, puisque cette souche, la plus fréquemment rencontrée dans les voies buccales de l'homme [12], a été isolée directement à partir de tissus leucémiques humains [13].

MATERIEL ET METHODES

Deux souches de mycoplasmes ont été étudiées:

—l'une, le *mycoplasme orale*, avait infecté spontanément des fibroblastes de souris PS [14]† et a été identifiée par le laboratoire de Mme de Ruys (Amsterdam),

—l'autre, le *mycoplasme salivarium*, a été inoculée systématiquement à des cellules diploïdes d'embryons humains ce type fibroblastique (souche L809, souche Wi 38).

L'absence de contamination par des mycoplasmes a été régulièrement contrôlée dans les souches cellulaires (L809 et Wi 38) par culture sur milieu de Hayflick. Ces cellules ont été entretenues avant l'inoculation, et, au cours de ces essais, sur un milieu sans antibiotique.

Les cellules en culture monocellulaire sont infectées à 37°C. Au bout d'une heure, elles sont lavées au tampon tris-phosphate, et le milieu d'entretien (milieu d'Eagle additionné de 2% de sérum de veau) est introduit, puis

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†Les auteurs remercient le Docteur et Madame MOURIQUAND de leur avoir donné cette souche à examiner.

renouvelé tous les jours. Les cellules sont fixées à la glutaraldéhyde à 5%, ensuite à l'acide osmique à 1% (Palade), pendant une heure, à 4°C. Les prélèvements sont effectués 2, 19, 26 et 48 heures, 5 jours et 7 jours après le début de l'expérience. Des cellules servant de témoins, ayant subi les mêmes manipulations sauf l'inoculation, sont également fixées au bout de 2 heures et 7 jours. L'inclusion à l'araldite [15] est effectuée après déshydratation progressive à l'alcool. Les coupes ultrafines ont été faites au couteau de verre avec l'ultramicrotome Porter-Blum, et colorées à l'acétate d'uranyle à 2%, puis examinées au microscope électronique Siemens Elmiskop II.

RESULTATS

1. *Mycoplasme salivarium*

Malgré les temps de prélèvements successifs, toutes les formations qui vont être décrites ont pu être observées dès la 2e heure, et jusqu'au 7e jour.

Les éléments sont soit *extra-*, soit *intracellulaires*, et l'on peut les répartir *en deux groupes*, suivant leur dimension qui, ainsi qu'il a été indiqué, relève soit de l'ordre de celle des bactéries (diamètre compris entre 1 et 3μ), soit de l'ordre de celle des virus (diamètre compris entre 0,1 et 0,3μ).

Dans chacun de ces groupes, le *polymorphisme* est extrême.

1.A. *Les éléments de grande taille*, c'est-à-dire dépassant le micron, comprennent essentiellement deux variétés.

(a) *les éléments à structure pseudo-bactérienne, et comportant une 'aire nucléaire'* (Fig. 1). Leur forme est souvent arrondie ou ovalaire; ils mesurent de 1 à 3μ de diamètre et possèdent une plage centrale à structure de type nucléaire caractéristique, formée d'un réseau de filaments, soit ténus, soit denses et osmiophiles, qui se détachent sur un fond clair. Il a été montré qu'un tel réseau, dont l'aspect est typique, correspondait à de l'ADN [16]. Autour de cette 'aire nucléaire', la zone cytoplasmique dense contient des granulations mesurant 100 à 200 Å de diamètre, ayant l'aspect habituel de ribosomes. La membrane de surface revêt le type classique des membranes cytoplasmiques à trois feuillets ('unit membrane' de Robertson).

Ces éléments ne se distinguent morphologiquement de vraies bactéries que par l'absence de paroi cellulaire rigide stratifiée. Ils sont, en revanche, comparables aux formes L de certaines souches bactériennes [17–19].

(b) *Les éléments sans structure nucléaire apparente, et ressemblant à du cytoplasme cellulaire* (Fig. 2). Leur forme est beaucoup plus variée que dans le cas précédent: arrondie, en haltère, en raquette etc.; leur cytoplasme est dense, riche en ribosomes; on ne distingue pas de réseau d'ADN. Les éléments de ce type peuvent être soit adjacents, soit distants de la membrane cellulaire, ou paraître se former par 'bourgeonnement' aux dépens de celle-ci (Fig. 2, 2). Dans ce dernier cas, ils se distinguent parfois difficilement de certains pseudopodes cellulaires (*c.f.* discussion).

A leur périphérie, on observe souvent la formation d'un petit bourgeon arrondi qui correspond à l'émission d'un des éléments de petite taille qui vont être décrits maintenant (Fig. 2, 2).

1B. *Les éléments de petite taille* (diamètre compris entre 0,1 et 0,3μ).

Leur forme est généralement régulière, arrondie ou ovalaire, parfois en virgule, ou en bâtonnets: on les a décrits sous le nom de 'corpuscules élémentaires' (Fig. 3, 1 et. Fig 4, 1 et 2). Leur structure est simple; elle comporte une membrane externe à triple feuillet et une zone centrale, soit très dense, au point que l'on ne peut distinguer sa structure, soit semblable au cytoplasme d'une cellule avec des ribosomes. Entre la membrane externe et la zone centrale, l'espace est infime et mesure environ 50 Å; la plupart du temps, il est à peine observable. Ces corpuscules élémentaires sont assez nombreux et souvent entremêlés avec des formes de grande taille ou des formes filamenteuses ou avec des villosités de la surface de la cellule (Fig. 8, 3).

Leur caractère remarquable réside dans les relations étroites contractées avec la membrane cellulaire. Non seulement ces corpuscules entrent en contact avec la membrane plasmatique, mais souvent ils paraissent même être en continuité directe avec elle. Au premier abord, il semble que l'on puisse suivre morphologiquement toute une série de stades qui suggèrent leur apparente formation par étranglement du pédicule d'un 'bourgeon' émis par le cytoplasme cellulaire (Fig. 4, 1 et 2), le phénomène étant comparable à celui observé pour certaines familles de virus (Fig. 4, 3). En fait, il est très difficile, malgré ces aspects très évocateurs, d'éliminer, sur ces images, la possibilité que les particules soient simplement très intimement accolées à la surface cellulaire. Celle-ci est mal définissable, soit parce qu'elle est recouverte d'une couche de substance particulière (mucopolysaccharides?), soit parce qu'il s'agit d'une fixation à la glutaraldéhyde, ou encore d'une coupe légèrement tangentielle. Mais ce qu'on peut affirmer c'est qu'il existe souvent, en regard de la particule, une densifi-

cation très caractéristique de la membrane cellulaire qui revêt parfois l'aspect d'un petit cône (Fig. 3, 1) ou d'une cupule (Fig. 5, 1) (pédicule? site récepteur?).

A l'intérieur de la cellule, les deux variétés d'éléments qui viennent d'être décrits se retrouvent.

Ils sont généralement contenus dans de grandes vacuoles, et donc séparés du cytoplasme environnant par une membrane. Ils sont immédiatement reconnaissables s'ils sont constitués par des amas de 'corpuscules élémentaires' ou par des éléments à l'aspect bactérien typique.

Dans de nombreux cas, leur structure parait beaucoup plus hétéroclite, très remaniée, et les aspects les plus polymorphes peuvent être décrits. Parmi les formes les plus fréquemment rencontrées, on signalera des corps sphériques ou ovalaires constitués par un matériel d'aspect floconneux de densité extrêmement variable, claire (Fig. 6, 1 et 2) ou au contraire plus ou moins opaque aux électrons (Fig. 6, 3 et 4).

A l'intérieur de ces structures complexes, deux caractères très particuliers sont fréquemment retrouvés. Il s'agit, dans un cas, de petites zones irrégulières, très osmiophiles, fréquemment allongées le long de la membrane vacuolaire (Fig. 6, 1, 3 et 4); dans d'autres cas, de véritables granulations denses arrondies cernées ou non d'une membrane (Fig. 6, 2), et qui ressemblent à des 'corpuscules élémentaires'.

Certains de ces corps arrondis peuvent prendre des aspects très semblables à ceux décrits pour les *lysosomes* (Fig. 6, 5). Cette identification est rendue d'autant plus plausible que certaines vacuoles, contenant des débris divers, présentent la structure typique de *vacuoles autophagiques*.

D'autres structures ovalaires et de formes assez régulièrement semblables apparaissent, soit vides, soit contenant des restes de membranes ou de glycogène. Elles sont caractérisées par leur tendance à conglomérer *en amas à contours polycycliques* (Fig. 6, 6).

En somme, la présence des mycoplasmes, au sein même de la cellule, revêt les aspects les plus divers. Mais, malgré ces variations, on remarquera que les structures du type 'corpuscules élémentaires', qui étaient si nombreuses à la surface, sont plus rarement observés à l'intérieur de la cellule, et qu'il s'agit presque toujours d'éléments classés ici dans la catégorie de grande taille. Ceux-ci paraissent ainsi avoir été englobés dans des vacuoles à pouvoir phagocytaire.

2. *Mycoplasme orale*

Dans cette souche, on retrouve soit à l'intérieur, soit à la surface de la cellule, les deux variétés d'éléments classés suivant leurs tailles.

Parmi les éléments de petite taille, à côté de corpuscules élémentaires tout à fait comparables à ceux décrits pour le *Mycoplasme salivarium*, il existe des formes d'un aspect très particulier qui n'a pas été décrit, jusqu'à présent, pour d'autres souches étudiées au microscope électronique (Fig. 7).

Il s'agit d'éléments denses en forme de courts serpentins et dont les limites sont irrégulières et anguleuses. Le diamètre des filaments est de 0,05 μ, leur longueur varie de 0,1 à 0,2 μ. Ils décrivent les courbes et angles les plus variés. Comme dans le cas des corpuscules élémentaires, ils semblent être émis à partir de la membrane de surface de la cellule.

3. *Modifications cellulaires observées dans les cultures infectées avec les deux souches*

Elles sont importantes quoique non constantes, certaines cellules paraissant tout à fait normales. De nombreuses lésions, cependant, peuvent être observées, dont il est impossible de dire si elles sont spécifiques ou si elles ne correspondent qu'à des symptômes de souffrance cellulaire.

L'ergastoplasme peut être modifié, hypertrophique, avec des ribosomes très osmiophiles et des citernes largement dilatées. A un degré extrême, il peut être constitué par de véritables flaques contenant une substance homogène, dense, beaucoup plus apparente que celle contenue d'habitude dans ses cavités (Fig. 8, 1).

Parfois tout le cytoplasme est constitué par un ergastoplasme ainsi remanié, et la cellule émet alors dans l'espace intercellulaire des portions entières de ce cytoplasme ergastoplasmique modifié (Fig. 8, 2); le processus est comparable à une clasmatose. Il est frappant de constater que les formations ainsi émises se disposent à la surface des cellules comme des mycoplasmes et peuvent être phagocytées comme ces organismes.

La quantité de glycogène est modifiée. On observe des amas denses et volumineux, et souvent des chaînons de particules (β), à l'intérieur des vacuoles claires à contours polycycliques (Fig. 6, 6).

Mais l'altération cytoplasmique la plus importante concerne la *membrane plasmatique*.

Elle est modifiée du fait de l'émission des filaments ou des particules correspondant aux corpuscules élémentaires décrits plus haut: fréquemment elle semble réagir par des

mouvements pseudopodiques d'une remarquable intensité et elle se hérisse de microvillosités soit longiformes (Fig. 9, 1) soit à base large (Fig. 9, 2).

Dans le noyau, aucune modification ultrastructurale n'a été observée ici, quoiqu'il ait été montré qu'une ségrégation des constituants nucléolaires pouvait s'observer [20].

DISCUSSION

Les aspects des mycoplasmes observés ici sont semblables à ceux décrits par de nombreux auteurs [1, 3, 13, 21–23] et, fait primordial pour leur identification, la plupart des formes observées sont semblables aussi à celles décrites en milieu acellulaire.

En ce qui concerne le *cycle de reproduction* des mycoplasmes, certains faits semblent bien établis.

La pénétration des formes, décrites ici sous la désignation de 'formes pseudo-bactériennes', a lieu certainement par phagocytose après une période d'attachement à la membrane cellulaire bien mise en évidence en contraste de phase par Fogh et Fogh [24], et au microscope électronique par Zucker-Franklin *et al* [25].

De même, à l'intérieur de la cellule, on peut observer que tous les stades de digestion progressive subie par ces micro-organismes aboutissent à la formation de lyosomes et de vacuoles autophagiques.

En revanche, aucune certitude n'est acquise sur les rapports séquentiels éventuels entre les formes 'pseudo-bactériennes' et 'pseudo-virales', car, jusqu'à présent, il semble que l'on ne soit jamais parvenu à relier certains aspects à des temps d'infection progressifs définis. Malgré ce manque de synchronisation, les images morphologiques sont très suggestives:

D'une part, la formation d'un élément de type pseudo-viral par un processus de 'bourgeonnement' à partir de la membrane externe d'une forme de type pseudo-bactérien est admise et bien illustrée par plusieurs auteurs [13, 23]. Il est plausible de postuler qu'un phénomène comparable peut avoir lieu à l'intérieur du mycoplasme puisque de nombreuses formes 'pseudo-bactériennes' contiennent en leur intérieur des particules ressemblant à des corps élémentaires.

D'autre part, le point sur lequel l'attention est attirée ici, et qu'illustrent les images des Fig. 3–5, est la possibilité de *formation de corpuscules élémentaires, directement* à partir de la membrane de la cellule infectée, sans qu'un stade intermédiaire (émission à partir d'une forme pseudo-bactérienne) soit nécessaire. Les raisons pour lesquelles un tel phénomène ne peut être affirmé sur les seules images morphologiques ont déjà été signalées (p. 430).

Ce mode de reproduction par bourgeonnement ne serait pas le fait des seuls corpuscules élémentaires, mais concernerait également certains des éléments de grande taille. Il n'a été observé ici que pour les formes cytoplasmiques sans aire nucléaire, mais le résumé d'un travail de Zeigel [26] laisse supposer que ces auteurs ont observé la formation des éléments à ADN également par un processus de bourgeonnement de la surface.

Ces observations concernant un mécanisme de reproduction possible de mycoplasme aux dépens des cellules en culture sont à rapprocher des études récentes de Fogh et Fogh [24] qui ont montré que la reproduction des mycoplasmes était très favorisée par la présence de cellules. Ces auteurs montrent que des cellules amniotiques humaines en culture, infectées par le mycoplasme, souche HT, colorées à l'orcéine, se prêtent à un examen direct au microscope en contraste de phase et à une étude numérique des colonies. Ainsi, ils ont pu observer que lorsqu'une association avec des cellules était possible, la multiplication des mycoplasmes atteignait des titres beaucoup plus élevés que sur simple bouillon, et que ces titres augmentaient avec le nombre de cellules.

Cependant, les observations rapportées ici sont, pour l'instant, d'ordre essentiellement morphologique, et il est donc important d'énumérer les arguments de cet ordre qui permettent de distinguer les mycoplasmes des pseudopodes cytoplasmiques ordinaires d'une part, des particules virus d'autre part.

En ce qui concerne les *éléments pseudo-viraux*, ils se distinguent des *microvillosités*, car celles-ci n'ont pas, en règle générale, les mêmes formes en virgule ou en raquette. Lorsqu'elles sont arrondies, elles n'ont jamais un centre dense et homogène, comme c'est le cas de corpuscles élémentaires (Fig. 8, 3 et Fig. 4, 1), et leur membrane de surface n'est pas séparée du cytoplasme par un espace clair.

Lorsqu'il s'agit de formes filamenteuses, les villosités ont des diamètres plus variables. On n'observe pas de segmentation en chaînette.

D'autre part, la différence essentielle avec une *particule virus* résidera en la ténuité de l'espace compris entre la membrane externe et la zone centrale du 'corpuscule élémentaire', L'espace compris entre la limite du nucléoïde et l'enveloppe du virus est beaucoup plus marquée que dans le cas des 'corpuscles élémentaires'; on comparera la Fig. 3, 1 et 2, à la Fig. 5, 2.

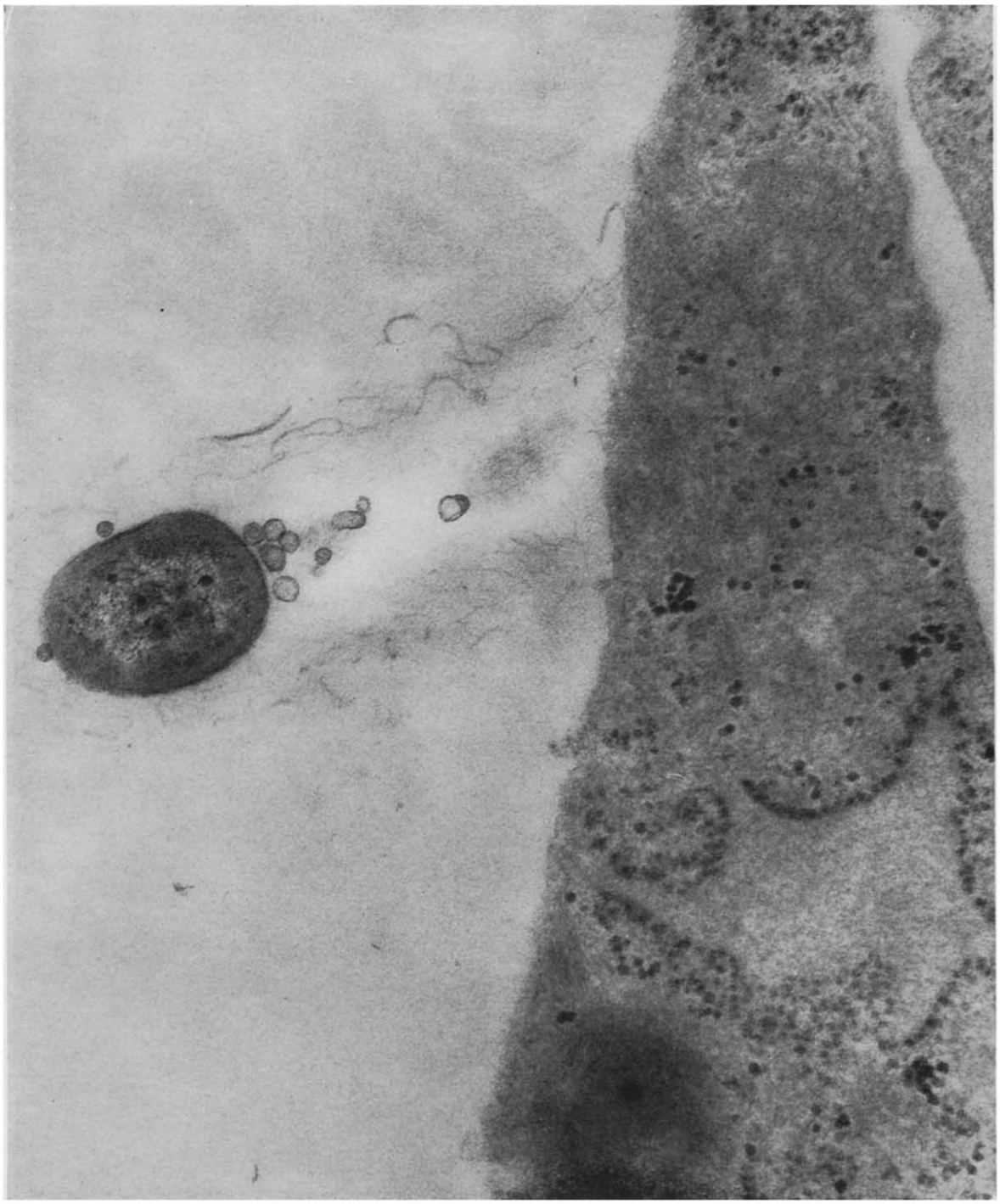


Fig. 1. Mycoplasme salivarium: *élément à structure pseudo-bactérienne. De forme ovalaire, il comporte une plage centrale formée d'un réseau de filaments ténus de type ADN, un cytoplasme périphérique présentant quelques granulations de ribosomes, une membrane de surface à trois feuillets.* (*Gr.* × 67.200).

(to face p. 432)

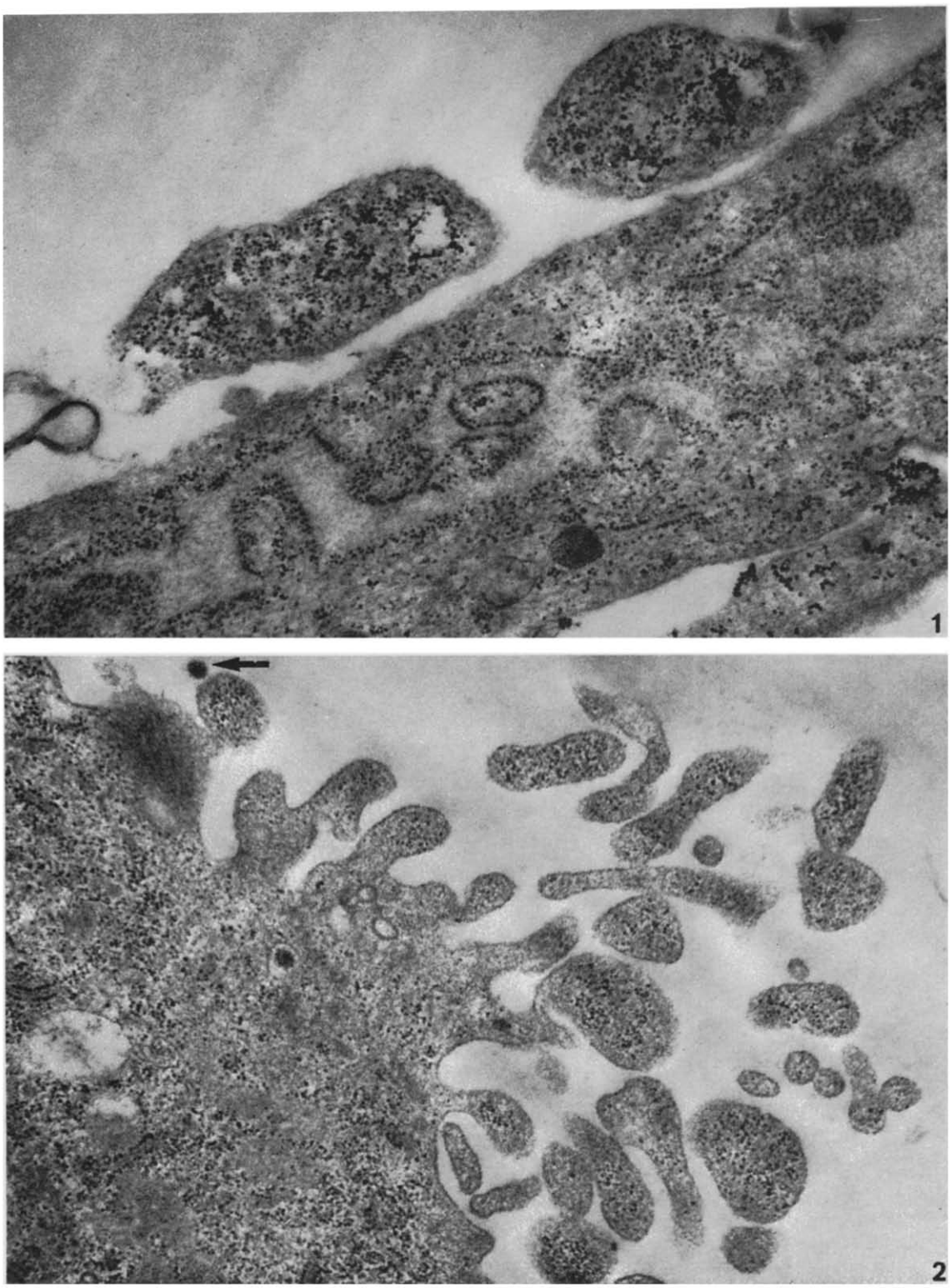


Fig. 2. (1) Mycoplasme salivarium: *eléments de grande taille sans structure nucléaire apparente: on observe deux éléments de forme allongée situés à la surface cellulaire.* (*Gr.* × 70.000).

(2) Mycoplasme orale: *eléments de grande taille sans structure nucléaire dont certains semblent 'bourgeonner' à partir de la membrane cellulaire. La flèche indique un corpuscule élémentaire* (*Gr.* × 35.000).

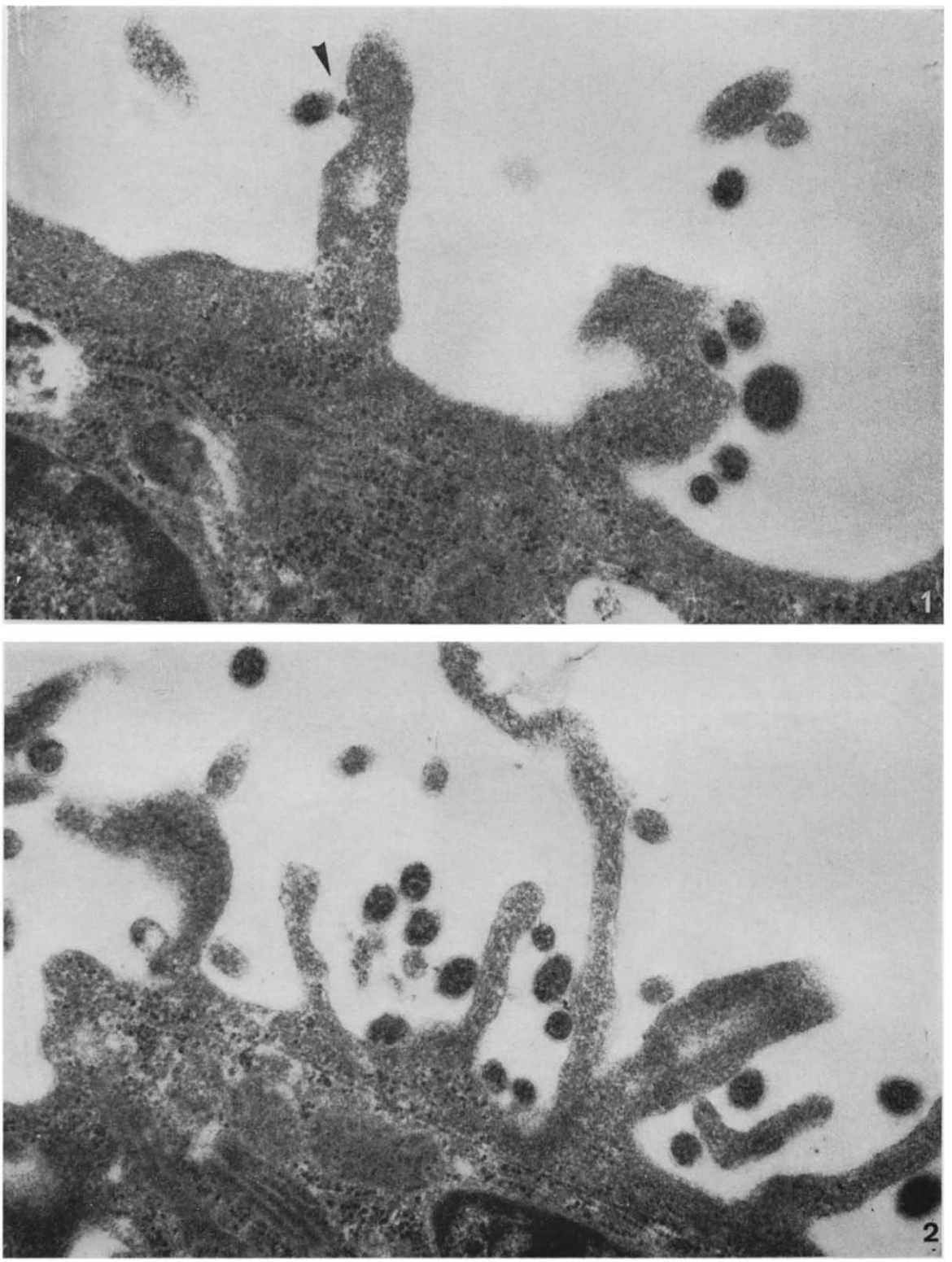


Fig. 3. (1 *et* 2). *Un des aspects caractéristiques des corpuscles élémentaires. On notera la densité de la zone centrale qui contraste avec la membrane externe laquelle apparaît comme un cercle clair très ténu. Comparer avec la zone claire qui entoure la nucléocapside d'un virus et qui est nettement plus large* (*Fig.* 5,2). *On notera sur* (1) *qu'un des corpuscules paraît rattaché à une villosité par une densification en forme de cône. De tels aspects sont fréquents* (1. *Gr.* × 48.000; 2. *Gr.* × 41.800).

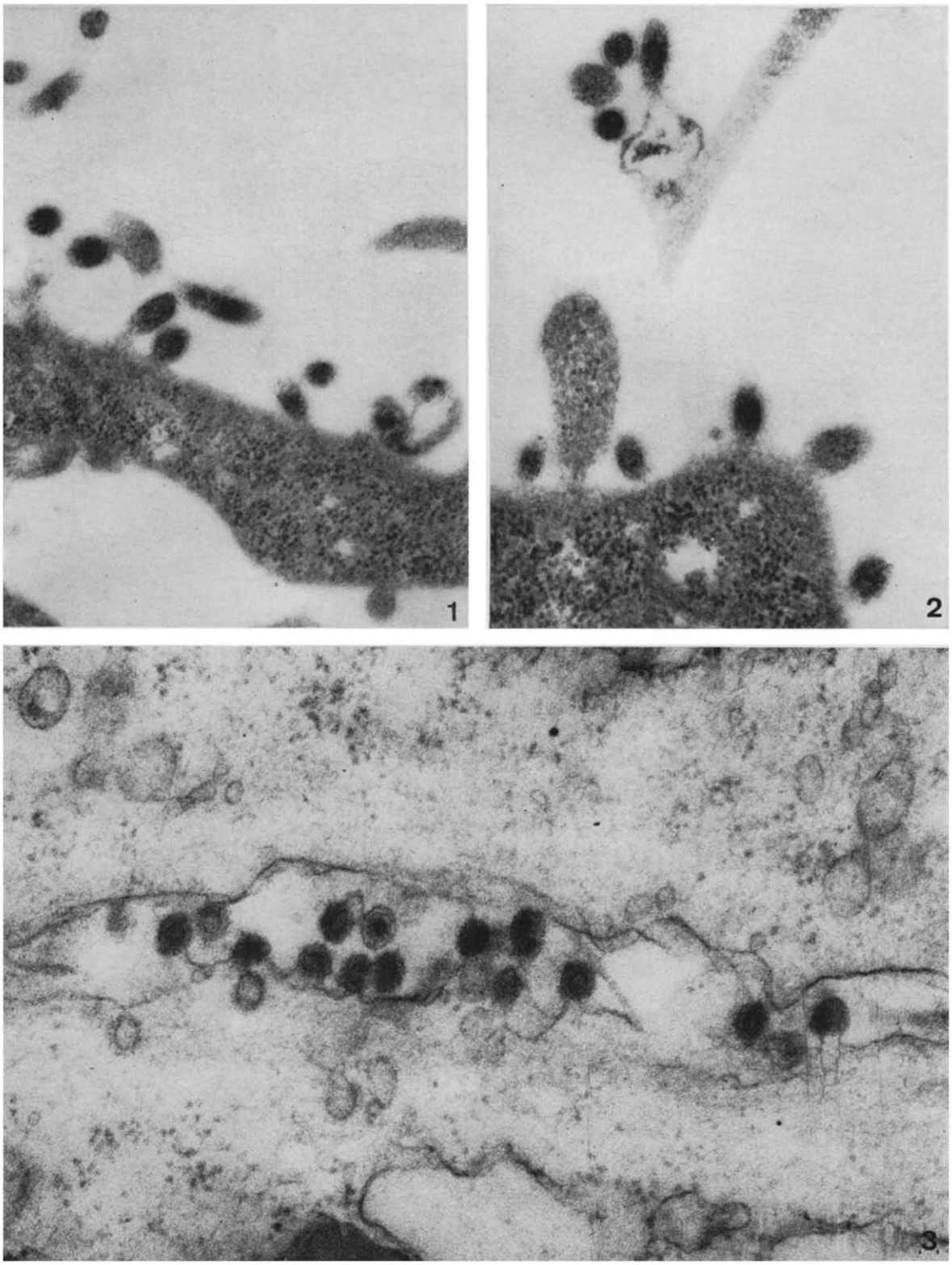


Fig. 4. (1 *et* 2). Mycoplasme orale: *corpuscules élémentaires en contact intime avec la membrane cellulaire. Adsorption ou bourgeonnement? Comparer avec* (3). *Bourgeonnement d'un virus du groupe leucosarcome aviaire* (*Sarcome de Rous*) (1. *Gr.* × 48.600; 2. *Gr.* × 48.400; 3. *Gr.* × 70.400).

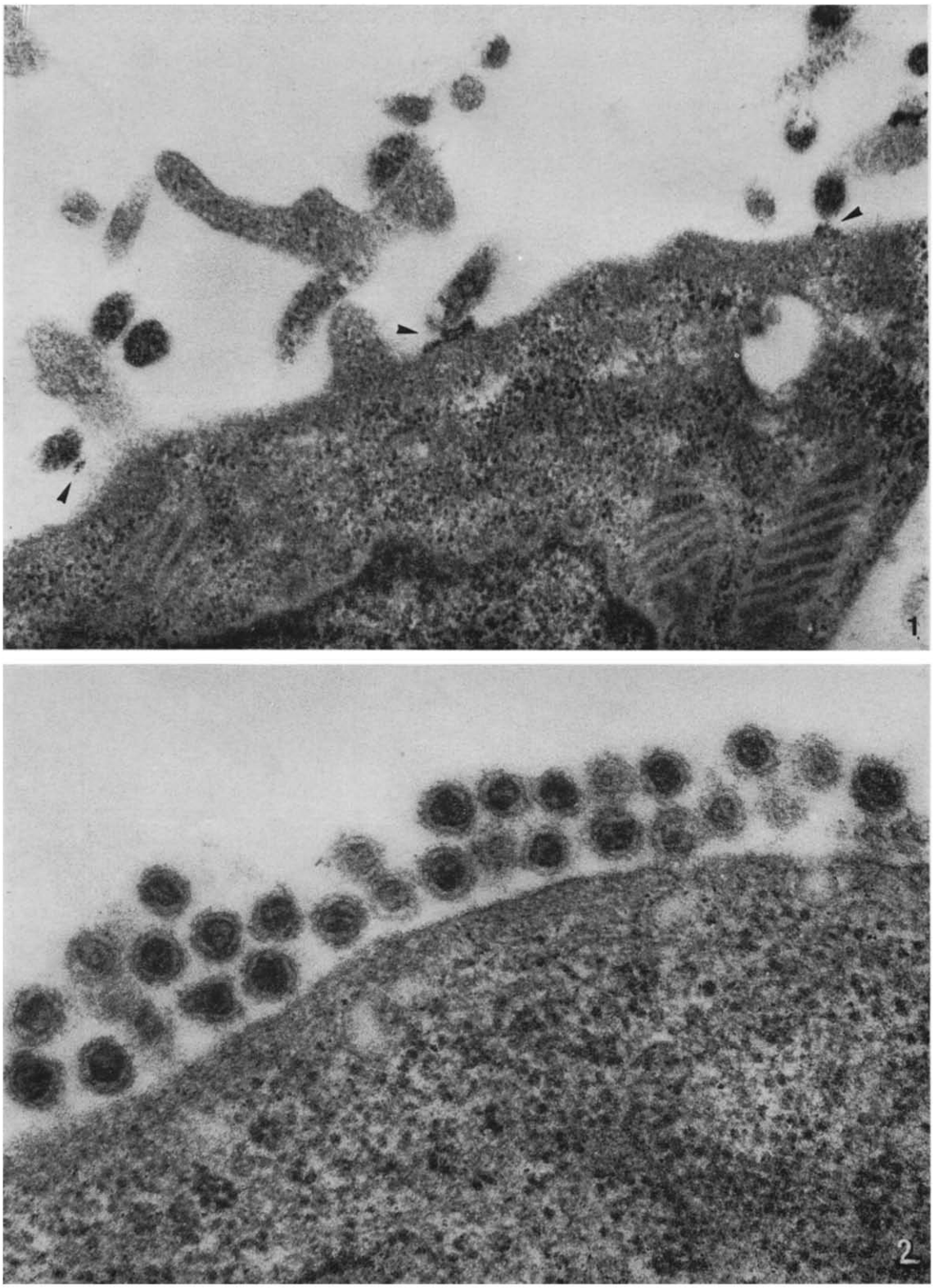


Fig. 5. (1). Mycoplasme orale: *on remarquera, au niveau de la surface cellulaire, l'existence d'une densification située en regard des corpuscles élémentaires* (*Gr.* × 45.000).

(2). *Virus de leucémie murine en culture* (*JLSV*5). *Noter l'épaisseur de la zone claire qui entoure la nucléocapside et comparer avec celle beaucoup plus mince des corpuscles élémentaires* (*Gr.* × 84.000).

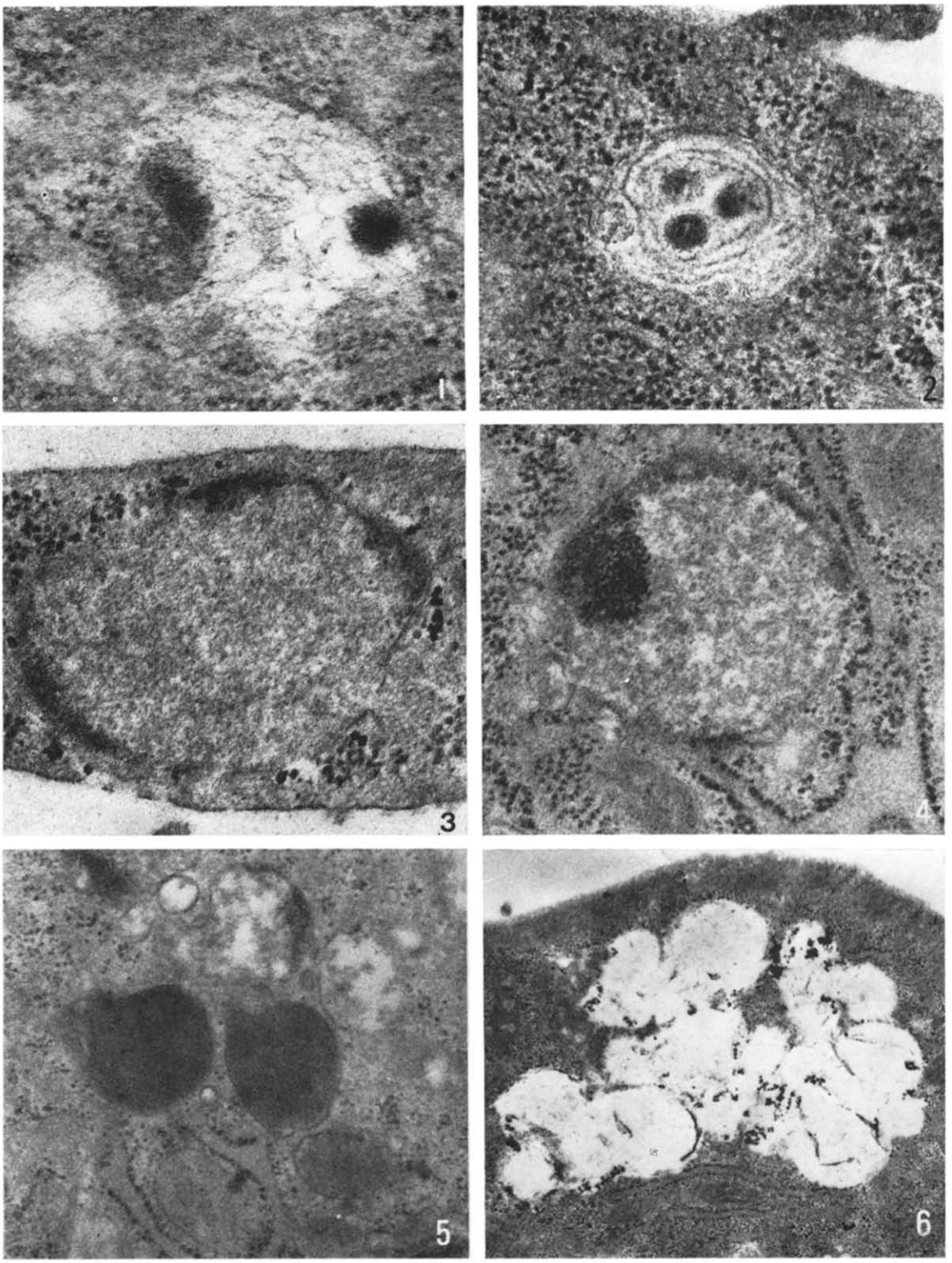


Fig. 6. (1–4). *Images intracellulaires sphériques ou ovalaires d'aspects floconneux et de densité variable* (1–4) *avec des zones osmiophiles allongées le long de la membrane vacuolaire* (1, 3 *et* 4) *et des granulations ressemblant à des corpuscules élémentaires* (2) (1. *Gr.* × 69.000; 2. *Gr.* × 84.000; 3. *Gr.* × 61.600; 4. *Gr.* × 48.600).

(5). *Structures de vacuolaires à côté d'images de lysosomes* (*Gr.* × 35.000).

(6). *Vacuoles en amas à contours polycycliques avec des restes de membrane et de glycogène* (*Gr.* × 20.700).

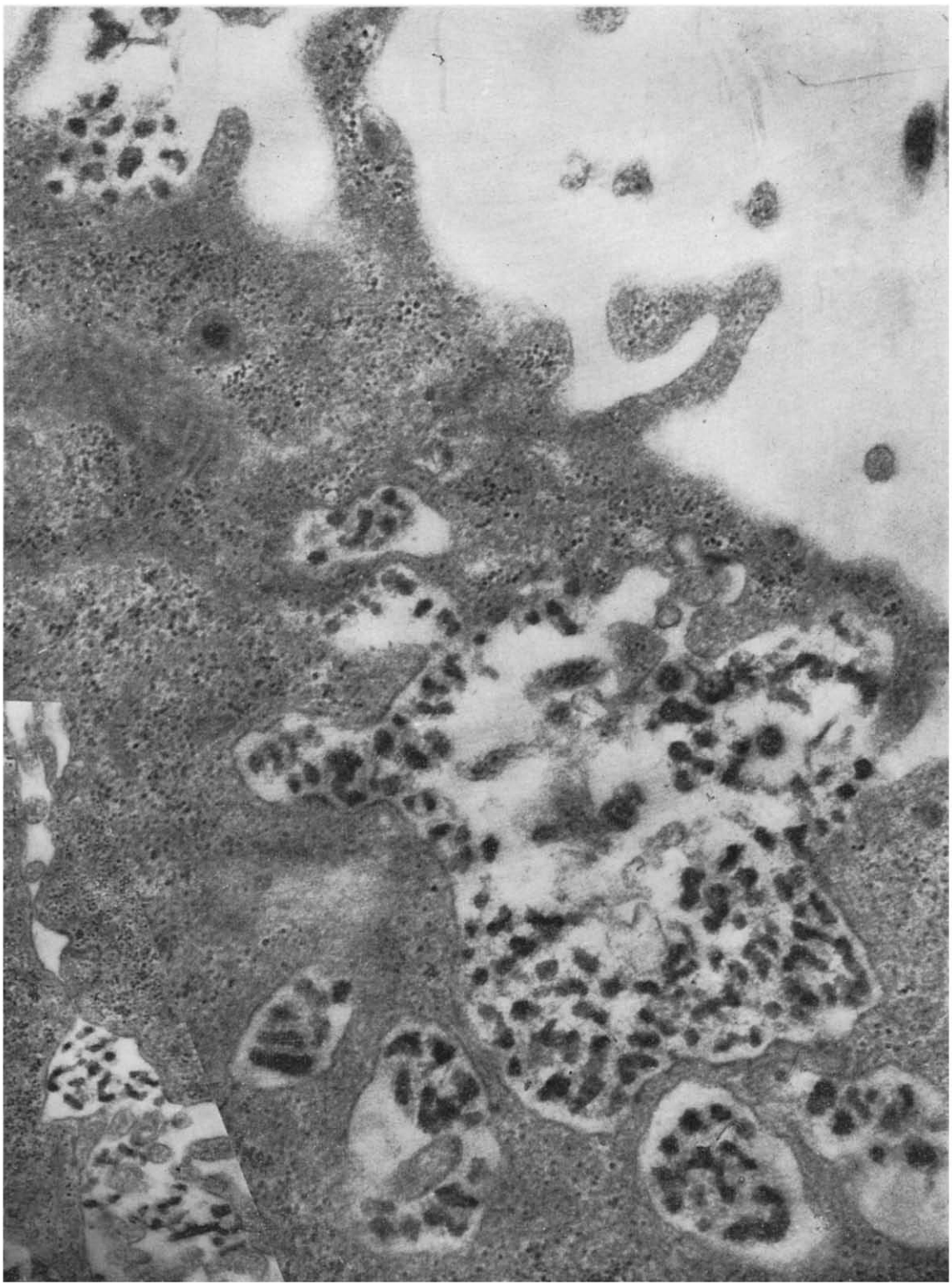


Fig. 7. Mycoplasme orale: *particules élémentaires de forme irrégulière anguleuse, les uns se trouvant à la surface cellulaire, les autres dans des vacuoles cytoplasmiques.* (*Gr.* × 45.000).

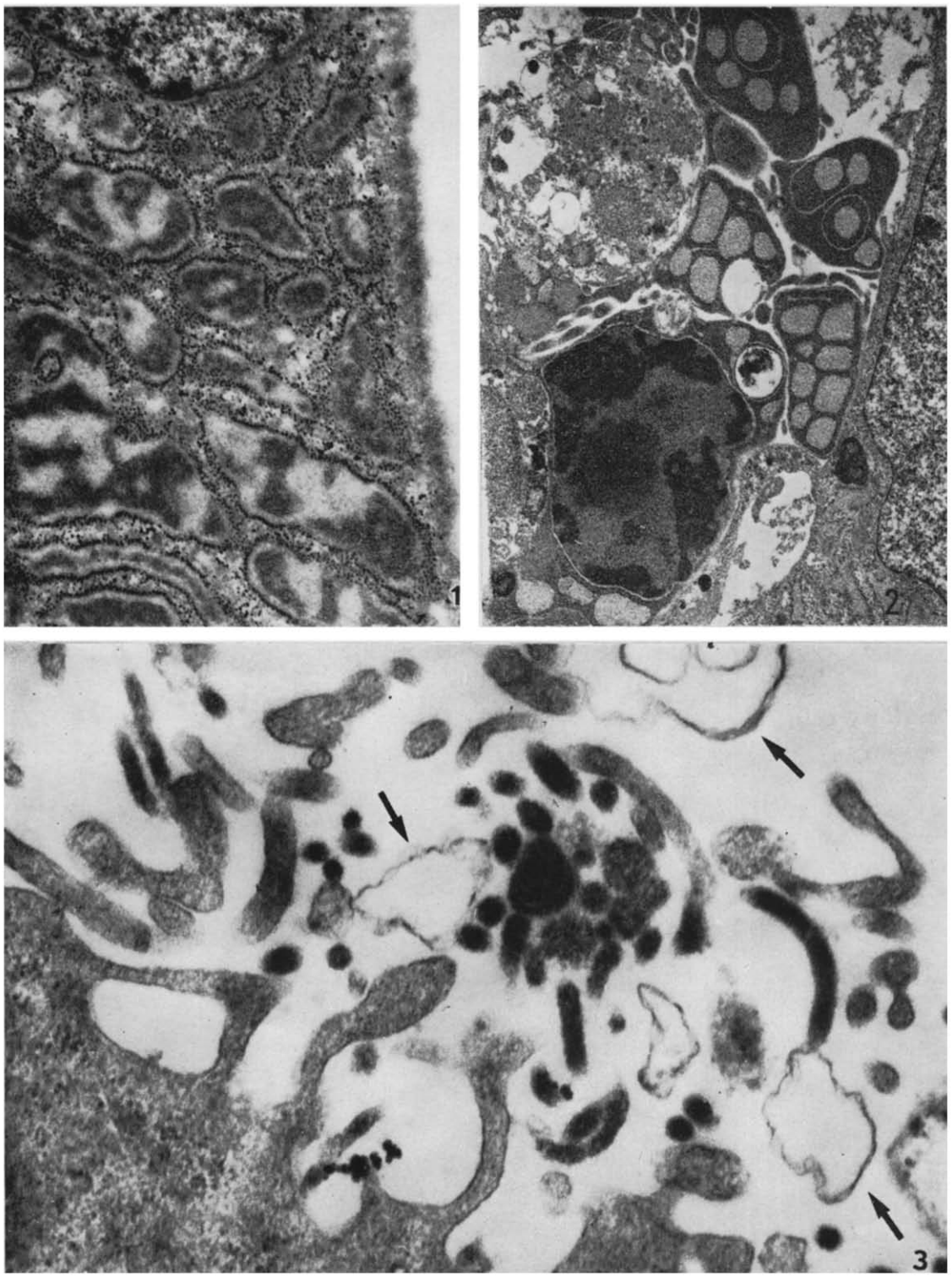


Fig. 8. (1). *Images de citernes ergastoplasmiques dilatées contenant des plages de substance dense* (*Gr.* × 23.400).

(2). *Présence dans l'espace intercellulaire de portions de cytoplasme ergastoplasmique modifié* (*Gr.* × 12.600)

(3). Mycoplasme orale: *corpuscules élémentaires, certains arrondis, d'autres ovalaires, d'autres encore étirés et incurvés. Ils sont entremêlés avec les villosités cellulaires qui sont moins denses. On remarquera* (→) *plusiers 'fantômes' correspondant à des vestiges d'éléments mycoplasmiques de grande taille* (*Gr.* × 42.000)

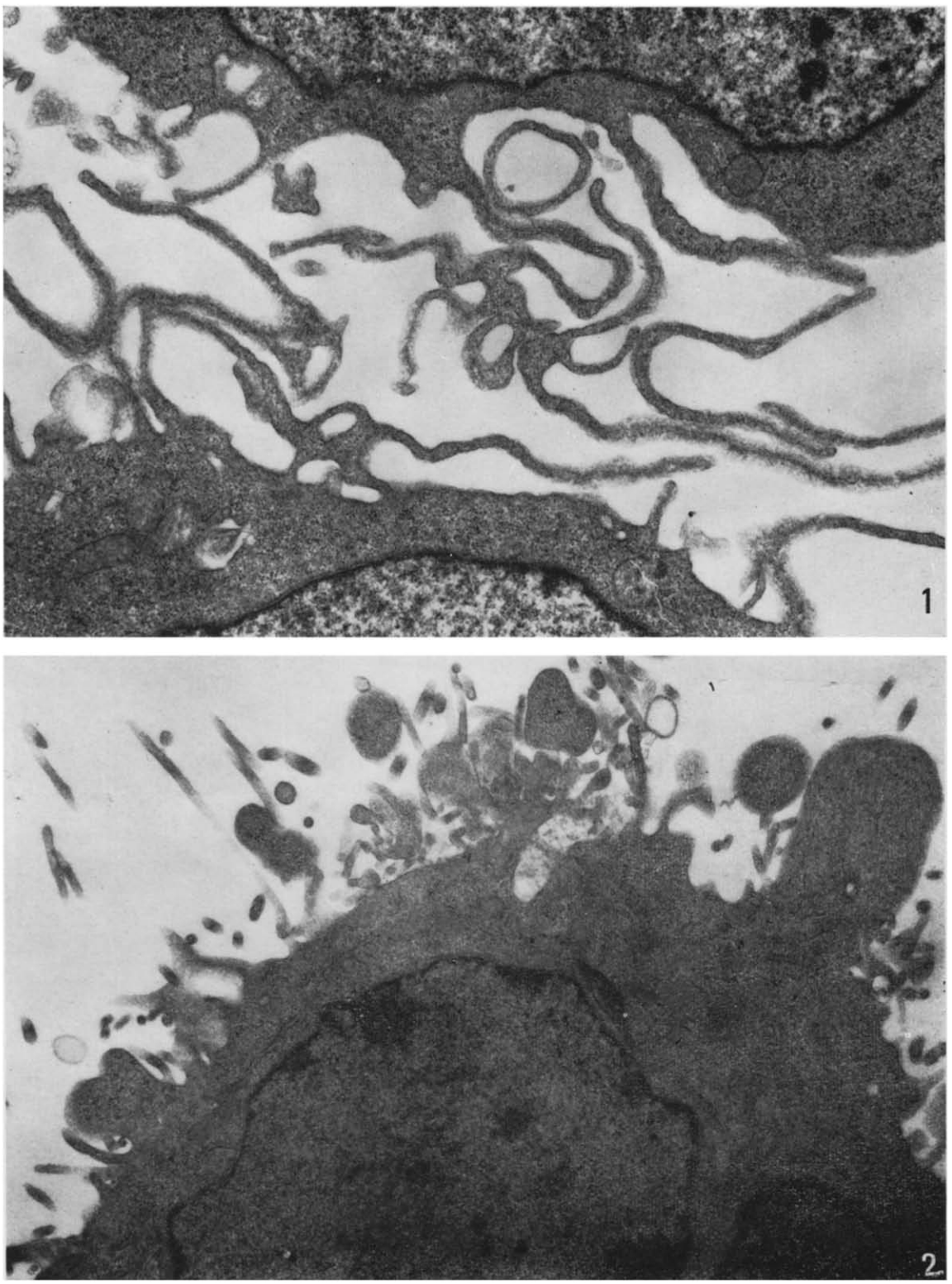


Fig. 9. (1). *Image de microvillosités filiformes à comparer avec la figure suivante.* (*Gr.* × 24.000).
(2). *Image de prolongements pseudopodiques d'une cellule infectée par des mycoplasmes. Noter la base large de certaines de ces protrusions.* (*Gr.* × 32.000).

Il n'est fait allusion ici qu'au cas des virus parvenus à maturité, car la distinction n'est pas à faire avec des virus 'jeunes' où il existe une membrane supplémentaire entre le nucléoïde et l'enveloppe.

L'importance du diagnostic différentiel avec les virus ne saurait être assez soulignée, et le nombre de travaux de microscopie électronique où les auteurs avaient cru identifier des particules virales dans les affections malignes humaines l'indique assez.

C'est le grand mérite d'Anderson [1] et d'Hummeler [3] d'avoir, les premiers, mis en garde contre l'apparence trompeuse au microscope électronique de certaines formes de mycoplasmes, et nous avons nous-mêmes, à l'occasion de nos propres recherches sur les leucémies humaines [27], insisté sur les pièges du diagnostic de 'particules' au microscope électronique [28, 29].

En ce qui concerne les *éléments pseudo-bactériens*, ils se distinguent aussi des pseudopodes et des villosités ordinaires, quoique leur diagnostic différentiel reste souvent très difficile dans le cas où il n'y a pas d'aire nucléaire. Leur forme arrondie et globuleuse est d'abord caractéristique (Fig. 9, 2). Elle n'est pas le fait de pseudopodes habituels qui sont rarement larges à leur base. Une fois émises dans les espaces intercellulaires, ces structures restent plus ou moins arrondies et peuvent présenter deux aspects qui signent leur nature mycoplasmique. Dans le premier cas, ils persistent avec une morphologie cytoplasmique, contenant toujours des ribosomes, très bien conservée. Autrement dit, ils ne dégénèrent pas comme le feraient des fragments de cytoplasme ordinaire, et ils sont même parfois phagocytés par d'autres cellules en restant intacts d'aspect. Dans d'autres cas, ils dégénèrent, et leur structure est alors absolument différente de celle d'un fragment cytoplasmique nécrotique. La portion cytoplasmique centrale disparaît entièrement, et il ne reste plus que le cadre périphérique qui conserve la forme initiale. Ces deux aspects, éléments à morphologie cytoplasmique intacte ou éléments représentés seulement par un 'fantôme' de membrane (Fig. 8, 3) sont semblables aux structure de mycoplasmes identifiées en milieu acellulaire.

En conclusion, il apparaît qu'une étude attentive au microscope électronique permet de distinguer, entre éléments mycoplasmiques, virus et villosités cellulaires, et de découvrir que des éléments mycoplasmiques peuvent simuler des corpuscules virus, non seulement par leur structure même, mais aussi par les rapports remarquables qu'ils peuvent contracter avec la membrane cellulaire.

RESUME

L'ultrastructure de deux souches de mycoplasmes (mycoplasme orale *et* mycoplasme salivarium) *en culture de tissu est étudiée avec la méthode des coupes ultra-fines.*

A côté d'éléments de grande dimension qui peuvent revêtir un aspect pseudo-bactérien avec leur plage centrale nucléaire, des éléments de petite taille, les 'particules élémentaires', revêtent un aspect pouvant simuler des particules virales d'autant plus que ces particules contractent des rapports étroits avec la membrane cellulaire, lesquels évoquent les aspects observés lors de la formation de certains types de virus. Les éléments de diagnostic différentiel au microscope électronique entre particules élémentaires, microvillosités, et particules virales, sont discutés.

SUMMARY

The ultrastructure of two strains of mycoplasmas (M. orale *and* M. salivarium) *has been studied in tissue culture by the ultra-thin section technique.*

This had not been done previously for mycoplasma orale *in spite of its frequent occurrence in human buccal cavity.*

Besides forms of large size similar in appearance to bacterial L forms, small elementary particules are described. These have already been shown by others to resemble virus particles and raise difficult problems in differential diagnosis.

This resemblance is further emphasized here especially since relationship with the cell surface may be compared from a morphological point of view with that observed with myxovirus and some RNA oncogenic virus.

The characteristics which will enable to distinguish between an elementary body, a microvillosity and a virus particle are described.

ZUSAMMENFASSUNG

Die Ultrastruktur von zwei Mycoplasma-Stämmen (M. orale und M. salivarium) in Gewebekultur wurde mit ultrafeiner Schnitt-Technik untersucht. Während größere Elemente mit zentral-nukleären Phänomena pseudobakterielle Bilder zeigten erinnerten kleinere 'Elementarpartikel' an virale Erscheinungsformen. Diese stehen, wie dies bei verschiedenen Virusarten der Fall ist, in engem Kontakt mit der Zellmembran. Die Möglichkeiten der elektronenoptischen Differentialdiagnose zwischen Elementarpartikeln, Mikrovilli und viralen Partikeln werden diskutiert.

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Résultats d'un Essai Thérapeutique Clinique sur une Association Radiothérapie et Chimiothérapie dans les Cancers Broncho-Pulmonaires

GROUPE COOPÉRATEUR d'ESSAIS THÉRAPEUTIQUES SUR LES CANCERS BRONCHO-PULMONAIRES*

Organisation Européenne de Recherche sur le Traitement du cancer (G.E.C.A.)

D'APRÈS certains travaux [1–4], les résultats de la radiothérapie dans les cancers bronchopulmonaires pourraient être meilleurs lorsqu'on lui associe une chimiothérapie. Cela nous a incités à entreprendre un essai thérapeutique pour comparer l'efficacité de la radiothérapie seule et de la radiothérapie associée à une chimiothérapie par la cyclophosphamide.

MATERIEL ET METHODES

Cet essai a commencé le 1er janvier 1963 et s'est terminé le 1er mai 1965. Quatre-vingt-quatorze malades y ont été inclus. Dans chaque hôpital, le traitement à appliquer à chaque malade a été déterminé selon une répartition au hasard. Finalement, 48 malades ont eu une radiothérapie seule, et 46 ont eu radiothérapie + chimiothérapie par la cyclophosphamide.

Un protocole définissait:—

(1) *Les critères d'inclusion dans l'essai.* Malades (a) ayant un cancer bronchique primitif au diagnostic établi avec certitude par l'histologie, ou avec suffisamment de présomption par la radiographie, l'endoscopie et la cytologie pour que le malade soit traité comme tel; (b) de sexe masculin et d'âge inférieur à 72 ans; (c) n'ayant pas reçu de traitement antérieur: chirurgie, radiothérapie ou chimiothérapie; (d) pour lesquels était portée une indication de radiothérapie.

(2) *Les causes d'exclusion.* (a) les cas chirurgicaux d'emblée; (b) les formes cavitaires et massives; (c) les cas avec épanchement pleural important; (d) les compressions caves; (e) l'existence de métastases; (f) l'association à une autre affection grave (infarctus du myocarde) ou à un autre cancer; (g) des conditions socio-psychologiques qui permettaient de prévoir que le traitement ne serait pas suivi régulièrement et complètement; (h) un état

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clinique tel que le malade ne pouvait pas supporter le traitement le plus agressif.

En tenant compte de toutes ces causes d'exclusion, on comprend que, pendant la période d'étude, 94 malades, soit 26% des cancers broncho-pulmonaires hospitalisés, entrèrent dans l'essai.

(3) *Les modalités de traitement.* Le principe général était le suivant: dans les deux séries, on faisait une radiothérapie aussi semblable que possible, à dose fixe et suffisante pour avoir une visée "cancéricide"; dans la série à traitement mixte, on ajoutait à la radiothérapie "ce que l'on pouvait de cyclophosphamide", dans les limites de tolérance clinique et hématologique. La dose totale de cyclophosphamide reçue était donc variable pour chaque malade.

(a) *radiothérapie:* il était administré une dose de 6.000 rads. à la tumeur (ou 5.500 rads si aucune correction n'était faite pour le poumon), à raison de 800 rads par semaine.

Des échanges réguliers avaient lieu entre radiothérapeutes et physiciens de chaque centre, de façon à standardiser les techniques et à confronter les procédés de dosimétrie.

(b) *administration de cyclophosphamide:* en fonction de la tolérance, on s'efforçait de se rapprocher du schéma suivant: 100 mg par jour, pendant 5 jours, durant la première semaine, puis 5 mg par jour, 5 jours par semaine, en injection intraveineuse lente, jusqu'à la fin de la période d'irradiation. L'injection était faite une heure avant la séance d'irradiation.

(c) *interruption du traitement:* elle était décidée en fonction de la chute des globules blancs:

—au-dessous de 3.000 globules blancs, on arrêtait la chimiothérapie, on continuait la radiothérapie; au-dessous de 2.000 globules blancs, on arrêtait la radiothérapie à son tour;

—on attendait la remontée leucocytaire qui, en principe, était assez rapide; au-dessus de 2.000 globules blancs, on reprenait la radiothérapie seule; à partir de 3.000, on reprenait la radiothérapie et la chimiothérapie à demi-dose;

—en cas de remontée leucocytaire trop lente (supérieure à 10 jours), on abandonnait définitivement la chimiothérapie.

(4) *Les modalités de surveillance.* Dans chaque service, un médecin responsable était chargé de conduire l'essai en liaison avec les médecins du service. Ce médecin responsable pratiquait, avant tout traitement, un bilan comportant un examen clinique, radiologique, endoscopique et hématologique.

Pendant le traitement, on faisait, chaque semaine au moins, un examen hématologique de surveillance, un examen clinique et une radiographie. En fin de traitement, un bilan complet, semblable au bilan initial, devait être fait. Puis les malades étaient revus régulièrement tous les 2 mois, pendant un semestre, tous les 3 mois, le semestre suivant, puis tous les 6 mois. Lorsqu'un malade ne se présentait pas à l'un des examens de surveillance on essayait d'avoir des nouvelles par le médecin traitant.

RESULTATS

1. *Comparabilité des malades des deux groupes*

(1) Nous avons d'abord vérifié qu'au moment de leur entrée dans l'essai les 2 groupes de malades étaient comparables quant à divers caractères tels que:

(a) l'*âge.* L'âge des malades s'échelonne de 37 ans à 72 ans;
l'âge moyen est, pour le groupe radiothérapie, 61 ans; pour le groupe radiothérapie + chimiothérapie, 60 ans.

La différence entre ces âges moyens n'est pas significative.

(b) *l'histologie.* Nous avons distingué 4 catégories: cancers épidermoïdes; cancers anaplasiques (à petites cellules et à grandes cellules) adénocarcinomes et autres variétés; pas de signes histologiques certains de nalignité (Tableau 1).

La répartition des catégories histologiques, dans les 2 groupes thérapeutiques, n'est pas significativement différente.

(c) *le coefficient de gravité.* Dans une étude pronostique en cours*, on a pu mettre en évidence un coefficient de gravité représentant une synthèse de l'information pronostique pour chaque malade. Ce coefficient tient compte des signes fonctionnels, des signes généraux, des extensions locales et à distance et de l'étendue des lésions appréciée sur la radio et en bronchoscopie. Il s'échelonne de 1 à 15. Il nous a paru opportun de comparer tout d'abord la gravité du groupe inclus dans l'essai à celui des malades vus dans les mêmes hôpitaux, mais exclus de l'essai, ceci en tenant compte des modalités thérapeutiques. Le tableau 2 montre que la "gravité" des malades de l'essai se situe entre celle des malades qui ont été opérés et celle des malades pour lesquels on a fait de la

*Résultats d'une enquête inter-hospitalière sur les cancers bronchiques primitifs: travail d'un Groupe d'hôpitaux de la région parisienne. A paraître.

Tableau 1.* *Répartition de l'histologie dans les deux groupes thérapeutiques. La répartition des variétés histologiques dans les 2 groupes est la même*

Histologie	Traitement	
	Radiothérapie	Radiothérapie + Chimiothérapie
Epidermoïdes	46% (22)	51% (24)
Anaplasiques	21% (10)	17% (8)
Autres cancers (adénocarcinomes . . .)	25% (12)	11% (4)
Pas de signes histologiques certains de malignité	8% (4)	21% (10)
Nombre total de malades	100% (48)	100% (46)

*Dans tous les tableaux, les chiffres entre parenthèses indiquent le nombre de cas.

chimiothérapie ou un traitement symptomatique.

Nous avons ensuite comparé la gravité des malades inclus dans l'essai selon qu'ils étaient dans le groupe avec radiothérapie seule ou radiothérapie + chimiothérapie. Dans le premier groupe, le coefficient de gravité est de 6,7, et, dans le second, il est de 6,0. La différence n'est pas significative. Hormis le traitement qu'on allait leur appliquer, les 2 groupes de malades étaient comparables pour les critères essentiels.

2. *Comparaison des traitements*

Pour des raisons diverses, et notamment une mauvaise tolérance clinique ou hématologique, le traitement a dû être interrompu chez certains malades. Nous avons donc étudié les traitements effectivement reçus dans les 2 groupes. Dans le groupe radiothérapie seule, la dose moyenne reçue est de 5.435 rads-tumeur, pendant une durée moyenne de 44,1 jours. Dans le groupe radiothérapie + chimiothérapie, la dose moyenne reçue est de 5.392 rads-tumeur, pendant une durée moyenne de 41 jours. Il n'y a pas de différence entre les doses d'irradiation reçues dans les 2 groupes. En ce qui concerne les doses de cyclophosphamide du deuxième groupe, chaque malade devait recevoir, selon le protocole prévu, 5,5 g en moyenne du produit, au total. Sur 46 malades, il y a 35 malades qui ont reçu la dose prévue, et 11 malades qui ont reçu moins de 5 g au total, du fait de signes de toxicité hématologique.

Remarquons, en ce qui concerne ce deuxième groupe, que, s'il avait effectivement reçu moins de rayons que le premier groupe, cela n'aurait pas faussé la comparaison des 2 traitements; l'essai, en effet, se plaçait sur un plan pragmatique, la question posée étant la suivante: y-a-t-il une différence entre radiothérapie à une certaine dose et entre radiothérapie plus cyclophosphamide à des doses variables, selon la tolérance du malade?

Tableau 2. *Coefficient de gravité dans les différentes catégories thérapeutiques*

Catégorie thérapeutique		Coefficient de gravité moyen
Malades chirurgicaux		3,8
Malades ayant eu radiothérapie	Inclus dans l'essai	6,5
	Exclus de l'essai pour des raisons prévues au protocole	7,8
Malades n'ayant eu que de la chimiothérapie ou un traitement symptomatique		8,4

3. *Comparaison de l'évolution immédiate des 2 groupes*

Elle a été jugée sur les données de l'examen radiologique et de la bronchoscopie.

(1) *L'évolution radiologique.* Elle a été appréciée sur des clichés standard, de face et de profil, faits les jours précédant le début du traitement, et immédiatement après la fin de ce traitement. La lecture des clichés et l'appréciation de l'évolution ont été faites par des médecins de chacun des services participants, qui avaient été réunis à cette occasion et qui jugeaient sans connaître la catégorie thérapeutique.

Le jugement porté pour chaque malade était: amélioration, état stationnaire, aggravation. Dans l'analyse statistique, nous avons groupé les états stationnaires et aggravations d'une part, et isolé les améliorations d'autre part. Les résultats figurent dans le tableau 3.

Il y a 70% d'amélioration dans le groupe radiothérapie + chimiothérapie et 58% dans le groupe radiothérapie seule. Cette différence n'est pas significative.

L'évolution radiologique, en fonction de l'histologie, a été étudiée. Il n'y a pas non plus de différence dans les deux groupes thérapeutiques quand on étudie séparément les carcinomes épidermoïdes et les carcinomes anaplasiques.

(2) *L'évolution bronchoscopique.* Elle a été mesurée par le même médecin faisant une bronchoscopie avant et après le traitement. Il n'était pas spécifié dans le protocole que le bronchoscopiste devait rester dans l'ignorance de l'existence ou non d'une chimiothérapie. En pratique, les conditions dans lesquelles se sont déroulés ces examens laissent penser que le jugement de l'observateur a été porté indépendamment du traitement réalisé.

Il n'y a que 35 malades qui ont en une bronchoscopie en fin de traitement et, pour ceux-ci, les résultats sont les suivants:

Il y a 83% d'amélioration bronchoscopique dans le groupe radiothérapie + chimiothérapie, et seulement 47 % dans le groupe radiothérapie. *La différence est significative.*

Tableau 3. Evolution radiologique dans les 2 groupes thérapeutiques

Evolution	Traitement		Signification
	Radiothérapie (48 cas)	Radiothérapie + Chimiothérapie (46 cas)	
Amélioration	58% (28)	70% (32)	NS
Stationnaire et aggravation	42% (20)	30% (14)	

4. *Comparaison de la tolérance clinique et hématologiqie:*

Nous avons enregistré, pour chaque malade, les signes cliniques et hématologiques d'intolérance.

Les signes cliniques d'intolérance ont été

Tableau 4. Evolution bronchoscopique dans les 2 groupes thérapeutiques

Evolution	Traitement		Signification*
	Radiothérapie (17 cas)	Radiothérapie + Chimiothérapie (18 cas)	
Amélioration	47% (8)	83% (15)	+
Stationnaire et aggravation	53% (9)	17% (3)	

*$p=0{,}04$.

nombreux et plus marqués dans le groupe qui recevait de la cyclophosphamide. Il s'agissait surtout de vomissements et nausées, mais leur intensité n'a jamais fait interrompre le traitement.

Les signes hématologiques de toxicité ont été plus marqués dans le groupe radiothérapie + chimiothérapie, bien que les différences en ce qui concerne les globules blancs et les plaquettes ne soient pas significatives (tableaux 5 et 6). Il est à noter, pour les plaquettes notamment, que les examens n'ont pas pu être faits régulièrement chez tous les malades.

5. *Comparaison des taux de survie*

On a pris, comme origine de survie, la date du bilan fait au début du traitement. Le point a été fait du 1er juillet 1967, date à laquelle tous les malades avaient au moins 24 mois de recul.

Le tableau 7 résume les pourcentages de survie de 6 mois, 12 mois, 24 mois et 36 mois. Il n'y a aucune différence significative entre les deux groupes, tout au moins avec les effectifs, assez faibles, que nous avons. Cependant, les pourcentages sont tellement semblables que nous ne pensons pas qu'avec plus de sujets nous aurions trouvé une différence importante.

Par ailleurs, nous n'avons trouvé aucune liaison entre l'évolution immédiate et la survie: les les malades, dont la bronchoscopie et la radiologie ont montré une amélioration immédiate, n'ont pas une meilleure survie. Il y a donc une discordance entre les résultats immédiats et lointains.

DISCUSSION

Evolution immédiate

Au premier abord, les résultats de l'évolution radiologique et ceux de l'évolution bronchoscopique ne sont pas semblables. Alors qu'il n'y a pas de différence dans l'évolution radiologique entre les 2 groupes, la différence est significative en ce qui concerne l'évolution bronchoscopique qui est la meilleure dans le groupe ayant eu radiothérapie + chimiothérapie.

Cependant, la bronchoscopie de contrôle n'a pas été faite à tous les malades, et on peut se demander si les meilleurs résultats bronchoscopiques observés pour le groupe radiothérapie + chimiothérapie ne sont pas l'expression d'un biais: en effet, dans le groupe ayant eu radiothérapie + chimiothérapie, on peut penser que la bronchoscopie n'aurait été faite que chez les malades peu éprouvés par le

Tableau 5. *Comparaison du taux des globules blancs dans les* 2 *groupes thérapeutiques*

Globules blancs*	Traitement		Signification
	Radiothérapie	Radiothérapie + Chimiothérapie	
Globules blancs supérieurs à 3000 par mm³	97% (38)	75% (33)	NS
Globules blancs inférieurs à 3000 par mm³	3% (1)	25% (11)	
Total	100% (39)	100% (44)	

*Valeurs les plus basses observées pendant et après le traitement.

Tableau 6. *Comparaison du taux de plaquettes dans les* 2 *groupes thérapeutiques.*

Plaquettes*	Traitement		Signification
	Radiothérapie	Radiothérapie + Chimiothérapie	
Taux supérieur à 120.000	85% (17)	79% (26)	NS
Taux inférieur à 120.000	15% (3)	21% (7)	
Total	100% (20)	100% (33)	

*Valeurs les plus basses observées pendant et après le traitement.

Tableau 7. Survie dans les 2 groupes.

6 mois		12 mois		24 mois		36 mois	
R	R+C	R	R+C	R	R+C	R	R+C
63% (48)	67% (46)	31% (48)	35% (46)	10% (48)	13% (46)	2% (40)	6% (33)

Les chiffres en bas et à droite de chaque case indiquent le nombre de malades qui avaient le recul suffisant.

traitement et améliorés quant à l'évolution de leur cancer. Dans le groupe radiothérapie seule, les malades sont en général moins fatigués par leur traitement, et la bronchoscopie aurait été faite aussi bien aux malades ayant une amélioration ou une aggravation de leur cancer; et de fait, comme le montre le tableau 8, parmi les malades ayant eu une bronchoscopie, il y a plus d'améliorations radiologiques (94%) dans le groupe radiothérapie + chimiothérapie que dans le groupe n'ayant eu que la radiothérapie (58%).

Pour éliminer ce biais possible, les faits suivants sont à noter: (a) il y a le même nombre de bronchoscopies faites dans les 2 groupes thérapeutiques, soit 17 dans le groupe ayant eu une radiothérapie seule, et 18 dans celui qui a eu radiothérapie + chimiothérapie; (b) s'il y avait eu une sélection des malades ayant eu une bronchoscopie en fin de traitement, portant sur le groupe ayant eu radiothérapie et chimiothérapie, le coefficient de gravité devrait être différent, plus faible que celui des autres malades. Or, il se trouve que, tant au début du traitement qu'à la fin, le coefficient de gravité moyen du groupe radiothérapie + chimiothérapie avec bronchoscopie en fin de traitement est le même que celui du groupe ayant eu la radiothérapie seule, avec bronchoscopie en fin de traitement. Ces 2 coefficients ne sont eux-mêmes pas différents de ceux des 2 groupes qui n'ont pas eu de bronchoscopie en fin de traitement. Etant donné qu'il ne semble pas y avoir de biais, on peut dès lors isoler les malades ayant eu une bronchoscopie en fin de traitement, et on étudiera l'évolution des deux critères radiologiques et endoscopiques.

1. *Comparaison de l'évolution radiologique, en tenant compte de l'évolution bronchoscopique.* Aussi bien parmi les aspects bronchoscopiques améliorés que parmi les aspects bronchoscopiques non améliorés, il y a plus d'améliorations radiologiques dans le groupe avec radiothérapie + chimiothérapie, et la différence est significative ($P=5\%$). Donc, quelle que soit l'évolution bronchoscopique, il y a plus d'améliorations radiologiques dans le groupe radiothérapie + chimiothérapie.

2. *Comparaison de l'évolution bronchoscopique en tenant compte de l'évolution radiologique.* Aussi bien parmi les aspects radiologiques améliorés que parmi les aspects radiologiques non améliorés, il y a plus d'améliorations bronchoscopiques dans le groupe radiothérapie + chimiothérapie. Donc, quelle que soit l'évolution radiologique, l'aspect endoscopique est plus souvent améliorée dans le groupe radiothérapie + chimiothérapie.

En conclusion, chacune des évolutions a une valuer propre indépendante pour comparer les 2 traitements. Parmi les 35 malades qui ont eu une bronchoscopie en fin de traitement, on peut résumer l'évolution immédiate en combinant, dans chaque groupe, le nombre d'améliorations. On compte 0 amélioration lorsqu'il n'y en a aucune, ni radiologique ni bronchoscopique; 1 amélioration lorsqu'elle a été ou radiologique ou bronchoscopique; 2 améliorations lorsqu'elle a été radiologique et bronchoscopique. Le tableau 9 résume cette information.

Finalement donc, l'amélioration, plus fréquente dans le groupe radiothérapie + chimio-

Tableau 8. Comparaison des pourcentages d'améliorations radiologiques selon que la bronchoscopie a été faite ou non

Bronchoscopie	Traitement	
	Radiothérapie	Radiothérapie + Chimiothérapie
Bronchoscopie faite	58% (10/17)	94% (17/18)
Bronchoscopie non faite	58% (18/31)	53,5% (15/28)

thérapie, est acquise dans les cas avec bronchoscopie faite en fin de traitement, cela probablement à la fois radiologiquement et à l'endoscopie.

La survie

La comparaison des taux de survie à 6 mois, 1 an, 2 ans et 3 ans, montre qu'il n'y a absolument aucune différence entre les 2 groupes. Rappelons que ces résultats ne valent que pour une certaine chimiothérapie, à savoir la cyclophosphamide, administrée seule, à une certaine dose, et pendant une période de 6 semaines en moyenne.

Certains travaux (4) ont trouvé des résultats différents. En utilisant du 5-fluoro-uracile, on a amélioré la survie du groupe avec chimiothérapie. Alors que, pour le groupe avec radiothérapie seule, la survie moyenne est de 6,2 mois $\pm 1{,}2$; dans le groupe radiothérapie + chimiothérapie, la survie moyenne est de 16,8 mois $\pm 5{,}1$. D'autres auteurs [5–8], en utilisant du 5-fluoro-uracile ont trouvé des résultats variables. Dans certains de ces derniers travaux cependant, la répartition des malades dans un groupe thérapeutique n'a pas été faite au hasard, ce qui enlève beaucoup de valeur aux résultats.

Un travail plus récent [9] comparant la radiothérapie seule à une association de radiothérapie +5-fluoro-uracile et radiothérapie +actinomycine, n'a pas montré de différence entre les 3 groupes.

D'autres travaux, concernant une association d'autres produits, n'ont pas mis de différence en évidence: les produits utilisés ont été soit une moutarde azotée [10], soit de la méchlorethamine [11], soit de la cyclophosphamide [12].

En conclusion, il semble qu'on puisse attribuer à l'association radiothérapie + cyclophosphamide, telle que nous l'avons faite, un avantage dans l'évolution immédiate de la tumeur, mais pas d'amélioration de la survie.

Cette discordance, qui pourrait paraître curieuse, est confirmée par l'absence de liaison entre l'évolution immédiate de la tumeur et la survie.

Ces résultats sont, dans l'ensemble, concordants avec les travaux récents d'autres auteurs [9]. Ils encouragent à étudier d'autres modes d'association de la chimiothérapie à la radiothérapie, à la fois quant à la nature des produits chimiques à utiliser, leur temps d'administration, leur chronologie par rapport à la radiothérapie.

Tableau 9. *Résumé de l'évolution immédiate dans les* 2 *catégories thérapeutiques*

Evolution	Traitement		Signification*
	Radiothérapie	Radiothérapie + Chimiothérapie	
2 améliorations	29 % (5)	78 % (14)	+++
0 et 1 amélioration	71 % (12)	22 % (4)	

*+++ : $p \leqslant 0{,}001$.

RESUME

Nous rapportons les résultats d'un essai thérapeutique clinique sur les cancers bronchiques primitifs. On a comparé l'évolution selon que le traitement comportait une irradiation seule ou une irradiation associée à une chimiothérapie.

Quatre-vingt-quatorze malades ont été inclus dans cet essai et ont été répartis pas tirage au sort en deux groupes: un groupe a reçu une irradiation de haute énergie, à une dose totale de 6.000 *rads, étalée sur* 6 *semaines; l'autre groupe a reçu la même irradiation associée à la cyclophosphamide, cette dernière étant administrée chaque jour, avant la séance d'irradiation, à raison de* 200 *mg par voie intraveineuse.*

Les résultats immédiats sur la tumeur, appréciés par l'examen radiologique et la bronchoscopie, sont meilleurs dans le groupe ayant eu une irradiation associée à la chimiothérapie. C'est dans ce groupe qu'on été observés des signes d'intolérance, digestifs et hématologiques, plus marqués. Ces effets toxiques n'ont cependant pas été très importants et ont eu une durée relativement limitée.

La comparaison des deux groupes, en ce qui concerne la survie, ne montre aucune différence. Ainsi, la survie à 2 ans est de 10% dans le groupe ayant eu de la radiothérapie seule, et de 13% dans le groupe ayant eu de la radiothérapie associée à la chimiothérapie.

Par ailleurs, la survie des malades ayant eu une régression tumorale immédiate importante n'est pas meilleure que celle des malades dont la tumeur n'avait pas régressé en fin de traitement.

Ces résultats encouragent à étudier d'autres modalités d'association de radiothérapie et de chimiothérapie.

SUMMARY

The results of a controlled therapeutic trial in primary bronchogenic carcinoma are presented. A comparison of the disease evolution is made according to the fact that patients were treated by means of radiotherapy alone, or cyclophosphamide in combination with radiotherapy.

Ninety-four patients were included in the trial and divided by randomization into two groups: the former was given a high voltage irradiation until a total accumulated dose of 6000 rad. over a period of 6 weeks, and the latter had the same irradiation plus cyclophosphamide consisting of 200 mg per day injected intravenously before daily irradiation.

The immediate results on the tumour, estimated by means of radiological examination and bronchoscopy were better in the group which received combined treatment. In this group, hematological and digestive side effects were more marked than in the other group. Those toxic reactions were however not very severe and their duration relatively short.

As far as survival is concerned, a comparison between the two groups showed no significative difference. Two-year survival rate: 10% in the group treated by irradiation only, compared to 10% in the other group.

Moreover, the survival of patients who showed an immediate important tumour regression was not better than the survival of patients whose tumour showed no regression at the end of treatment.

These results are an encouragement to study other combinations of radiotherapy and chemotherapy.

ZUSAMMENFASSUNG

Es werden Ergebnisse einer therapeutisch-klinischen Untersuchung mit primären Bronchialkarzinomen beschrieben. Ein Vergleich des Krankheitsverlaufs wird durchgeführt bei Patienten, die entweder nur mit Radiotherapie oder mit Radiotherapie und Chemotherapie behandelt wurden.

In diese Untersuchung wurden 94 Patienten einbezogen und durch Auslosung in zwei Gruppen geteilt: die erste Gruppe bekam eine Hochenergiebestrahlung mit einer Totaldosis von 6000 rad, auf 6 Wochen verteilt; die zweite Gruppe bekam die gleiche Bestrahlung verbunden mit einer täglichen Dose von 200 mg Cyclophosphamid, intravenös, vor der Bestrahlung.

Die sofortigen Wirkungen auf den Tumor, durch radiologische und bronchoskopische Untersuchungen beurteilt, sind besser in der Gruppe, die eine Bestrahlung verbunden mit einer Chemotherapie erhalten hat. In dieser Gruppe wurden aber Verdauungs—und hämatologische Störungen beobachtet. Diese toxischen Wirkungen waren jedoch nicht sehr bedeutend und von relativ begrenzter Dauer.

Der Vergleich des Überlebens an beiden Gruppen zeigt keinen Unterschied: zweijähriges Überleben wurde bei 10% in der Gruppe, die nur mit Radiotherapie behandelt wurde, und bei 13% in der mit den zwei Therapien festgestellt. Ausserdem ist das Überleben der Patienten, die eine sofortige tumorale Regression hatten, nicht besser als der Patienten, deren Tumor nicht am Ende der Behandlung zurückgegangen war.

Diese Ergebnisse regen zu einem Studium anderer Verfahren der Kombination der Radiotherapie mit der Chemotherapie an.

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Letter to the Editor

Prolonged Lactation in Nodule Bearing Mice and its Effect on Mammary Tumor Genesis

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IT HAS been well established that lactation protects the mammary gland of the mouse against the tumor-producing action of hormones since in glands in which lactation is prevented by thelectomy or litter removal tumors appear earlier than in glands from which milk is removed [1–4].

In mice subjected to prolonged unilateral lactation a low incidence of hyperplastic nodules was found in glands which had a history of prolonged lactation, whereas contralateral thelectomized glands contained many nodules [4].

In view of the fact that in the study of the processes leading to tumor formation in the mammary gland a fruitful distinction has been made between the formation of a hyperplastic nodule and its subsequent outgrowth into a tumor [5], the question arises whether lactation affects besides nodule genesis also the transformation of the nodule into a tumor.

In the present study, female ($C_3H \times 020$) F_1 hybrid mice (carrying the mammary tumor virus) were used. The animals had been forced bred during 5 months by housing them 12 to a cage together with one male at the age of 6 weeks. The litters were removed shortly after parturition.

Two groups of mice were studied.

a. Eight macroscopically tumor-free mice, individually housed after the last parturition and the mammary glands allowed to regress. Five to six weeks later, the animals (of which 4 had developed a tumor) were autopsied and whole mounts of the mammary glands were prepared to ascertain the presence of nodules.

b. Thirty-five macroscopically tumor-free mice which had received 2 isologous pituitary glands under the kidney capsule during the last pregnancy. At the same time, the nipples were removed on one side and following parturition prolonged unilateral lactation was instituted as described earlier [4].

In 24 of these mice, the first tumor found was removed surgically and a second tumor was allowed to develop.

Table 1 shows that the mammary glands of the forced bred animals contained many hyperplastic nodules. It can be assumed that in the animals used in the second experimental group, a similar number of nodules was present in the glands at the beginning of the lactation period.

Table 2 shows that in 35 unilaterally lactating mice the rate of tumor appearance in the lactating and thelectomized glands did not differ significantly ($P=0{\cdot}3$, sign test). In contrast, data from a previous study [4], also included in the Table, show that when lactation is initiated at the age of 10–12 weeks, the first tumors appear invariably in the thelectomized glands.

The appearance of a second tumor, which was studied in 24 of these 35 animals, also indicates that lactation has no effect on the site of tumor appearance.

Apparently the primary effect of lactation

on tumor genesis is the inhibition of nodule genesis [4]. The transformation of a nodule into a tumor occurs with comparable frequency in the control animals in which the glands were regressing and in the lactating and thelectomized glands of the lactating animals.

Table 1. *Incidence of hyperplastic nodules in mammary glands of forced bred* (♀C_3H × ♂020) F_1 *mice**

No. of animals	No. of animals with nodules	No. of glands inspected	No. of nodules gland (range)
8	8†	64	2·8 (0–7)

*Forced bred during 5 months, regression during 5–6 weeks.
†Four animals developed one tumor each during the regression period.

Table 2. *Site of appearance of first and second mammary tumor in* (C_3H × 020) F_1 *mice bearing* 2 *pituitary grafts and subjected to prolonged unilateral lactation*

No. of animals	Age at onset of lactation	No. of animals with tumor in thelectomized gland	Latence period after parturition weeks (range)	No. of animals with tumor in lactating gland	Latency period after parturition weeks (range)
13*	10–12 weeks	13†	23,4 (14–33)	0	—
35	24–26 weeks	21†	5,2 (2–14)	14	5,7 (2–11)
24‡	24–26 weeks forced bred	13§	8,1 (4–13)	11	8,2 (4–14)

*Data from [4].
†First tumor.
‡Animals are also included in the group of 35 animals mentioned above (see also text).
§Second tumor.

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Announcements

International Group for the Study of Gastric Cancer*

IN JULY 1965, the International Federation of Surgical Colleges, through the European Group of the Committee on Research, set up a Working Party* to conduct an investigation into malignant disease of the stomach as an international project. This disease was selected for study because of the extreme and changing variations in its incidence throughout the world, and because of the uniformly high mortality rate associated with the disease. The need for such an investigation had been stressed by the British surgeon Professor Digby Chamberlain at the Fourth Annual Meeting of the Federation in Oslo in 1961.

The first meeting of the Working Party was held in London on 27th July, 1965, with representatives from five European countries present. The selected members of the Working Party were all practising surgeons with backgrounds of research in malignant disease. An exploratory discussion took place and a programme for a prospective investigation worked out. It was considered that an international investigation of this nature provided the opportunity of:

(1) Demonstrating geographical differences in the modes of presentation, diagnosis and management of the disease.

(2) Appraising current methods of therapy and the reason for variations between the countries concerned.

(3) Studying a large number of patients in a relatively short period of time and thus obtaining information which could lead to earlier diagnosis of the disease.

There is no doubt that throughout the world one of the main reasons for the high mortality rate is the large proportion of patients with non-resectable lesions when first seen by the surgeon.

With these objectives in mind is was decided to develop a comprehensive questionnaire for the collection and collation of clinical data from patients treated in the five hospitals represented by the members of the Working Party. A questionnaire was prepared so as to include information relating to possible aetiological factors, predisposing states, epidemiological features, symptomatology, clinical findings, special investigations, therapy and results of treatment.

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The prepared questionnaire was considered in detail at a second meeting in December 1965 and after some amendments was ready for use on January 1st, 1966.

Progress of the Study

Bi-annual meetings of the Working Party have taken place, the last in December 1967. When the investigation had been in progress one year the Working Party was enlarged by members from Holland and Poland, thus increasing the number of countries represented to seven.

Almost 800 patients have been included in the first two years of the study and it is proposed to continue collecting data from new patients for at least one more year.

The questionnaire was prepared as a tri-lingual document (English, French and German). The accumulating data is stored on computer tape in the Karolinska Institute Stockholm. During the course of the study it has been necessary to make a few further amendments to the questionnaire, but this has not interfered with the collection of data. Analysis of the material will begin in January 1969.

During the past two years the members of the Working Party have given much consideration to the necessity of a Pathological investigation into gastric cancer, to develop in association with the clinical study. Private and collective discussions with expert Pathologists have given high hopes that such a study will materialise. Attention will be paid to the development of an internationally acceptable histopathological classification of the disease and to the correlation of results of treatment with histological features of the cancer.

Eventually the Working Party intend to extend their activities to promote more basic research into gastric cancer and hope to provide a comprehensive pattern of the disease as it exists in Europe and perhaps other parts of the world.

For further information concerning the investigation, please contact the Hon. Secretary of the Working Party on Research, J. Ian Burn, FRCS., International Federation of Surgical Colleges, Royal College of Surgeons of England, Lincoln's Inn Fields, London, W.C.2.

Establishment of an Information Office on the Clinical Therapeutical Trials within the Field of Cancer

AN INFORMATION Office on Clinical Therapeutical Trials within the field of Cancer has been established by the Committee on Controlled Therapeutical Trials of the International Union Against Cancer.

Those who are planning to start a trial will thus be able to obtain from this Office information on similar trials which could be in progress elsewhere.

If required, the Office will also facilitate contacts between investigators.

It is clear that the Office will be able to perform its various duties only if, in return, information on current trials are regularly sent to it. *The more information you will transmit to the Office, the better will be the service it can provide.*

Such information may be sent to the Office or to any member of the Committee.

Address of the Office

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Création d'un Bureau d'Information sur les Essais Thérapeutiques Cliniques en Cancérologie.

UN BUREAU d'information sur les Essais Thérapeutiques Cliniques en cancérologie a été créé par le Comité des Essais Thérapeutiques Cliniques de l'Union Internationale contre le Cancer.

Ceux qui envisagent d'entreprendre un essai pourront ainsi obtenir, du Bureau, des renseignements sur des essais analogues qui pourraient être en cours de réalisation ailleurs. Si nécessaire, le Bureau pourra également faciliter des contacts entre les Chercheurs.

Bien entendu, ce Bureau ne pourra remplir ces différentes tâches que si, en retour, des informations lui parviennent régulièrement sur les essais en cours. *Plus vous lui adresserez de renseignements, plus il sera à même de rendre des services.*

Ces renseignements peuvent être envoyés au Bureau, ou à tout membre du Comité.

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Errichtung Eines Informationsbüros über Kontrollierte Klinische Prüfungen im Krebsgebiet

Das UICC-Komitee für die Versuchsplanung auf dem Krebsgebiet hat ein Informationsbüro über kontrollierte klinische Prüfungen errichtet.

Hierdurch können alle Kollegen, die einen klinischen Versuch unternehmen wollen, sich über Projekte gleicher oder ähnlicher Art vorher informieren. Wenn es nötig ist, kann das Büro auch Verbindungen zwischen verschiedenen Forschern herstellen.

Natürlich kann das Büro diese verschiedenen Aufgabrn nur erfüllen, wenn ihm Auskünfte über laufende Versuche regelmässig erteilt werden. *Je mehr Informationen das Büro erhalten wird, desto besser kann es Informationen erteilen.*

Informationen können dem Büro oder einem der Komiteemitglieder eingesandt werden.

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Europ. J. Cancer Vol. 4, pp. 455–458. Pergamon Press 1968. Printed in Great Britain

Third International Symposium on the Biological Characterization of Human Tumours

Preliminary Notice

THE THIRD INTERNATIONAL SYMPOSIUM on the Biological Characterization of Human Tumours* will take place from 14–16 April 1969 in the Superior Council of Scientific Investigation, Madrid, Spain and with the support of the Spanish Association against Cancer.

Enquiries should be made from the Secretary:

Dr. W. Davis
International Agency for Research on Cancer
16, avenue Maréchal Foch
69-Lyon 6e
France

or from the Organising Secretary:

Dr. N. Retana
Asociación Española contra el Cancer
Amador de los Rios, 5
Madrid 4
Spain

*The proceedings of the Second Symposium were published in the *European Journal of Cancer* **3,** 4/5 (1967).

Troisième Symposium International sur la Caractérisation Biologique des Tumeurs Humaines

Note Préliminaire

Le Troisieme Symposium International sur la Caractérisation Biologique des Tumeurs Humaines* se tiendra, du 14 au 16 avril 1969, dans les locaux du Conseil Supérieur de la Recherche Scientifique, à Madrid, Espagne, avec la collaboration de l'Association Espagnole contre le Cancer.

Les demandes de renseignements doivent être adressées au Secrétaire:
le Docteur W. Davis
Centre International de Recherche
sur le Cancer
16, avenue Maréchal Foch
69-Lyon 6e.
France

ou au Secrétaire chargé de l'organisation du Symposium:
le Docteur N. Retana
Asociación Española contra el Cancer
Amador de los Rios, 5
Madrid 4
Espagne

*Le compte-rendu du Second Symposium a été publié dans la *Journal Européen de Cancérologie* **3,** 4/5 (1967).

Drittes Internationales Symposium über die Biologische Charakterisierung Menschlicher Tumoren

Vorgängige Notiz

Das Dritte Internationale Symposium über die Biologische Charakteriesierung Menschlicher Tumoren* wird mit Unterstützung der Spanischen Gesellschaft für Krebsbekämpfung vom 14–16 April 1969 im Oberrat für Wissenschaftliche Forschung in Madrid, Spanien stattfinden.

Anfragen sind zu richten an den Sekretär:

Dr. W. Davis
International Agency for Research on Cancer
16, avenue Maréchal Foch
69-Lyon 6e
Frankreich

oder an den Organisationssekretär:

Dr. N. Retana
Asociación Española contra el Cancer
Amador de los Rios, 5
Madrid 4
Spanien

*Die Vorträge des Zweiten Symposiums sind in der *Europäische Zeitschrift für Cancerologie* **3,** 4/5 (1967) erschienen.

Tercero Symposium Internacional sobre Caracteristicas Biologicas de Tumores Humanos

Informacion Preliminar

El Tercero Symposium Internacional sobre Características Biológicas de Tumores Humanos* se celebrará del 14 al 16 de Abril de 1969 en el Consejo Superior de Investigaciones Científicas, Madrid-España, patrocinado por la Asociacion Española Contra el Cáncer.

La información que se desee deberá ser solicitada al Secretario:

Dr. W. Davis
International Agency for Research on Cancer
16, avenue Maréchal Foch
69-Lyon 6e
Francia

ó al Vice-Secretario de la Organización:

Dr. N. Retana
Asociación Española contra el Cancer
Amador de los Rios, 5
Madrid-4
España

*Las Actas del Segundo Symposium fueron publicadas en el *European Journal of Cancer* **3,** 4/5 (1967).

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Studies of Specific Properties of Tumour Cell Membranes using Stereoscan Microscopy

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INTRODUCTION

STUDIES of cell movements and cell contacts in tissue culture have shown that the movements of normal fibroblasts are controlled by contact with neighbouring cells (contact inhibition) [1]. When the actively moving membrane of a cell makes contact with a neighbouring cell, an adhesion generally forms between the two cells and the ruffling activity of the membrane ceases. This control of movement by contact is lost in anaplastic sarcoma cells which move freely over each other and over normal cells. Contact inhibition in normal fibroblasts eventually leads to a characteristic growth pattern in dense culture of the type shown in Fig. 1a. This is due to the elongated form of the cells and contact inhibition operating between the sides of the long cells. Loss of contact inhibition in sarcoma cells leads to a more random growth pattern, as shown in Fig. 1b. These changes in cell behaviour are partly dependent on changes in the cell surface structure. This has been studied both with various types of sarcoma cells and by comparisons between epithelial cultures and carcinoma cells [2]. In the present investigation use has been made of a new microscopic technique, Stereoscan microscopy, to study the nature of the cell movements and cell membrane properties which are responsible for the changed behaviour of tumour cells.

METHODS

(a) *Stereoscan microscopy*

A Cambridge Instrument Company's microscope was used for these experiments. It utilises a fine pencil of electrons which scans the surface of the specimen. The secondary electrons so produced are collected by a coarse grid. The signal from this grid is used to modulate the beam of a television tube which scans in synchrony with the beam in the electron microscope. The image so produced is similar to a television image. When objects are seen with the naked eye in visible light the image is generally formed by diffuse reflection of light from the object. This reflected light is collected by the eye and focused on the retina. The object is seen as a whole with a 3-dimensional appearance. The image formed by the Stereoscan microscope is in certain respects rather similar to this image and also gives a 3-dimensional impression, but it is of course obtained at much greater magnification with the help of an electronic presentation.

(b) *Method of preparing the specimens*

For the study of cells in culture we have grown the specimens on small circular coverslips placed in Falcon flasks.

Immediately before fixation the cultures were washed in synthetic culture medium at 37°C to remove protein. The short exposure to pure synthetic medium did not produce noticeable changes in cell appearance or behaviour. Two methods of fixation were used:

(1) glutaraldehyde fixation as for conventional electron microscopy
(2) freeze substitution.

For freeze substitution the specimen was

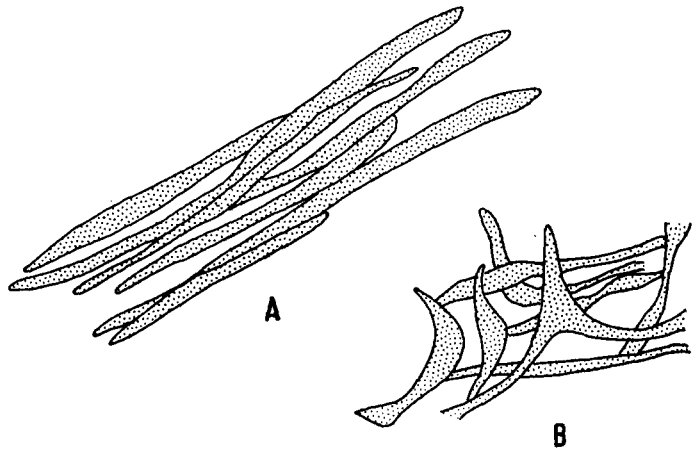


Fig. 1. *A. Growth pattern of a confluent culture of untransformed* C_{13} *strain hamster fibroblasts. B. Growth pattern after transformation with polyoma virus.*

washed with synthetic culture medium at 37°C as before, but was then plunged immediately into a boiling tube containing liquid pentane immersed in liquid nitrogen. This gave rise to almost instantaneous fixation of the specimen by freezing. The specimen was then removed and placed immediately in alcohol at −40°C surrounded by solid carbon dioxide in a thermos flask. Freeze substitution was then effected by the gradual penetration by ethyl alcohol. After 12 hr the specimen was allowed to warm up slowly to room temperature and the alcohol was allowed to evaporate. All specimens were coated with a thin film of gold–palladium in high vacuum to render them conducting before observation in the microscope. When placed in the microscope the specimens were inclined at various angles (up to 72°) to the incident beam. This is possible in Stereoscan microscopy owing to the great depth of focus. This technique enabled the lower surface of the cell to be examined from the side.

(c) *Cell culture*

Two types of cell culture were used in these experiments; the C_{13} clone of BHK21 hamster embryonic fibroblasts and the PYY clone of similar cells after transformation to malignant cells with polyoma virus (the original clones were obtained from Professor M. A. Stoker). In each case the cell lines were grown from single cells. In the case of the transformed cells, these were isolated from infected cultures also grown up from single cells. The characteristic growth patterns are shown in Fig. 1a and b. The cells were grown on small glass coverslips placed in the bottom of Falcon flasks. In addition to growing the cells on glass, they could also be grown on the surface of Millipore filters and on sintered glass of varying texture. The sheets of Millipore were cemented on to the base of a Dural ring using ethyl acetate to render the Millipore adhesive. This prevented the Millipore from floating to the surface and kept it on the bottom of the culture flask. The sintered glass was obtained by fracturing various discs of sintered glass of the type used for filtration.

RESULTS

(a) *Behaviour of* C_{13} *fibroblasts*

Figure 2 shows a C_{13} fibroblast in the Stereoscan microscope (×2250). It is growing on a smooth glass surface and the glass surface is inclined at an angle of 60° to the vertical. It is therefore possible to see partly underneath the cell. Figure 3 shows a higher power view (×7500) of the edge of the cell seen at a steeper angle of 72°. The presence of large gaps between the cell and the glass and of a region of close contact can be clearly seen. Microvillae of 2–3000A° dia. can be seen to be attached to the glass. The ends of microvillae can be seen to lie on the surface of the glass.

Figure 3 shows the effect of growing the C_{13} cells on fine Millipore (GS) of porosity 0·2μ. This is a very low power picture (×225) and it can be seen that groups of cells are aggregating together to form a massive colony. They are attaching preferentially to each other rather than to the Millipore surface. These colonies are capable of growing into large masses of compact cells with up to ∽1,000,000 cells in a colony. No separate cells are then found on the surface of the Millipore.

Figure 4 shows contacts between C_{13} cells and a fine Millipore surface. The presence of microvillae can be seen but there are no regions of close contact between the cell

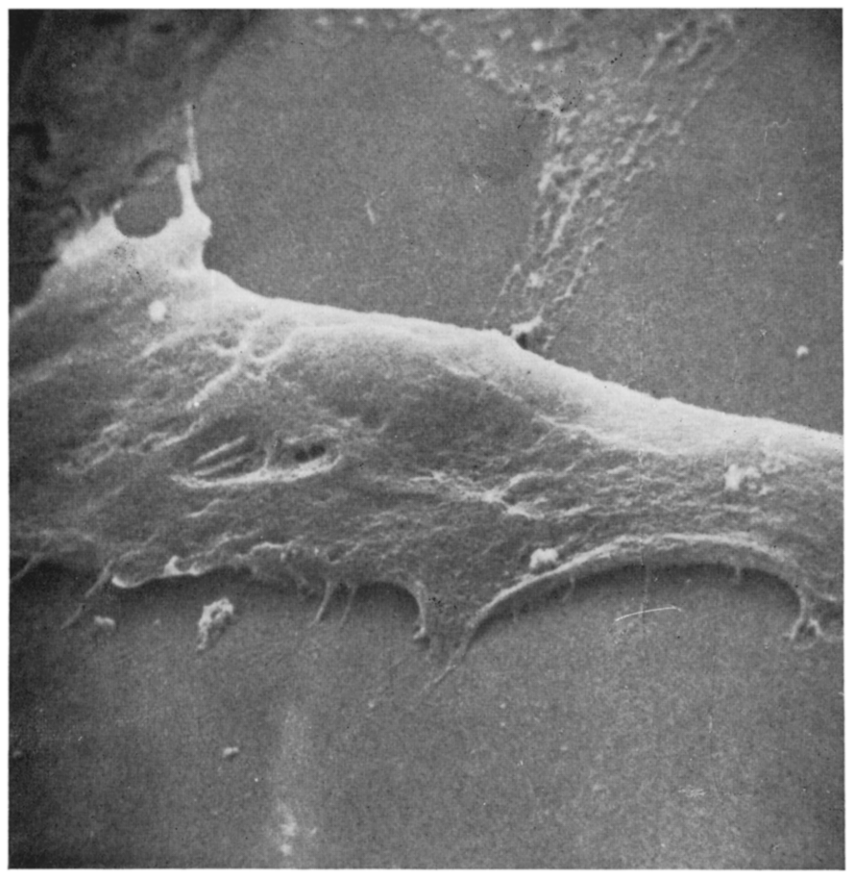


Fig. 2. Stereoscan picture of C_{13} fibroblast growing on smooth glass surface. Freeze substituted. Inclined at 60° to beam. ×2250.

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Fig. 3. *As Fig.* 2. *Inclined at* 72°. ×7500.

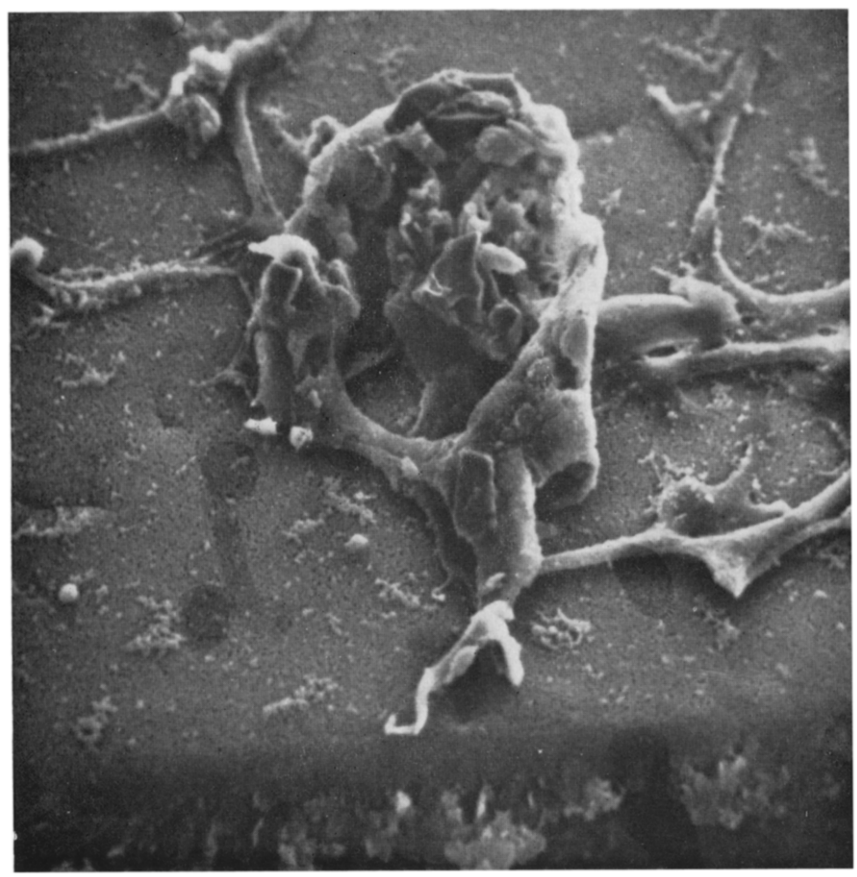


Fig. 4. C_{13} *cells growing on fine Millipore* (0·4 μ*pore size*) *forming colonies at* 60°. ×225.

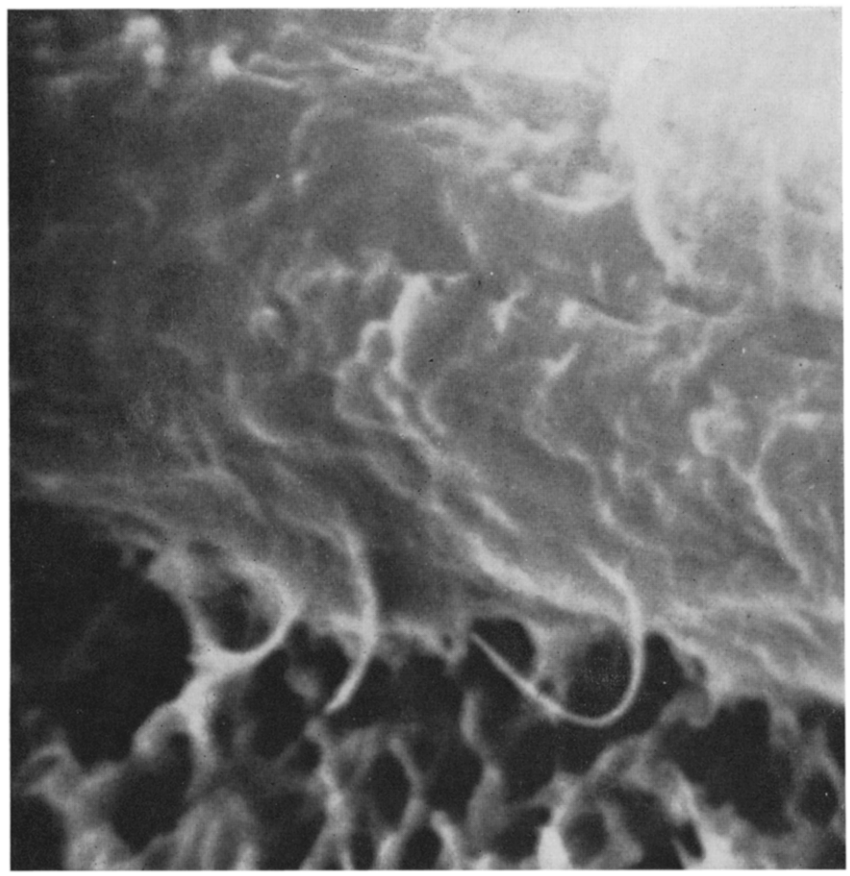


Fig. 5. *C_{13} cell on fine Millipore. Viewed at 72° under cell Millipore at bottom. Fine non-adhering microvillae on lower surface of cell.* ×8000.

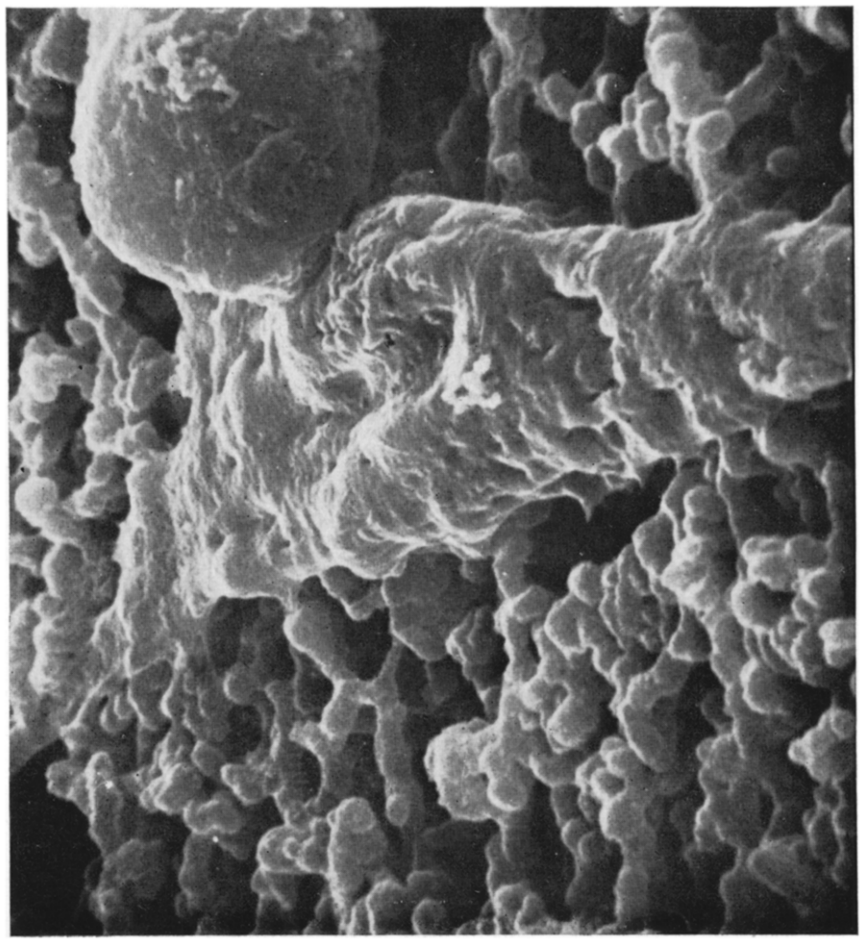


Fig. 6. *C_{13} cell on coarse Millipore* ($0{\cdot}8\mu$ *porosity*) *at* 60°. $\times 7800$.

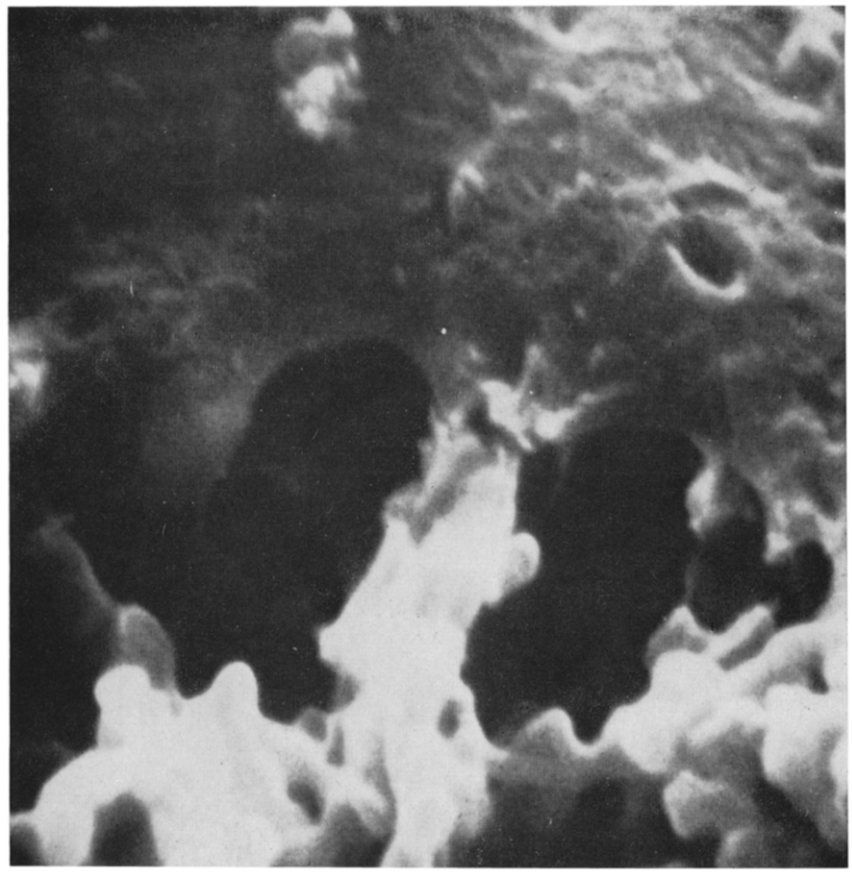


Fig. 7. C_{13} *cell on coarse Millipore at* 60° *showing contacts between lower surface of cell and Millipore (Millipore below).* ×8000.



Fig. 8. *PYY transformed cell spreading on fine Millipore at* 60°. ×5000.

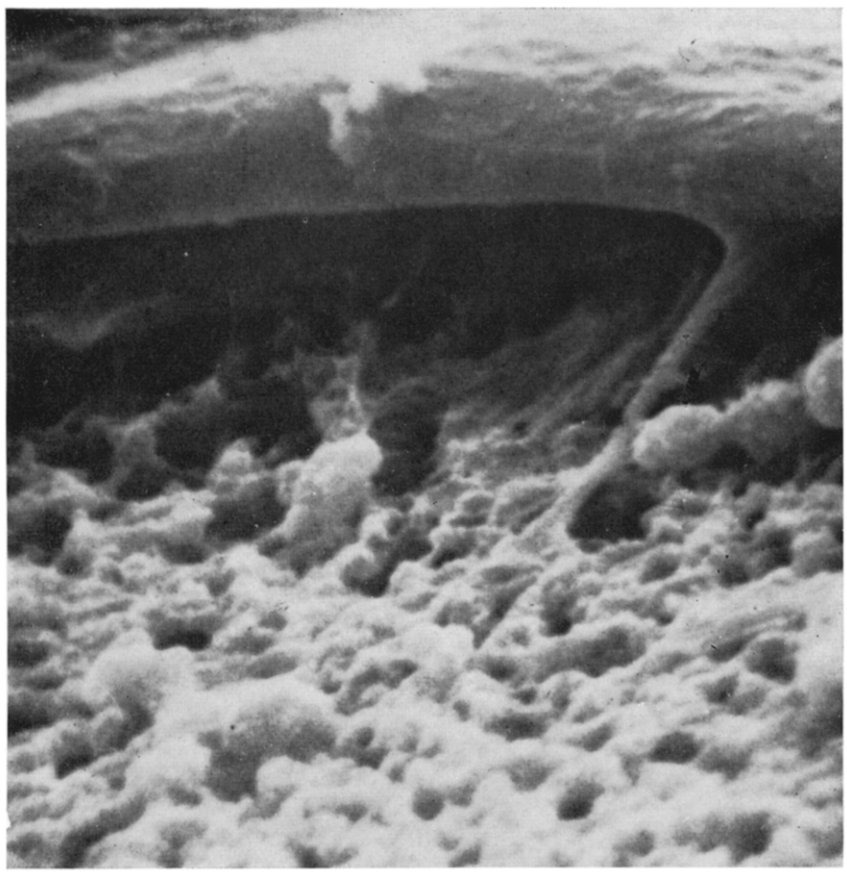


Fig. 9. *PYY on fine Millipore showing membranes attaching to Millipore surface at* 60°. ×8000.

and the open structure of the Millipore.

Figure 6 shows a C_{13} cell growing on the surface of coarse Millipore (RA) of porosity 0·8μ. The cell is spreading on this surface in a similar way to glass. Figure 7 shows the contacts between the cell and the Millipore, at magnification of ×14,000. On this surface the membrane of the C_{13} cells is able to make more extensive contacts with the Millipore and to bridge the gaps between the irregularities.

Growth of the cells on the intermediate grade of Millipore gave cells which partly formed colonies as on GS Millipore and grew partly as spread out cells as on RA Millipore.

On sintered glass, a similar behaviour was observed. Cells growing on fine sintered glass produced dense colonies and did not spread whereas those grown on coarse sintered glass were able to spread once more. Intermediate textures gave partly colony formation and partly spreading cells.

(b) *Behaviour of PYY transformed fibroblasts*

Figure 8 shows a transformed cell growing on GS Millipore, the surface on which the C_{13} cells form colonies and do not spread. The transformed cells are able to spread on the surface equally as well as they spread on smooth glass. Figure 9 shows a view of the contacts between the transformed PYY cells and the fine Millipore. It can be seen that the cell is putting out many spreading membranes which are making extensive contacts with the Millipore surface.

It was found that the transformed cells were able to attach to all surfaces—smooth glass, fine medium and coarse sintered glass and fine, medium and coarse Millipore. In no case was colony formation observed.

DISCUSSION

Earlier studies of the locomotion of tissue cells on glass using interference microscopy [3] and a surface contact microscope [4] which enabled the regions of contact between the underside of the cell and the glass to be observed, had indicated that tissue cells migrate as a consequence of the undulations of their cell membranes. These undulations on the lower surface give rise to intermittent contact between the cell and the glass surface as the cell moves forwards. As can be seen in Figs. 2 and 3 the Stereoscan microscope has enabled these contacts to be observed directly; the presence of a small region of contact between the cell and the glass suggesting a wave-like movement of the membrane can be clearly seen. The results of the observations with the Stereoscan microscope are in good agreement with the earlier observations using optical methods with the moving cells. But the results described for the behaviour of the cells on different surfaces indicate that there is a critical surface texture on which the untransformed cells are unable to adhere. On GS Millipore, the strong *intercellular* adhesions caused the cells to associate in the form of dense colonies, but PYY cells were able to attach to all the surfaces examined. The examination of Figs. 5 and 9 indicate that the type of membrane movement shown by the non-malignant and the malignant cells differ. These differences are evidently due to changes in behaviour of the cell membranes and not to chemical changes in the solid substrate. This is shown by the similar behaviour on fine Millipore and on sintered glass of the two cell types. The transformed cells are producing expansive undulations of their membranes which are able to bridge the gaps in the irregular surface of the GS Millipore. When the gaps are again increased, as on RA Millipore, the movements of the untransformed cells are again able to obtain a purchase on the elements of the filter.

These observations provide additional evidence for an altered structure of the tumor cell surface. In this respect the *cell–substrate* adhesions differ from the *cell–cell* adhesions.

Transformation to malignancy is generally accompanied by a decreased area of stable intercellular contact and failure of contact inhibition of movement. Evidence from cell electrophoresis has also indicated that many malignant tissue cells show an increased electrical surface charge density [5, 6] which would lead to increased repulsive forces between the cells and hence to reduced adhesions. Although the main repulsive forces between cells are due to repulsion between like charges as is the case with simple colloidal particles, the question of whether cells adhere or not is also known to involve other factors including reactions with calcium ions. Calcium ions are able to form bridges between negatively charged groups $-COO^- \; Ca^{2+} \; {}^-OOC-$, either between groups on the same cell or between cells. The special arrangement of the negatively charged groups on the cell surface will therefore be important in determining whether calcium bridges can form or not.

The interesting feature of the malignant cells studied in these experiments is their more versatile adhesiveness to all the surfaces examined. The normal cells, although able to

form strong attachments to each other were only able to adhere to a limited range of surface textures. This ability of the malignant cells to adhere more generally to surfaces accompanied by weakened adhesion to neighbouring tumour cells [8] may provide them with an increased capacity to invade and penetrate normal tissues.

Acknowledgements—This investigation has been supported by grants to the Chester Beatty Research Institute (Institute of Cancer Research: Royal Cancer Hospital), from the Medical Research Council, the British Empire Cancer Campaign for Research and by the Public Health Service Research Grant No. CA-03188-10 from the National Cancer Institute, U.S. Public Health Service.

RESUME

L'étude des mouvements des cellules normales et cancéreuses, en culture, a mis en évidence des différences dans leur surface cellulaire et leurs contacts intercellulaires. En faisant croître les cellules sur des surfaces de différentes textures, ce contraste entre les cellules normales et les cellules malignes devient très frappant, et peut être facilement observé au microscope stéréoscanique.

SUMMARY

Studies of the movements of normal and malignant cells in culture have indicated differences in their cell surface and intercellular contacts. By growing the cells on surfaces of different textures this difference between normal and malignant cells becomes very striking and can be seen very clearly by studies with the Stereoscan microscope.

ZUSAMMENFASSUNG

Studien über die Bewegungen normaler und maligner Zellen in vitro *haben Unterschiede in ihrer Zelloberfläche und dem Zellkontakt aufgezeigt. Diese Unterschiede werden sehr deutlich, wenn man die Zellen auf Oberflächen verschiedenen Gefüges wachsen lässt, und können mit dem Stereoscan-Mikroskop sehr klar erkannt werden.*

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Lysosomal Enzyme Activity in the Interstitial Fluid of Solid Mouse Tumour Transplants

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The Cancer Research Division of Radiumhemmet, Karolinska Institute, Stockholm 60, Sweden

THE LARGE increase in enzymatic activity of cell-free interstitial fluid (IF) of solid tumours has been reported with particular reference to carbamide bond splitting enzymes [1, 2]. Previous assays included cathepsins; most of the other enzymes investigated, like peptidases and lactate dehydrogenase, represented soluble cytoplasmic ones. Further information of other lysosomal enzymes in tumour IF is thus needed since their activity may be operative in relation to tumour cell physiology, the detachment of tumour cells and tumour invasion [3–5]. These parameters will be discussed below and comparison will be made with granuloma fluid [6] and ascites tumour fluid [7].

The data to be reported will summarize our experience from a spectrum of different mouse tumour fluids, all of which have been assayed in a nearly native state. The mode of presentation, techniques of microsampling and ultramicroassays, as well as the reservations inherent in the assays methods as such, follow the pattern of the previous report [2]. No enzyme separations have so far been made from tumour IF, nor have isoenzymes been considered. The occurrence of a cathepsin inhibitor is mentioned [8], but specific information as to other enzyme inhibitors possibly present is lacking.

MATERIAL AND METHODS

Cell-free tumour IF has been assayed only from *single cases* of solid tumours either from their peripheral or central regions or both. No cases of pooled fluid have been used. Comparison is made with similar assay data performed on mouse blood plasma and intraperitoneal (IP) fluid of normal mice of the same strains. Data on ascites tumour fluid are cursorily mentioned.

Tumour lines. The relevant growth characteristics, sizes and ages of the tumour material are compiled in Table 1. Only progressively growing tumours were included. Terminal cases and those with extensive cystic necrosis were excluded. Tumour glycolysis was not accelerated by glucose administration prior to sampling as previously described [1, 2].

It might be added that we have failed to obtain sufficient amounts of IF from some very dry tumours, such as the Harding-Passey melanoma. Some rat tumours, as well as some human mammary carcinomas, have presented other difficulties of sampling due to a high fat content clogging the capillaries (*cf.* the low cholesterol figure of tumour IF as sampled by the "chamber technique" of Gullino *et al.* [9].

In randomly selected cases ordinary histology was performed on unfixed fresh-frozen cryostat sections (Table 1). The regional distribution and relative amounts of active macrophages was registered in other sections treated by histochemical procedures for the demonstration of acid phosphatase activity acc. to both the Gomori and the naphtol AS-MX standard technique [10].

Sampling. Blood plasma, IP and ascites

Table 1. *Specifications of present tumour material*

Type of tumour	Mouse strain	Spontan. or transpl.	Age (days)	Size ranges (mm)	No. of cases investigated	Gross morphology and characteristics of fluid obtained	Histology
A. Solid mouse tumours							
ELD Solid, from ascites form	Albino stock and C3H mice	Tr.	10	5–10	29	Young transplants only; slowly growing. Clear IF, no marked necrosis.	Anaplastic hyperdiploid carcinoma. Some macrophages throughout the tumours.
Mammary carcinoma	C3H	Spontan.	Unknown	10–40	7	Gelatinous brownish fluid in cystic compartments. Some necrosis.	Slowly growing multicystic regular carcinoma, rich in macrophages.
MCIS Sarcoma from ascites from	C3H	Tr.	8–11	5–20	40	Rapidly growing w. multifocal necrosis. IF rich in cells w. some admixed blood.	Anaplastic rhabdomyosarcoma
MCAC Sarcoma	A (Snell)	Tr.	13	5–15	7	Rapidly growing solid tumours without marked necrosis. IF moderately turbid.	Anaplastic fibrosarcoma
MC 57 Sarcoma, methylchol. induced	C 57 BL	Tr.	6–27	10–20	24	Rapidly growing fibrosarcoma, mostly small-sized. No cystic necrosis. IF w. some blood admixture.	Anaplastic fibrosarcoma
Malignant lymphoma	A (Snell) and FI hybrids	Tr.	7–14	10–30	13	Flat disk-shaped subcut rapidly growing tumours. Small focal necroses. IF mainly clear.	Compact foci of diffusely invading anaplastic lymphoma cells.
					120		
B. Mouse ascites tumours							
ELD Ascites	C3H	Tr.	6–16		16	Turbid ascites fluid without marked admixture of blood cells.	Anaplastic hyperdiploid carcinoma.
MCIS Ascites	C3H	Tr.	6–16		12	Thick brownish fluid, rich in detritus and admixed blood.	Ascites form of the rhabdomyosarcoma mentioned above.
					28		

*Most of these tumour lines were obtained by courtesy of Prof. G. Klein, Institute of Tumour Biology.

Table 2. Methods of assay

Enzyme	Dilution of original sample: Plasma, IP fluid, ascites and peripheral tumour IF	Central IF	Buffer	Substrate	Substrate conc.	Activator added final conc.	pH of react. mix.	Incub. temp.	Incub. time	References
AcNPhPhase	1+1	1+4	Citrate 0·05 M	*p*-nitrophenyl phosphate (C. F. Boehringer)	0·0055 M	None	4·8	37°	30 min	Andersch and Szczypinski [11]
β-Glucuronidase	1+1	1+4	Acetate 0·1 M +KCl 0·2 M	Phenolphthalein-β-glucuronide (Sigma)	0·0015 M	None	4·6	37°	30 min	Fishman and Bernfeld [12]
Aryl sulphatase	1+1	1+4	Acetate 0·2 M	*p*-nitrocathechol sulphate (Sigma)	0·02 M	None	5·5	37°	1 hr	Roy [13]
Catheptic activity *cf.* text	1+3	1+9 or 1+19	Acetate 0·2 M	Edestine (Hopkin & Williams)	2%	1. No addition 2. Cysteine 0·004 M + EDTA 0·5 M	5·0	40°	1 hr	Anson's method as modified by Ottoson and Sylvén [14]

tumour fluid as well as IF from the peripheral and central regions of solid unicentric tumour transplants was sampled without addition of citrate or other anti-clotting agents. The technique routinely used was the same as previously described [1, 2] with the aid of thin capillaries under direct visual control. This avoids the introduction of secondary sieving effects by membraneous and other filter chambers surrounded and covered by closely attached cells (*cf.* [9] and *Discussion* in [2]). Cells and other admixed debris were then immediately removed by high-speed centrifugation [1]. Phase-contrast and electron microscopy of such clear fluids have been performed in previous series [1]. All fluid samples from tumours normally contained clear fluid, which never clotted as previously reported [1]. Samples occasionally contaminated with fresh blood were discarded; but samples brownish coloured from partially digested haemoglobin were included (Table 1). Some markedly destructive tumours, like the MCIS and MC 57 sarcomas, normally open-up smaller vessels and are thus often contaminated by some red cells.

No pretreatment of fluid samples. Our aim was to perform all enzyme assays on the native fluid as close to their *in vivo* conditions as possible. The tumour IF may contain some undamaged lysosomes which very likely could have been spun down by further centrifugation or we could have released their hydrolases by addition of Triton or other detergents. This has so far not been systematically studied. The activity ranges presented will thus illustrate the freely available activities under nearly native conditions.

Methods of assay. The protein content was determined by the micromethod of Nayyar and Glick [15] on samples diluted 10 times. The microtechniques for assay of some selected lysosomal enzymes are listed in Table 2, *cf.* [6]. All assays have been performed in triple tests against an appropriate blank containing the test material and substrate solutions kept separately in the water bath during the incubation time. All calibration curves have been carefully checked with reference to time of incubation and dilution of samples as previously mentioned [2, 14]. The observed activity figures have been recalculated and expressed as standard units (U) or per cent hydrolysis of the substrate per 1 or 10 µl original fluid and per unit time, unless otherwise stated (Tables 4 and 5). This is in conformity with previous data on granuloma fluid [6] and will ensure direct comparison between these compartments except in the case of the total catheptic activity. For comparison with previous total catheptic activity data [6] on granuloma IF, it should be observed that those were assayed at pH 4·1; since the figures were low they had to be expressed per 10 µl but those on tumour IF are per 1 µl as in Table 3 of ref. [2].

Some modifications have been introduced in the present assays of the total catheptic activity. First, the substrate has been changed from urea-denatured haemoglobin (which is often contaminated by trace amounts of cathepsin) to *urea-denatured edestine.* This hemp seed globulin, partially degraded by 6·6M urea to a mol. wt. of about 49,000, is free of spontaneous degradation thus presenting low and consistent blank figures. Secondly the pH of assays was shifted from 4·1 to 5·0. In previous assays attention was focused on one activity peak at pH 4·1 representing a mixture of mainly the pepsin-like cathepsin D (which is not influenced by added cysteine) and the trypsin-like cathepsin B. The latter has, in a purified state, a pH optimum of activity around 5·6 [16] and forms, therefore, from a biological point of view, a more interesting proteinase. In order to detect more of this activity, still to some part mixed with D, the relative activities of both normal IP and tumour fluids were compared *at pH* 5·0. It follows that present catheptic activity data cannot be directly compared with those previously published [2]. This new set of data is recorded together with other lysosomal enzymes; a more detailed study of cathepsin B activity and its plasma inhibitor will appear [16]. The cathepsin C (EC 3, 4, 4, 9) activity against GlyPhe-amide in tumour IF was previously mentioned [2].

It should be added that the catheptic activity of normal plasma at pH 5·0 is too low to be correctly measured by the present technique. The plasma samples cannot be sufficiently diluted in order to avoid technical difficulties, and hence these data are omitted in Table 4.

Role of ionic strength and activators. In general, all fluid samples have been diluted with buffers only (Table 2), thus reaching an ionic strength of about 0·1. Further addition of salt was used only in the case of the β-glucuronidase assays [17] although this did not lead to any activity increase. However, it may be of advantage to add 0·2M KCl in order to increase the solubility of suspended proteins and the degree of enzyme dissociation. This addition may therefore be recommended in cases of turbid IF.

*Table 3. Loss of enzyme activity by storage**

Enzyme assay	Fresh material Rel. activity/sample	Refrigerated at +4°C. overnight % loss	Deep-frozen at −28°C. overnight % loss
AcNPhPhase	0·6 U/10 µl/30 min	40	30
β-Glucuronidase	5·8 U/10 µl/min	12	12
Aryl sulphatase	1·4% hydrol./10 µl/hr	9	0
Cathepsin (mainly D); assayed without addition, at pH 4·1	17% hydrol./µl/hr	25	11

*Examples chosen from one series of experiments performed on cell-free fluid from one ELD ascites tumour 5 days old.

A new factor introduced into the cathepsin assays at pH 5 is incubation with *cysteine + EDTA* (Tables 4 and 5) in order to reach a high degree of activation of available cathepsin B [8, 16]. This implies that previous assays of the total catheptic activity—performed on urea-denatured proteins with the addition of cysteine only—on biological materials, where cathepsin B forms a component part, have been too low *cf.* [1, 2, 7, 14, 18–21].

Stability of interstitial fluid enzymes. The necessity to use fresh materials for the assay of AcNPhPhase and cathepsin activity is evident from the experiments recorded in Table 3.

Treatment of data. The average activity figures are reported on a per volume basis since we are dealing with fluid transport compartments more or less in a state of flux. Standard deviations are not given since tumour fluids in general present very marked activity increases; the individual variations are represented by the activity ranges. The blood plasma levels constitute the natural baseline presenting the largest pool and the smallest individual variations. Different normal interstitial fluid compartments vary in their composition; for instance the enzymatic figures per µl of subcutaneous IF are generally larger than those of the IP fluid, those of the thoracic lymph are different from those of the main lymph from the legs, etc. Therefore, it seemed appropriate to correlate activity data of tumour IF (Table 5) to those of normal blood plasma of the corresponding strain of mice (Table 4). The only correlation possible in the case of the catheptic activity was, however, to that of IP fluid (T/IP in Table 5) since reliable plasma data are lacking as mentioned above. Data on central tumour IF were also correlated to tumour size and degree of necrosis by plotting individual activity data diagrammatically with respect to the characteristics of each tumour of its class. The results are not illustrated but cursorily mentioned.

Another baseline presently omitted is activity data correlated to a per unit amount of total protein content. Since the latter figures are mentioned in the Tables such correlations will be easily available to the readers.

RESULTS

Present activity data refer to levels of "free" enzymatic activity available as such to substrate interaction under *in vivo* conditions in topographically specified fluid compartments. The assays of normal IF (Table 4) were performed under conditions strictly identical with those of tumour compartments (Table 5). The results may thus be directly compared with each other as in a host of other chemical assays on clinical and impure materials.

Plasma and IP fluid. All lysosomal enzyme activities were low in plasma and IP fluid of healthy mice; some slight strain and sex differences are omitted. A moderate activation of the total proteinase activity occurred following the addition of SH+EDTA suggesting that some cathepsin B activity might be present in IP fluid. When compared at a per protein content the IP activity data become increased by a factor of 2 times.

Peripheral tumour fluid. The edema fluid at and outside the cortical well-vascularized tumour zones uniformly showed slight or moderate increase in lysosomal enzyme activity as compared with blood plasma (Table 5; Section A). The IF from the rapidly growing and most destructive tumour, the MCIS sarcoma, also presenting a macrophage reaction, showed the highest AcNPhPhase, and aryl sulphatase figures. This may be compared with the data on ascites fluid (Table 5; Section C) expected to present a similar degree of capillary filtration and rate of exchange as the IF at the tumour periphery [22]. The catheptic activity of ascites fluid was, however, markedly increased about 10 times, *cf.* [7].

It thus appears that the actual pool of

Table 4. Activity levels in normal mouse plasma and intraperitoneal (IP) fluid

	No. of samples invest.	Protein content (%)	Acid NPhPhosphatase (U/10 µl/30 min)	β-Glucuronidase* (U/10 µl/30 min)	Aryl sulphatase* (% hydrol./10 µl/hr)	Cathepsin at pH 5·0 Edestine (ΔE/µl/hr)	Cathepsin at pH 5·0 Edestine Cyst.+EDTA (ΔE/µl)/hr
Plasma							
Albino stock mice	14	5·3 (5·0–5·6)	0·68 (0·10–0·80)	4 (2·5–8)	0·5 (0·2–1·5)	—	—
C3H strain	12	6·05	0·44 (0·05–0·55)	2 (0·5–9)	0·4 (0·3–0·8)	—	—
C57 BL	7	6·3 (5·8–6·7)	0·64 (0·12–0·85)	3 (1–6)	0·3 (0·2–0·5)	—	—
IP Fluid							
Albino stock mice	26	3·16 (2·60–3·60)	0·72 (0·15–1·50)	10 (6–17)	0·7 (0·3–1·4)	<0·01	0·037 (0·021–0·048)
C3H strain	14	2·80 (2·15–3·15)	0·44 (0·10–0·55)	4 (0·5–9)	0·4 (0·1–0·6)	<0·01	0·036 (0·032–0·041)
C57 BL	8	3·4 (2·9–3·8)	0·14 (0·06–0·44)	3 (1–6)	0·5 (0·2–0·7)	<0·01	0·023 (0·012–0·047)
*Correlation factors for recalculation into IU (µmoles substrate/ml/min)			$f = \times 3 \cdot 3$	$f = \times 0 \cdot 5 \times 10^{-3}$	$f = \times 1 \cdot 7 \times 10^{-3}$		

Ranges in brackets when available. U=units. ΔE=extinction increase.

Table 5. Protein content and enzymatic activity of cell-free interstitial tumour fluid

	AcidNPhPhosphatase*			β-Glucuronidase*		Aryl Sulphatase*		Cathepsin		
	Protein content (%)	(U/10 µl/30 min)	Ratio T/pl.	(U/10 µl/30 min)	Ratio T/pl.	(% hydrol./ 10 µl/hr)	Ratio T/pl.	Ed. without addition (ΔE/µl/hr)	Ed.+Cyst +EDTA (ΔE/µl/hr)	Ratio (T/IP)
A. Peripheral tumour fluid										
ELD Carcinoma	3·8 (3·25–4·40)	1·2 (0·35–1·75)	2	2·9 (1·5–4·8)	1	1·0 (0·4–1·8)	2	0·05 (0·01–0·08)	0·11 (0·07–0·16)	3
MCIS Sarcoma	4·9 (3·44–7·0)	1·5 (1·08–1·80)	3	8·0 (6·5–12·3)	4	2·4 (1·5–2·7	6	—	—	—
MC 57 Sarcoma	4·2 (4·20–4·25)	0·7 (0·48–1·04)	1	7·0 (3·1–8·0)	2	0·6 (0·5–0·75)	2	0·04 (0·01–0·07)	0·10 (0·08–0·16)	4
Lymphoma	2·4 (2·35–2·45)	0·7 (0·12–1·40)	>1	18·5 (4·8–40·0)	6	0·1 (0·05–0·15)	<1	~0·02 (0·01–0·04)	0·10 (0·06–0·14)	3
B. Central tumour fluid										
ELD Carcinoma	4·1 (2·60–5·10)	1·2 (0·38–2·0)	2–3	20·8 (11·8–47·0)	5–10	4·5 (2·6–8·0)	9	0·33 (0·27–0·38)	>0·48 (0·36–0·55)	>13
Spontaneous mammary carcinoma	9·0 (8·5–9·5)	21·4 (9·0–22·8)	49	130·0 (16·0–170·0)	65	4·5 (2·1–7·2)	11	0·14 (0·03–0·25)	>0·65 (0·36–1·27)	>18
MCIS Sarcoma	4·6 (2·00–6·35)	6·0 (0·44–20·1)	15	29·0 (6·5–66·5)	15	11·7 (1·4–26·0)	29	0·32 (0·16–0·56)	0·43 (0·23–0·64)	12
MCAC Sarcoma	5·2 (4·30–7·0)	3·6 (1·33–7·5)	8	95·0 (63·5–125·0)	47	19·0 (11·0–27·0)	48	0·38 (0·18–0·65)	>1·85 (0·44–3·88)	>50
MC 57 Sarcoma	5·1 (2·70–8·25)	10·7 (0·46–22·8)	17	48·0 (8·0–133·0)	16	6·1 (2·1–11·0)	20	0·15 (0·02–0·32)	0·43 (0·12–0·84)	18
Lymphoma	4·7 (3·30–6·45)	7·8 (1·1–11·6)	17	91·0 (65·0–118·0)	30	1·4 (0·5–2·3)	3	0·17 (0·13–0·20)	0·23 (0·13–0·30)	10
C. Ascites tumour fluid										
ELD Hyperploid	3·7 (2·25–4·65)	0·7 (0·04–1·65)	1–2	4·1 (1·6–9·5)	1–2	0·8 (0·2–2·5)	2	0·07 (0·05–0·15)	0·35 (0·15–0·63)	10
MCIS Sarcoma	3·6 (2·15–4·65)	2·3 (0·35–9·0)	5	2·9 (0·6–7·0)	1–2	1·2 (0·2–2·4)	3	0·05 (0·01–0·11)	0·34 (0·13–0·52)	10
*Correction factors for recalculation into IU (µmoles substrate/ml/min)		f=x 3·3		f=x $0·5\times10^{-3}$		f=x $1·7\times10^{-3}$				

Average data and ranges in brackets. Ratio T/pl. expresses the rise in average enzymatic activity in tumour IF over the corresponding activity in blood plasma in normal mice of the same strain.

The numbers of cases investigated are given in Table 1.

The marked increase in total catheptic activity at pH 5·0 following the addition of cysteine +EDTA should be noted.

lysosomal enzyme activity might be correlated both to the growth characteristics and stromal cell reactions at the tumour periphery.

Central tumour fluid. These presented more distinctive activity patterns (Table 5; Section B) to be evaluated with close attention to the histology of tumour groups. The general characteristics included very considerable increases in all lysosomal hydrolases, and a remarkable activation of cathepsin activity at pH 5·0.

The question then arose if some leading pattern in these variations could be found. For this purpose, in each tumour type, the observed data were subgrouped together according to tumour size, extent of necrosis and degree of active macrophage reaction. The ELD solid carcinoma series all represented young tumors with a sparse macrophage reaction and insignificant necrosis: the figures showed the slightest rise in lysosomal enzyme activity without very large peak figures (*cf.* ranges in brackets). This carcinoma may be compared with the spontaneous mammary with the opposite characteristics (Table 1); the latter presented most considerable increases in lysosomal activity, particularly referring to the AcNPhPhase, β-glucuronidase and total catheptic ones. The lymphomas in this series formed a group distinct from the others, possibly due to their diffuse growth pattern, variable admixture of macrophages and also high incidence of tumour cell death.

The sarcoma group comprised one tumour type with excessive necrosis (MCIS) while the two others had less necrosis. The relative enzymic data are evident from Table 5. The most anaplastic and destructive sarcoma was probably the MCAC which also showed the most enormous increase in catheptic activity with a maximum in one case of 50 times the normal IP fluid, that is over 100-fold that of the plasma level at pH 5·0. Admixture of fresh blood generally tended to diminish the rise in catheptic activity possibly due to added amounts of the plasma inhibitor [8].

Remarks relating to catheptic activity. Among the lysosomal hydrolases so far studied in tumour IF the cathepsins have presented the greatest obscurity due to lack of insight into the nature of their different forms as studied in sufficiently isolated states, *cf.* [23]. The very marked rise in acid proteinase activity previously recorded at pH 4·1–4·5 may be ascribed mostly to cathepsin D [1, 2, 21]. Despite careful search for the trypsin-like cathepsin B with proteins and BANA as substrates no significant activity was found so far; the presence of a potent inhibitor was predicted [3]. Cathepsin B has now been sufficiently purified [16]; optimum activity was about pH 5·6 and a marked activation occurred following addition of cysteine + a chelating agent like EDTA. Cathepsin D as such was not influenced by added cysteine or EDTA. In the present assays at pH 5·0 we are thus still dealing with *a mixture of cathepsin D and B.* The very marked activation demonstrated in Table 5 clearly indicates, for the first time, *that cathepsin B is present in an active form in most tumour IF.*

DISCUSSION

The important point is that solid tumour IF presented an extracellular milieu characterized by large increases in lysosomal hydrolase activity. The remarkable cathepsin activation following the addition of cysteine + EDTA further forms the first evidence for the presence in tumour IF of cathepsin B.

We are dealing with two topically different types of tumour IF: one is the peripheral at and outside the growing tumour border. The cortical tumour zone is well vascularized and a driving force in the form of a capillary pressure is provided for flow of IF. Excessive edema may occur, cellular reactions, mainly macrophages, and cell death complicate the picture. The fluid and added proteins are continuously carried away to the draining lymph trunks [22]. This is the "milieu externe" which faces and influences the surrounding host cells and fibrous proteins. In this edematous zone slight or moderate increases in lysosomal enzyme activity were noted per µl. If the fluid became stagnant for some time, or if central IF was sieved through to some extent, the activity figures would rise. Our present figures are only average ones representative of the whole compartment as such. The actual activity figures at cellular interphases are unknown depending upon individual degrees of activation and inhibition in each microcompartment. Even small increases in average activity of deleterious enzymes normally inhibited (or enzymes with a specific destructive action) might therefore have important physiological implications.

The central IF, on the other hand, is mainly modelled by the biology of older tumour cell generations and their decay. This IF is largely stagnant [22] and active macrophages are generally scant. This compartment uniformly presented the most enormous increases in activity of all the enzymes investigated both at a per volume and a per protein basis. IF from

young non-necrotic tumours showed less marked rises in lysosomal enzyme activity *cf.* [2]. Individual activity variations are possibly due to differences in enzyme stability, inactivation by environmental factors, etc. all of which cannot be discussed for lack of data. A remarkable finding was the very large rise in the available cathepsin B activity as suggested by the new activation procedure. The enzyme is apparently present although its activity level in its natural surroundings seems moderate without maximal activation. Chelation similar to that obtained by added EDTA may to some extent be produced by lactate *cf.* [3], but the actual -SH level is probably low [21] and remains to be studied.

Sources of enzymic contributions. The postulation is justified that lysosomal proteins and enzymes must be added in order to explain the major part of the local activity increases observed in tumour IF. The dual origin of such added enzymic terms, both from "leaky" vital tumour cells [24] as well as resulting from injury and/or autolysis on the part of tumour and host cells, have been discussed in previous articles [2–5]. Since the rate of fluid transport away from the tumour periphery is considerable [22], as compared with the more stagnant central IF, the amounts of enzyme proteins added per unit time into the former compartment must be larger than the enzyme activity figures bear witness of.

The role of macrophages as contributors of lysosomal hydrolases mainly at the tumour periphery is of special interest since macrophages sometimes occur in significant numbers in this region. Some information as to the role of macrophages and leukocytes may be obtained from recent enzyme assays on talcum granuloma fluid in rats [6]. The enzymic assays of non-infected granuloma IF demonstrated no rise in AcNPhPhase, high levels of β-glucuronidase and aryl sulphatase, moderate rise in catheptic activity at pH 4·1 and no increase at all in the dipeptidase activity. This was the pattern characteristic of a nearly pure (90%) macrophage population. Even the infected granuloma IF with excessive death of leukocytes and macrophages was almost lacking in dipeptidase activity (no inhibitor present). This leads to the conclusion that (1) the macrophage contributions may explain part of the increase in β-glucuronidase and aryl sulphatase, but not that of AcNPhPhase and dipeptidase activity and (2) very large numbers of macrophages must be present to effect the increases in enzyme activity of tumour fluid. In other words, the granuloma IF presented an enzyme pattern rather distinctive from that of tumour fluid.

Therefore, it seems reasonable to assume that tumour IF may receive some contributions mainly of β-glucuronidase and aryl sulphatase from macrophages, and perhaps some AcNPhPhase from leukocytes (turbid IF), but contributions from inflammatory cell reactions can hardly explain the whole enzyme composition of tumour IF. Moreover, the numbers of macrophages present in central tumour regions seem much too small. It may thus be inferred that the major parts of enzyme additions are derived from the tumour cells [24] previously shown to be rich in peptidases [21]. More specific correlations between enzymic data and the different regional cell types in this tumour material will be discussed in connection with a forthcoming histochemical study (Niemi and Sylvén, in preparation).

Biological implications. The main issue is the finding of considerable amounts of lysosomal hydrolases freely available to substrate interaction in the native IF of solid tumours. Previous data [1, 2, 7, 14] covered a host of enzymes splitting carbamide bonds rendering a large number of substrates (various peptides, amides, etc.) open to attack. This referred mainly to nutritional aspects and was thought to explain to some extent the "metabolic superiority" of tumour cells. Other substrates and enzyme reactions will now become similarly involved by the demonstration of additional lysosomal enzymes, although it may still be too early to ascribe a special biological significance to any one of the AcNPhPhase, β-glucuronidase or aryl sulphatase.

If these three enzymes contribute little to our understanding of cellular behaviour we have to consider much wider digestive and biological interactions in the case of the cathepsin B activity. The demonstration in tumour IF of this "trypsin-like" effect was finally made thanks to change of substrate and the introduction of cysteine + a chelating agent. The actual activity levels between pH 5–7 depend upon the presence of proper activators. Moreover, a specific cathepsin B plasma inhibitor exists belonging to, or associated with, the haptoglobins [8]. If proper activation occurs and inhibitor material is insufficiently represented then a strong trypsin-like protease effect will become active up to pH 7. This would obviously open new perspectives explaining basic events like the detachment of tumour cells, loss of cohesion [25], capacity of invasion [26], destructiveness directed against host proteins [21], and other factors locally involved

in metastasis formation *cf.* [3–5]. The marked digestive action of liver proteins by lysosomal hydrolases has been described [27] and the degradation of cartilage matrix, muscle proteins and collagen, *cf.* [18], may be mentioned. A slight increase in the rate of cellular detachment was observed following addition of impure lysosomal material [28]. The use of weak trypsin solutions for the detachment of cells from each other and from glass surfaces *in vitro* is of long standing *cf.* [29]. The *in vivo* effects would most likely be similar by local activation of cathepsin B present in tumour IF. Present data will thus tentatively lead to the conclusion that discharge of lysosomal enzymes—possibly associated with lysosomal changes [30, 31] to be further studied—may constitute important and still unrecognized operative factors in tumour growth, invasiveness and destructiveness.

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RESUME

Ce travail relate des expériences sur l'activité des hydrolases lysosomiques du liquide interstitiel acellulaire, provenant de régions périphériques et centrales de divers transplants de tumeurs solides, chez la souris. Ces activités furent comparées à celles observées dans le plasma normal et le liquide intrapéritonéal des mêmes souches de souris. Toutes les activités enzymatiques furent exprimées par rapport à un volume de 1 *ou* 10 µl *de liquide de la tumeur d'origine. L'observation caractéristique—et quelque peu inattendue—fut que le liquide de la portion centrale des tumeurs avait, en général, une forte augmentation de la nitrophénylphosphatase acide, de la* β*-glucuronidase, de l'arylsulphatase et de l'activité cathepsinique à pH* 5,0. *On put mettre aussi en évidence l'existence d'une activité de la cathepsine B lors de l'addition de cystéine et d'EDTA.*

L'origine différente des protéines enzymatiques ajoutées, provenant soit de la tumeur, soit des cellules de l'hôte, est discutée. Parmi les hydrolases lysosomiques décrites, l'intérêt physiologique le plus grand est attribué à l'activité de la cathepsine B, ressemblant à l'activité de la trypsine. Cette activité pourrait être la cause de la libération des cellules tumorales, favorisant ainsi l'invasion néoplasique et la formation de métastases.

SUMMARY

This study embodies lysosomal enzyme assays on cell-free interstitial fluid from peripheral and central regions of various solid mouse tumour transplants. Comparison was made with the corresponding activities of normal plasma and intraperitoneal fluid from the same strains of mice. All enzymatic activities have been expressed per a volume basis of either 1 *or* 10 µl *of original tumour fluid. The characteristic and somewhat unexpected feature was that central tumour fluid presented, on an average, very large increases in acid nitrophenyl-phosphatase,* β*-glucuronidase, aryl sulphatase and total catheptic activity at pH* 5·0. *Evidence was further presented for the occurrence of cathepsin B activity following the addition of cysteine + EDTA.*

The dual origin of added enzymatic proteins both from tumour and host cells is discussed. Among the lysosomal hydrolases described the greatest physiological interest is attributed to the trypsin-like cathepsin B activity. This activity seems to be operative in the detachment of tumour cells, thus favouring invasion and the formation of metastasis.

ZUSAMMENFASSUNG

Die Arbeit befaßt sich mit Bestimmungen von einigen lysosomalen Hydrolasen in zellfreier interstitieller Flüssigkeit aus peripheren und zentralen Regionen verschiedener solider Impftumoren bei Mäusen. Verglichen wurde mit den entsprechenden Aktivitäten von normalem Plasma und von intraperitonealer Flüssigkeit desselben Mäusetumors. Alle Enzymaktivitäten sind auf Volumen bezogen und zwar auf 1 *oder* 10 µl *Tumorflüssigkeit. Das charakteristische und etwas unerwartete Ergebnis war, daß die zentrale Tumorflüssigkeit im Durchschnitt eine Erhöhung der Aktivität der sauren Nitrophenylphosphatase,* β*-Glycuronidase, Arylsulphatase und totalen Kathepsin Aktivitäten zeigte. Es konnte ferner das Auftreten von Kathepsin B-aktivität nach Zugabe von Cysteine+ EDTA gezeigt werden. Der doppelte Ursprung der zugefügten Enzymproteine nämlich sowohl aus Tumor- als auch aus Wirtszellen wird diskutiert. Unter den lysosomalen Hydrolasen kommt das größte physiologische Interesse dem trypsinähnlichen Kathepsin B zu. Diese Aktivität scheint an der Ablösung von Tumorzellen mitzuwirken und begünstigt so das invasive Wachstum und die Metastasenbildung.*

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A Blockage in the S-phase of HeLa Cells Produced by a Human Tumor Polypeptide

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INTRODUCTION

IN PREVIOUS articles the purification of a diffusible polypeptide from tumor fluids was described [1, 2]. The molecular size of the positively charged polypeptide was estimated by gel filtration to be 1900–2000. Qualitative amino acid analyses revealed the presence of alanine, arginine, cysteine, glutamic acid, glycine, leucine, tyrosine, and valine [1, 2]. The polypeptide inhibited the growth rate of four different asynchronously growing cell strains and induced a depression in the mitotic frequency of synchronized L cells [3]. It was suggested that the depression in the mitotic frequency was due to an effect on some premitotic process.

The present paper describes experiments designed to discover in which phase of the life cycle the cells were most sensitive to the addition of the polypeptide. By studying the accumulation of mitoses of HeLa cell populations incubated in the presence of colcemid [4] it is shown that the effect of added polypeptide at the used dose level is limited to the S-phase and that this effect can be completely abolished by the addition of deoxyribonucleosides.

MATERIALS AND METHODS

Cells

Stock cultures of PPLO-free HeLa (Gey) cells were growing in stationary culture flasks containing Eagle's medium supplemented with 10% calf serum, antibiotics and glutamine [3]. The cells were harvested for experiments by trypsin treatment, centrifuged and resuspended in fresh culture medium and were then seeded out on Petri dishes (7 cm dia.) at a final total cell number of 10^6 cells/dish and with a total volume of 10 ml medium/dish. The cells were incubated at 37° for about 18 hr in humidified air containing 5% CO_2 in order to re-enter the logarithmic phase of growth.

Partially synchronized cultures

After 18 hr the medium was replaced by fresh medium containing thymidine (TdR) at a final concentration of 2mM [5] and the cells were incubated for another 36 hr. After this time the cells were washed three times with prewarmed and pregassed medium in order to release the thymidine blockage. The Petri dishes were then divided into two series, one of which was given 5 ml/dish of fresh prewarmed and pregassed medium only and the other 5 ml/dish of prewarmed and pregassed medium containing polypeptide at a final concentration of 60μM, calculated as leucine equivalents as determined by a ninhydrin method [3, 6]. Colcemid, dissolved in a small amount of culture medium, was added at zero time to 10 dishes of each series at a final concentration of 0·25μg/ml. All Petri dishes were then reincubated at 37°C and one Petri dish of each of the control and the polypeptide-treated colcemid-containing series was sampled every ½ hr with start at 1·0 hr (Fig. 1). At 5·0 hr a new set of 10 dishes were given colcemid with the first sample taken at 6·0 hr; 10 hr after

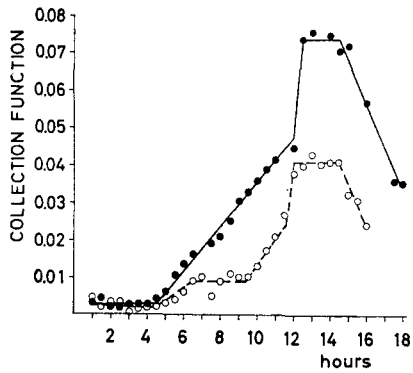


Fig. 1. The collection function of mitoses of partially synchronized HeLa cells with and without addition of polypeptide.

● ——● control o – – o polypeptide added at zero time at a final concentration of 60μM, expressed as leucine equivalents. Partial synchronization was induced by blockage with 2mM TdR for 36 hr and the release of that blockage by three washings in culture medium. The accumulation of mitoses was obtained by colcemid added as described under Materials and Methods.

Both the control series and the polypeptide series show an initial lag in the accumulation of mitoses during 0 to 4·5 hr. This period of time represents the duration of $G_2+\frac{1}{2}$ M. The control series then accumulate mitoses in a linear fashion between 4·5 and 12 hr, which interval represents the duration of the S-phase. Subsequent to the linear portion a burst in mitoses occurs, representing those cells which were at the boundary of G_1/S at start of the reincubation. In the polypeptide series the cells originally present in the S-phase shows a lag in the collection function between 6·5 and 9·5 hr.

reincubation a third set of dishes were given colcemid for sampling between 11·0 and 15·5 hr, and so forth. By this relay method [4], the accumulation of mitoses could be studied on samples in ½ hr intervals during 18 hr without errors due to cytotoxic effects caused by colcemid [7]. Zero time was chosen when half the number of dishes in each series were reincubated.

Asynchronous populations

Petri dishes with growing cells were divided into four series and the medium was replaced according to the following scheme: *Series* 1: fresh medium only, *Series* 2: fresh medium containing 60μM polypeptide, *Series* 3: fresh medium containing 0·1 mM of each of TdR, adenosine deoxynucleoside (AdR), cytidine deoxynucleoside (CdR), and guanosine deoxynucleoside (GdR), *Series* 4: fresh medium containing 0·1 mM of each of TdR, AdR, CdR, and GdR together with 60μM polypeptide. All media were prewarmed and pregassed. The total volume of medium in each dish was 5 ml. To all series colcemid was added at a final concentration of 0·25 μg/ml at the time of medium replacement. The Petri dishes were incubated at 37° and one dish of each of the four series was sampled every ½ hr during 5·5 hr. Zero time was set when half of the dishes of each series were reincubated.

In order to determine the length of the G_2-phase, the collection function [8] for mitoses was calculated from a separate experiment with a random population to which 2 mM TdR was added together with colcemid [4].

Chemicals and polypeptide

The deoxynucleosides were purchased from P-L Biochemicals (Milwaukee, Wisc.). Colcemid was presented as a gift by the Ciba Co., Basle. Polypeptide was prepared from the ascites fluid of human patients carrying inoperable ovarial carcinomas according to previously described methods [1, 3].

Mitotic counts

The medium of each Petri dish was collected separately. The cells growing in the Petri dish were treated for 5 min at 37° with 3 ml 0·03% trypsin dissolved in physiological saline. The trypsinized cells were thoroughly resuspended and centrifuged together with the supernatant culture medium. The cell pellet was immediately fixed in ethanol–acetic acid (3:1) and smeared on microscope slides. After drying in air at room temperature, the cells were stained by haematoxylin and mounted. The frequency of mitoses was counted on 1000 cells in each preparation. The microscope slides were coded for the cell counting technician. The collection function for mitoses was calculated according to Puck [8] as the $\log(1+N_M)$, where N_M is the mitotic frequency.

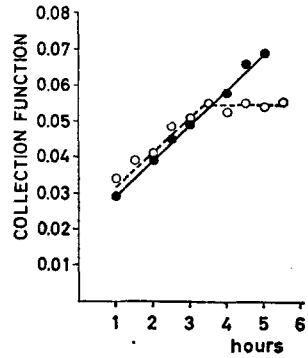


Fig. 2. The collection function of mitoses of asynchronously dividing HeLa cells with and without polypeptide.
●——● *control* o – – o 60μ*M polypeptide added at zero time.*
In the control series the collection function for mitoses is linear with time. When polypeptide is added a delay in the arrival in metaphase occurs at 3·5 hr. This delay corresponds to the lag in the S-phase demonstrated in Fig. 1.

RESULTS

Partially synchronized populations

The collection function for mitoses of a partially synchronized HeLa population is seen in Fig. 1 (solid line). The mitotic collection showed an initial lag [4] corresponding to the duration of $G_2+1/2$ $M=4·5$ hr. After this initial lag a linear accumulation of mitoses started, and proceeded for a time corresponding to the duration of the S-period=7·5 hr. About 12 hr after reincubation a burst of mitoses was obtained, representing those cells which became concentrated at the boundary of the G_1 and the S-phases due to the TdR-treatment. Subsequent to this burst, a rapid decline of the mitotic collection occurred. This final decrease in the collection function represented those cells which were in the G_1-phase at the end of the TdR-treatment.

When 60μM polypeptide, calculated as leucine equivalents, was added at the time of reincubation to the HeLa population which was previously partially synchronized, the mitotic collection was apparently identical with that of the control cells for the first 4·5 hr (Fig. 1 hatched line). The accumulation of mitoses for the first succeeding 2 hr (ranging between 4·5 and 6·5 hr) of the S-phase proceeded at a rate slightly slower than that of the control. These cells represented that portion of the population which was at the end of the S-phase at zero time. After this 2 hr interval a polypeptide induced lag (occupying the time portion between 6·5 and 9·5 hr) appeared in the accumulation of mitoses caused by a delay in the arrival of S-cells in mitosis. The polypeptide-induced lag had a duration of approximately 3 hr at the polypeptide dose given. The accumulation of mitoses then started again and proceeded at a linear fashion, at a rate equal to that of the control. At about 12 hr after reincubation, a burst of G_1/S-cells

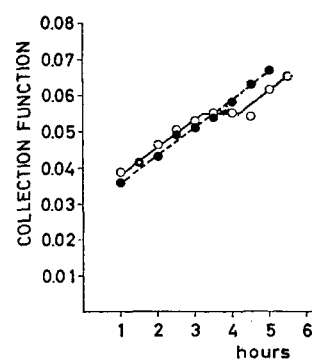


Fig. 3. The collection function of mitoses of asynchronously dividing HeLa cells in the presence of TdR+AdR+CdR+GdR with and without polypeptide.
o——o 0·1*mM of each of TdR+AdR+CdR+GdR added at zero time.* ●– – ● 0·1*mM of each of TdR+AdR+CdR+GdR together with* 60μ*M polypeptide. The additions were made at zero time.*
When deoxynucleosides are added together with polypeptide the collection function of mitoses is linear with time as in the simultaneously performed control of Fig. 2. Deoxynucleosides alone induce a brief delay (between 3·5 and 4·5 hr) in cell arrival in metaphase followed by an accumulation of mitoses of a normal rate.

occurred, however, not as large as in the control population. The final decline in the mitotic collection from polypeptide - treated cells appeared to be of the same rate as in the control population.

Asynchronously dividing populations

The collection function for mitoses of an asynchronous HeLa population was found to be definitely linear with time between 1 hr and 5·0 hr after colcemid addition (Fig. 2 solid line). Samples taken at 0 and at 0·5 hr were not found to fit into the expected linearity and those values were therefore excluded from the diagrams. Such deviations from the linearity have been observed in some cell types and have been interpreted to reflect the penetration rate of colcemid into the nucleus [7, 8]. Deviations from the linearity do also occur if the incubation time in presence of colcemid should be extended over 5·5 hr due to degeneration of mitotic figures [7, 9].

The addition of 60μM polypeptide at zero time, together with colcemid, resulted in a mitotic collection which was normal only for a time period up to 3·5 hr (Fig. 2 hatched line). This time period corresponded to the duration of G_2 as revealed by a separate experiment where 2 mM TdR was added together with colcemid. The curve for polypeptide-treated cells then levelled off when the last G_2-cells had passed on to mitosis, indicating that the polypeptide induced a delay in cell arrival from the S-phase into mitosis. This delay corresponded to the polypeptide-induced effect obtained in the S-phase of the synchronized cell population described above. In experiments where lower doses of polypeptide were used, the delay starting at 3·5 hr could later be overcome. In those cases, the delay produced decreased in duration time with decreasing polypeptide dose and was followed by a linear collection function of a normal slope.

A lag in the mitotic accumulation of cells, originally present in the S-phase, would indicate that a process closely linked to the DNA synthesis was affected. This assumption was tested by the addition of the deoxynucleosides TdR+AdR+CdR+GdR at a final concentration of 0·1 mM of each simultaneous to the addition of 60μM polypeptide. In these series the mitotic collection was linear with time up to 5·0 hr (Fig. 3 hatched line) and the curve had a slope of the same magnitude as that of the control series. The addition of TdR+AdR +CdR+GdR only at a final concentration of 0·1mM of each to 10^5 cells/dish induced a a normal collection function for 3·5 hr followed by a brief delay (time period between 3·5 and 4·5 hr) and a subsequent normal accumulation of mitoses (Fig. 3 solid line).

DISCUSSION

According to the experiments where 2mM TdR was added to an asynchronously dividing population the duration of the G_2-phase was estimated to be 3·5 hr. In the partially synchronized control population the initial lag in the collection function corresponded to the duration of $G_2+\frac{1}{2}M$ (i.e. the mitotic time excluding the duration of anaphase and telophase). This initial lag occupied a time period of 4·5 hr, the duration of $\frac{1}{2}M$ would then by subtraction be 1·0 hr. From the slope of the collection function in the control series of the random population (Fig. 2), the generation time, T, could be calculated. This slope is equal to $0{\cdot}301/T$ [8]. The calculation gave a value of $T=30$ hr for the total life cycle of this cell strain. Thus, the pertinent phases in the life cycle of the HeLa strain used were found to have the following durations: $S=7{\cdot}5$, $G_2=3{\cdot}5$ and $\frac{1}{2}M=1$ hr. These values must, however, be regarded as approximations since no complete life cycle analysis was performed.

The collection function for mitoses in the polypeptide-treated synchronously dividing population indicated that the polypeptide did not interfere to a measurable extent with the duration of the G_2-phase at the given dose. The first portion of cells arriving from the S-phase in mitosis seemed to be only slightly affected by the polypeptide addition. This might indicate that the portion of cells present near the end of the S-phase at the time of polypeptide addition were less sensitive to the polypeptide than those cells which caused the lag appearing later in the collection function of cells in the S-phase. The polypeptide-induced delay in the accumulation of mitoses of the cells in the S-phase had, for the given polypeptide dose, a duration of approximately 3 hr. The results obtained with asynchronously dividing populations treated with varying polypeptide doses strongly indicated that the length of the observed lag in the S-phase was dose-dependent. Therefore, it is to be expected that the final linear rise in the collection function (between 9·5 and 12 hr) of polypeptide-treated synchronized cells will be shortened at higher polypeptide concentrations giving an even smaller burst of G_1/S-cells. This would mean that the synchronic wave of mitoses is also dependent on added amount of polypeptide, a result already obtained in earlier synchronization experiments [3]. The second linear rise

in the collection function of cells arriving from the S-phase in mitosis in polypeptide-treated cultures had a slope approximating to the slope of the collection function of S-cells in the control. Thus, the polypeptide did not affect the rate of transfer of these cells to metaphase at the concentration studied. The most likely explanation would be that too small amounts of polypeptide were present at the site of action within these cells to give a measurable change in the rate of accumulation of mitoses.

The final burst of mitoses of polypeptide-treated cells represented a mean value of 9·8% mitoses, a value which, compared to the 18·5% in the control population, indicated a loss of about 47% of the potentially dividing fraction of the population. The time portion occupied by the polypeptide-induced lag in the collection function between 6·5 hr and 9·5 hr in the S-phase represented 40% of the entire length of the S-phase. These two figures indicate that the cells affected by the polypeptide failed entirely to reach mitosis within the time of the experiment. One may suggest that these cells, which develop a disturbed DNA metabolism, sooner or later undergo an irreversible injury detectable by dye exclusion tests. These cells would thus contribute to the increased cell mortality in suspension cultures observed after three-day incubation in presence of polypeptide [3].

The small delay in the accumulation of mitoses of random cells in the presence of 0·1mM of each of the deoxynucleosides indicated a cessation in the progression of a portion of cells from the S-phase in mitosis due to a slowing down in the rate of DNA synthesis. That such a delay can be expected is supported by the observation that a total of 3×10^6 strain L cells were significantly growth inhibited by 0·1mM TdR [10]. It may also be added that 2μM/ml of deoxyadenosine have been observed to inhibit the synthesis of DNA in Ehrlich ascites carcinoma cells and that this amount of AdR was suggested to be able to inhibit mitosis of certain cells *in vitro* [11].

The addition of deoxynucleosides together with the polypeptide completely abolished the effect of the polypeptide. This result indicates that the polypeptide interferes with the metabolism of cells in the S-phase in some process(es) closely linked to the DNA synthesis, but at a level where the effect can be counteracted by deoxynucleosides.

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RESUME

La cinétique de l'entrée des cellules en mitose, en présence de Colcémide, a été étudiée sur des populations de cellules HeLa traitées par un polypeptide diffusible, dérivé de liquide d'ascite néoplasique humain. L'accumulation des mitoses de cellules partiellement synchronisées a montré que le polypeptide, à la dose utilisée, n'a d'effet que sur les cellules en phase S du cycle mitotique. Par l'étude de l'accumulation des mitoses de cellules en croissance asynchrone, on a pu montrer que des désoxyribonucléosides étaient capables de neutraliser l'action du polypeptide. On en a conclu que le polypeptide empêchait la multiplication des cellules en entravant des processus étroitement liés à la synthèse de l'acide désoxyribonucléique.

SUMMARY

The kinetics of cell arrival in mitosis in the presence of colcemid was studied on HeLa cell populations treated with a diffusible polypeptide derived from human tumour ascites fluid. The accumulation of mitoses of partially synchronized cells revealed that the polypeptide, at the given dose level, affected cells only in the S-phase of the life cycle. By studying the accumulation of mitoses of asynchronously growing cells it could be shown that deoxyribonucleosides could counteract the effect of the polypeptide. It is concluded that the polypeptide impaires cell multiplication by interfering with processes closely linked to the DNA synthesis.

ZUSAMMENFASSUNG

Bei einer HeLa-Zellpopulation, welche mit einem diffusiblen Polypeptid aus menschlichem Tumorascites behandelt war, wurde die Kinetik der Erreichung der Mitose in der Gegenwart von Colcemid studiert. Die Ansammlung von Mitosen bei teilweise synchronisierten Zellen

zeigte, dass das Polypeptid mit der verwendeten Dosis die Zellen nur in der S-phase des Zyklus beeinflusste. Bei asynchron wachsenden Zellen konnte gezeigt werden, dass Desoxyribonukleoside dem Effekt des Polypeptid entgegenwirken. Hieraus wird geschlossen, dass das Polypeptid die Zellvermehrung hemmt, indem es in Prozesse eingreift, welche mit der DNS-Synthese in Zusammenhang stehen.

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Onze Observations d'un Modèle Précis D'évolution Caryotypique au Cours de la Leucémie Myéloïde Chronique

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Les évolutions clonales, caractérisées par des transformations systématisées du caryotype, sont le mieux observées dans la leucémie myéloïde chronique (LMC), soit durant la phase chronique, soit, beaucoup plus souvent, lors de la transformation blastique.

Elles semblent se faire selon certains modèles [1, 2] qui sont, du plus simple au plus complexe: (1) La duplication du chromosome Ph^1, (2) L'acquisition d'un surnuméraire. (3) L'acquisition et la duplication de plusieurs surnuméraires. (4) La perte de chromosomes. (5) L'acquisition et la perte de chromosomes. (6) L'acquisition de chromosomes marqueurs autres que le Ph^1, associée à l'un ou l'autre des modèles précédents. (7) La polyploïdisation.

Le modèle 6 comprend un type particulier d'évolution clonale dont la fréquence, par rapport aux autres modèles d'évolutions clonales, est probablement très élevée. Elle est caractérisée par la présence du Ph^1, par l'acquisition d'un chromosome marqueur médiocentrique de la taille d'un *C*, par la perte d'un chromosome 17–18, et, éventuellement, par d'autres remaniements numériques.

Pedersen [3, 4] a, le premier, réuni 3 cas de LMC comportant la perte d'un 17–18 et l'acquisition d'un C. Grouchy *et al.* (1) ont rapporté 3 cas semblables. Puis Engel [5, 6] et Stich *et al.* [7] ont attiré l'attention sur la présence du marqueur et proposé l'hypothèse qu'il s'agit d'un isochromosome pour le bras long du 17–18 perdu.

Nous avons étudié 24 cas d'évolutions clonales [8], la plupart pendant la transformation d'une LMC. Parmi ces 24 cas, 11 sont compatibles avec ce type précis caractérisé par la perte d'un 17–18 (probablement un 17), l'acquisition d'un marqueur médiocentrique, l'acquisition d'un ou plusieurs *C*. Ce modèle représenterait peut-être celui selon lequel se font la moitié des évolutions clonales.

Nous rapporterons ici les observations cytogénétiques faites au cours de ces 11 évolutions clonales. Les observations 1, 3 et 5 ont été incluses dans une publication précédente [1].

Observations cliniques

Cas 1. Homme âgé de 45 ans. Pas d'antécédent pathologique. Début clinique à 42 ans d'une LMC. Traitement par le busulphan. Transformation aiguë après trois ans d'évolution. Traitement par le dibromomanitol. Décès trois mois plus tard. Examen caryotypique lors de la transformation aiguë.

Cas 2. Homme âgé de 23 ans. Pas d'antécédent pathologique. LMC en évolution depuis 5 ans, traitée par radiothérapie (2.300 R sur le corps caverneux, 1.200 R sur la région sacrée) et busulphan. Examen

caryotypique lors de la transformation aiguë. Décès trois mois plus tard.

Cas 3. Homme âgé de 21 ans. Antécédents de néphrites et d'angines. LMC évoluant depuis trois ans et traitée par le busulphan. Etude chromosomique au cours de la transformation aiguë. Traitement par la prednisone et la 6-mercaptopurine. Apparition d'adénopathies cervicales considérables. Nombreux accidents infectieux. Décès par septicémie.

Cas 4. Homme âgé de 39 ans. A l'âge de 31 ans épithélioma cutané traité par aiguilles de radium. A 36 ans, découverte d'une LMC. Traitement par le busulphan. Transformation aiguë à 38 ans. Traitement par la 6-mercaptopurine. Décès l'année suivante. Anomalie de Pelger-Huet. Présence de cellules malignes évoquant des cellules réticulaires jeunes.

Cas 5. Homme âgé de 53 ans. A subi, douze ans auparavant, 80 radioscopies, après la découverte d'une masse médiastinale d'allure kystique sans aucun retentissement fonctionnel. LMC se transformant en leucémie aiguë après huit ans d'évolution. Traitement: busulphan, prednisone et 6-mercaptopurine.

Cas 6. Homme âgé de 60 ans. LMC évoluant vers la transformation blastique en trois ans Traitement: busulphan et méthylglyoxal. Etude caryotypique pendant la phase aiguë.

Cas 7. Homme âgé de 34 ans. Etude chromosomique faite un mois $\frac{1}{2}$ après le diagnostic clinique et après huit jours de traitement par le dibromomanitol.

Cas 8. Homme âgé de 46 ans. Antécédents de tuberculose. LMC découverte à 38 ans lors d'un examen systématique. Examen cytogénétique pendant la phase blastique. Traitement par le busulphan et le méthylglyoxal. Décédé après quelques mois.

Cas 9. Homme âgé de 44 ans. Etude cytogénétique trois mois après le diagnostic. Le traitement par le busulphan s'est révélé inefficace et le décès est survenu peu de temps après.

Cas 10. Homme âgé de 45 ans. LMC en évolution depuis sept ans. Transformation aiguë. Traitement: busulphan, prédnisone, méthylglyoxal, 6 mercaptopurine. Décès survenu rapidement après l'examen caryotypique.

Cas 11. Femme âgée de 73 ans, ayant subi à l'âge de 34 ans une hystérectomie pour fibrome. Diagnostic clinique d'une LMC porté à l'âge de 70 ans. Transformation aiguë trois ans plus tard. Traitement: busulphan, prednisone. Décès par colapsus peu après la transformation aiguë.

Les données cliniques et hématologiques de ces malades apparaissent dans les Tableaux 1, 2 et 3.

MÉTHODES

Les études chromosomiques ont été faites selon des techniques précédemment décrites [9]. Elles comprennent, pour tous les malades, un ou plusieurs examens de la moelle (examen direct ou culture à court terme de 24, 48 ou 72 h) et, pour certains malades, une culture de sang à court terme (Tableaux 4 et 5).

Tableau 1

Cas no.	Age	Sexe	Nombre d'examens	Phase	Traitement	Durée d'évolution
1	45	M	1	aiguë	BUS	3 ans
2	23	M	1	id	R.I., BUS	5 ans
3	21	M	2	id	BUS, PRE, 6-MP	3 ans
4	39	M	1	id	R.I., BUS, 6-MP	3 ans
5	53	M	2	id	R.I., BUS, PRE, 6-MP	8 ans
6	60	M	2	id	BUS, MGAG	3 ans
7	34	M	1	chronique	BUS	début
8	56	M	1	aiguë	BUS, MGAG	8 ans
9	44	M	2	chronique	BUS	3 mois
10	45	M	1	aiguë	BUS, PRE, 6-MP	7 ans
11	73	F	1	id	BUS	3 ans

Age, Sexe, évolution et traitement: BUS=Busulphan, PRE=Prednisone, 6-MP=6-Mercaptopurine, MGAG=Methylglyoxal, R.I.=radiation.

Tableau 2

Cas no.	G.R. $\times 10^6/mm^3$	G.B. $\times 10^3/mm^3$	Plaquettes $\times 10^3/mm^3$	Lympho-cytes	Mono-cytes	Polynucléaires Neutro.	Eosi.	Baso.	Méta-myélo.	Myélocytes Neutro.	Eosi.	Baso.	Promyélo.	Blastes	PAL.
1	2,230	3,2	117	14	1	36	1	1	17	23				2	
2	2,890	22,3	81	3	2	59		5	5	24				2	0
3	1,950	18,8	98	3	1	25		3	7	21			8	31	165
	2,600	1,9	120	19	6	54	1		8					9	
4	3,070	47	104			23	1	1	13	37			2	6	81
5	2,570	29,2	70	3		30	3		26	16	7		3	11	38
	2,230	7	39	7	7	17			5	16	9	5	7	27	
6	3,820	132	201	6	2	5	4	1							
7	3,410	398	100	2	3	28	2	2	10	48			3	2	0
8	4,140	9,6	140	4		30		10	8	12				36	
9	5,350	29	226	8	4	66		4	3	13			1	1	0
10	2,660	79	12	7	1	4			2				2	83	10
11	2,120	27,6	107	6		16	3	16		5	1	15		80	161

Hémogrammes
PAL: valeurs normales: 20 à 60 phosphatase alcaline leucoytaire)

Tableau 3

Cas no.	Méga-caryocytes*	Cellules réticulaires	Série granulocytaire									Séries	
			Polynucléaires				Myélocytes						
			Neutro.	Eosi.	Baso.	Métamyélo.	Neutro.	Eosi.	Baso.	Promyélo.	Myéloblastes	Monocytaire	Erythro-blastique†
1			14			20	2	8		2	10	42	24,6
2	135		13,5	1,5	5	16	40	4,5	1,5	5	8	5	2
3	1		26	3		12	26	8	1	8	8	8	3
			10,5	1,5	5,5	0,5	5			13,5	40,5	15	4
4		65,5	1		0,5	0,5	12		0,5	2,5	17,5		3,5
5		0,5	4,5	3,5	40,5	1,5	7,5	11	9	7,5		2,5	3,5
6	96		15	8	12	9,5	17	19	2	1,5	14,5	1,5	32,5
7	1		30,5	0,5	1,5	16,5	41	1,5		3,5	1	4	2,5
8	8		4,5	1	7,5	3,5	31,5	26	2	5,5	15	2	11,5
9	5		51,5	1	0,5	14	28			1	1	3	8
	1		45	1	2	5,5	24		0,5	1	2	19	18,5
10			2				1,5	2,5	0,5	1,5	90	1	10,5
11			6,5	3	13		2,5	11	14	2,5	47		9

Myélogrammes.
*Sur toute la lame.
†Par 100 éléments blancs.

Tableau 4

Cas no.	Examen M	Examen S	Heures de culture	PHA	Nombre de chromosomes ⩽44	45	46	47	48	49⩾	4N	Nb de cellules +Ph¹%	Nb de cellules Total
	+		2	−	2		2				2	100	6
1		+	48	−		2	4			1		100	7
		+	72	+	3	3	18	1				32	25
	+		48	−	7	15	2				2	100	26
2		+	id	−		11	3			1		100	15
	+		48	−	23	13	27	88		1	2	100	154
I	+		id	+	17	12	54	67		2	9	79	161
		+	id	+	18	10	28	58		1	1	100	116
3													
II	+		id	+	2	3	25	2			1	12	33
		+	id	+	1	7	20	1				45	29
	+		2	−	5	7	21	8	2	3	12	76	58
4	+		48	−	6	2	3	2	1	2	17	100	33
	+		id	+	5	2	6	4	3	10	16	91	46
I	+		72	+	2	1		3	8	23		100	37
5													
		+	48	−	1	1	3	8	2	16		100	31
II		+	id	+		1	4	9	7	26		100	47
		+	72	+	2	1		9	9	13		100	34
I	+		48	− +	7	2	10	1	5		1	100	26
6													
	+		24	−		2	3	1			1	100	7
II	+		48	−		1	3	1		4		78	9
	+		id	+	3	1	3				4	82	11
	+		48	+	4	4	11		1			75	20
7		+	id	−	3	7	13	3	2			58	28
	+		2	−			1					100	1
8	+		48	−			1	4				100	5
	+		id	+	3	2	4	7			1	65	17
I		+	48	+	2	2	8					100	12
9													
II	+		3	−	13	5	26					98	44
10	+		48	−	2		3	12	3			100	20
11	+		48	+		2	11	1				100	14

Distribution des nombres chromosomiques.

Tableau 5

Cas no.	Nb de caryo-types	Nb de chromo-somes	Chromosomes absents Groupe A	B	C	D	E 16	E 17	F	G	Chromosomes surnuméraires Groupe A	B	C	Mar	D	E	F	G	Ph¹
	1	47			+									+				+	+
1	1	46						+					+						+
	1	46						+	+					+				+	+
	7	46						+						+					+
	2	46																	+
	2	45						+											+
	7	45						++						+					+
2	1	44			+			++						+					+
	2	44				+		++						+					+
	1	43						++	+	+				+					—
	1	46						++					+	+					+
	1	49						++					+	+			++		++
	2	46																	+
	2	47												+					+
	10	46						+						+					+
	1	45						+		+				+					+
	1	45	+					+						+					+
	1	43			+			++	+					+					+
	2	46						+		+			+	+					—
	1	46						+		+			+	+					+
3	1	46						+	+				+	+					+
	1	46						++					+	+					+
	1	46					+	+					+	+					+
	2	46				+		+					+	+					+
	1	46		+				+					+	+					+
	20	47						+					+	+					+
	1	46						++					++						+
	2	47												+					+
	2	46																	+
	1	47												+					+
	1	46						+						+					+
	1	45						+		+				+					—
	1	45			+			+						+					+
	1	45						++						+					+
	2	46						++					+	+					+
	1	46					+	+					+	+					+
4	3	47					+	+					++	+					+
	1	48						+					++	+					+

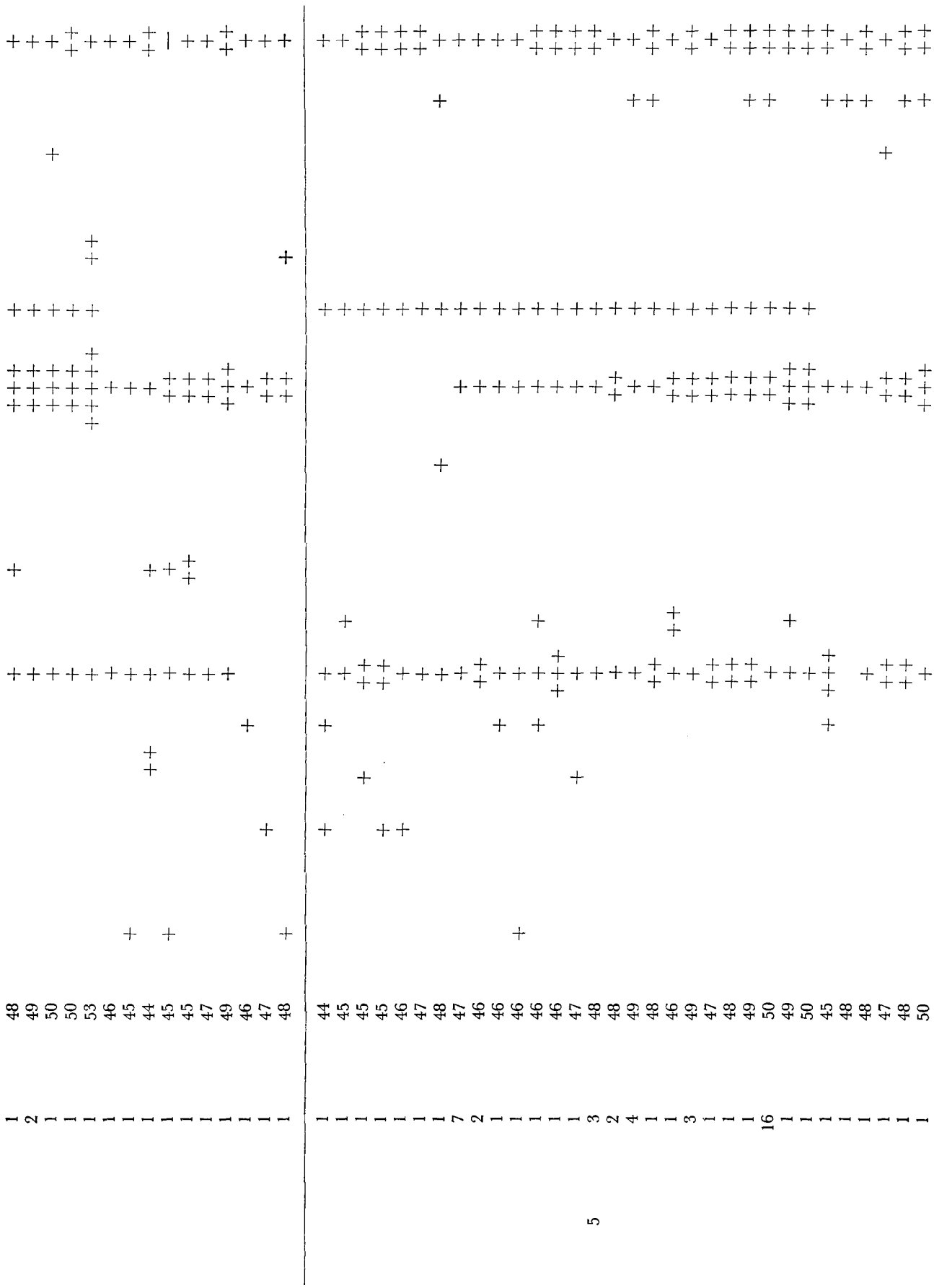

48 49 50 50 53 46 45 44 45 45 47 49 46 47 48
1 2 1 1 1 1 1 1 1 1 1 1 1 1 1
44 45 45 45 46 47 48 47 46 46 46 46 46 47 48 48 49 48 46 49 47 48 49 50 49 50 45 48 48 47 48 50
1 1 1 1 1 1 1 1 7 2 1 1 1 1 1 3 2 4 1 1 3 1 1 1 16 1 1 1 1 1 1 1 1
5

Tableau 5 (suite)

Cas no.	Nb de caryotypes	Nb de chromosomes	Chromosomes absents Groupe A	B	C	D	E 16	E 17	F	G	Chromosomes surnuméraires Groupe A	B	C	Mar	D	E	F	G	Ph¹
	1	49						+	+				++					++	++
	1	50						+					+++					+	++
	1	50											++					+	++
	2	46																	+
	1	46						+						+					+
	1	45						++						+					+
	1	45						+	+					+					+
6	1	46						+	+					+				+	+
	1	46						+	+				+					+	+
	1	46			+			+						+				+	+
	1	47						+						+				+	+
	1	47				+		+					+	+					++
	2	48						+					+	+					++
	1	47				+								+					++
	1	48											+	+					+
	4	46																	+
	1	45						+											+
	1	47						+					+	+					+
7	1	48						+					++	+					+
	1	50											+++	+					+
	1	60											+++++++	+	+++			+	+
8	3	46																	+
	6	47						+					+	+					+
	1	45						+											+
	1	44			+			+	+					+					+
9	1	45						+	+					+					+
	1	45						+		+				+					+
	3	46						+						+					+
	1	46					+	+					+	+					+
10	1	47						+					+	+					+
	2	48						+					+	+	+				+
	1	45			+			+						+					+
11	3	46						+						+					+
	1	47						+					+	+					+

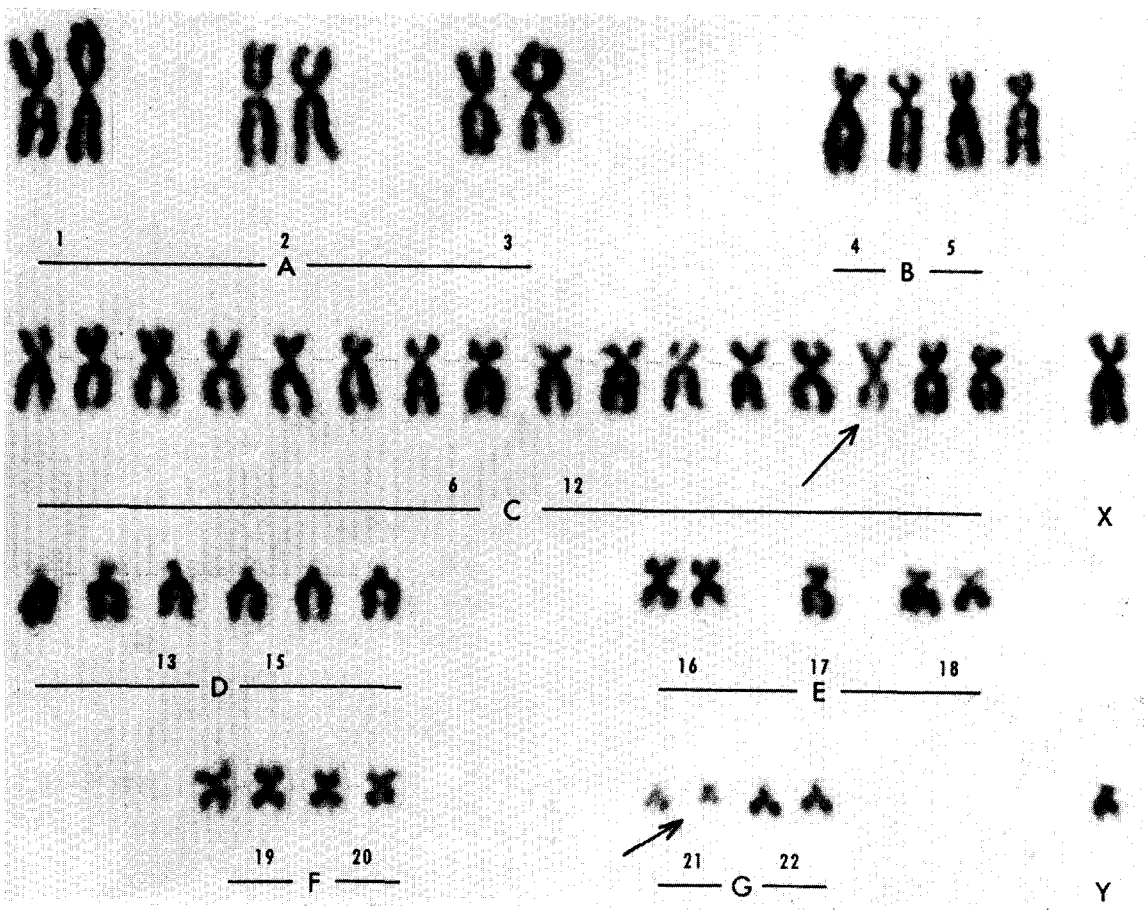


Fig. 1. *Caryotype à* 4 *chromosomes montrant le Ph*^{1}*, la perte d'un* 17*, l'acquisition d'un marqueur médiocentrique de la taille d'un* C. *Noter la constriction particulière de ce dernier.*

(to face p. 488)

RESULTATS

Les études caryotypiques ont montré, pour tous les malades, des caractères communs qui sont: la présence du Ph¹, la perte d'un 17–18, et l'acquisition d'un chromosome marqueur médiocentrique de la taille d'un C (Fig. 1). Les caractères propres à chaque malade sont les suivants (Tableaux 4, 5).

Cas 1. Mode à 46 chromosomes. Dans une cellule, on note la présence du marqueur sans perte d'un 17–18.

Cas 2. Mode à 45 chromosomes par perte de deux 17–18.

Cas 3. Deux modes, l'un à 46 chromosomes, l'autre à 47 chromosomes par acquisition d'un C surnuméraire apparemment normal. Deux cellules ont acquis le marqueur, sans avoir perdu le 17–18.

Cas 4. Pas de mode évident. Nombreuses cellules hyperdiploïdes avec 47 à 53 chromosomes et polyploïdes. Dans une cellule, acquisition du marqueur sans perte d'un 17–18. Acquisition successive de 1 à 5 C surnuméraires. Duplication du Ph¹.

Cas 5. Mode à 50 chromosomes. Duplication du Ph¹. Existence d'une translocation $t(Dq-; 2p+)$. Présence du marqueur. Perte d'un, de deux et même de trois 17–18. Acquisition de 1 à 3 C surnuméraires, et d'un *G* surnuméraire. Dans 9 cellules, perte d'un ou deux 17–18, sans acquisition du marqueur.

Cas 6. Mode à 46 chromosomes. Certaines cellules ont perdu le 17–18, sans avoir acquis le marqueur. Inversement, d'autres ont acquis le marqueur sans avoir perdu le 17–18. Acquisition d'un C et duplication du Ph¹.

Cas 7. Dans deux cellules, acquisition du marqueur sans perte du 17–18.

Cas 8. Mode à 47 par acquisition d'un *C* surnuméraire.

Cas 9. Lors d'un premier examen, mode à 46 chromosomes avec perte du 17–18 et acquisition du marqueur. Dans une cellule non caractérisée par ces anomalies, apparition d'un grand marqueur anormal ($2p-$, $q+$). Lors d'un deuxième examen, ce deuxième type cellulaire constituait le clone dominant (98% des cellules).

Cas 10. Acquisition d'un C surnuméraire.

Cas 11. Acqusition d'un C surnuméraire.

DISCUSSION

L'ensemble de ces observations est caractérisé par trois 'variants communs' [10]: le Ph¹, la perte d'un 17–18 et l'acquisition d'un marqueur.

Si le Ph¹ ne pose pas de problème particulier, les deux autres 'variants communs' posent: (1) le problème de savoir si la perte affecte un 17 ou un 18, (2) le problème de la nature du chromosome marqueur et de son éventuelle parenté avec le chromosome 17 ou 18 perdu. En d'autres termes, le problème se pose de savoir si le chromosome marqueur est un isochromosome pour le bras long du 17–18 manquant, ainsi que l'avait proposé Engel [5, 6] ou s'il résulte d'un autre remaniement.

L'hypothèse de l'isochromosome demande: (*a*) que l'on fournisse la preuve que le marqueur est bien un chromosome médiocentrique, (*b*) que l'on identifie le chromosome manquant à un 17 ou à un 18, et que la taille du marqueur soit compatible avec celle du chromosome manquant, 17 ou 18, et non avec l'autre paire.

Les trois chromosomes du groupe 17–18, ainsi que la marqueur, ont été mesurés chez trois malades (cas 3, 4 et 5). L'analyse statistique des mensurations a été faite par individu.

(*a*) Il est impossible de démontrer statistiquement que les deux bras du marqueur ont la même longueur, car on ne peut orienter ce chromosome. On peut cependant déterminer, par une analyse de la variance, si la variabilité entre cellules est plus ou moins grande que celle qui existe entre les mesures des deux bras dans une même cellule. A partir de l'analyse de la variance, il a été possible de calculer des coefficients de corrélation intra-classe, ces coefficients mesurant 'la ressemblance entre les deux mesures'. Pour les trois malades, ces coefficients étaient respectivement: 0,886, 0,877 et 0,689. Ces trois valeurs sont statistiquement significatives: $P<0{,}01$. Elles sont compatibles avec l'hypothèse que la marqueur est bien médiocentrique.

(*b*) Dans chaque cellule, les 3 chromosomes 17–18 ont été classés par ordre de tailles croissantes, soit $E_1 \leqslant E_2 \leqslant E_3$. Il n'est pas possible alors d'identifier un chromosome isolé et une paire de chromosomes semblables. On peut toutefois placer le marqueur dans l'une ou l'autre de quatre positions: marqueur $<E_1$; $E_1<$marqueur$<E_2$; $E_2<$marqueur$<E_3$ $E_3<$marqueur.

En cas d'ambiguïté, on partage le classement du marqueur entre les 2 ou 3 classes possibles. Les résultats suivants ont été obtenus:

La répartition entre les quatre classes est inégale. La taille du marqueur se répartit également entre marqueur $<E_3$ et marqueur $>E_3$ (29 et 37, les chiffres ne diffèrent pas entre eux). Il n'y a pas d'hétérogénéité entre les trois individus. Ces résultats sont dès lors compatibles avec l'hypothèse que le marqueur est un isochromosome pour le bras

Malade	Mar $<E_1$	$E_1<$Mar$<E_2$	$E_2<$Mar$<E_3$	Mar$>E_3$	X_2	p
Cas 3	3	3	4	11	8,52	0,05
Cas 4	1,5	3,5	5	15	17,32	0,001
Cas 5	2	2	5	11	10,80	0,02
Total	6,5	8,5	14	37	35,79	0,001

long de E_3, c'est-à-dire d'un 17. Dans un dernier temps, on peut encore comparer la taille moyenne des bras du marqueur au bras long de E_3 (le 17) et à la moyenne des bras longs de E_1 et E_2 (les 18). Les résultats ne sont pas significatifs pour les comparaisons entre le marqueur et E_3, chez les trois sujets étudiés, alors qu'ils sont significativement différents pour les comparaisons entre marqueur et moyenne de E_1-E_2.

En conclusion, l'ensemble de l'analyse statistique est compatible avec l'hypothèse que le marqueur est un isochromosome et qu'il a été formé à partir du bras long d'un 17.

Ainsi que le montre le Tableau 5 et la Fig. 2, il existe, chez un certain nombre de malades, des cellules qui paraissent à première vue incompatibles avec l'hypothèse discutée ici. Ce sont, soit des cellules ayant perdu un 17, sans avoir acquis le marqueur, soit, inversement, des cellules ayant acquis le marqueur sans avoir perdu le 17. Ces cellules sont rares: elles représentent, au total, 30 cellules sur 198 cellules analysées, soit 15%.

On peut cependant, dans l'hypothèse de l'isochromosome pour un 17, expliquer les cellules ayant le marqueur sans avoir perdu le 17 par une duplication du 17 restant, donc, en définitive, par une 'tétrasomisation' pour le bras long du 17. Inversement, les cellules ayant perdu un 17, sans avoir acquis le marqueur, pourraient être des cellules 'complémentaires' des cellules du clone majoritaire. Il semble que ces cellules puissent du reste évoluer à leur propre compte (Fig. 2).

On sait que les chromosomes marqueurs tendent à devenir disomiques [11, 12]. Or, chez aucun des malades étudiés ici, on n'a observé la duplication du marqueur. Ce fait pourrait être un argument en faveur de l'hypothèse d'un isochromosome. En effet, le marqueur représentant du matériel chromosomique, déjà à l'état disomique, n'aurait plus à subir une duplication qui entraînerait une

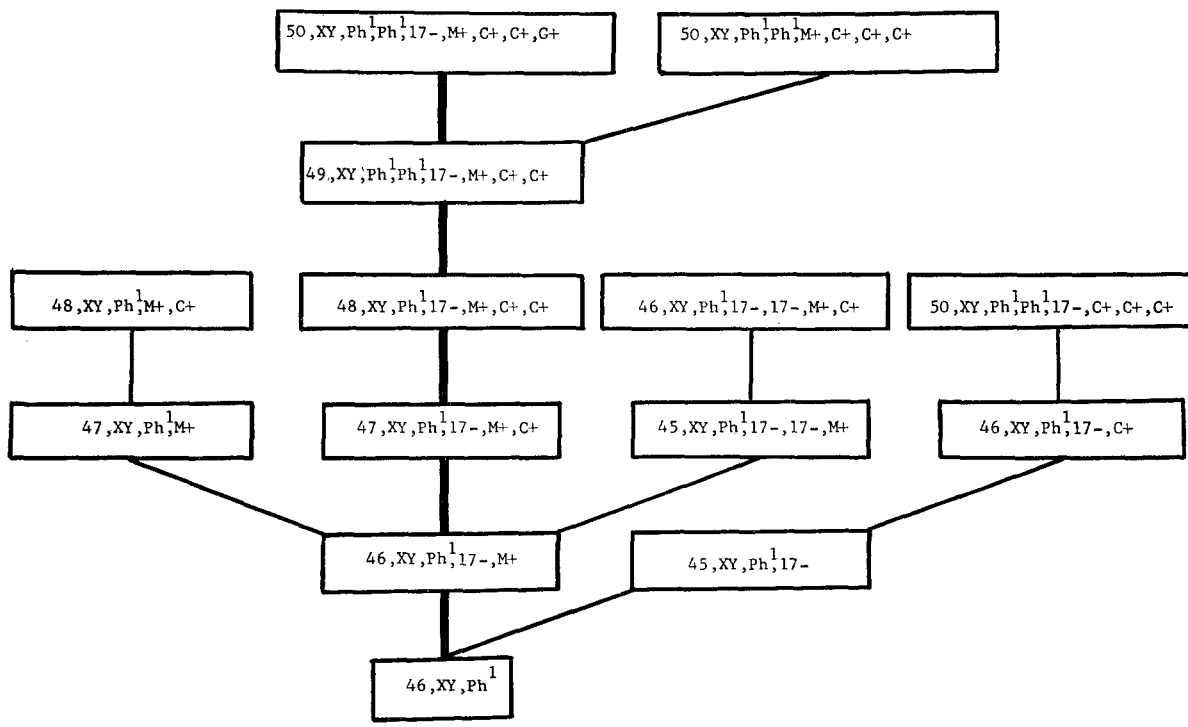


Fig. 2. Schéma de l'évolution clonale. Le trait plus épais indique la voie évolutive principale, caractérisée par le Ph¹, la perte du 17, l'acquisition d'un marqueur (M) et de plusieurs C surnuméraires. Les autres voies sont des voies minoritaires. On note, à gauche, l'acquisition du marqueur sans perte du 17, et, à l'extrême droite, la perte du 17 sans acquisition du marqueur (voir texte). Dans cette dernière voie, les cellules intermédiaires entre les clones à 46 et 50 chromosomes ont été observées.

'pentasomisation'. En revanche, la duplication du 17 restant conduit à un équilibre par tétrasomie.

En définitive, les observations décrites ici sont compatibles avec l'hypothèse de la formation d'un isochromosome à partir du bras long d'un 17 [5, 6]. Elles ne la démontrent pas. Il est certain, d'autre part, que le chromosome marqueur peut être le résultat d'un grand nombre de remaniements différents ne concernant pas nécessairement le chromosome 17. Dans ce cas, la perte du 17 et l'acquisition du marqueur seraient deux événements distincts. Les cellules 'incompatibles' avec l'hypothèse de l'isochromosome, seraient compatibles avec cette deuxième hypothèse.

Si le Ph^1, la formation du marqueur, et la perte du 17 constituent le thème central de l'évolution clonale, il existe des cellules qui témoignent de nouvelles tentatives évolutives. Elles sont représentées dans la Fig. 2. On assiste en particulier à la duplication du Ph^1 et à l'acquisition de plusieurs C surnuméraires.

Du point de vue clinique, il n'a pas encore été possible de mettre en évidence des caractères propres à ce groupe de malades.

Ces 11 malades se répartissent en 10 hommes et 1 femme. La signification de cette 'sex-ratio' anormale n'est pas claire; on doit remarquer en effet que dans la totalité des malades atteints de LMC et étudiés dans notre laboratoire, la 'sex-ratio' est de 2 hommes: 1 femme.

Parmi les 11 malades, 1 (cas 5) avait reçu des doses importantes de rayons X et 2 avaient été traités par radiothérapie (cas 2 et 4).

Tous les malades ont été traités par chimiothérapie. L'un d'eux a été l'objet de l'étude caryotypique huit jours après le début du traitement (cas 7).

La raison d'être de modèles différents d'évolutions clonales n'est pas claire. Dans 1 cas, il semble que l'on puisse lier un modèle particulier à une caractéristique clinique particulière: c'est le cas du modèle 4 où la perte d'un G ou d'un Y est associée à une évolution clinique prolongée chez des malades toujours du sexe masculin [13, 14].

Il devient donc nécessaire d'accumuler le plus grand nombre possible d'observations afin de chercher à mettre en évidence des relations entre tel ou tel modèle et telle ou telle particularité clinique.

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RESUME

Sur un total de 24 *cas d'évolution clonale observés au cours d'une leucémie myéloïde chronique (LMC),* 11 *obéissaient à un même modèle, défini par la présence du chromosome* Ph^1, *la perte d'un chromosome* 17–18, *et l'acquisition d'un marqueur médiocentrique de la taille d'un C.*

La majorité des cellules observées (85%) *sont statistiquement compatibles avec l'hypothèse que le chromosome marqueur est un isochromosome pour le bras long d'un* 17. *Il existe cependant* 15% *de cellules qui ont perdu un* 17, *sans avoir acquis le marqueur, ou inversement. Elles peuvent correspondre, respectivement, à des cellules complémentaires du clone majoritaire, ou à des duplications du* 17 *restant.*

La possibilité d'autres remaniements est également discutée pour expliquer la perte du 17 *et la formation du marqueur.*

Ce modèle paraît très fréquent. Il pourrait représenter la moitié des évolutions clonales au cours des LMC. Les particularités cliniques sont discutées.

SUMMARY

Out of a total 24 *cases of clonal evolutions studied in the course of chronic myeloid leukemia (CML),* 11 *followed an identical pattern defined by the presence of the chromosome* Ph^1, *the loss of a chromosome* 17–18, *and the acquisition of a mediocentric marker chromosome the size of a* C.

A majority of cells (85%) *are compatible with the hypothesis that the marker chromosome is an isochromosome for the long arm of a chromosome* 17. *Some* 15% *of the cells have lost a chromosome* 17 *without having acquired the marker, or vice versa. These cells may however correspond, respectively, to cells complimentary to the major clone or to a duplication of the remaining* 17.

The possibility of other rearrangements is also discussed.

This model seems to be very frequent. It could represent half of the clonal evolutions occurring during CML. Its clinical particularities are discussed.

ZUSAMMENFASSUNG

Von 24 *Klonen, die in Verlauf einer chronisch-myeloischen Leukämie (CML) untersucht wurden, wiesen* 11 *das gleiche Karyogramm auf. Es war charakterisiert durch das Vorhandensein des Ph¹-Chromosoms, den Verlust eines Chromosoms* 17–18 *und das Auftreten eines metacentrischen 'marker'-Chromosoms von der Grössenordnung* C.

Die statistische Auswertung zeigte, dass für die Merhzahl der untersuchten Zellen (85%) *die Hypothese zutrifft, dass das 'marker'-Chromosom ein Iso-chromosom aus den langen Armen von* 17 *darstellt. Andererseits haben* 15% *der Zellen* 17 *verloren, ohne ein 'marker'-Chromosom zu besitzen, oder umgekehrt. Sie können demnach komplementären Zellen des Hauptklons oder Verdopplungen des* 17 *entsprechen.*

Ausserdem werden Möglichkeiten anderer Umordnungen diskutiert, um den Verlust von 17 *und die Bildung des 'marker'-Chromosoms zu erklären.*

Das beschriebene Modell tritt sehr häufig in Erscheinung. Es entspricht der Hälfte der Klone, die sich im Verlaufe der Entwicklung der CML bilden. Die klinischen Besonderheiten werden diskutiert.

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Theory of Tumour Initiation by Chemical Carcinogens:

Dependence of Activity on Structure of Ultimate Carcinogen

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SINCE the first demonstration by Kennaway of carcinogenic activity in a pure chemical compound [1], many attempts have been made to correlate chemical structure with carcinogenic potency. These attempts have met with only limited success, largely because of the tacit assumption that the administered compound was carcinogenic *per se*, or that the distinction between a carcinogen and a non-carcinogen was at the level of metabolic conversion to a reactive metabolite. It has become increasingly apparent in recent years that covalent binding between a carcinogen (or some species derived therefrom) and DNA, RNA and protein occurs *in vivo* even when the administered carcinogen is not itself chemically reactive [2]. In such cases, therefore, a metabolic conversion to a chemically reactive species must occur *in vivo*.

In a series of closely related compounds (*e.g.* the twelve monomethylbenz[*a*]anthracenes) it is reasonable to assume that all members would be subjected to essentially the same metabolic reactions. Thus, *the distinction between carcinogens and non-carcinogens would most likely be a consequence of the chemical structure of the reactive intermediate rather than of the structure of the parent compound.* It is necessary, therefore, to know the nature of the ultimate carcinogen (i.e. the species which reacts with the essential cellular receptor) in order to fully understand the relationship between structure and carcinogenic potency. In cases where the administered compound is not chemically reactive the identity of the ultimate carcinogen is not clearly established. However, it now seems that only a limited number of possibilities need be considered. This conclusion follows from consideration of the extensive studies of the metabolism of various types of chemical carcinogen, e.g. aromatic hydrocarbons [3], aromatic amines [4, 5] and dialkylnitrosamines [6].

Another factor of great importance will be the relationship between the site of production of the reactive metabolite within the cell and the location of the essential cellular receptor. It is known that whereas the hydroxylating enzymes are located on the endoplasmic reticulum, the conjugating enzymes are soluble. Less evidence is available to define the location of the essential cellular receptor but this receptor is generally believed to be either DNA or a specific protein [2, 3, 5, 7].

For the sake of clarity of presentation it will be assumed that the essential cellular receptor is located within the nucleus and that the reaction between the ultimate carcinogen and this nuclear receptor involves an ionic mechanism of one of the types represented in Fig. 1. Consideration of the alternative view, that the receptor is not located in the nucleus or that

Ultimate carcinogen type A

$$RX \xrightarrow[k_1]{} R^+ + X^- \xrightarrow[k_2]{Y^-} RY + X^-$$

If $k_1 \gg k_2$ then second order kinetics would apply.
If $k_2 \gg k_1$ then the first order kinetics would apply.

$$RX \xrightarrow{Y^-} RY + X^-$$

Second order kinetics.

Ultimate carcinogen type B

$$R_1^+ \xrightarrow{Y^-} R_1Y$$

Fig. 1.

other types of reaction mechanisms are involved, will be given later.

In Fig. 1, reactive metabolite, type A, could be represented by, for example, epoxides which Boyland has proposed as possible carcinogenic metabolites of the polynuclear aromatic hydrocarbons [3], or by the esters of arylhydroxylamines which the Millers have proposed as reactive metabolites of the aromatic amines and amides [8]. Such species could react with cellular nucleophiles by the mechanisms indicated in Fig. 1. The nature of the species, R^+, in the examples quoted above, is such that it would not be expected to be particularly stable, i.e. $k_2 \gg k_1$. Thus, the rate determining step for the first mechanism in Fig. 1 would be the ionization of RX. For a given member of a series of closely related compounds, this rate would be proportional to the stability of the cation, R^+, i.e. *the more stable* R^+, *the more reactive* RX *would be.* Exactly the same situation obtains in the second mechanism proposed in Fig. 1, where the more stable is R^+, the more polarized would be the bond between R and X. The proposed metabolites, RX, would be generated in the cytoplasm, and the essential receptor is being considered as located within the nucleus. If the stability of RX were sufficiently limited to be a significant factor determining its ability to penetrate to the nuclear receptor, then the more stable was R^+, the less would be the carcinogenic potency of the administered compound. Alternatively, if the lifetime of RX were not of the same order of magnitude as the time required for this molecule to diffuse through the intracellular distances involved, minor structural changes might not be expected to influence carcinogenic potency.

The second type of reactive metabolite considered (type B, Fig. 1) is a positively charged species, R_1^+, directly generated by metabolism. (If, however, k_1 were much greater than k_2 in Fig. 1, i.e. R^+ were much more stable than RX, the following discussions could apply to species R^+ arising from the dissociation of RX in the cytoplasm). The ultimate carcinogens of type B would in general be expected to be of more transient existence than the ultimate carcinogens of type A. There would also be another major distinction. In a series of related compounds, *the more stable was* R_1^+, *the less reactive it would be,* and therefore the greater chance it would have of penetrating to the nuclear receptor. Thus, for this type of ultimate carcinogen increased carcinogenic potency would be associated with increased stability of R_1^+.

One way to distinguish which type of ultimate carcinogen was involved in carcinogenesis by a particular class of compound would be to determine the lifetimes of the appropriate species RX and R_1^+. These could then be compared with the time required for diffusion of the appropriate reactive species from the sites of their metabolic generation to the essential cellular receptor. This approach at present lacks appropriate experimental data. However, a distinction between these types of ultimate carcinogen can be made by consideration of the relationship between the carcinogenic potencies of the parent compounds and the stabilities of the positively charged species, R^+ or R_1^+, derived from them. The ultimate carcinogens of type B would be indicated to be involved if an increase in carcinogenic potency were found to be associated with increased stability of R_1^+. The ultimate carcinogens of type A would be indicated if increased carcinogenic potency were associated with decreased stability of R^+.

UNSUBSTITUTED AROMATIC HYDROCARBONS

It is with this particular class of compounds that theoretical chemists have had most success

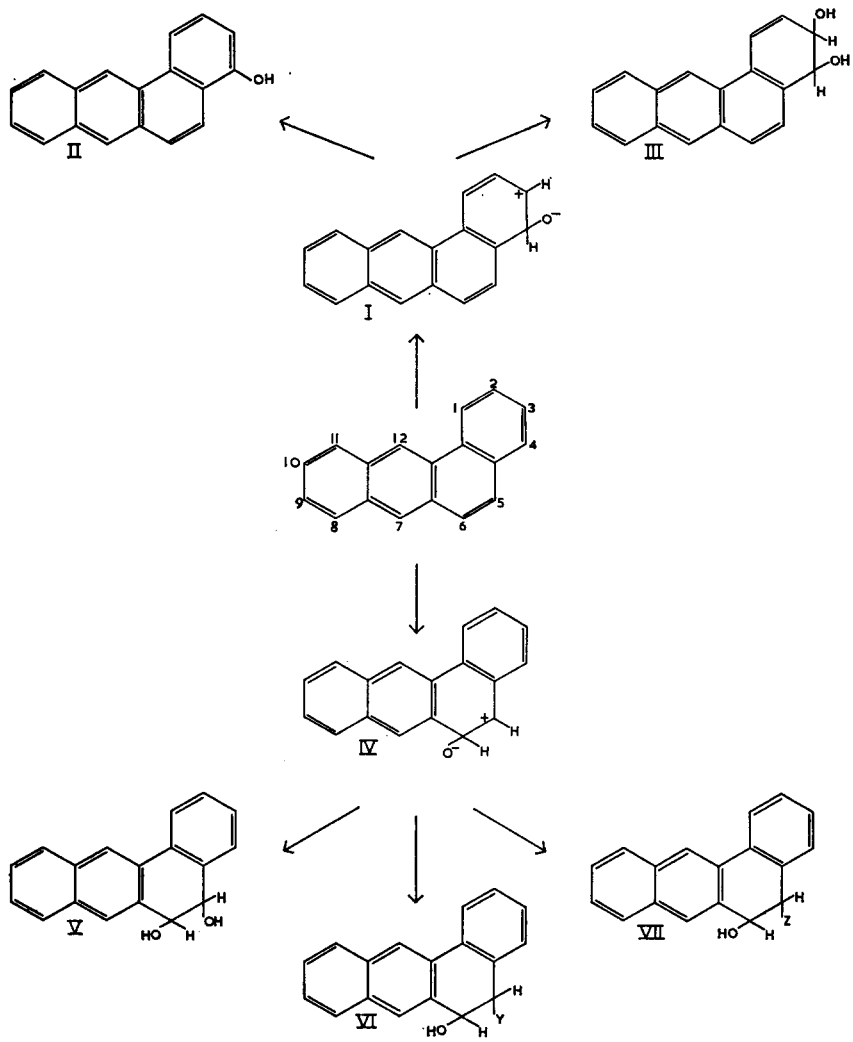


Fig. 2.

in correlating structure with carcinogenic potency. The calculations of the Pullmans have shown that a chemically reactive *K* region (i.e. regions corresponding to the 5,6-bond in benz[*a*]anthracene, Fig. 2) together with a chemically unreactive *L* region (i.e. regions corresponding to the 7 and 12 positions in benz[*a*]anthracene, Fig. 2) is required for carcinogenic potency in an extensive series of compounds in this class [9].

The only proposed reactive metabolite for which any experimental support is available is the *K* region epoxide (type A, Fig. 1) proposed by Boyland [3]. However, Sims [10] has pointed out that the initial intermediates of metabolism of these compounds are probably of the type designated as I and IV in Fig. 2. These will be considered as potential ultimate carcinogens of type B (Fig. 1). The oxygen atom in such metabolic intermediates would no doubt direct approaching nucleophiles in such a way that products of *trans* configuration would be obtained.

In order to display carcinogenic activity, it is generally accepted that an unsubstituted aromatic hydrocarbon must contain a double bond of low bond localization energy. The reason for this is fairly clear. Intermediates such as I would readily undergo an elimination reaction to give a phenol, II, as this would result in a considerable gain in resonance energy to the system. The speed of this elimination would allow only a small extent of reaction of the carbonium ion, I, with cellular nucleophiles. Such reaction would involve only nucleophiles readily available at the site of carbonium ion formation. Amongst these water would presumably predominate giving rise to small yields of dihydrodiols, III. The low bond localization energy of a *K* region bond would considerably slow down the rate of the elimination reaction in species of the type IV. Reaction with cellular nucleophiles would then become the predominant reaction pathway for such a species. This would lead to products of the type V, diols from reaction with water; VI,

products from reaction with other cellular nucleophiles such as glutathione; and possibly, VII, products from reaction with the essential cellular receptor, $\mathcal{Z}$. These same considerations apply equally to the postulated epoxides as pointed out by Boyland [3]. Thus, whichever type of proposed ultimate carcinogen is considered, only that formed at a bond of sufficiently low bond localization energy would be capable of reacting to any great extent with cellular nucleophiles, and therefore of reacting at all with the essential cellular receptor. Sims has found dihydrodiols and a mercapturic acid, but not phenols as products of metabolism of the 1,2-bond of anthracene [11]. Thus it would appear that a compound containing within its structure a double bond of bond localization energy equal to or lower than that of the 1,2-bond in anthracene could be considered as potentially carcinogenic. The carcinogenic potency of such compounds would be expected to depend upon the stability of the derived carbonium ion (type IV), and for the two types of ultimate carcinogen under consideration, R^+ derived from the epoxide and R_1^+ directly generated are identical.

It follows from the previous general argument that if the epoxides (type A) are the ultimate carcinogens, stability of the carbonium ion (type IV) will correlate negatively with carcinogenic potency. Conversely, if the directly generated carbonium ion is the ultimate carcinogen, this correlation will be positive.

It has been shown that for a series of related compounds, a correlation exists between the stability of carbonium ions and the differences in π-energies between these cations and the corresponding carbinols [12]. Further, Dewar has shown that, to a first approximation this energy difference is correlated with the NBMO coefficient, a_{or}, at the site of carbonium ion formation [13]. Thus, a broad correlation was found between the rates of solvolysis of a series of arylmethyl halides and the appropriate NBMO coefficients a_{or}. The rates increased with decreasing values of a_{or}. The experimental data extended only up to hydrocarbons containing four fused benzene rings and even in this limited series the predicted order of reactivities was not in all cases consistent with experiment [14].

Despite these limitations and the lack of relevant experimental data, we have followed this method to assess the expected relative stabilities of the carbonium ions derived from the carcinogenic aromatic hydrocarbons. The influence of the oxygen atom adjacent to the site of carbonium ion formation has been ignored and the oxygen has been considered

Table 1. *Relationship between carcinogenic potency and* NBMO *coefficients for suggested carbonium ion metabolites of unsubstituted aromatic hydrocarbons*

Parent compound	*r*	1-a_{or}	Carcinogenic potency	
			a	*b*
Phenanthrene	9	0·283	0	0
Benzo[*c*]phenanthrene	6	0·329	+	+
Benz[*a*]anthracene	5 (6)	0·288	?	0 (skin), ++ (s.c.)
Dibenz[*a,j*]anthracene	5	0·291	+	+
Dibenz[*a,h*]anthracene	6	0·306	++	++
Pyrene	4	0·299	0	0
Dibenzo[*a,l*]pyrene*	8 (9)	0·302	+++(?)	1 tumour in 1 of 2 mice
Benzo[*a*]pyrene	4 (5)	0·312	+++	++++
Dibenzo[*a,i*]pyrene	6 (7)	0·319		++++
Dibenzo[*a,h*]pyrene	5	0·436	++++	++++
Chrysene	5	0·374	0	0
Benzo[*c*]chrysene	1	0·311	+	+
Benzo[*g*]chrysene	9	0·393	+	++

The nonbonding molecular orbital (NBMO) coefficients, a_{or}, were calculated by the method of Longuet-Higgins. [21] The results are expressed as a delocalization coefficient, 1-a_{or}, where a_{or}=NBMO coefficient at the site of carbonium ion formation. The atom '*r*' is that in the *K* bond with the higher cation delocalization energy. *Chemical Abstracts* nomenclature has been used throughout.

Carcinogenic potencies: *a* from Pullman and Pullman; [19] *b* from Hueper and Conway. [22] Words such as moderate, potent etc. used to describe carcinogenic potency have been replaced by plus signs according to the definition given by Badger. [23] s.c. indicates subcutaneous.

*The compound thought to be dibenzo[*a,l*]pyrene in the references quoted for this table has since been shown to be dibenzo[*a,e*]fluoranthene. The true dibenzo[*a,l*]pyrene is now known to be a very potent carcinogen (++++). [24]

Table 2. The delocalization coefficients ($1\text{-}a_{or}$) *for suggested carbonium ion metabolites of non-carcinogenic aromatic hydrocarbons and the presence of regions of low* para *localization energy in these carbonium ion metabolites*

Parent compound	r	$1\text{-}a_{or}$	Presence of *L* region
Anthracene	2	0·412	+
Naphthacene	2	0·434	+
Benzo[*e*]pyrene	4	0·292	—
Benzo[*a*]naphthacene	6	0·326	+
Pentacene	2	0·458	+
Pentaphene	6	0·291	+
Picene (Benzo[*a*]chrysene)	6	0·359	—
Dibenzo[*c,g*]phenanthrene	6	0·331	—
Benzo[*b*]chrysene	5	0·382	+
Dibenzo[*b,g*]phenanthrene	7	0·339	+
Dibenzo[*a,e*]pyrene	13	0·391	—
Naphtho[2,3-*a*]pyrene	4	0·321	+
Dibenzo[*def,mno*]chrysene	5	0·329	—
Tribenz[*a,c,h*]anthracene	15	0·299	—
Dibenzo[*a,j*]naphthacene	6	0·317	+
Dibenzo[*a,l*]naphthacene	6	0·307	+
Anthraceno(1′,2′:1,2)anthracene	8	0·383	+
Dibenzo[*b,k*]chrysene	6	0·438	+
Benzo[*c*]pentaphene	7	0·310	+

The symbols have the same meaning as in Table 1. Where there is present in the residual aromatic system a region (or regions) of relatively low *para* localization energy across which delocalization has to occur, a plus sign has been entered in the last column of the Table. A minus sign has been entered where no such region is present or where the positive charge is formally assigned to a carbon atom directly joined to one of the carbon atoms of the *L* region. *Chemical Abstracts* nomenclature has been used throughout.

to be attached to that carbon atom of the *K* region double bond which has the lowest reactivity number [15]. In several cases the two carbon atoms are equivalent in this respect e.g. benz[*a*]anthracene, Fig. 2. In this particular case it has been established [16] that the mercapturic acid formed by metabolism has the structure VI depicted in Fig. 2 when $Y = -S \cdot CH_2 \cdot CH(NHCOCH_3)\ CO_2H$.

The results of these calculations are presented in Table 1 which compares carcinogenic potency with a delocalization coefficient, $1\text{-}a_{or}$, for carbonium ions of the general type, IV of Fig. 2. Increasing values of $1\text{-}a_{or}$ should indicate increasing stability of the corresponding carbonium ions.

In general, an increased carcinogenic potency seems to correlate with an increased value of $1\text{-}a_{or}$, although chrysene is definitely an exception to this. Benzo[*c*]phenanthrene is known to be non-planar [17] and thus the value of $1\text{-}a_{or}$ assigned to this compound is undoubtedly overestimated. In fact the values of $1\text{-}a_{or}$ for all compounds are probably overestimated as a result of the assumptions involved in their calculation [18]. The Hamiltonian integral between orbitals r and s were given the same value if atoms r and s were bonded, whereas this integral is dependent on bond length. Also, this bond integral was taken to be zero when atoms r and s were not bonded. Thus, these approximations do not take into account specific structural features in the residual conjugated systems under consideration. The presence of a *K* region in the residual conjugated system might be expected to reduce the conjugating ability of the system somewhat, and the presence of an *L* region would be expected to reduce this conjugating ability to an even greater extent. It is notable therefore that in the hydrocarbons which display carcinogenic activity, with the exception of benzo[*c*]phenanthrene and dibenzo[*a,h*]pyrene, there is no *L* region present in the residual conjugated systems. In the case of benzo[*c*]phenanthrene, if the positive charge were generated on the 5-position, the charge would no longer have to be delocalized across an *L* region. Such a species might, in fact, be a more stable carbonium ion than that where the positive charge is formally assigned to the 6-position, even though the calculated a_{or} values indicate the contrary. The case of dibenzo[*a,h*]pyrene is similar in this respect, in that the positive charge is considered to be formally located on a carbon atom directly linked to one of the *L* region carbon atoms.

In Table 2 the delocalization coefficients for

a wide series of non-carcinogenic hydrocarbons are presented. These include all the hydrocarbons dealt with by the Pullmans [19] with the exception of those which do not contain a bond of lower bond localization energy than that of the 1,2-bond of anthracene. Where the residual conjugated system is such that delocalization has to occur across a region of relatively low *para* localization energy, this has been noted in Table 2. No attempt has been made to quantitate the effect such structural features would have upon the delocalization coefficients. It is considered, however, that where such structural features are present in the residual conjugated systems, the delocalization coefficients presented should be regarded as being more highly overestimated than in cases where such *L* regions are absent. Thus, it is reasonable to expect that the delocalization coefficients for non-carcinogenic compounds, where no *L* region is present in the proposed carbonium ion intermediates, should be lower than those found for the carcinogenic hydrocarbons in Table 1. This is the case for benzo[*e*]pyrene and tribenz[*a,c,h*]anthracene. Dibenzo[*c,g*]phenanthrene is known to be non-planar [20] and thus the stabilization of the derived carbonium ion will be much less than the delocalization index suggests. Dibenzo [*def,mno*]chrysene (anthanthrene) is an exception, in that it would be expected to be carcinogenic, as would dibenzo[*a,e*]pyrene, which has been considered to be active by some workers and inactive by others [19]. Picene (benzo[*a*] chrysene) is also exceptional in common with chrysene. All chrysene derivatives in fact have high values of $1\text{-}a_{or}$, and it is possible that structural features of the chrysene nucleus might decrease the delocalization to well below that indicated by the calculated coefficients.

Thus, the correlation described above gives a very similar prediction of carcinogenic potencies to those of the Pullmans but the basis of the present correlation is much more specific in biochemical terms. In fact it could be considered that the Pullmans' requirements for carcinogenic potency are indirectly defining the stability of the carbonium ion intermediates proposed here.

It appears then, that for carcinogenic compounds an increase in the predicted stability of the derived carbonium ion (type IV) in general correlates with an increase of carcinogenic potency. This indicates that the ultimate carcinogens in this series of compounds would be expected to be the carbonium ions (type B of Fig. 1), rather than the proposed epoxides (type A).

METHYL SUBSTITUTED AROMATIC HYDROCARBONS

It has been shown [25] that the carcinogenic potencies of the methylbenz[*a*]anthracenes correlate positively to some extent with the conjugating abilities of the positions in the benz[*a*] anthracene nucleus to which the methyl groups are attached. This could be considered to indicate that carbonium ions, of the type postulated as ultimate carcinogens for unsubstituted aromatic hydrocarbons, are also the ultimate carcinogens for this class of compounds. However, many exceptions to the above correlation were noted. It is reasonable, therefore, to consider an alternative type of ultimate carcinogen.

Some methylbenz[*a*]anthracenes are known to be converted metabolically into the corresponding hydroxymethyl derivatives [26, 27]. It is not unreasonable to expect that other methyl substituted aromatic hydrocarbons would be subjected to similar metabolism. Thus, it could be considered that the ultimate carcinogens for this series of compounds arise, *in vivo* via such metabolism. If this metabolism involved an attack by an electrophilic oxygen, as is considered to be the case for the metabolism of double bonds, then two distinct mechanisms could be proposed. The electrons from a carbon–hydrogen bond could be considered to be transferred into a new bond between the electrophilic oxygen and the carbon atom, thus generating a hydroxymethyl derivative and a proton. The hydroxymethyl derivative could then be converted to a reactive metabolite of type A (Fig. 1) e.g. a sulphate ester. Alternatively, it could be considered that the approach of the electrophilic oxygen would result in the transfer of the electrons from one of the carbon–hydrogen bonds into a new bond between the oxygen and hydrogen. This would directly generate a formal positive charge on the extranuclear carbon atom, and such an arylalkyl carbonium ion could be considered as an ultimate carcinogen of type B (Fig. 1). No experimental evidence is currently available to distinguish between these two mechanisms for metabolism of methyl groups.

Thus, the ultimate carcinogens could again be either of type A or type B, and a distinction between the two could perhaps be made by a consideration of the relative stabilities of the appropriate arylalkyl carbonium ions. The appropriate delocalization coefficients, $1\text{-}a_{or}$, were calculated as previously described and are presented, together with carcinogenic potencies taken from the literature, in Table 3.

It is evident that for the methylbenz[*a*]

Table 3. Relationship between carcinogenic potency and NBMO *coefficients for suggested arylalkyl carbonium ion metabolites of methyl-substituted aromatic hydrocarbons*

Parent compound	1-a_{or}	Carcinogenic potency		
		a	*b*	*c*
3-Methylbenz[*a*]anthracene	0·271	0	0	0
1-Methylbenz[*a*]anthracene	0·297	0	+	0
9-Methylbenz[*a*]anthracene	0·297	+	+	0
2-Methylbenz[*a*]anthracene	0·307	0	0	0
10-Methylbenz[*a*]anthracene	0·307	+	+	0
4-Methylbenz[*a*]anthracene	0·324	0	+	0
5-Methylbenz[*a*]anthracene	0·361	+	+	0
11-Methylbenz[*a*]anthracene	0·361	+	0	0
6-Methylbenz[*a*]anthracene	0·361	+	+++	+++
8-Methylbenz[*a*]anthracene	0·369	++	+++	+++
12-Methylbenz[*a*]anthracene	0·417	++	+++	+++
7-Methylbenz[*a*]anthracene	0·439	+++	++++	++++
		a	*e*	
2-Methylbenzo[*a*]pyrene	0·250	++++	+	
8-Methylbenzo[*a*]pyrene	0·273	0		
10-Methylbenzo[*a*]pyrene	0·329			
11-Methylbenzo[*a*]pyrene	0·329			
9-Methylbenzo[*a*]pyrene	0·346	0		
4-Methylbenzo[*a*]pyrene	0·389			
5-Methylbenzo[*a*]pyrene	0·389	++		
7-Methylbenzo[*a*]pyrene	0·389	++		
12-Methylbenzo[*a*]pyrene	0·400			
3-Methylbenzo[*a*]pyrene	0·424		++++	
1-Methylbenzo[*a*]pyrene	0·433			
6-Methylbenzo[*a*]pyrene	0·500	++++		
		e		
3-Methylbenzo[*c*]phenanthrene	0·275	+		
2-Methylbenzo[*c*]phenanthrene	0·281	+		
6-Methylbenzo[*c*]phenanthrene	0·318	++		
1-Methylbenzo[*c*]phenanthrene	0·318			
4-Methylbenzo[*c*]phenanthrene	0·324	+		
5-Methylbenzo[*c*]phenanthrene	0·333	++		
		e	*a*	
2-Methylchrysene	0·266			
3-Methylchrysene	0·291			
4-Methylchrysene	0·312	0	+	
5-Methylchrysene	0·312	+++	+++	
1-Methylchrysene	0·331	0		
6-Methylchrysene	0·359	0	+	
		e		
3-Methyldibenz[*a*,*h*]anthracene	0·267	++		
1-Methyldibenz[*a*,*h*]anthracene	0·298			
2-Methyldibenz[*a*,*h*]anthracene	0·298	++		
4-Methyldibenz[*a*,*h*]anthracene	0·325			
6-Methyldibenz[*a*,*h*]anthracene	0·349	++		
5-Methyldibenz[*a*,*h*]anthracene	0·361			
7-Methyldibenz[*a*,*h*]anthracene	0·397	++++		

Carcinogenic potencies: *a*, from Badger; [23] *b*, Dunning and Curtis; [28] *c*, Huggins *et al.*; [29] *e*, from Hueper and Conway [22]. Words such as moderate, potent etc. used to describe carcinogenic potency have been replaced by plus signs according to the definition of Badger [23].

Chemical Abstracts nomenclature has been used throughout.

anthracenes there is a positive correlation between carcinogenic potency and the delocalization coefficients of the derived carbonium ions for all compounds except the 2-methyl derivative.

For the methylbenzo[*a*]pyrenes, again with a single exception, this positive correlation is apparent. In contrast the Pullmans found it "extremely puzzling that whereas benzo[*a*] pyrene and its 6-, 5-, 3-, 2- and 7-methyl derivatives are carcinogenic, the 8- and 9-methyl derivatives are inactive" [19].

The correlation in the methyldibenz[*a*,*h*] anthracenes is again positive.

No clearly defined correlation is evident from the data concerning the methylbenzo[*c*]phenanthrenes. However, the non-planar nature of the conjugated system [17] was not considered in the calculations and thus the coefficients obtained are of dubious significance.

As was found for the unsubstituted hydrocarbons, chrysene derivatives appear to be exceptions to the general correlation. This, again, suggests that certain structural features of the chrysene nucleus perhaps invalidate the methods used to assess the stabilities of the derived carbonium ions.

In summary, although some exceptions are found, the overall positive correlation between carcinogenic potencies and predicted stabilities of the proposed arylmethyl carbonium ion metabolites is good. Therefore, these are indicated to be the ultimate carcinogens.

Introduction of a second methyl group into monomethyl substituted hydrocarbons would be expected to further stabilize the arylmethyl carbonium ions and therefore an increase in carcinogenic potency might be expected. In agreement, several dimethylbenz[*a*]anthracenes were more active in inducing tumours in skin than were either of the corresponding monomethyl derivatives. However, when these compounds were administered subcutaneously the dimethyl derivatives were generally of lower carcinogenic potency than either of the corresponding monomethyl derivatives [22]. It appears, therefore, that factors other than the stabilities of the proposed arylalkyl carbonium ions may be predominant in determining the carcinogenic potencies of these compounds when administered by the subcutaneous route.

OTHER CHEMICAL CARCINOGENS

The concepts proposed for the aromatic hydrocarbons could well be extended to a consideration of other chemical carcinogens. Quantitative theoretical treatment of molecules containing elements other than carbon and hydrogen is difficult. The effect of substitution by various groups on the stability of cations derived from aromatic compounds can however be considered in a purely qualitative fashion. Substituents with overall electron withdrawing properties, —Cl, —Br, —I, —NO_2 and —CF_3 (i.e. substituents which deactivate benzene towards electrophilic substitution [30, 31] would be expected to decrease this stability whilst substituents with overall electron donating properties, —OH and —CH_3 (i.e. substituents which activate benzene towards electrophilic substitution [30, 31]) would be expected to increase this stability. A fluoro-substituent does not affect the reactivity of benzene towards electrophilic attack [30] and would not therefore be expected to influence this stability to any great extent.

Several aromatic amines and amides are known to be carcinogenic and for these compounds it is well established that metabolism is a prerequisite of biological activity [4, 5]. The Millers showed that 2-acetylaminofluorene is metabolically converted to the *N*-hydroxy derivative in animal species susceptible to this carcinogen [32]. Furthermore, this *N*-hydroxy compound was found to be a more potent carcinogen than the parent compound [33]. The *N*-hydroxy derivatives of many other aromatic amines and amides have since been implicated as proximate carcinogenic agents [5]. Thus, it has been proposed that arylhydroxylamines [5, 34] or reactive esters derived therefrom [5], could be the ultimate carcinogens. These reactive species are of the type A of Fig. 1. However, it would be of interest to consider also, species of the type B of Fig. 1, $ArNR^+$ for example, where Ar=aryl and R=H, CH_3, C_2H_5 etc., which might possibly be generated directly by metabolism in some cases. Again, a distinction between the type A and type B ultimate carcinogens might follow from a consideration of the relationship between carcinogenic potency and the stability of the appropriate $ArNR^+$ species.

2-Acetylaminofluorene produces tumours at several sites and the main target organ varies according to species. Different mechanisms could be involved in different cases and thus the following discussion is confined to carcinogenic potencies reported for rat liver [22].

2-Acetylaminofluorene is a more potent liver carcinogen than the 1-, 3-, and 4-acetylaminofluorenes which give tumours largely at sites other than the liver. Simple resonance theory predicts that a formal positive charge on the nitrogen in the 1- and 3-substituted derivatives would be largely confined to the *N*-substituted ring, whereas in the 2- and 4-substituted derivatives it could be stabilized by both aromatic rings. Thus, only the properties of the 1-, 2-, and 3-substituted derivatives are consistent with a positive correlation between carcinogenic potency, and stability of the $ArNR^+$ species under consideration. However, the effects of 7-chloro-, 3- and 7-iodo-substituents in reducing the carcinogenic potency of 2-acetylaminofluorene support this positive correlation. Also consistent is the fact that the monofluoro-2-acetylaminofluorenes all exhibit carcinogenic potencies comparable to that of the unsubstituted compound. The reduced

activity of 7-hydroxy-2-acetylaminofluorene represents another exception to the correlation but as this compound is a known metabolite of the parent compound it may be more readily excreted [35]. Replacement of the methylene bridge, embodying the 9-position of 2-acetylaminofluorene, by other groups or atoms generally leads to compounds of lower carcinogenic potency. In some cases e.g. 4-acetylaminobiphenyl, the interaction between the two aromatic rings would be reduced with concomitant decrease in the stabilization of the proposed cation.

The positive correlation, between carcinogenic potency for the liver and stability of the postulated $ArNR^+$ species, which emerges from these somewhat superficial considerations, cannot perhaps be considered as highly significant. However, it does suggest that these $ArNR^+$ species (Ar=2-fluorenyl) should be considered as possible ultimate carcinogens. The nature of the R-group would depend upon the mechanism by which the reactive species were generated. If they were considered to arise as intermediates in metabolic deacetylation, then R would be a hydrogen atom. Consistent with this suggestion is the finding that 2-diacetylaminofluorene, for which deacetylation presumably must precede *N*-hydroxylation, is a more potent liver carcinogen than the monoacetyl derivative [36]. Furthermore, Kriek [37] has found that injection of isotopically labelled 2-acetylaminofluorene into rats led to binding to liver RNA of a metabolite containing the acetyl group, whereas the metabolite bound to DNA lacked this group. Direct attack of the $ArNH^+$ species on the DNA of the nucleus is clearly consistent with this finding.

In the series of hepatocarcinogenic azodyes, typified by 4-dimethylaminoazobenzene ($ArNMe_2$), the presence of at least one methyl substituent on the amino group is considered to be essential for high carcinogenic potency [38]. Thus, it is conceivable that an ultimate carcinogen of type B of Fig. 1, e.g. $ArNMe^+$ could be generated as an intermediate of the metabolism of such methyl groups.

Substitution of the primed ring of dimethylaminoazobenzene by electron withdrawing groups generally leads to compounds with lower carcinogenic potency than the parent compound [39]. Monofluoro derivatives generally exhibit carcinogenic potencies comparable to, or greater than that of the parent [39]. These findings are again consistent with a positive correlation between carcinogenic potency and stability of the postulated $ArNMe^+$ species, thus indicating that such species may be the ultimate carcinogens.

Methyl substituents in the aromatic rings can either enhance carcinogenic potency as at the 3′-position, or diminish it, as at all other positions [39]. Evidently, for these compounds, no correlation with the stability of the $ArNMe^+$ species exists. This suggests consideration of alternative reactive species, such as the corresponding arylmethyl carbonium ions. With the methyl substituent at the 2′-, 4′-, or 3-position, the cation would be expected to exist largely as the quaternary ammonium form and to be correspondingly unreactive. This form would not contribute appreciably to the structure of carbonium ions generated on a 3′-, or 2-methyl substituent. The lack of carcinogenic potency of the 2-methyl derivative is inconsistent with such an interpretation, but recent studies have indicated that this compound may well be capable of initiating the carcinogenic response [40].

In the series of carcinogenic azotoluenes [22], carbonium ions generated on the carbon atom of the methyl groups could similarly be considered as possible ultimate carcinogens.

Consideration of the naphthylamines appears to present a case where stability of the corresponding $ArNH^+$ species correlates negatively with carcinogenic potency. In man, 2-naphthylamine is a more potent bladder carcinogen than is 1-naphthylamine [41], whereas the delocalization coefficients, $1\text{-}a_{or}$, calculated for the corresponding naphthylmethyl carbonium ions, 0·273 and 0·329 respectively, indicate that the species $ArNH^+$ should be more stable when Ar=1-naphthyl than when Ar=2-naphthyl. The indication here, though again of limited significance, is that the ultimate carcinogen is of the type A of Fig. 1, e.g. a naphthylhydroxylamine or reactive derivative thereof.

For the carcinogenic aliphatic nitrosamines, nitrosamides and nitrosamidines [42], and for the carcinogenic alkylating agents [43], carbonium ions have long been considered to be the ultimate reactive species. Alkyl carbonium ions, i.e. ultimate carcinogens of type B of Fig. 1, would be expected to be too unstable to diffuse from the cytoplasm to the nuclear receptor. However, for the alkylating agents, the nitrosamides and the nitrosamidines, which can yield carbonium ions without the intervention of metabolic processes [43, 44], the administered compounds themselves, or metastable derivatives thereof, e.g. cyclic immonium ions from nitrogen mustards [45], or alkyldiazohydroxides from nitrosamides or nitrosamidines [44], could diffuse to the nuclear

receptor. The required carbonium ions could then be generated at the essential site. Although metabolic activation is indicated to be a prerequisite for carcinogenic action of the dialkylnitrosamines [42], the significant metabolic products have been considered to be either monoalkylnitrosamines [6] or alkyldiazohydroxides [46]. These again could be regarded as the metastable species which diffuse to the nuclear receptor.

Thus, these examples illustrate the general applicability of the concept that a major determining factor for carcinogenic potency will be the structure of the reactive intermediate rather than the structure of the parent compound.

DISCUSSION

If covalent binding between a chemical carcinogen or a metabolite thereof and an essential cellular receptor is a necessary step in the carcinogenic process, then a correlation between the structure of the ultimate carcinogen and the carcinogenic potency of the parent compound might be expected. Such correlations were in fact found for several chemically diverse groups of carcinogens when any of the limited number of probable ultimate carcinogens was considered. Furthermore, if the essential cellular receptor were considered to be located within the nucleus, and the stability of the ultimate carcinogen was considered to be a significant factor determining its ability to penetrate to this nuclear receptor, the nature of the correlation observed indicated the type of ultimate carcinogen likely to be involved. The possible ultimate carcinogens considered were selected on the basis of studies of metabolism which have been reported in the literature. Where no such studies were available it was assumed that metabolic reactions analogous to those reported for similar compounds would be involved.

Boyland has pointed out that for both phenanthrene and benz[*a*]anthracene the *K* region is the bond subjected to the most extensive metabolic attack [3]. Thus, for unsubstituted hydrocarbons the proposed ultimate carcinogens, in the first instance (Tables 1 and 2) were all considered to arise as a result of metabolic attack at that bond which, according to the Pullmans [19], has the lowest bond localization energy. The concepts presented here only require, however, that the bond involved should have a bond localization energy lower than that of the 1,2-bond in anthracene. The fact, then, that Sims [47] has so far failed to detect metabolism at the *K* regions of benzo[*a*]pyrene i.e. the 4,5- and 11,12-bonds, is not inconsistent with these concepts. Extensive dihydrodiol formation, but no phenol formation, was detected at the 9,10-bond [47, 48]. This 9,10-bond could well be the bond involved in the generation of the ultimate carcinogen from benzo[*a*]pyrene. The corresponding delocalization coefficient, $1\text{-}a_{or}$, where r indicates the 10 position, is 0·423. It is clear that the correlation (Table 1) would be improved if benzo[*a*]pyrene were assigned this delocalization coefficient. If the sites of carbonium ion formation were considered, by analogy with the benzo[*a*]pyrene case, to be the 1 position of dibenzo[*a,i*]pyrene ($1\text{-}a_{or}$= 0·459); the 10 position of dibenzo[*a,l*]pyrene ($1\text{-}a_{or}$=0·382); the 12 position of dibenzo[*a,e*] pyrene ($1\text{-}a_{or}$=0·402); and the 4 position of dibenzo[*a,h*]pyrene ($1\text{-}a_{or}$=0·441), similar improvements in the observed correlation would be found. Dibenzo[*def,mno*]chrysene (anthanthrene), which can be considered as a substituted benzo[*a*]pyrene, does not possess a bond analogous to the 9,10-bond of benzo[*a*]pyrene; its lack of carcinogenic potency might then be accounted for if a similar failure to metabolize the *K* regions were to be found.

For the methyl substituted benz[*a*]anthracenes, benzo[*a*]pyrenes and dibenz[*a,h*]anthracenes, the correlation between carcinogenic potencies and delocalization coefficients for the corresponding arylmethyl carbonium ions was good. This indicated that the reaction with the nuclear receptor involved such species predominantly. However, in the case of some methylbenz[*a*]anthracenes at least, metabolism at the methyl groups has not been found to be the exclusive metabolic reaction [26,27]. A contribution to the reaction with the cellular receptor could conceivably be made, therefore, by the type of carbonium ion metabolite proposed for unsubstituted hydrocarbons. The extent of this contribution would be dictated by the relative extents of metabolic attack at the two appropriate sites, and also by the relative stabilities of the two carbonium ions generated.

The carcinogenic aromatic amines and amides were considered in a purely qualitative fashion. The considerations did indicate, however, that species of the general type, $ArNR^+$ could be ultimate carcinogens in hepatocarcinogenesis by *N*-substituted compounds. A suggestion that such species could arise from metabolic de-acetylation or metabolic de-methylation could account for many of the known structural requirements for hepatocarcinogenic potency. This suggestion

also places primary aromatic amines, in general, in a category separate from the *N*-substituted compounds. The naphthylamines, 4-aminobiphenyl, 4-aminostilbene, and 2-aminofluorene have in fact been classified as carcinogens with a higher specificity for the bladder or ear duct than for the liver [35]. In the case of the naphthylamines, the structure–activity relationship did suggest that the ultimate carcinogen for the bladder might be of the type A of Fig. 1, rather than of the type B. If sufficient experimental data were available, it would be of interest to determine, therefore, whether type A ultimate carcinogens were always indicated for primary aromatic amines, irrespective of the target organ involved, and correspondingly, for *N*-substituted compounds, whether type B ultimate carcinogens were indicated for all organs, or whether this was only the case for the liver.

On the basis of the proposed scheme in which the receptor is located in the nucleus, the positive correlations between carcinogenic potency and stability of the cationic species, clearly support the view that such cationic species are the ultimate carcinogens. However, the positive correlations only exclude ultimate carcinogens of the type A of Fig. 1, if it is assumed that their stabilities limit their ability to penetrate to the nuclear receptor. Alternatively, if the type A ultimate carcinogens were of a somewhat higher order of stability, a positive correlation might also be expected. It should then follow that the type A ultimate carcinogens themselves, or their immediate metabolic precursors, would be more potent carcinogens than the parent compounds. This interpretation is unacceptable for the hydrocarbons, since the *K* region epoxides [49–51] and the hydroxymethylbenz[*a*]anthracenes [50, 52] have been found to be less potent carcinogens than the parent compounds. On the other hand some *N*-hydroxy compounds and their esters have been assessed as more potent carcinogens than the parent aromatic amines or amides [53]. Whilst these findings have been considered to support the view that *N*-hydroxy compounds or their esters are the probable ultimate carcinogens, they cannot be considered to exclude the involvement of the proposed $ArNR^+$ species in the hepatocarcinogenic action of *N*-substituted compounds in the rat. As already mentioned the experimental findings, regarding the nature of the metabolite of 2-acetylaminofluorene bound to liver DNA *in vivo*, and the specific requirement for an *N*-methyl group for hepatocarcinogenic potency in the azo-dyes, are consistent with the involvement of the proposed $ArNR^+$ cations as ultimate carcinogens. For liver carcinogenesis by these compounds, it is difficult to see how the *N*-hydroxylation hypothesis could account for these facts. An acceptable overall concept of aromatic amine carcinogenesis for the rat would be, therefore, that the *N*-hydroxyamine is the ultimate carcinogen in all organs for primary amines, and also in all organs, except those capable of metabolically generating the $ArNR^+$ species, for carcinogenic *N*-substituted amines.

Although the nucleus has so far been regarded as the location of the essential cellular receptor, the interpretation of the observed correlations would remain the same for any location distant from the site of metabolic generation of the ultimate carcinogen. However, if the receptor were considered to be readily available at this site, the ability of any highly reactive ultimate carcinogen either of type A or type B to react with this receptor would be independent of structure. Again, for a somewhat stable type A ultimate carcinogen a positive correlation between structure and activity might be expected. This possibility has already been discussed, and considered not to apply, particularly for the hydrocarbons.

The ultimate carcinogens of type B of Fig. 1 have, so far, been regarded as cations. For a given hydrocarbon, the degree of stabilization of the proposed cations or of analogous radicals would be the same [21]. The effect of electron withdrawing substituents on the stability of cations and radicals is, however, different in that such substituents do not stabilize cations but do stabilize radicals [21]. Radicals are not indicated, therefore, to be the ultimate carcinogens in hepatocarcinogenesis by 2-acetylaminofluorenes or 4-dimethylaminoazobenzenes, but insufficient experimental evidence is available to enable such distinction to be made for the hydrocarbons.

The most important factor determining carcinogenic potency is indicated by the present considerations to be the stability of the ultimate carcinogen. Other factors must presumably be involved, e.g. solubility; molecular size; molecular shape; the possibility that a specific complex formation between the ultimate carcinogen and the cellular receptor might occur; and the ease with which a particular species can elicit a carcinogenic response once bound to the cellular receptor. The contribution by such factors cannot be large, however, in the cases discussed here.

The present considerations have depicted for the first time a common factor between

chemically different groups of carcinogens. This common factor is the stability of the ultimate carcinogens generated *in vivo*. Though not all alternative possibilities can definitely be excluded, these considerations indicate that the ultimate carcinogens for the carcinogenic hydrocarbons are carbonium ions, generated directly by metabolism; that for hepatocarcinogenesis by *N*-substituted amines, cationic species again are possibly the ultimate carcinogens; and that the most likely location of the cellular receptor is within the nucleus.

These conclusions are, therefore, consistent with a requirement for a chemical reaction between the ultimate carcinogen and DNA as an essential stage in carcinogenesis i.e. a mutational hypothesis of tumour initiation.

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RESUME

Pour plusieurs groupes de produits, comprenant des hydrocarbures aromatiques, des amines aromatiques et des colorants azoïques, il a été établi que c'est la structure du cancérogène modifié, plutôt que celle du composé administré, qui est le déterminant le plus important du pouvoir cancérogène. L'interprétation des relations entre la structure et l'activité qui ont été mises en évidence dans ce travail permet de proposer un mécanisme moléculaire possible de la cancérogenèse.

SUMMARY

Support for the concept that the structure of the ultimate carcinogen, rather than that of the administered compound, will be the more significant determinant of carcinogenic potency has been deduced for several groups of compounds, including aromatic hydrocarbons, aromatic amines and azo-dyes. The interpretation of the structure–activity relationships found in this investigation suggests a possible molecular mechanism for tumour initiation.

ZUSAMMENFASSUNG

Für mehrere Gruppen von Verbindungen (aromatische Kohlenwasserstoffe, aromatische Amine und Azofarbstoffe) wird gezeigt, dass die Struktur des endgültigen Carcinogens und nicht die der zugeführten Verbindung für die carcinogene Wirkung entscheidend ist. Die Interpretation der Struktur-Wirkungs-Beziehung, die in diesen Untersuchungen gefunden wurde, ergibt einen möglichen molekularen Mechanismus der Tumorerzeugung.

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Co-Existence of Allogeneic Tumour Growth and Homograft Immunity in Man*

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INTRODUCTION

THE CONCEPT that cancer cells possess neo-antigens which excite an immunologic host defense reaction has been well established during the past decade [1,2] but the progressive growth of untreated autochthonous cancer to a fatal termination remains as inevitable as ever. The unavoidable conclusion therefore is that cancer can grow progressively in the face of an anti-tumor immune reaction. Reconciliation of these apparently contradictory phenomena is possible if a normal rejection response is ineffectual because of some characteristic of the tumor, or if the host reaction is not of a type which causes rejection of antigenically foreign cells. Characteristics of a tumor which might protect it from normal rejection reactions are its location in a site such as eye or brain which is relatively inaccessible to antibodies and immunocytes, or some characteristic of the individual tumor cells which endow them with resistance to the rejection mechanisms. Immune reactions such as enhancement and specific immune tolerance which do not cause rejection of antigenically foreign cells are well known, although imperfectly understood. A third explanation for the concurrence of progressive tumor growth and anti-tumor immune reactions might be that although the immune reactions are of the types that kill antigenically foreign cells and the cells are normally susceptible to these reactions, the rate of propagation of the tumor cells exceeds the rate of destruction. The phenomenon of co-existing tumor growth and anti-tumor immune reactions, and its possible explanations, seems to parallel the phenomenon of progressive growth of certain homotransplanted tissues, except that the foreign cellular antigens in the latter are normal components of the transplanted cells rather than neo-antigens acquired during malignant transformation.

Woodruff [3, 4] has reviewed several situations in man and experimental animals in which homografts of normal tissues (skin and bone marrow) achieve continuous growth. Amongst the hypotheses which he considered as possible explanations for such failure of rejection was a change in the characteristics of the transplanted cells ('adaptation') whereby they lost their vulnerability to immunologic attack, or an inhibition of the efferent phase of the host rejection reaction. Phillips [5] made quantitative studies of primary and repeat

**Author's note.* These studies were carried out in a clinical research unit as part of an extensive study of homo- and autotransplantation of human cancer cells which has been reported in part in other publications. All recipients were volunteers who knew the nature of the procedures and that they were performed as experiments with no expectation of therapeutic effect.

Editor's note. Clinical observations have demonstrated the danger of transplanting a tumor from one person to another. This may result in proliferation and dissemination of the tumor in the recipient with resulting death [E. F. Scanlon, Roger A. Hawkins, W. W. Fox and W. Scott Smith, Fatal homotransplanted melanoma *Cancer* **18,** 782 (1965)].

homografts of mouse tumors and demonstrated that with certain inoculum doses, transplant sites, and time relationships, allogeneic tumor growth progressed in the face of homograft immunity. Furthermore, in several of the mice which received five or ten simultaneous tumor transplants, some tumor nodules were rejected after a period of growth while others in the same individual mice continued to increase in size (Phillips, personal communication, 1967).

During studies of cancer cell homotransplants in human volunteers one recipient was observed who clearly exhibited the coexistence of allogenic tumor growth and homograft immunity. The course of these transplants is described here because such an observation in man is unique and because it is a further demonstration that immunologic mechanisms can be responding to and actually inhibiting the growth of antigenically foreign tumor cells, and yet fail to prevent progressive growth of an established tumor.

METHODS

The cells for homotransplantation were grown as monolayers on glass in a nutrient medium consisting of 50% human ascitic fluid, 45% Gey's solution and 5% chick embryo extract. They were harvested from the culture bottles by 0·25% trypsin in isotonic saline (0·85% NaCl), washed twice with isotonic saline to remove the trypsin and culture medium, and were then suspended in plain Gey's solution for inoculation. Two cell lines were used: HEp 2, an epidermoid cell line from larynx cancer, and HEp 3, an epidermoid cell line from cancer of the mouth [6].

Each homotransplant was performed by injecting 0·5 ml of a cell suspension subcutaneously at two sites on the anterior surface of one thigh. This use of duplicate transplants permitted excision of one of the nodules for histologic study without termination of the experiment. The transplant sites were examined two or more times each week. The maximum and minimum diameters of each nodule were measured with a cm ruler and recorded. The two nodules of each transplant set gave nearly identical results so the mean diameter for each day is recorded in Fig. 1.

The recipient was a 58 year old woman with squamous carcinoma of the breast which had disseminated widely to skin and lymph nodes after radical mastectomy, and had continued to progress in spite of intensive X-ray therapy to the chest wall. The patient received no cytostatic drugs or hormones, and the last X-ray treatment was given 15 weeks before the homotransplant study. The total and differential leukocyte counts were normal at the time of and following homotransplantation. Paper electrophoresis of serum proteins showed moderate elevation of α1 and α2 globulins, and normal levels of β- and γ-globulins (0·39, 1·19, 1·03, and 0·90 g/100 ml respectively) on the day of the first transplant. She received experimental therapy with Mengo virus and several arboviruses during the period of the homotransplantation studies [7] but these therapeutic attempts had no apparent influence on the course of her own cancer or the homotransplants.

RESULTS

Figure 1 illustrates the course of transplant growth. The first transplant consisted of two subcutaneous implants, each consisting of $1{\cdot}8 \times 10^6$ cells. Both produced subcutaneous nodules which grew steadily to a diameter of 3 cm at 5 weeks, when one was completely excised for histologic study. It showed an almost pure population of the transplanted cells with a few scattered lymphocytes at the periphery of the nodule (Fig. 2). During the sixth week the remaining primary transplant nodule decreased significantly in size and

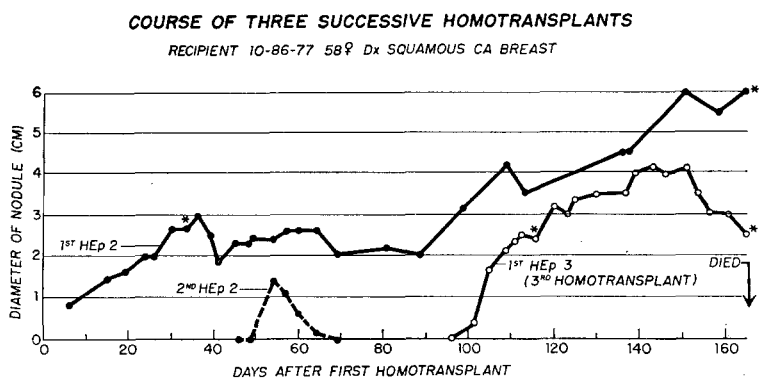


Fig. 1. *Course of the three successive homotransplants. Asterisks indicate times of histologic examinations.*

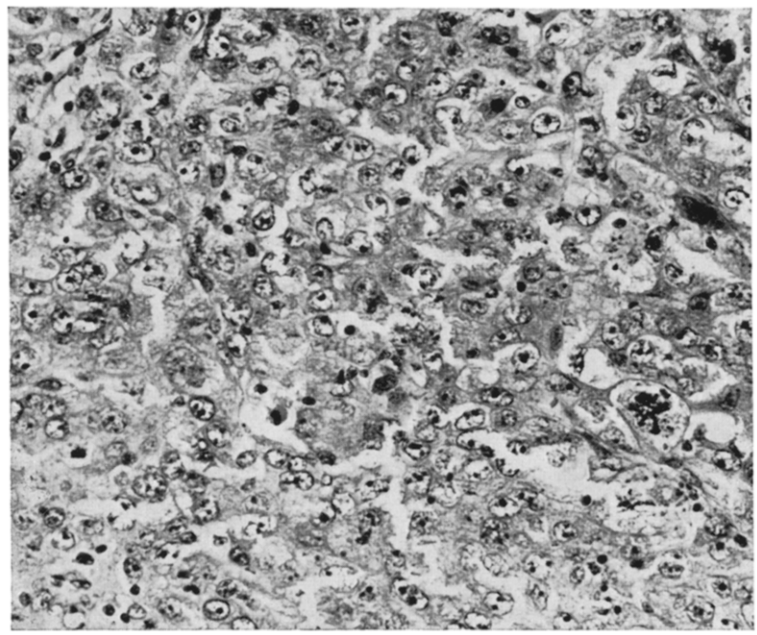


Fig. 2. *Histology of the primary (HEp* 2) *homotransplant nodule on day* 34. *There is almost pure growth of the allogeneic cancer cells and no evidence of a rejection reaction.*

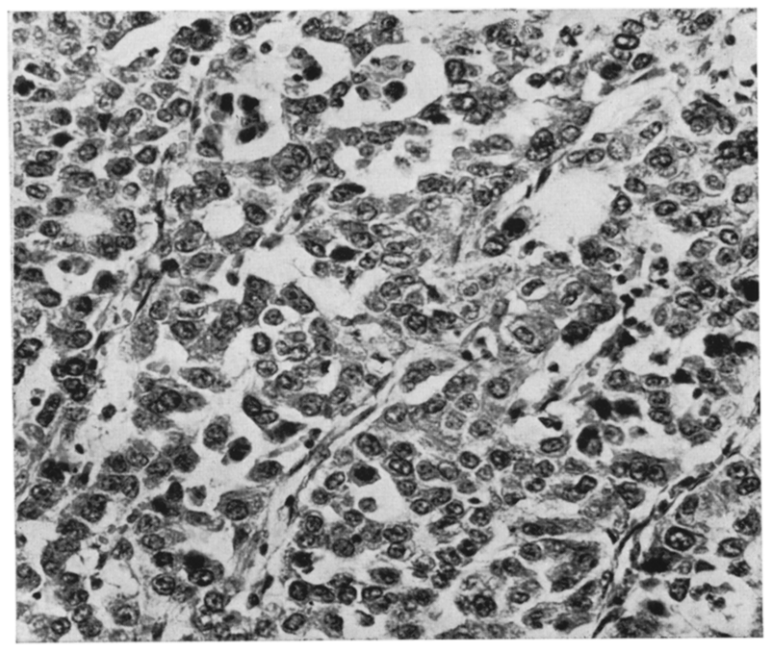


Fig. 3. *Histology of the primary (HEp* 2) *homotransplant nodule on day* 167 (*autopsy*) *showing the non-necrotic peripheral zone which is composed almost entirely of healthy-appearing HEp* 2 *cells.*

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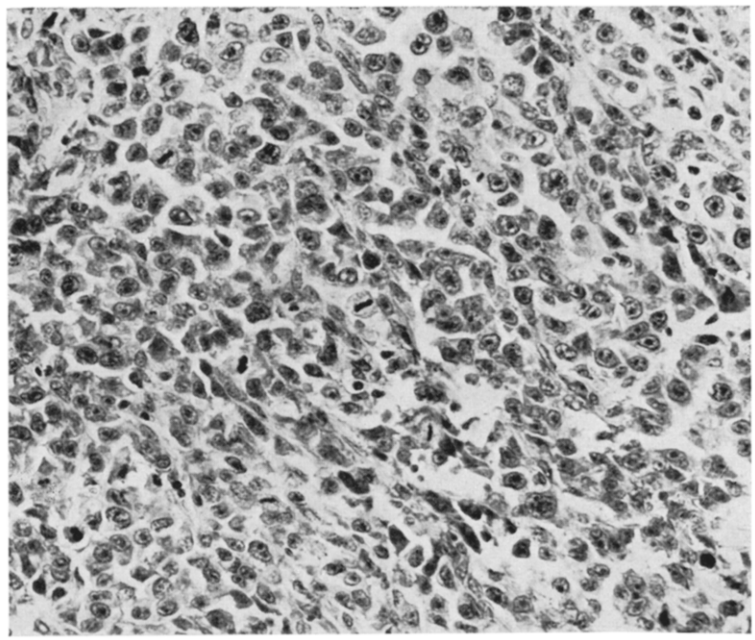


Fig. 4. *Histology of the third (HEp* 3) *transplant on day* 20 (*day* 114 *after the primary homotransplant*). *There is active propagation of the allogeneic cancer cells and no evidence of a homograft rejection reaction.*

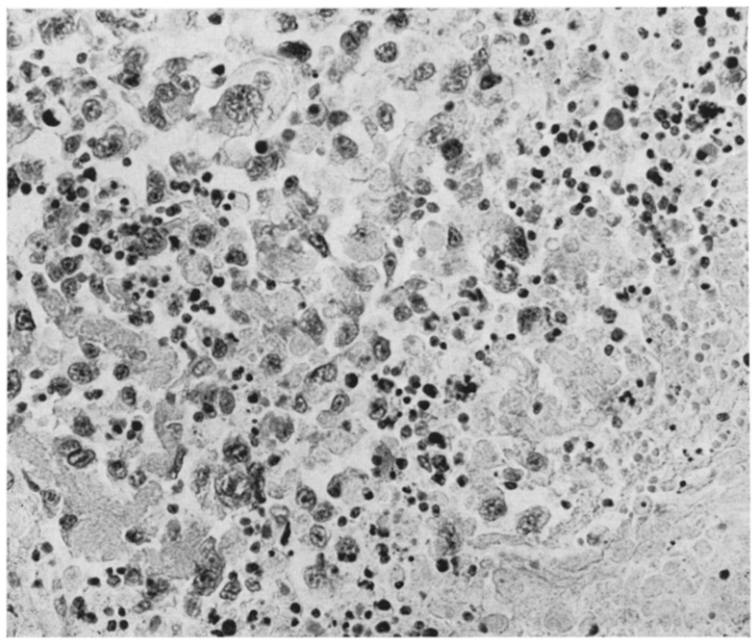


Fig. 5. *Histology of the HEp* 3 *implant on day* 70 (*autopsy*) *showing an area in which HEp* 3 *cells are still identifiable, although most show degenerative changes. Lymphocytes are scattered throughout the field and were also concentrated prominently at the periphery of the nodule.*

became redder and slightly fluctuant. Thereafter however it remained quite constant in character and size until the 14th week when it started to grow again and it continued to increase in size until the patient died of widespread cancer and sepsis 23 weeks after initiation of the primary transplant. At autopsy this HEp 2 nodule was approximately $3 \times 5 \times 7$ cm and was well demarcated from the surrounding subcutaneous tissues. There was a soft necrotic center but this was surrounded by a 1 cm zone of firm healthy-appearing tissue. Microscopically (Fig. 3) the outer zone consisted almost entirely of tumor cells of the transplanted type. The central area showed necrotic epithelial cells with no evidence of bacteria or host-cell reaction. At the periphery there were scattered small collections of lymphocytes, but scarcely more numerous than in the day 34 biopsy. There was a thin capsule of connective tissue. There was no infiltrative growth into the host tissues. (The same HEp 2 cell suspension which was used for these transplants was used simultaneously in the same manner in another patient with advanced cancer. It produced palpable nodules by day 6 which reached a maximum diameter of 1·6 cm on day 17 and had regressed completely by day 47. One of the nodules was biopsied on day 14 and showed proliferating HEp 2 cells with a peripheral collection of lymphocytes).

The second transplant was injected 47 days after the primary transplant—the time when the primary transplant nodule appeared to be undergoing rejection. It too consisted of two injections, each containing 4×10^6 HEp 2 cells and was implanted 5 cm proximal to the primary transplant sites. After 7 days this second set of transplants had formed small but distinct subcutaneous nodules, but they regressed rapidly during the next week and had disappeared completely by 23 days after inoculation. No microscopic studies of this transplant were made. (The same cell suspension which was used as the inoculum for this second homotransplant was also used as primary homotransplants in another patient with advanced cancer. In this recipient, subcutaneous nodules became palpable after 5 days and increased to a maximum diameter of about 1 cm between the second and fifth week. One nodule was excised on day 28 and the other on day 35. Both showed a good growth of the HEp 2 cells and the latter had areas of lymphoid infiltration.)

A third homotransplant was injected at two subcutaneous sites in the other thigh 95 days after the first transplant (51 days after the second), using $2 \cdot 0 \times 10^6$ of a different cell line, HEp 3. Previous homotransplantation studies have shown that the HEp 2 cell line produces homograft immunity [8] and serum antibodies [9] that cross-react with the HEp 3 cell line. The HEp 3 cells produced subcutaneous nodules which became palpable between 6 and 9 days and increased progressively to a maximum diameter of 4 cm at 6 weeks. One of the nodules was excised for histological study on the 20th day after it was implanted. It revealed (Fig. 4) active propagation of the HEp 3 cells with no evidence of a rejection reaction. The remaining HEp 3 nodules decreased rapidly in size after six weeks but was still 2·5 cm in diameter when the patient died. At autopsy, this nodule consisted almost entirely of necrotic tissue with numerous areas of lymphoid infiltration and only rarely could groups of degenerating HEp 3 cells be identified (Fig. 5).

DISCUSSION

The impaired rejection of primary homografts of cancer cell lines in patients with advanced cancer is a frequent phenomenon which reflects an impairment of certain immunologic mechanisms in such patients. It has been shown to be related to the presence of advanced neoplasia *per se* [10], but it is well known that intensive radio therapy and chemotherapy may also cause an immunosuppression that will impair homograft rejection. The cause of the impairment in this patient however is not pertinent to the following considerations.

The course of the three successive homotransplants in this recipient clearly revealed that the immunological processes of graft rejection were impaired but not absent. Because of the impairment the primary transplant apparently grew without eliciting a significant host reaction for about five weeks (in contrast to the marked rejection reaction which occurs almost immediately in normal recipients). By five to seven weeks the patient's immune mechanisms had responded to the foreign (allogeneic) tissue antigens, as indicated by the temporary decrease in size of the primary transplant nodule and the rapid rejection of the second HEp 2 transplant, but this immune response was quantitatively insufficient to completely reject the primary transplant. The fact that, by this time, the primary homotransplant nodule contained in the magnitude of 10^{10} cells (assuming 10^6 cells/mg of tissue), whereas the repeat homograft inoculum

was a few million cells, may explain this discrepancy in response of the primary and repeat homografts of HEp 2 cells.

The growth of the third set of homotransplants was also restrained by a host rejection reaction. In view of the previously demonstrated cross-resistance induced by homografts of HEp 2 and HEp 3 cells, the growth of this third set of homotransplants was much greater than would have been predicted. Nevertheless the nearly complete regression of the HEp 3 nodule in the two weeks before the patient's death, is clear evidence that there was an immunologic rejection of the HEp 3 cells.

The interplay of several factors probably determined the actual behavior of the third (HEp 3) homograft: the great growth potential of the HEp 3 cell line; antigenic differences between HEp 2 and HEp 3 cells such that the cross-reactions did not provide maximal immunity; quantitative and qualitative impairment of immune responses in the recipient such that even after primary stimulation they were not maximally effective; a "preoccupation" of immune responses with the primary transplant such that they could not be concentrated against the new homotransplant for maximum effect (i.e., a state of antigen excess).

It is evident from these observations of tumor cell homotransplants that, in man as in experimental animals, the continuing growth of an established tumor does not indicate an absence of host defense reaction against the tumor, but merely that such anti-tumor reactions as do occur are insufficient to cause complete destruction of the tumor. By analogy, the fact that clinical cancer almost always runs a progressive course means only that host defenses against cancer are insufficient to stop the growth of a tumor mass after it is well established. We need not dismiss the possibility that there are host defense reactions against autochthonous human cancers and that such reactions may prevent the growth of small numbers of residual cells after therapy, or the establishment of metastatic implants.

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RESUME

Un malade, atteint d'un cancer épithélial généralisé, a reçu trois homotransplantations sous-cutanées successives de cellules cancéreuses humaines (lignées de culture de tissus HEp 2 et HEp 3). La première produisit un gros nodule local qui persista jusqu'à ce que le malade décède de son cancer, six mois plus tard, en dépit du fait, démontré par les deux transplantations suivantes, que la première transplantation avait induit une immunité à l'homogreffe. Le phénomène est interprété comme un déséquilibre entre la capacité des cellules tumorales de se propager et celle de l'hôte de rejeter les cellules étrangères. La réponse immunitaire était suffisante pour rejeter une quantité faible de cellules, mais non pour détruire une masse tumorale bien établie. Des courbes de croissance et des constatations histologiques sont présentées. Des analogies entre ce phénomène et l'évolution naturelle de cancers spontanés sont discutées.

SUMMARY

A patient with disseminated squamous cancer received three successive subcutaneous homotransplants of human cancer cells (tissue culture lines HEp 2 and HEp3). The primary homotransplant produced a large local nodule which persisted until the patient died of her cancer 6 months later, in spite of evidence from two subsequent homotransplants that the first transplant had elicited homograft immunity. The phenomenon is interpreted as an imbalance between the capability of the tumor cells to propagate and the capability of the host to reject the foreign cells. The immune response was sufficient to reject a relatively small number of allogeneic tumor cells, but was not sufficient to destroy a well established tumor mass. Growth curves and histologic findings are presented. Possible parallels between this phenomenon and the natural history of autochthonous cancer are discussed.

ZUSAMMENFASSUNG

Eine krebskranke Patientin erhielt drei subcutane Transplantationen menschlicher Krebszellen (Linien HEp 2 and HEp 3). Das erste Transplantat bildete einen grossen umschrie-

benen Knoten, der bis zum Tode der Patientin nach 6 Monaten bestand, obschon zwei weitere Transplantationen zeigten, dass das erste Transplantat Immunität erzeugt hatte. Das Phänomen wird als ein Ungleichgewicht angesehen zwischen der Wachstumstendenz der Tumorzellen und der Fähigkeit des Wirtsorganismus Fremdzellen abzustossen. Die Immunreaktion war ausreichend eine kleine Zahl allogener Tumorzellen abzustossen, aber nicht ausreichend eine ausgebildete Tumormasse zu zerstören. Wachstumskurven und histologische Ergebnisse werden dargestellt. Mögliche Parallelen zwischen diesem Phänomen und der Entwicklungsgeschichte autochthonen Krebses werden diskutiert.

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Fine Structure of Primary Rat Hepatoma Induced by 2-Acetylaminofluorene Feeding

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INTRODUCTION

ALTHOUGH the fine structure of several transplantable out hepatomas has been described in a number of studies [1–6], relatively little attention has been paid to the electron microscopy of primary, chemically-induced hepatomas [7, 8]. The use of transplantable tumours, especially of the "minimal deviation" type discussed by Potter [9], is convenient for biochemical studies but has the disadvantage that the most highly differentiated of these has probably undergone de-differentiation to a greater extent than a similar but, perhaps, non-transplantable tumour. It seemed desirable, therefore, to examine the fine structure of some chemically-induced, liver cell tumours growing *in situ*. Some indication of the growth rate of these tumours would have to be inferred from their histological appearance, rather than from direct measurements. On the other hand, it would be possible to compare the fine structure of the tumour cells, not merely with that of normal rat hepatocytes, but also with that of the cells of the pre-neoplastic liver resulting from treatment with the same carcinogen.

In a previous study [10] it was shown that prolonged feeding of the carcinogen, 2-acetylaminofluorene (2-AAF), induces a number of ultra-structural changes in the hepatocytes of the Leeds strain of rat, which appear to be permanent and independent of the continued feeding of the carcinogen. Liver tumours arising at a later stage in rats treated with the same dose of 2-AAF, having different degrees of invasiveness and anaplasia, have now been investigated. An attempt has been made to correlate the fine structure of these tumours with their apparent degree of differentiation and invasiveness.

MATERIAL AND METHODS

Male rats of the Leeds strain, weighing approximately 200 g, were fed a standard diet to which had been added 0·05% by wt. of 2-acetylaminofluorene (2-AAF). Feeding was continued for 10 months, after which time the animals were returned to a carcinogen-free diet. They were sacrificed at intervals between 2 and 8 months after the end of treatment. The rats were killed by stunning, and samples of every tumour nodule present in the liver of each rat were rapidly excised for processing for electron microscopical and histological examination. Histological specimens were fixed in 10% formaldehyde–saline, embedded in paraffin wax and stained with haematoxylin and eosin.

For electron microscopy the tissue was finely minced and fixed in cacodylate-buffered 4% glutaraldehyde for 4 hr [11, 12], washed in cacodylate-sucrose overnight and post-fixed in 1% osmium tetroxide [13]. The material was embedded in Epon 812 [14] and thin sections were stained with lead tartrate [15] and examined in an RCA EMU-3G electron microscope at 100 kV.

On the basis of histological examination, three groups of tumours were selected for electron microscopy. The first group consisted

of well-differentiated, non-invasive hepatomas; the second group included more anaplastic and clearly invasive tumours; the third group consisted of extremely anaplastic tumours and showed evidence of intensive mitotic activity and a high degree of invasiveness. For descriptive convenience these groups were designated as A, B and C, respectively.

RESULTS

During the 6 months' sampling period that followed the feeding of 2-AAF, the liver of each rat killed contained increasing numbers of individual tumours, with the passage of time, and a larger proportion of the tumours were more anaplastic. Whilst most of the tumours were clearly of hepatic cell origin, there was some doubt in the case of Group C tumours, owing to their extreme anaplasia. However, no cholangiomata were found at any stage.

Light microscopy

Group A tumours were cystic, and there was some evidence that the cysts were formed by centrifugal degeneration of the tumour cells. The tumours were well-differentiated, although there was some variation in the size and arrangement of their cells. Mitotic figures were frequent, but there was no evidence of any invasion of the surrounding liver tissue, which was compressed to form a capsule. The tumour cell nuclei were variable in size and generally contained prominent nucleoli.

Group B tumours had the appearance of malignant hepatomas or hepatocarcinomas. They were clearly of hepatic cell origin, but more anaplastic than those of Group A and showed definite invasion of the liver tissue. Mitotic figures were observed more frequently, suggesting a more active cell proliferation.

Group C tumours appeared to be rapidly-growing, highly-invasive and extremely anaplastic. Their structure was disorganized, and they contained numerous areas of focal degeneration. These tumours could not be identified with certainty as being of hepatic cell origin. Their inclusion in the present study was justified only by the circumstantial evidence that only tumours of hepatocyte origin arose in Leeds rats after 2-AAF feeding, in this experiment.

Electron microscopy

The main ultrastructural features of the tumours have been summarised in Table 1, which also includes, for comparison, a summary of the fine structure of pre-neoplastic liver cells [10].

Group A tumour cells. In general, these cells appeared to be well-differentiated, in agreement with the histological findings. The rough-surfaced form of the endoplasmic reticulum (Fig. 1) was present in quantities approaching those of normal rat hepatocytes. The membranes of each cisterna were closely spaced except at the edges of these elements, where coarse granules were often found within the lumen (Fig. 3). Large numbers of ribosomes were attached to the cisternae in all cells, as well as to the cytoplasmic aspect of the nuclear membrane. Tangential sections of the granular endoplasmic reticulum showed that the attached ribosomes were generally arranged in rosettes, spirals and chains (Fig. 2).

Free ribosomes were present as single particles as well as in aggregated forms and their quantity appeared to be only slightly greater than in normal hepatocytes.

The cytoplasm contained areas of agranular endoplasmic reticulum, consisting of rather loosely-packed, irregularly-branching tubules, associated with a small amount of glycogen (Fig. 2). It was localised in relatively small areas and, in general, resembled that of normal liver cells. The quantity of glycogen present was also similar to that of normal rat hepatocytes, large glycogen areas being observed (Fig. 1). Individual glycogen rosettes tended, however, to be rather smaller than usual.

The tumour cell mitochondria were abnormally variable in size and were usually smaller than the hepatocyte mitochondria of the normal rat. Although elongated forms were commonly present and, occasionally, their cristae were circular or triangular in cross-section, their internal structure was generally normal. Grossly abnormal or degenerate forms of mitochondria were seldom observed.

The microbody population appeared to be normal with respect to numbers and morphology.

The Golgi zones were well-developed and contained abundant granules within their membranes. The saccules of these organelles sometimes had the appearance of ring-shaped figures, composed of double membranes. In addition to lysosomes of normal morphology and abundance, Group A tumour cells possessed a few vacuoles of a more pliomorphic nature. Some were membrane-bounded structures, containing large, irregular granules and amorphous material, while others were largely empty and contained only small amounts of disorganised membranes. These structures, some of which were probably autophagic

Table 1. *The ultrastructure of hepatic tumours and the pre-neoplastic hepatocytes induced by 2-AAF compared to normal hepatocytes*

Cell component	Pre-neoplastic liver [10]	Group A Tumours	Group B tumours	Group C tumours
Granular endoplasmic reticulum	Decreased quantity, single short cisternae	Normal but elongated cisternae	Small quantity, single, short, dilated cisternae—abnormal lipid inclusions	Scarce—bizarre forms only
Golgi zones	Hypertrophied, contain numerous granules	Hypertrophied, also abnormal forms	Atrophied, few granules	Atrophied, no granules
Lysosomes	Normal (autophagic vacuoles also present)	Normal (autophagic vacuoles also present	Few	Few
Free ribosomes	Small increase (aggregated)	Small increase (aggregated and single)	Increased (aggregated and single)	Numerous (single)
Microbodies	Normal	Normal	Normal	Absent
Glycogen	Normal	Normal	Normal	Absent
Agranular endoplasmic reticulum	Abundant, dilated	Normal	Normal	Absent
Mitochondria	Normal, but smaller	Normal, but smaller	Normal, but smaller	Few and small
Nucleus	Normal	Normal, but 'blebbing' of membrane	Normal, but lipid inclusions	Variable shape, variety of inclusions
Nucleolus	Hypertrophied	Hypertrophied	Normal (some hypertrophy)	Variable
Cell surface	Variable separation of cells, increase in desmosomes. Dilated bile canaliculi	As in the pre-neoplastic hepatocytes	More irregular	Variable—extremely irregular

vacuoles, together with the presence of glycogen and other cellular debris in the lumen of the sinusoids, were the sole indications of possible degenerative changes in these tumours.

The nuclei of the tumour cells contained large, diffuse nucleoli of skein-like appearance. Only a minority of the cells examined did not appear to possess hypertrophied nucleoli. The outermost membranes of the perinuclear cisternae were occasionally expanded locally, to form blebs or dilatations which protruded into the cytoplasm.

The cell membranes of adjacent tumour cells were usually closely apposed. An unusual feature, which was noted in many cells, was the presence of short cisternae of the granular endoplasmic reticulum closely applied to the cytoplasmic aspects of the cell membranes of adjacent cells at the same locus.

Structures resembling bile canaliculi were present, but they were greatly extended along the line of separation between the cells (Fig. 4). Abundant microvilli were present at these sites. The cell membrane was often interrupted by narrow invaginations, many of which contained cytoplasmic protrusions from adjoining tumour cells. Cell surfaces bordering on the space of Disse (Fig. 1) possessed numerous pinocytotic pits.

Group B tumour cells. Although Group B tumour cells, in general, resembled those of Group A with respect to their glycogen content, agranular endoplasmic reticulum, mitochondria and microbodies, there was evidence of decreased differentiation at the ultrastructural, as well as the microscopical, level of organisation.

The cells contained only small amounts of the granular endoplasmic reticulum, and that which was present was abnormal in many respects. This organelle was usually present as single, short, cisternal profiles, either free in the cytoplasm or encircling the mitochondria and microbodies. Although the cisternae possessed numerous attached ribosomes, they were irregularly dilated. The extent of this dilatation varied, especially from one cell to another. Some cisternae were almost normal in appearance, while other possessed a widened lumen and appeared to branch and anastomose freely (Fig. 7).

Golgi zones were few in number and generally small in size, containing relatively few granules (Fig. 7). Only occasionally were Golgi zones observed which presented an 'active' appearance. However, the cytoplasm sometimes contained smooth, membraneous structures which consisted of small vesicles and saccules, and which may have been degenerate Golgi zones. The tumour cells contained few lysosomes, and only occasional autophagic vacuoles were present (Fig. 7).

An unusual characteristic of the cells of Group B hepatomata was the presence of numerous, membrane-enclosed, cytoplasmic lipid inclusions (Figs. 5 and 9). The single membranes which bounded these inclusions appeared to be derived from dilated cisternae of the granular endoplasmic reticulum, since there were many points of continuity between them. The appearance was, therefore, quite atypical of normal cytoplasmic lipid, which is not membrane-bounded. Coarse, electron-dense granules were often found within the tubules of the agranular endoplasmic reticulum (Fig. 6) but there was no evidence of any association between these and the abnormal lipid inclusions.

The relationship between the cell membranes of adjacent tumour cells was more irregular than in Group A tumours. Minor dilatations of the intercellular spaces were present, often containing granular material. The cell surfaces bordering on these spaces sometimes possessed small numbers of pits or depressions, which resembled those seen on the surface of many cells during pinocytosis. In some areas, however, larger dilatations of the intercellular space were observed, having the appearance of greatly distended bile canaliculi. These spaces were limited by tight junctions and desmosomes, and possessed relatively few microvilli. The surrounding cytoplasm often contained fine, fibrillar material. Cytoplasmic invaginations and protrusions of the type which was found in Group A tumour cells, were present in small numbers.

The tumour tissue did not possess sinusoids of normal appearance—instead there were rather irregular, blood-filled spaces. Numerous microvilli were present on the cell surfaces which bordered on these spaces.

Binucleate cells were often observed in these tumours (Fig. 5), and nuclei frequently contained small lipid inclusions. The nucleoli were usually normal in appearance, hypertrophied forms being less common than in Group A.

The cytoplasm contained more free ribosomes than that of either normal liver cells or Group A tumour cells, but their state of aggregation was similar to that found in Group A.

Group C tumour cells. The pronounced anaplasia noted in histological preparations of these tumours was reflected in their somewhat

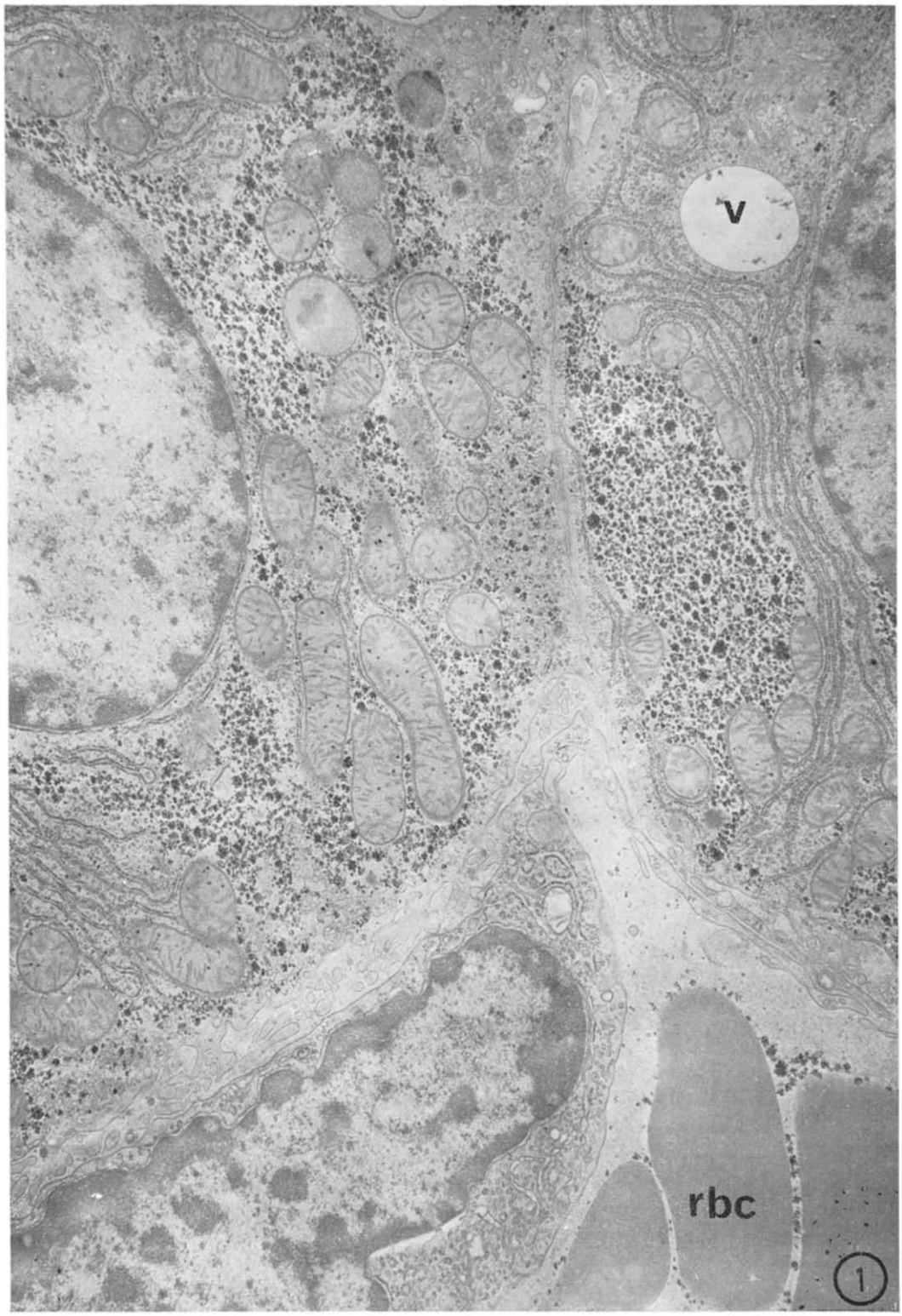


Fig. 1. *Group A tumour.*
The general structure of the cytoplasm is shown. Note the large glycogen areas and elongated, granular endoplasmic reticulum. At the upper part of the figure both cells contain a Golgi zone associated with a lysosome in one cell, and with a membrane-bounded vacuole (v) *in the other. The lower part of the field shows part of a sinusoid with its lining Kupffer cell. The lumen contains erythrocytes* (*rbc*) *and some glycogen. The tumour cell surfaces bordering on the space of Disse bear numerous pinocytotic pits.*
Magnification × 15,000.

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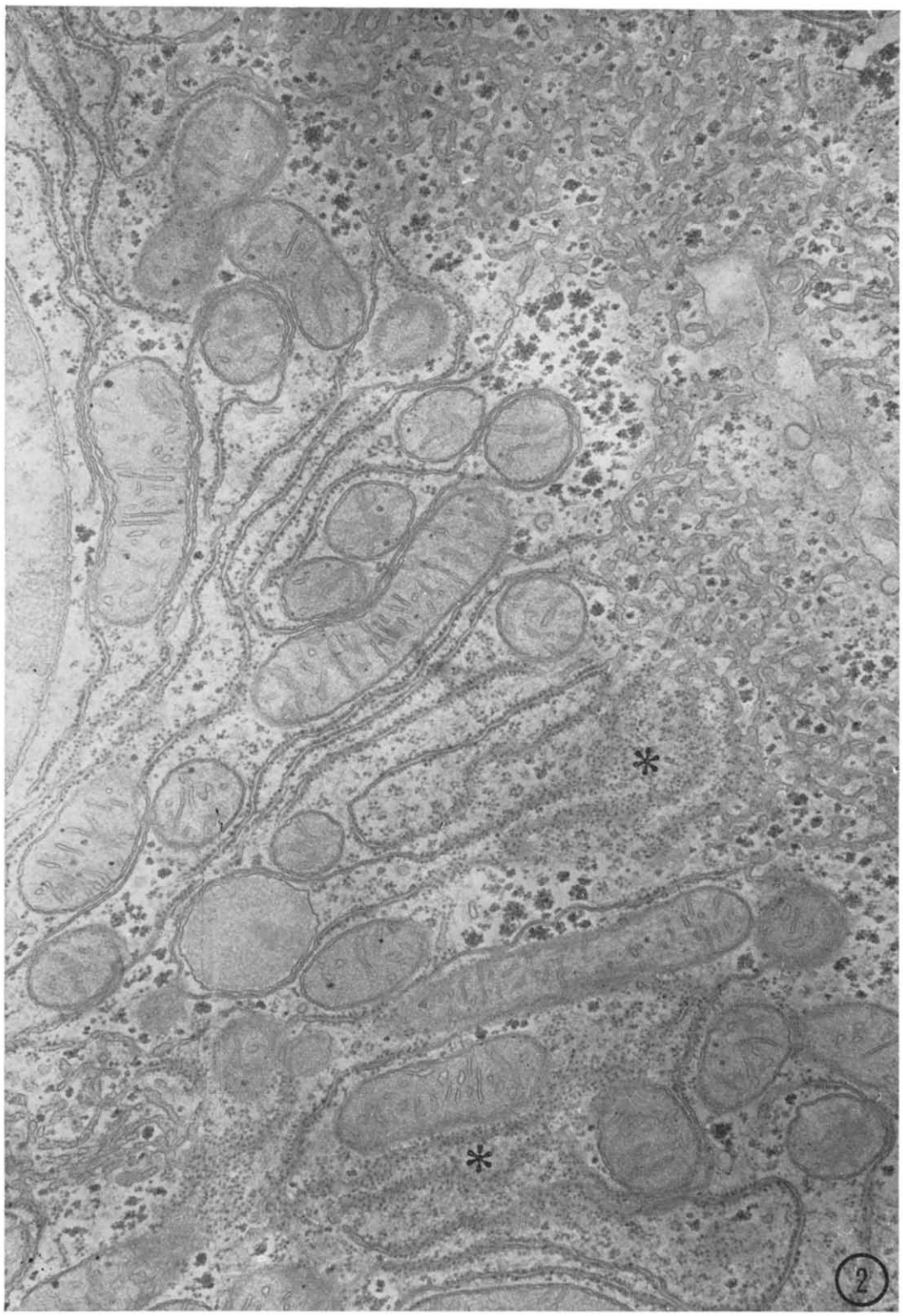


Fig. 2. Group A tumour.

Portion of tumour cell cytoplasm, showing characteristic, well-organised granular endoplasmic reticulum continuous at its cisternal edges with the tubular, agranular endoplasmic reticulum. Where it has been sectioned tangentially, the granular endoplasmic reticulum reveals the rosette-like and spiral arrangment of its attached ribosomes (*). Some cristae mitochondriales are circular or triangular in cross-section. ***Magnification*** × 30,000.

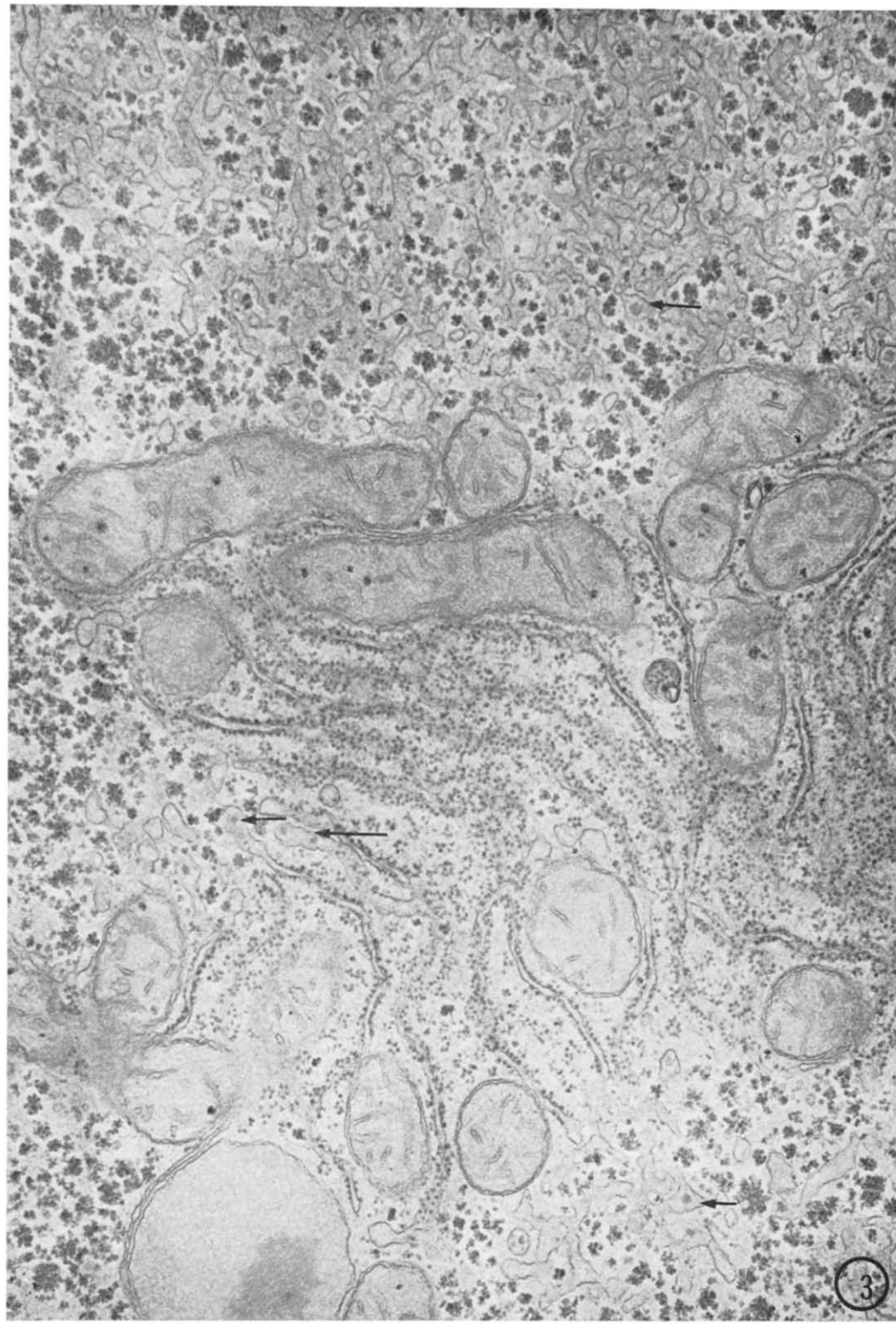


Fig. 3. *Group A tumour.*
Cytoplasmic detail of a tumour cell showing the small size of the mitochondria relative to the microbody (lower left). The presence of dense granules is shown (arrows) within the agranular endoplasmic reticulum and in the terminal dilatations of the rough-surfaced cisternae.
Magnification × 35,000.

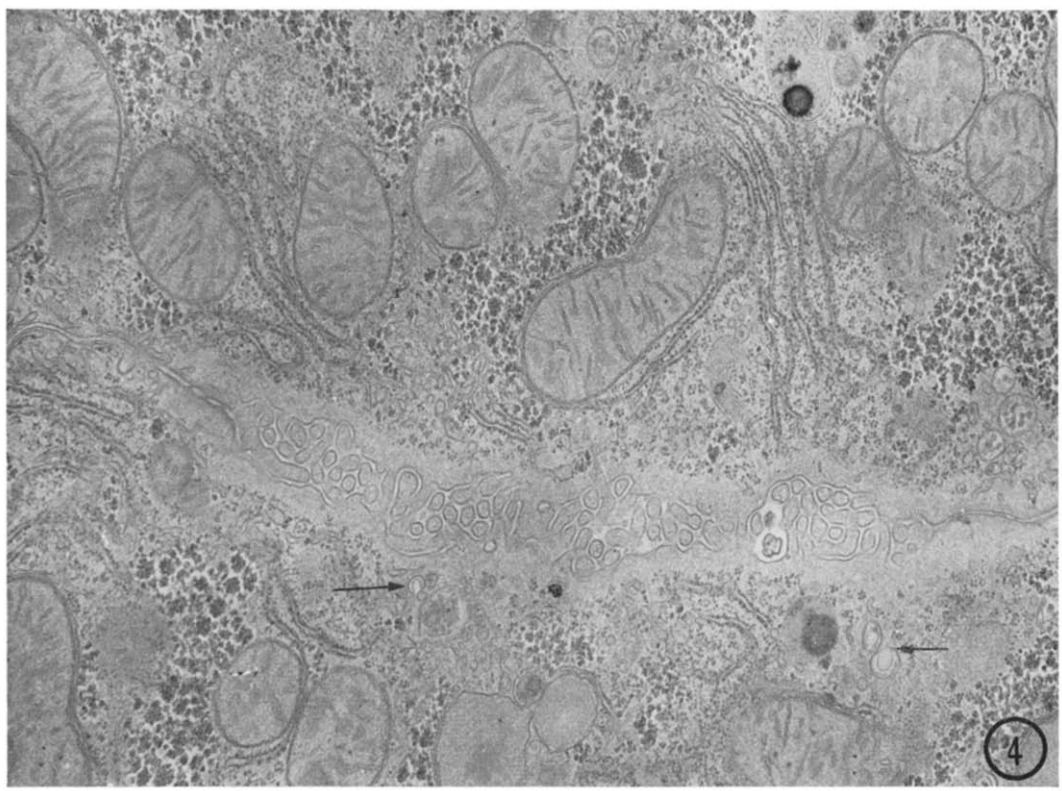


Fig. 4. Group A tumour.
An extended form of bile canaliculus is shown, limited by desmosomes. The adjoining cytoplasm contains some tubular invaginations (arrows).
Magnification × 22,500.

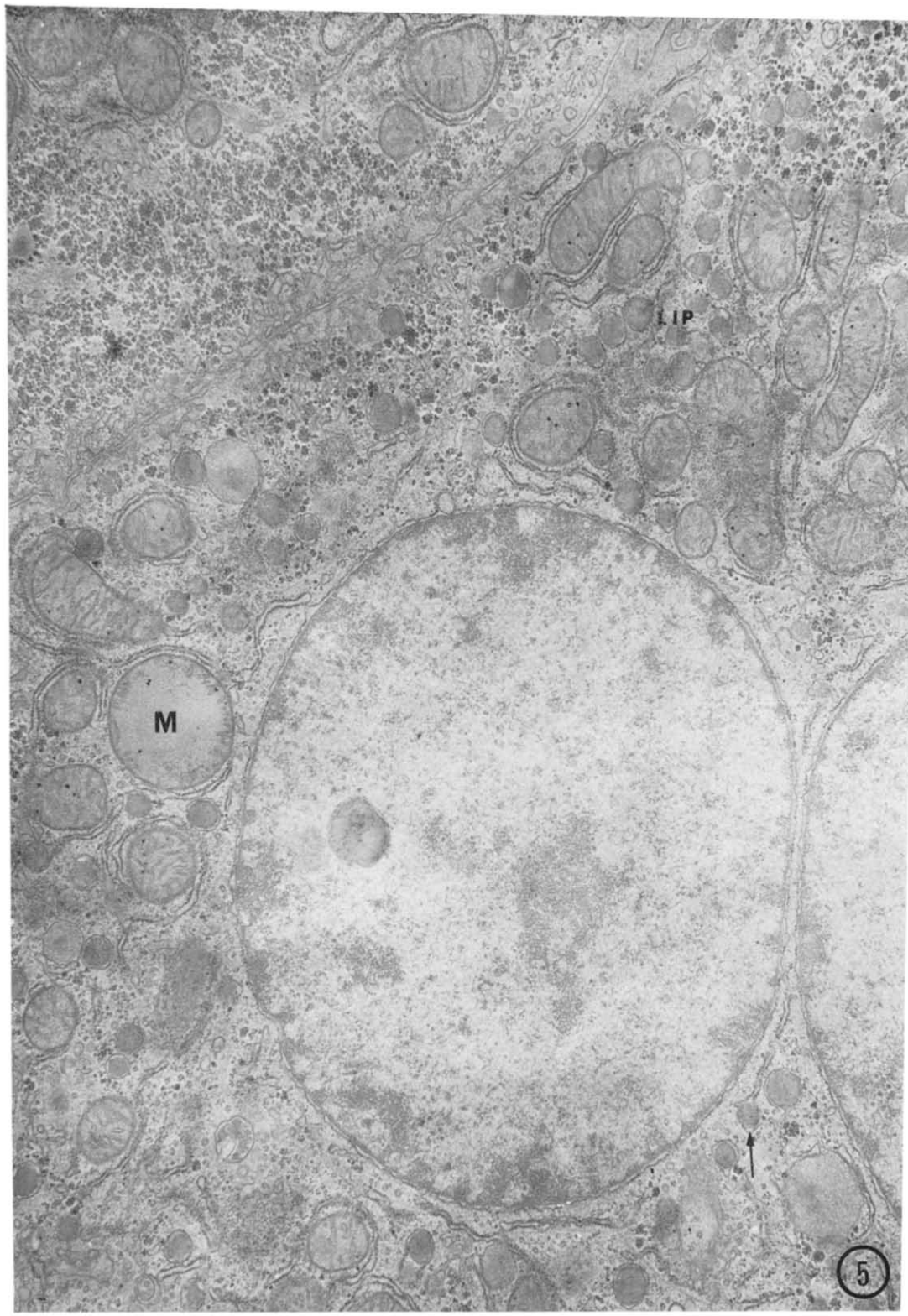


Fig. 5. *Group B tumour.*
The electron micrograph shows part of a binucleate cell. The cytoplasm contains abundant, lipid droplets (lip) which are bounded by single membranes, derived from the endoplasmic reticulum (arrow). A nuclear, lipid inclusion is also present. A large mitochondrion (m) with short cristae is shown in the juxtanuclear region.
Magnification × 15,000.

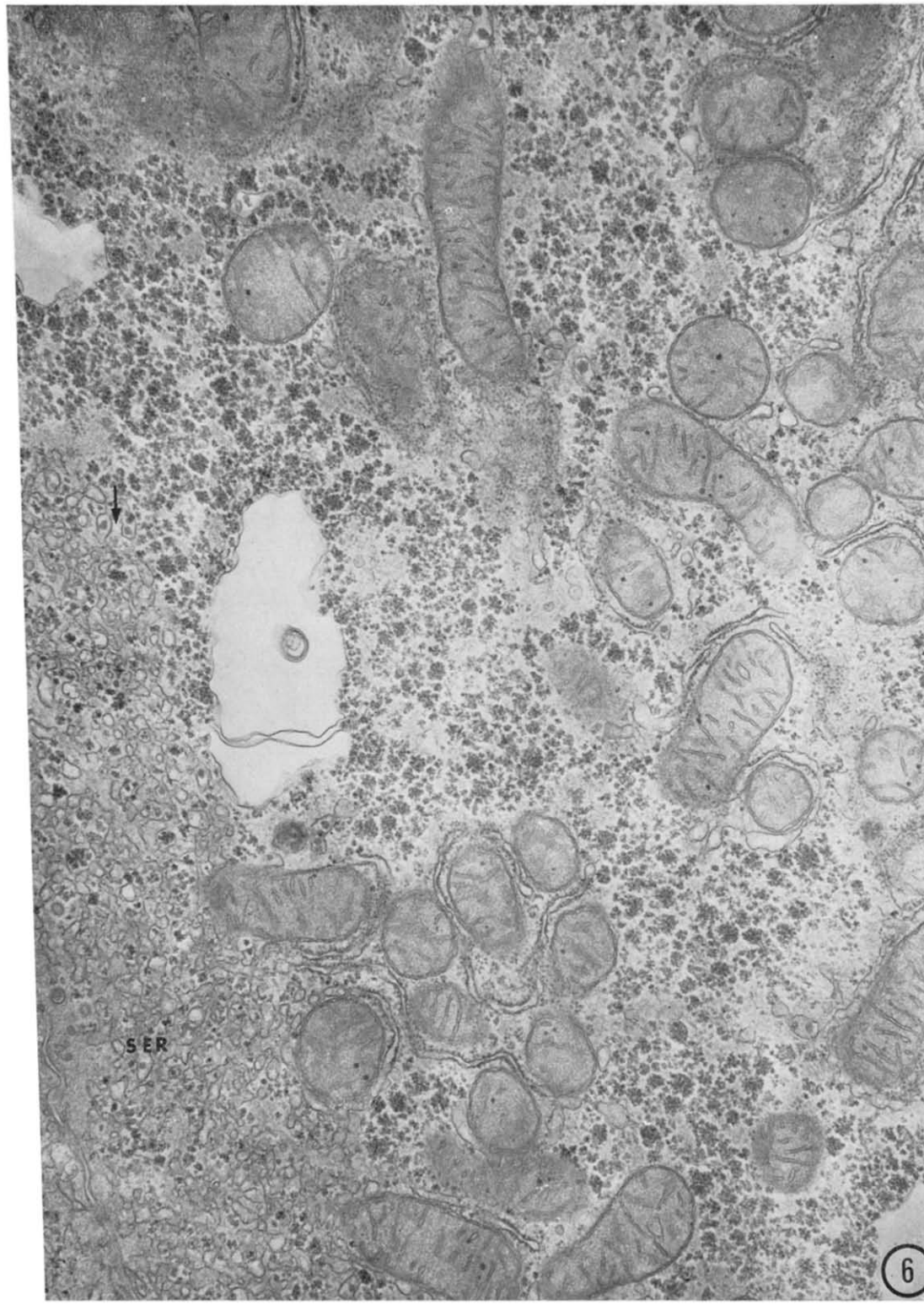


Fig. 6. *Group B tumour.*
Part of the peripheral cytoplasm is shown. Areas of glycogen rosettes are present, bordering on an area of agranular endoplasmic reticulum (ser), the elements of which contain coarse granules (arrow). Two membrane-bounded vacuoles are also shown.

Magnification × 22,500.

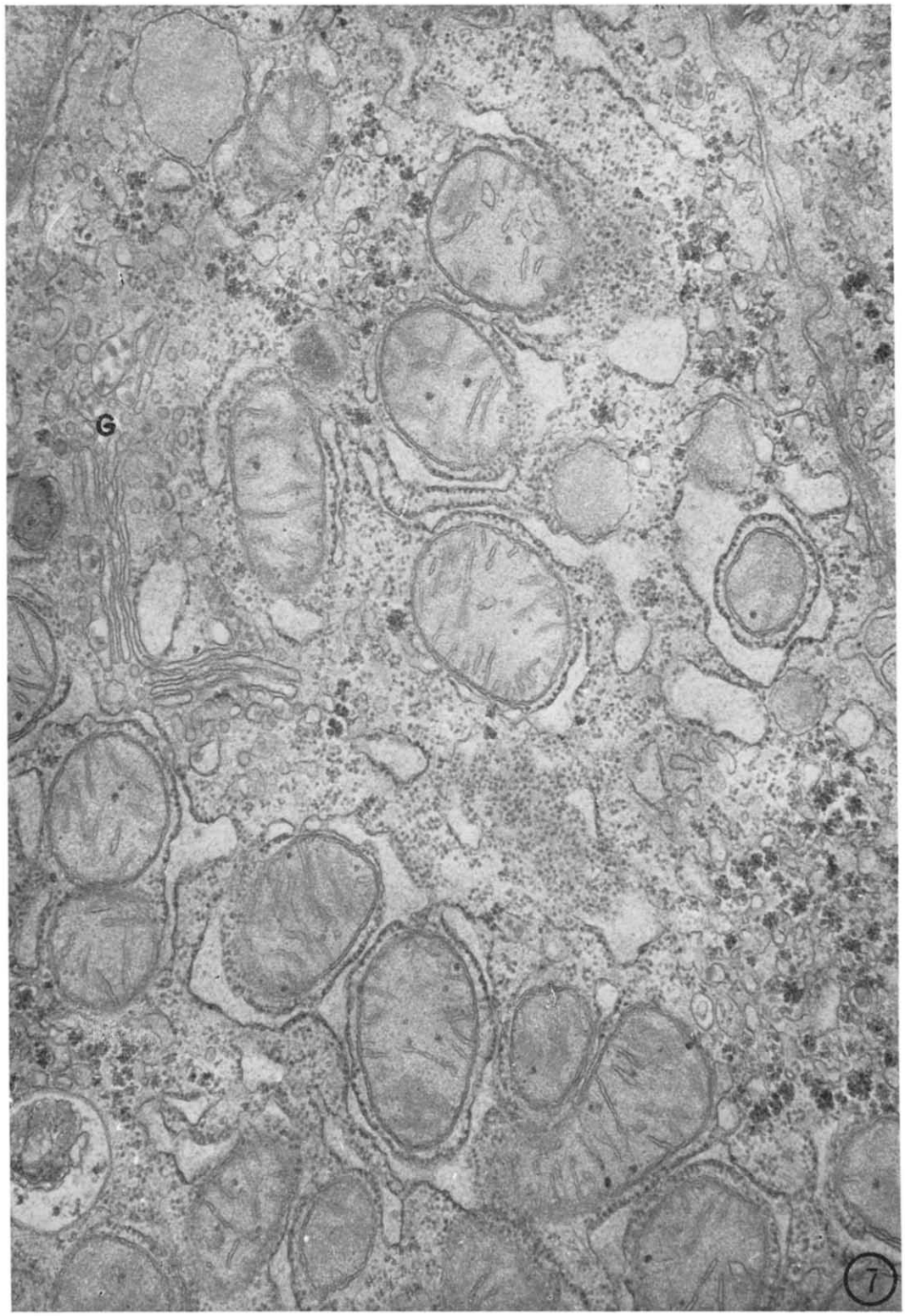


Fig. 7. Group B tumour.
The granular endoplasmic reticulum is irregularly dilated, the cisternae branching and anastomosing. A small Golgi zone (G), *containing a number of granules, is shown. At the lower left of this figure there is a small, autophagic vacuole. The cytoplasm contains abundant free ribosomes.*
Magnification × 30,000.

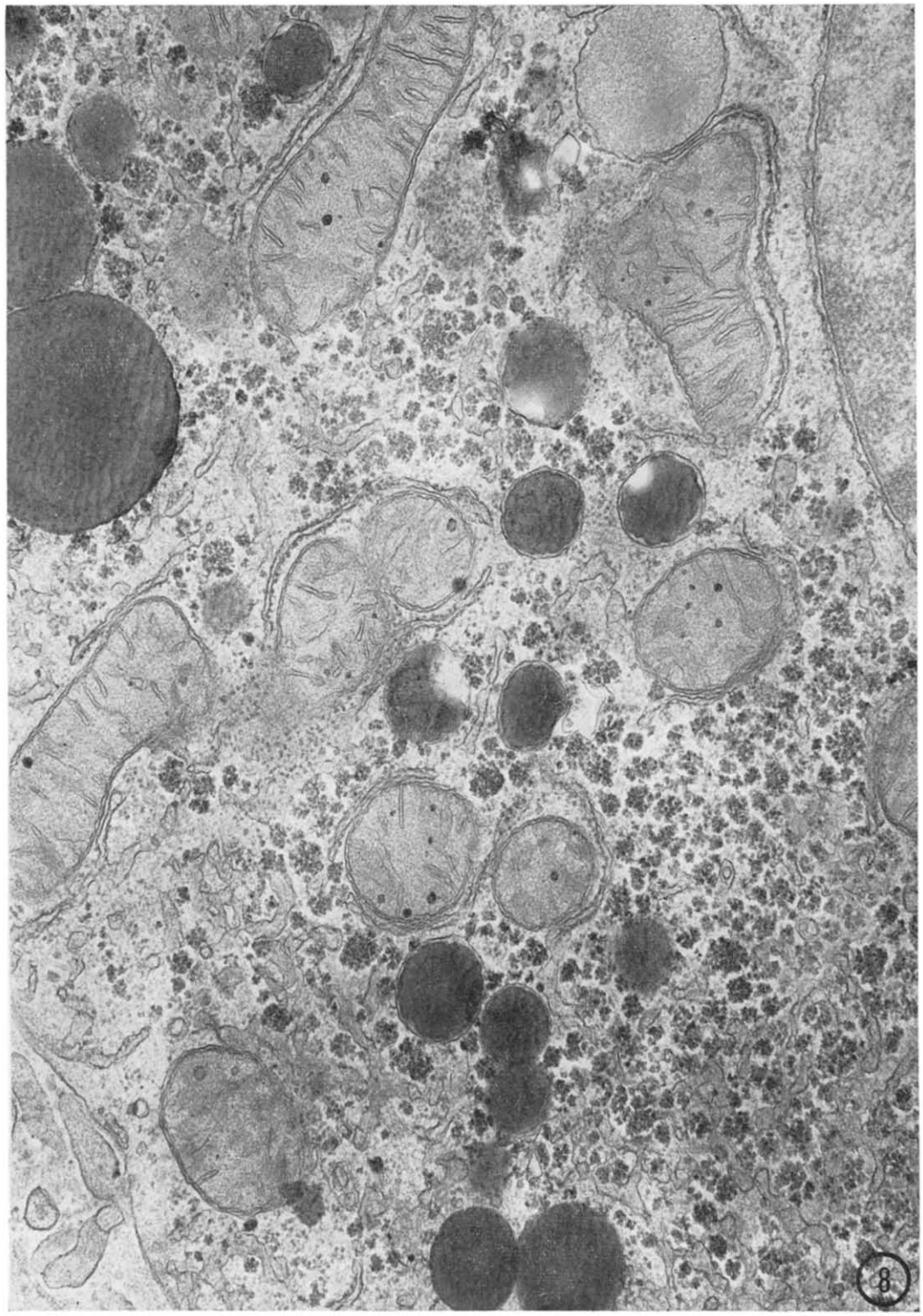


Fig. 8. *Group B tumour.*
A number of densely-stained, lipid droplets are present. some of which show a limiting membrane. Some cristae mitochondriales are dilated.
Magnification × 30,000.

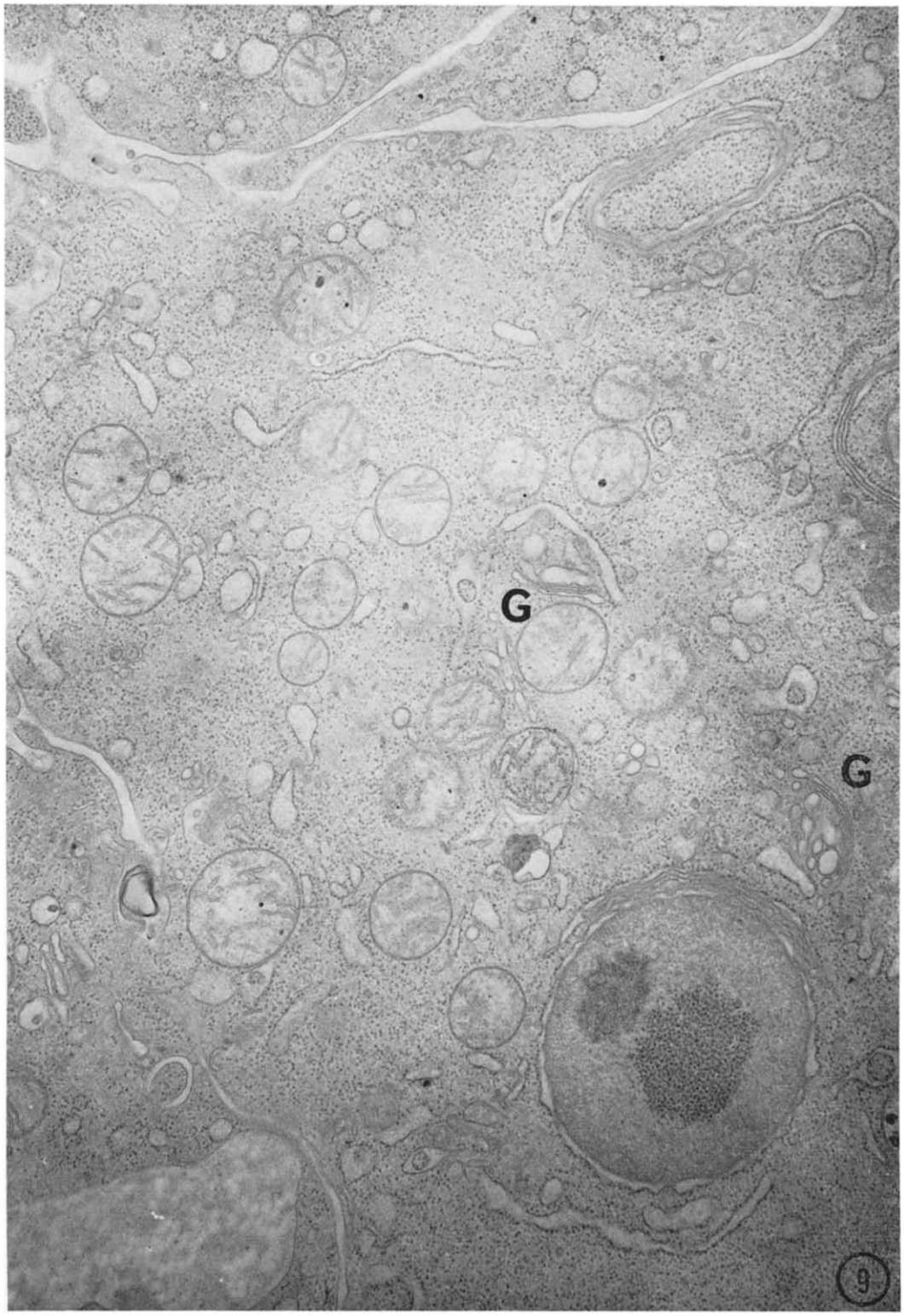


Fig. 9. *Group C tumour.*
The nucleus is unusually regular in outline for this type of tumour. Bizarre forms of granular endoplasmic reticulum are shown, as well as two atrophic Golgi zones (G). *The mitochondria are small and the cytoplasm is packed with numerous free ribosomes.*
Magnification × 15,000.



Fig. 10. *Group C tumour.*
Portions of several tumour cells are shown, containing abnormal forms of granular endoplasmic reticulum. The irregular outlines of the cell surfaces are shown.
Magnification × 22,500.

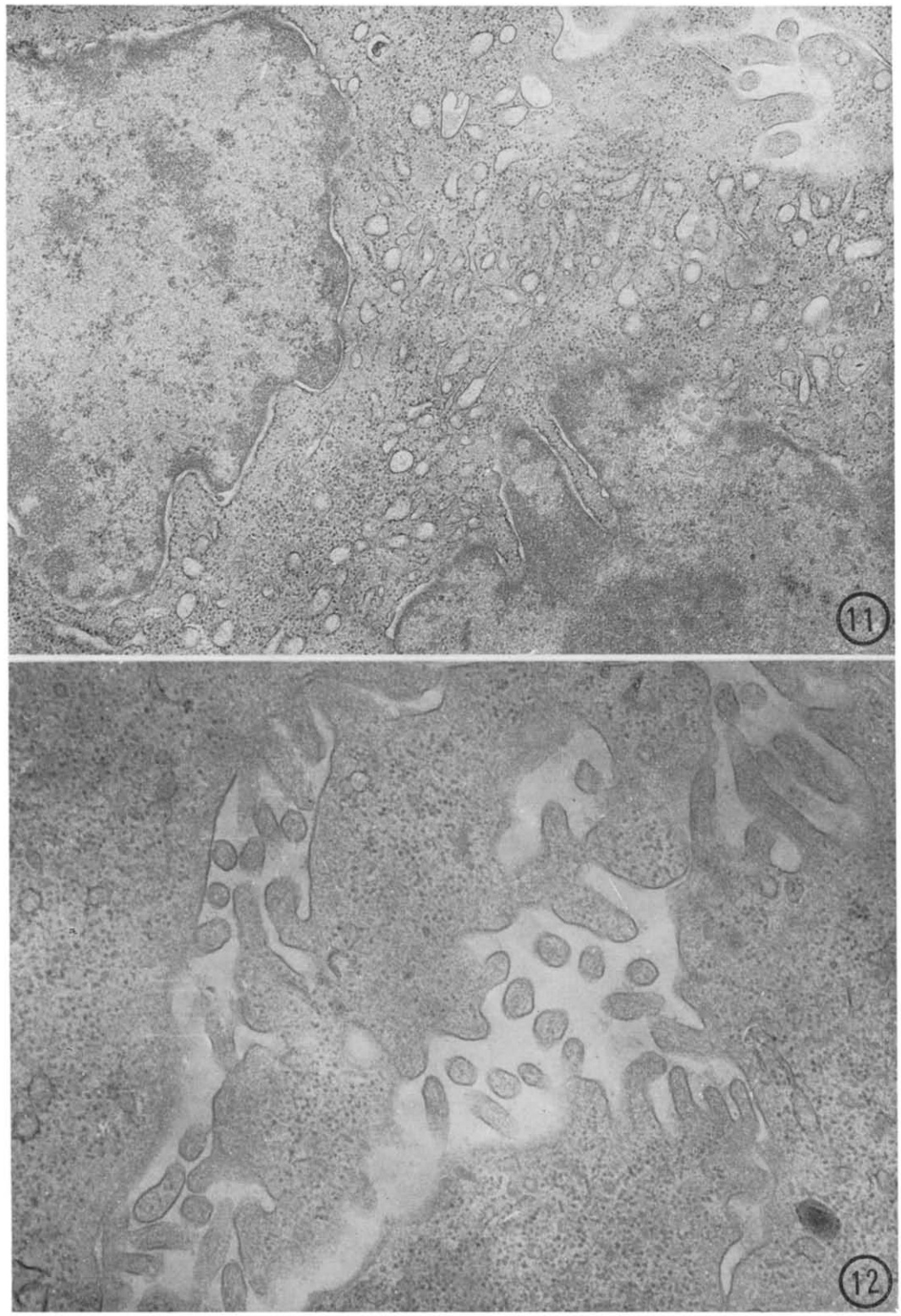


Fig. 11. *Group C tumour.*
Part of a binucleate tumour cell is present, showing the deep, nuclear indentations. The cytoplasm contains a number of rough-surfaced, vesicular elements.
Magnification × 22,500.

Fig. 12. *Group C tumour.*
Cell surface showing local abundance of microvilli of varying sizes.
Magnification × 30,000.

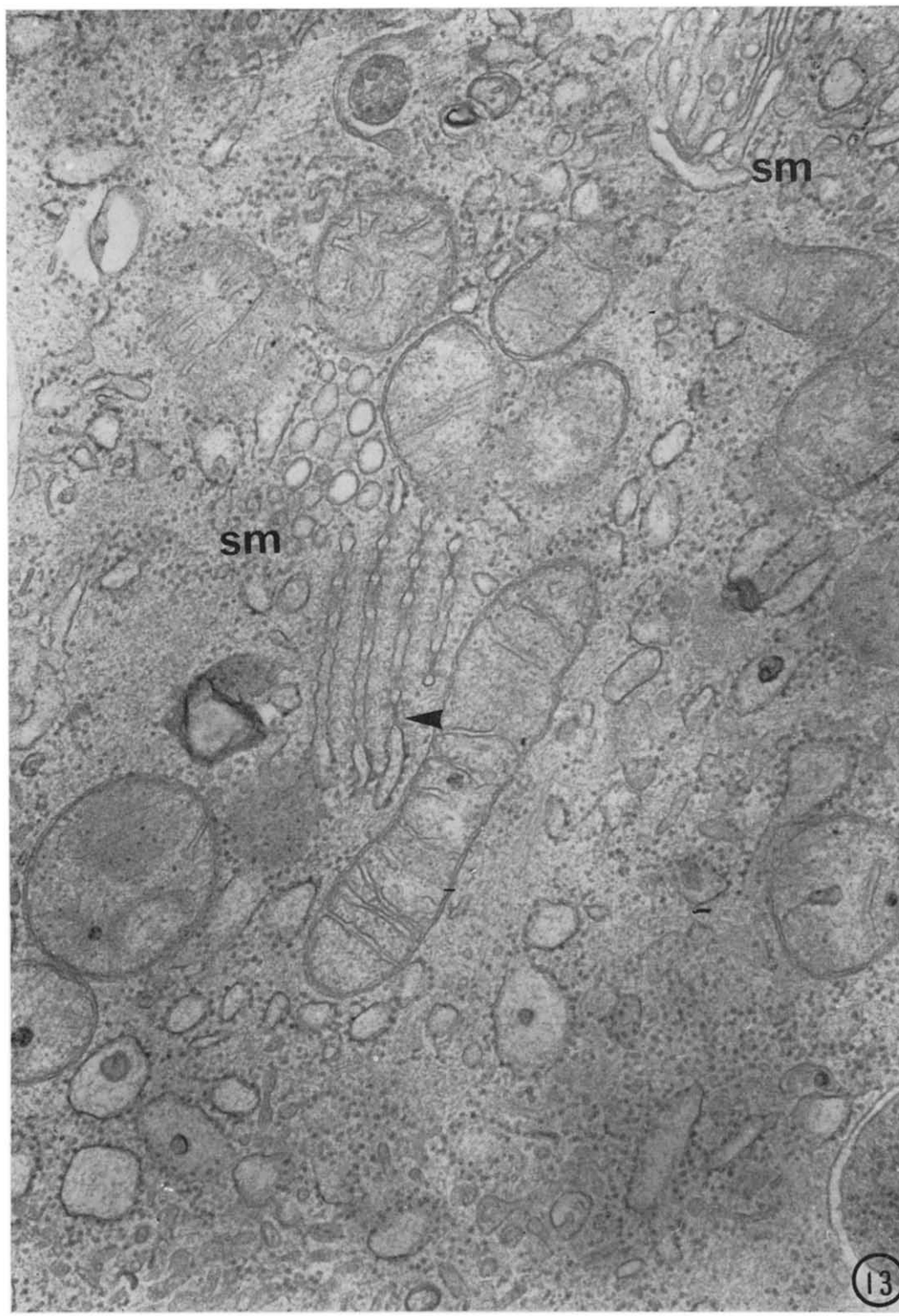


Fig. 13. *Group C tumour.*
The cytoplasm contains an array of fenestrated membranes, apparently derived from the granular endoplasmic reticulum (arrow). Two groups of smooth, membraneous vesicles (sm) are present, one of which has been sectioned at right angles to its long axis. Some of the rough-surfaced vesicles contain coarse, dense granules.
Magnification × 45,000.

'simplified' ultrastructure. Glycogen, agranular endoplasmic reticulum and microbodies appeared to be totally absent from their cells. The cells varied greatly in size and shape, as did their nuclei.

The nuclei were characteristically irregular in outline, frequently possessing deep indentations (Fig. 11). The perinuclear cisternae were often dilated and were interrupted by abundant, nuclear pores. Their cytoplasmic aspects bore numerous attached microsomes. The nucleoplasm sometimes contained abnormal inclusions, apparently of cytoplasmic origin, which appeared to have arisen by the pinching off of the deep, nuclear indentations.

The granular endoplasmic reticulum bore little resemblance to that which was present in the previous groups of tumours. It appeared as distended vesicles and cisternae of variable shape and size, some of which contained large, electron-dense granules, and also as pairs of closely-apposed cisternae (Figs. 9–11). Attached ribosomes were absent from the surfaces in apposition. These unusual quadruple-membrane structures were often annular in form. Apart from these two forms of granular endoplasmic reticulum, other bizarre types were also observed. These consisted of single annular or anastomosing, cisternal profiles, small arrays of annulate lamellae or fenestrated cisternae (Fig. 13) and various other structures.

The mitochondrial population was small in number. The mitochondria were also small in size relative to those of normal rat hepatocytes. Their intramitochondrial granules were often larger than those of normal, hepatic cell mitochondria.

The Golgi zones were very small and atrophic in appearance and rarely contained granules (Fig. 9). They usually consisted of short, parallel saccules associated with a few, small vesicles. Parallel groups of small, membraneous vesicles in linear arrays were also present (Fig. 13). These were peculiar to Group C tumours and have no apparent counterpart in normal liver or other tissues.

Few lysosomes were observed, but those which were present had the morphology of normal, rat hepatic lysosomes.

The cytoplasm of Group C tumour cells had a granular appearance, owing to the presence of numerous, free ribosomes, most of which were not aggregated with polysomal clusters. Free ribosomes were, quantitatively, the dominant structures of the cytoplasm.

The tumour cell surfaces were usually complex in form (Fig. 10). They often possessed large digital processes in addition to a few microvilli (Fig. 12). The wide intercellular spaces frequently contained amorphous material resembling a diffuse, basement membrane.

DISCUSSION

The relatively frequent recurrence of the anaplastic types of tumour, towards the end of the 6 month sampling period suggests that there may be some progression of the tumours in the direction of greater de-differentiation.

The present study has shown that there are some points of similarity between the fine structure of the hepatic tumour cells and that of the hepatic cells of pre-neoplastic liver, which have been described previously [10]. These common features include nucleolar hypertrophy (although this is not a constant feature of Groups B and C), the possession of abnormally small mitochondria, increased free ribosomes and abnormalities of the cell surface.

Any change in the relation between the surfaces of adjacent cells, which leads to a less intimate contact between the cells, might contribute to the disorganisation of tissue growth that is a part of the process of neoplasia. In view of the present findings, it would seem that the change in cellular contact is probably much more complex than a simple decrease in surface adhesion. It is interesting that alterations in the morphology of the hepatic cell surface are among the changes produced in the rat liver by prolonged treatment with 2-AAF [10], and, therefore, are probably specific to its carcinogenic action. They appear to be progressive in nature, the degree of disturbance of cell contact being related to the malignancy of the hepatoma.

The state of the granular endoplasmic reticulum may be regarded as one of the indices of differentiation in hepatic cells, since it has a central role in the production of proteins for extracellular distribution, including lysosomal enzymes [16–19]. During 2-AAF treatment of the rat, this organelle is depleted and disorganized and does not recover its normal form in 'preneoplastic' liver cells [10], although it may be functionally active in the latter. It is somewhat surprising that Group A tumour cells should possess approximately normal quantities of granular endoplasmic reticulum, arranged in parallel arrays of cisternae (although these are rather elongated), when the surrounding hepatic cells do not. On the other hand, Group B tumour cells have relatively little granular endoplasmic reticulum and what there is tends to be present as single, short cisternae. In Group C this

progression reaches a climax, the organelle being disorganized and severely depleted. This agrees with the general concept that loss of differentiation accompanies increasing malignancy and appears to be correlated with a progressive decrease in number, and atrophy of the Golgi zones, and a decline in the lysosome population, as well as a variable decrease in nucleolar hypertrophy (which is a constant feature only in Group A and preneoplastic hepatic cells). The functional relationships of these cellular components have been discussed previously [10] and changes affecting them have been related to possible changes in the protein-synthetic activity of the hepatic cells. Thus, Group B tumour cells appear to show more loss of specialised function, in so far as the production of 'export' proteins is concerned, than do Group A cells. They also contain more free ribosomes in their cytoplasm, which indicates an enhanced rate of synthesis of cellular proteins, probably related to the higher growth rate of these tumours. In Group C, the loss of specialised cytoplasmic structures is pronounced. The dominant presence of free ribosomes in the cells of these tumours suggests that the major, if not entire, part of their metabolic activity is probably linked to cell growth.

There was little evidence to suggest that the cells of Group A tumours were substantially less well differentiated than those of the surrounding, pre-neoplastic liver [10]. Comparison between the morphology of the endoplastic reticulum in the two types of tissue suggests, if anything, the contrary. It would seem, therefore, that the change from the pre-neoplastic state to the neoplastic, is not necessarily accompanied by any obvious ultrastructural changes.

The presence of small mitochondria was noted in all the tumours and Group C cells, in addition, contained relatively few of these bodies. Small mitochondria have been observed in other hepatomata, such as the Morris 3683 [3], and the relative lack of mitochondria observed in Group C is similar to that observed in rapidly-growing, transplantable tumours [1, 3]. This latter feature may be indicative of the possession of an energy metabolism which is less dependent on oxidative pathways than is that of normal liver cells.

Each group of tumours had certain individual peculiarities of ultrastructure, not all of which had obvious counterparts in the normal, rat hepatic cell. For example, Group B tumour cells possessed an abundance of membrane-enclosed, cyoplasmic, lipid inclusion. Lipid inclusions normally possess no bounding membranes, an exception being found in normal, intestinal, epithelial cells during fat absorption [20]. What relevance this may have to the metabolic activity of Group B tumours is not clear, because there is little evidence that the granular form of the endoplasmic reticulum, which provides the enclosing membranes of the lipid inclusions, is in any way concerned with lipid metabolism. It is possible that this accumulation of lipid was derived from extracellular sources, but there is no evidence to suggest the route by which the lipid could enter the granular endoplasmic reticulum.

Trotter [21] has shown that the liver cells of the fasting animal may take up quantities of lipid from the blood, by a form of pinocytosis, which subsequently appear within smooth-surfaced cytoplasmic vesicles. However, the relationship of the membrane-enclosed lipid of Group B tumour cells to the granular form of the endoplasmic reticulum suggests that a rather different situation may obtain here, although the fine structure of the enclosed material resembled that of the lipid which is found in various normal tissues. It did not appear to be identical with the coarsely granular material which was found in the rough-surfaced cisternae of the cells of Group A tumours, which probably represented secretory products. Granules of a similar appearance and distribution have been observed in rat liver cells and considered to be lipoprotein synthesised in the liver for subsequent release into the blood [22, 23].

Group C cells often had unusual nuclear inclusions which probably resulted from the pinching off of the deep nuclear invaginations, small areas of cytoplasm thus being trapped. Leduc and Wilson [24] considered that such invaginations of the nuclear envelope may represent attempts by enlarged cells to maintain the normal ratio of nuclear surface area to volume.

Quadruple-membrane structures, and other bizarre forms of endoplasmic reticulum, similar to those found in Group C cells, have been observed in other cells such as HeLa [25], as well as in various, rapidly-growing, transplantable hepatomas [5]. Buck [26] found such structures in mitotic cells of the Walker 256 carcinoma and considered them to be precursors of the forming nuclear envelope. There was no evidence, however, for any such functional significance in the present study.

The absence of microbodies, together with the occurrence of annulate lamellae, paired cisternae and numerous free ribosomes which

were observed in Group C tumours, have also been described in a number of transplantable hepatomas [5].

The discontinuous nature of tumour progression [27] would account for the presence of aberrations of ultrastructural morphology peculiar to individual tumours; it is those progressive and/or persistent abnormalities which are common to many tumours, within a particular experimental system, which merit further study.

It appears that a number of ultrastructural differences between the tumours which have been described may be correlated with increasing malignancy and anaplasia. The most obvious of these are the increasing numbers of free ribosomes, together with a decline in their state of aggregation, greater irregularity of the cell surface, progressive alterations in the endoplasmic reticulum, and Golgi zones, and the apparently associated decline in the lysosome population. The precise functional significance of many of the morphological abnormalities remains somewhat obscure, in the absence of appropriate biochemical data. However, it may be of interest that many of the abnormal features of the tumour cells are similar to those found in pre-neoplastic liver cells. This suggests that the pre-neoplastic liver may be a more suitable material, in some respects, for investigations concerned with the essential changes produced by carcinogens, than are the so-called minimal deviation hepatomas.

To a limited extent, the ultrastructural organisation of Group A tumour cells more closely resembled that of normal rat hepatocytes than that of the pre-neoplastic liver cells of 2-AAF treated rats. However, this resemblance between normal and tumour cells may be regarded as somewhat superficial, depending as it does on the comparatively normal abundance and fine structure of the two forms of the endoplasmic reticulum in Group A tumour cells, and of considerably less significance than the persistence of much of the pre-neoplastic lesion in the latter. The persistence of the cell surface changes may be of particular importance since it is possible that they may be accompanied by alterations in the permeability of the plasma membrane. Any increase in the permeability of the cell surface might permit an abnormally high rate of loss of tissue chalones from the cells, resulting in a breakdown of tissue homeostasis [28] which would favour tumour promotion and progression. The presence of certain pathological lesions in both tumour cells and pre-neoplastic liver cells indicates that these represent stable hereditable changes, apparently irreversible in character [10], which are the criteria used by Foulds [20] to denote a somatic mutation, whether intra- or extrachromosomal. It is possible that among these changes may be those which are essential to the process of carcinogenesis.

In so far as any recovery from the pre-neoplastic lesion does occur in the experimental system used in the present study, this appears to be partial and to result in tumour formation. Although the possibility that some of the pre-neoplastic liver cells might give rise to normal hepatocytes cannot be excluded, there is no evidence, direct or circumstantial, that this process occurs in other than a minority of cases, if at all.

Acknowledgements—The author wishes to thank Miss Jennifer A. Moody for her valuable technical assistance and the Yorkshire Council of the British Empire Cancer Campaign for its financial support.

RESUME

L'auteur décrit la structure fine de trois types de tumeurs différant par leur degré d'invasivité et d'anaplasie, mais produites par le même agent, le 2-acétylaminofluorène (2-AAF). A part certaines anomalies de l'ultrastructure, propres à chaque groupe de tumeurs, une certaine corrélation existe entre les différences de la structure fine et le degré de différenciation histologique. Certains composants cellulaires présentent des modifications progressives en rapport avec un accroissement de la malignité. Certaines de ces anomalies ressemblent à celles qui ont été précédemment décrites dans les cellules hépatiques prénéoplasiques.

SUMMARY

The fine structure has been described of the cells of three groups of tumours, differing in their degree of invasiveness and anaplasia but induced by identical treatment with 2-acetylaminofluorene (2-AAF). Apart from some ultrastructural abnormalities peculiar to the individual tumour groups, there appeared to be some correlation between their fine structural differences and their degree of histological differentiation. It was noted that

certain cellular components underwent progressive changes with increasing malignancy and that some of the abnormalities resembled those found in the previously described pre-neoplastic liver cells [10].

ZUSAMMENFASSUNG

Es wird die Feinstruktur von 3 Gruppen von Tumorzellen beschrieben, die sich durch den Grad der Anaplasie und die Fähigkeit zur Infiltration unterscheiden, die aber durch gleiche Behandlung mit 2-Acetylaminofluoren erzeugt worden waren. Neben einigen Anomalien der Ultrastruktur, die für die einzelnen Tumorgruppen typisch waren, schien eine Beziehung zwischen ihrer Feinstrukturdifferenz und dem Grad histologischer Differenzierung zu bestehen. Einige Zellkomponenten zeigten mit steigender Malignität fortschreitende Veränderungen und einige der Anomalien ähnelten denen in zuvor beschriebenen präneoplastischen Leberzellen [10].

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Estrogen Metabolism in Postmenopausal Women with Primary and Recurrent Breast Cancer

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IT HAS long been thought that there is a causal relationship between estrogen secretion and the etiology and spread of mammary cancer in man, but there is no direct evidence [1]. Published information concerning estrogen excretion and metabolism by patients with breast cancer is conflicting in spite of the fact that it is almost 80 years since Schinzinger, in 1889 [2], first suggested an endocrinological background in human breast cancer.

Elevated urinary estrogen concentrations have been found in patients with carcinoma of the breast [3–6]. Reduced levels have also been reported [7–9]. Some investigators have been unable to find any differences compared with healthy women [10–12].

The estrogen metabolism of patients with carcinoma has been studied also on the basis of steroid loading tests. After an administration of polyestradiol phosphate, Rauramo and Grönroos [13] observed that postmenopausal patients with cancer of the cervix excreted smaller quantities of estrone, estradiol and estriol than a control series. In a similarly planned test, Brown [14] found that the ratio estriol/total estrogens was higher in mammary cancer patients than in healthy subjects. Elevation in the urinary estrogen concentrations has often been established after administration of androgens [11, 15–17].

The varying and conflicting results have been attributed mainly to differences in the methods of estimation and the heterogenous age of the populations studied. An endeavour was made in the present work to eliminate the age factor and the factors of error caused by the variations in the cyclical function by selecting only postmenopausal women for the test.

MATERIAL AND METHODS

The series comprised 34 postmenopausal women. Fourteen of them (Group A) were primary, still untreated cancer cases and 10 (Group B) were recurrences. The control group (Group C) consisted of 10 non-cancerous women. The time since the menopause was 2–15 years. The mean age was practically the same in all the groups: 63·6 years in Group A, 60·2 years in Group B and 63·8 years in Group C.

Estrone, estradiol and estriol were determined by the method of Brown [18], with the saponification step added [19], as modified by Diczfalusy and Westman, and some changes in the chemicals and their concentrations and purification used [20]. In addition, the slightly more accurate method of carrying out the Kober reaction, introduced by Nocke [21] was used. Basal excretion from 48 hr urine was determined first. The test subjects were then given an intramuscular injection of 80 mg of polyestradiol phosphate (Estradurin, Leo), after which the quantities of estrogens were determined from the next 48 hr output of urine. The estrogen determinations were made after clinical diagnosis by palpation and thus without disturbing operative stress. The carcinoma diagnosis was verified later histologically.

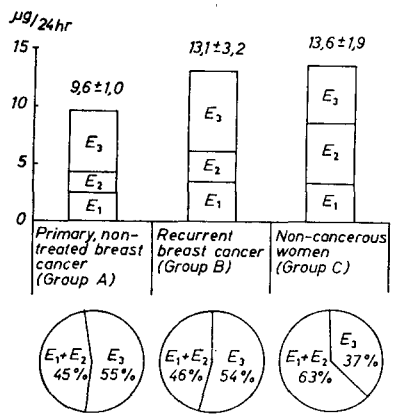


Fig. 1. The basal urinary excretion of estrone (E_1), estradiol (E_2) and estriol (E_3) in various groups of postmenopausal women. Mean values in µg/24 hr and percentages.

RESULTS

Basal estrogen excretion. Figure 1 shows the basal excretion of total estrogens and the three 'classical' estrogens in the urine in both absolute and relative terms. The mean total estrogen excretion in Group A was 9·6±1·0 µg/24 hr, which was lower than the level for the controls, 13·6±1·9 µg/24 hr ($0{\cdot}025<p<0{\cdot}05$). In Group B (recurrent cases) the mean total estrogen excretion was 13·1±3·2 µg/24 hr, which was practically the same as the control value. Of the different fractions, the estradiol excretion by cancerous patients was smaller than that of non-cancerous women: Group A 1·8, Group B 2·6 and Group C 5·2 µg/24 hr; $p<0{\cdot}005$ and $0{\cdot}01<p<0{\cdot}025$, respectively. The proportion of the sum of the biologically more active components, estradiol and estrone, to the total also appeared to be lower in cancerous women. It was 45% in Group A, 46% in Group B and 63% in Group C. But there was no certain statistically significant difference.

Estrogen excretion after the loading test. Figure 2 shows the absolute and relative excretion values. The lowest total estrogen excretion figures were recorded again for patients with untreated primary breast cancer, mean value 15·6±2·3 µg/24 hr. This differs significantly from the excretion by recurrent patients and the controls: 32·5 ± 6·6 µg/24 hr and 29·7 ± 3·5 µg/24 hr; $0{\cdot}0005<p<0{\cdot}005$ and $p=0{\cdot}005$, respectively. The same relatively low excretion rate was recorded for all the fractions in Group A. The total estrogen excretion of patients with recurrent breast cancer corresponded to that established in healthy women. Nor was there any difference between these groups in the absolute or relative estriol excretion values.

The increase in total estrogen excretion

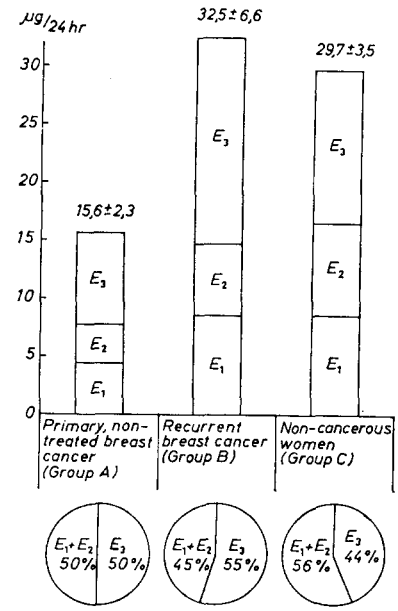


Fig. 2. The urinary excretion of estrone (E_1), estradiol (E_2) and estriol (E_3) after the estradiol loading test. Mean values in µg/24 hr and percentages.

after estradiol loading was significantly smallest in Group A as compared with the controls and the recurrent group; $p=0{\cdot}025$ and $0{\cdot}01<p<0{\cdot}025$, respectively (Fig. 3). The last two groups responded in almost the same way. The increase in estriol excretion was especially pronounced and significant.

DISCUSSION

Basal estrogen excretion. Patients with untreated, primary breast cancer excreted less estrogens than non-cancerous women. A similar observation was made by biological methods by e.g. Pincus and Pearlman [7], and later by Cramer and Wildner [9]. In contrast, the results of more recent studies, by Marmorston *et al.* [22] and Marmorston

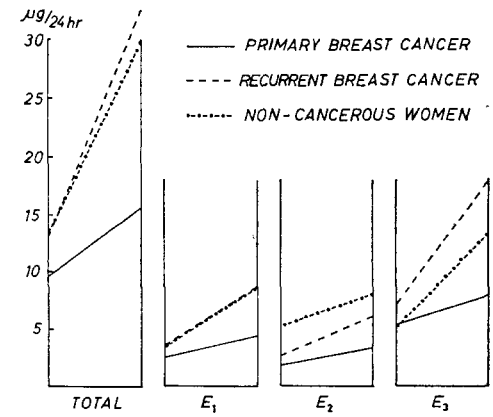


Fig. 3. The average increase in the urinary excretion of estrone (E_1) estradiol (E_2), estriol (E_3) and total estrogens (Total) during the loading test with 80 mg of polyestradiol phosphate.

[6], indicate an increase—though not a significant one—in the mean excretion of total estrogens in postmenopausal breast cancer patients after chemical separation of estrogen, estradiol and estriol and their quantification by bioassay. We do not know the reasons for the lowered estrogen excretion in the present study. It is possible that in cancerous patients the conjugation mechanism of estrogens with proteins is disturbed, or retention in the tissues is increased, a possibility already referred to by Loewe *et al.* [23]. A simple reason, of course, could be impaired function of the adrenals or ovaries.

In the present study, the proportion of estriol (54–55%) in total estrogens appeared to be higher in patients with cancer than in the control group (37%), although the difference was not significant. This observation concurs with those of Brown [14], Nissen-Meyer and Sanner [24], Persson and Risholm [25], and Stern *et al.* [26]. It thus seems that patients with cancer of the breast tend to excrete their estrogen as the biologically least active of the three fractions. The question is, what is the physiological significance of the raised proportion of estriol and has it any correlation with the course of the disease, to which Bulbrook [27] in his extensive survey indicates. The decrease in estradiol noted in the present study is in good agreement with the increase in estriol excretion. However, Marmorston *et al.* [22] did not observe a corresponding phenomenon in excretion of estradiol and the possibility of precursors of estriol other than estrone or estradiol has been suggested as an explanation. In any case, the fluctuations in the mutual proportions of the fractions are suggestive of changes in the estrogen metabolism of cancerous patients.

The loading test. The lowest total estrogen values, like the basal excretion values, were obtained for patients with untreated, primary breast cancer. Recovery after estradiol injection, correspondingly, was also the poorest. This tends to confirm the suspicion that there are disturbances in the elimination of estrogens in mammary carcinoma patients. Similar views were propounded by Rauramo and Grönroos [13] concerning uterine cancer: these patients excreted smaller quantities of estrogens than the controls.

It was interesting to note that the estrogen excretion of the recurrent cancer patients in the loading test—in contradistinction to the women with untreated primary breast cancer —resembled the values reported for the control group. In patients with recurrent cancer and in healthy women the organism tended to eliminate exogenous estradiol most actively, especially by converting it into less active estriol (Fig. 3). This tendency was not so pronounced in patients with primary breast cancer. We have found no mention in the literature of a phenomenon of this kind in patients with tumour recurrences.

RESUME

L'excrétion urinaire basale de l'oestrone, l'oestradiol et l'oestriol, et l'excrétion postérieure au test de fatigue effectué avec l'oestradiol phosphate ont été étudiées chez des femmes postménopausiques. Un premier groupe était composé de femmes ayant un cancer du sein primaire, non encore soigné, le deuxième comprenait des malades présentant une récidive de cancer du sein, et le troisième groupe, groupe témoin, était constitué de femmes non cancéreuses.

L'excrétion basale des oestrogènes totaux a été la plus faible chez les femmes atteintes de cancer du sein primaire. L'excrétion de l'oestradiol chez les malades cancéreuses a été inférieure à celle des témoins. La proportion de l'oestriol, la fraction la moins active biologiquement, a accusé une légère augmentation chez les patientes cancéreuses, mais la différence n'a pas été statistiquement significative.

Les malades atteintes de cancer du sein primaire ont présenté les taux les plus faibles d'oestrogène total, et ce également après le test de fatigue. La même excrétion, relativement faible, a été enregistrée dans toutes les fractions. L'excrétion élevée des oestrogènes totaux et de l'oestriol, chez les femmes atteintes de récidive, n'a pas différé, de façon significative, des taux correspondants chez les femmes en bonne santé, et la tendance d'éliminer l'oestrogène exogène a été correspondante dans ces deux groupes. L'augmentation de l'excrétion de l'oestriol, chez les malades atteintes de récidive, a pourtant été supérieure, de façon significative, au taux correspondant chez les patientes ayant un cancer primaire non encore soigné.

SUMMARY

The basal urinary excretion of estrone, estradiol and estriol and the excretion after loading with estradiol phosphate was studied in postmenopausal women. One group had primary, still untreated breast cancer, the second had recurrence of breast cancer and the third group, the controls, consisted of non-cancerous women.

The basal excretion of total estrogens was the lowest in women with primary breast cancer. The estradiol excretion of the cancer patients was smaller than that of the controls. The proportion of estriol, the biologically least active fraction, appeared to increase in cancer patients, but the difference was not statistically significant.

The lowest total estrogen levels after the loading test were also observed in patients with primary breast cancer. The same relatively low excretion was recorded for all fractions. The high total estrogen and estriol excretion in the recurrent cases did not differ significantly from the corresponding quantities for healthy women and the tendency to eliminate the exogenous estrogen resembled thus each other in these groups. The increase in estriol excretion of recurrent cases was, however, significantly higher than the respective value by still untreated cancerous patients.

ZUSAMMENFASSUNG

Bei postmenopausalen Frauen wurde die Harnausscheidung von Östron, Östradiol und Östriol—und die Ausscheidung nach einer Belastungsprobe mit Östradiol—gemessen. Die Untersuchungspersonen waren in Gruppen aufgeteilt, von denen die eine ein primäres, unbehandeltes, die andere ein rezidivierendes Mammakarzinom hatte. Die dritte, zur Kontrolle herangezogene Gruppe bildeten Frauen ohne Karzinom.

Es wurde festgestelt, dass bei den Frauen mit primären Mammakarzinom die Ausscheidung von Totalöstrogenen am niedrigsten war. Bei den Patientinnen mit Karzinom wurde weniger Östradiol ausgeschieden als bei den Kontrollpersonen, Der Anteil von Östriol der biologisch am wenigsten aktiven Fraktion, schien bei den Patientinnen mit Karzinom vermehrt zu sein—doch war der Unterschied nicht signifikant.

Auch nach der Belastungsprobe wurde bemerkt, dass die Patientinnen mit primärem Mammakarzinom die geringsten Mengen von Totalöstrogenen aufwiesen. Dieselbe, relativ geringe Ausscheidung konnte bei allen Fraktionen festgestellt werden. Die hohe Ausscheidung von Totalöstrogenen und Östriol in rezidivierenden Fällen unterschied sich nicht signifikant von den entsprechenden Quantitäten bei den gesunden Frauen und die Tendenz exogenische Östrogensubstang zu eliminieren war entsprechend in diesen beiden Gruppen. Die Vermehrung der Ausscheidung von Östriol in rezidivierenden Fällen war jedoch signifikant höher als die entsprechende Quantität bei den Patientinnen mit noch unbehandelten primärem Karzinom.

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Etude Ultrastructurale de la Moelle Hématopoïétique au Cours de la Régénération après Irradiation

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INTRODUCTION

Au cours des dernières années, différentes recherches ont été consacrées aux cellules lymphoïdes de la moelle osseuse. Plusieurs auteurs ont étudié le comportement de ces cellules chez les animaux soumis à une irradiation corporelle totale.

Chez les souris irradiées à 400 R, Brecher *et al.* [1] avaient déjà signalé, au cours de la régénération des moelles osseuses, une accumulation de cellules mononucléées dont la plupart ressemblent a des lymphocytes. Des observations similaires ont été faites chez le cobaye par Harris *et al.* [2, 3] et par Yoffey *et al.* [4, 5]. Cette accumulation précède la régénération érythropoïétique et granulopoïétique. Haot *et al.* ont étudié, au moyen d'un compteur électronique de particules, les variations quantitatives de deux populations cellulaires de volumes différents dans la moelle de souris CBA irradiées à différentes doses de rayons X [6, 7]. Ils ont montré que, pour des doses variant de 100 à 400 R, la population de petites cellules augmente nettement au cours de la régénération et atteint, aux environs du 11e jour, une valeur double de la normale.

Il est intéressant de noter qu'une accumulation de petites cellules mononucléées a été signalée aussi au cours de la régénération médullaire chez le rat traité par busulphan [8], et chez le chien traité par une moutarde azotée [9].

Le rôle des cellules lymphoïdes de la moelle est encore discuté. On a noté qu'à côté des lymphocytes apparemment analogues à ceux des organes lymphoïdes, il apparaît, dans diverses conditions expérimentales, des éléments mononucléés légèrement plus grands. Ils se distinguent des lymphocytes par une basophilie cytoplasmique plus marquée et par un aspect plus dispersé de la chromatine; ils synthétisent du DNA et se divisent activement [10, 11]. Yoffey *et al.* [4, 5] ont donné à ces éléments le nom de 'transitional cells'.

Le rôle et la fonction de ces cellules sont encore controversés; nous avons essayé de préciser leur nature en étudiant, au microscope électronique, les cellules médullaires au moment où, chez l'animal irradié, la population de petites cellules atteint une valeur importante.

TECHNIQUE

Seize souris R3 femelles âgées de 2 mois sont soumises à une irradiation corporelle totale de 500 R (Appareil Picker 200 kV, 15 mA, distance focale 50 cm, filtre 0,25 mm Cu).

Les animaux sont sacrifiés du 10e au 12e jour après l'irradiation. La moelle d'un fémur est prélevée par rinçage de la cavité médullaire au moyen de liquide de Hanks (1 ml). Une partie de la suspension est utilisée pour établir une courbe de distribution des volumes cellulaires à l'aide d'un compteur électronique de particules (Coulter modèle B). Le restant de la suspension est centrifugé et les cellules sédimentées, remises en suspension dans du sérum, servent à la préparation de frottis qui sont colorés par la méthode de May Grunwald Giemsa.

Le moelle du deuxième fémur est prélevée après ouverture longitudinale de la cavité médullaire et des fragments sont fixés pendant

45 min à la température ordinaire dans de la glutaraldéhyde diluée dans un tampon phosphate à pH 7,4; ils sont post-fixés pendant 15 min dans de l'acide osmique à 1% tamponné selon Millonig [12]. L'inclusion se fait à l'épon; les coupes sont colorées pendant 30 minutes dans une solution aqueuse d'acétate d'Uranyle à 5%, puis pendant 20 min, dans une solution de citrate de plomb selon Reynolds [13].

L'examen des spécimens se fait à l'Elmiskop I Siemens.

Pour l'étude détaillée au microscope électronique, 4 animaux ont été sélectionnés en fonction des courbes obtenues au compteur. Ont été choisies les souris dont la moelle contenait une population de petites cellules atteignant 50–60% de l'ensemble des éléments médullaires.

Six souris témoins, non irradiées, ont été étudiées dans les mêmes conditions; les courbes de distribution des volumes cellulaires et les myélogrammes ayant fourni des résultats comparables, les moelles de trois d'entre elles, prises au hasard, ont été examinées au microscope électronique.

RESULTATS

Distribution des volumes cellulaires

La figure 1 montre la distribution des volumes chez les 4 souris irradiées dont les moelles ont été étudiées au microscope électronique; la courbe témoin figurant sur le même graphique est la courbe moyenne établie à partir des 3 souris non irradiées, également utilisées pour l'étude ultrastructurale.

On voit que, chez la souris normale, les cellules ayant de 100 à 180 μ3 représentent environ 25% de l'ensemble de la population médullaire, tandis que les cellules mesurant de 200–300 μ constituent environ 38% de cette même population. Dix à douze jours après une irradiation de 500 R, le rapport est considérablement modifié, puisque la première population atteint 50–60% de l'ensemble alors que la deuxième oscille autour de 20%. Le groupe de cellules mesurant de 100 à 180 μ3 comprend les lymphocytes de la moelle. On peut se demander si l'augmentation numérique de cette population chez l'animal irradié est due à une accumulation de 'lymphocytes' ou de cellules plus volumineuses. Nous avons, dès lors, calculé le pourcentage de cellules de la

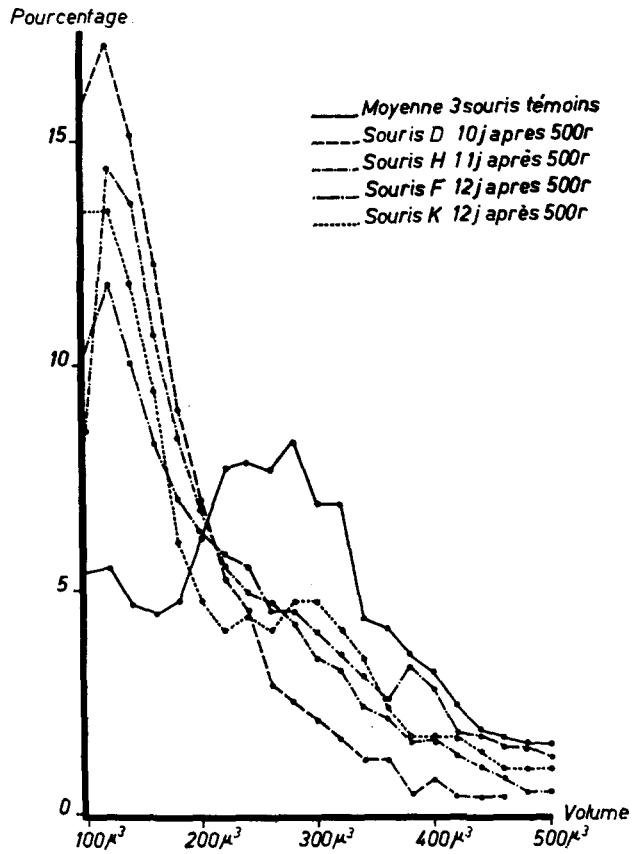


Fig. 1. *Distribution des populations cellulaires de la moelle osseuse observées au compteur électronique chez* 3 *souris témoins et chez les souris sacrifiées* 10–12 *jours après une irradiation de* 500 *R.*

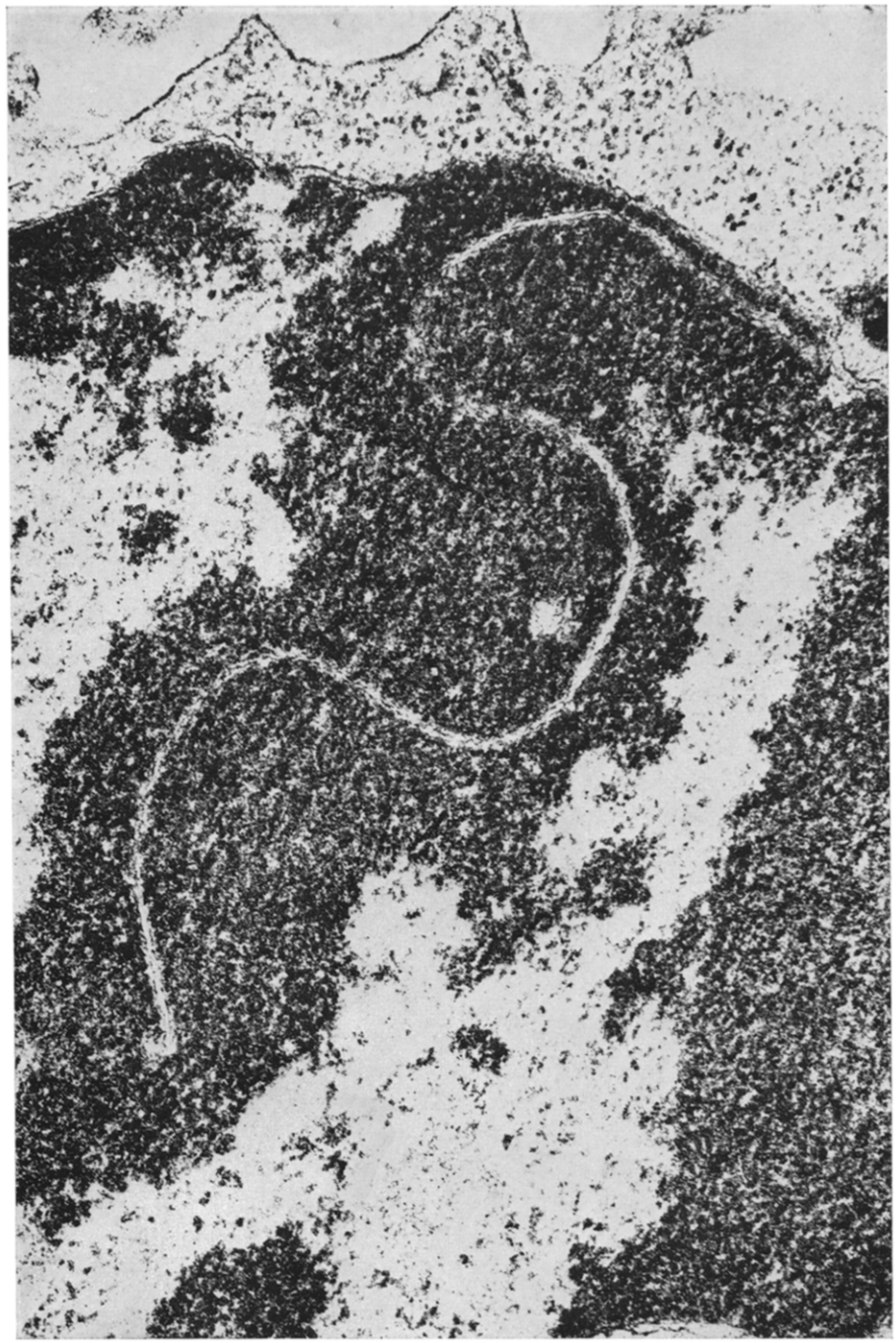


Fig. 2. Détail du noyau d'un lymphocyte médullaire. Une fine fente bordée par une membrane simple pénètre profondément dans la masse dense de la chromatine. Elle pourrait correspondre à une invagination de la membrane nucléaire ($\times$ 32,000).

(to face p. 530)

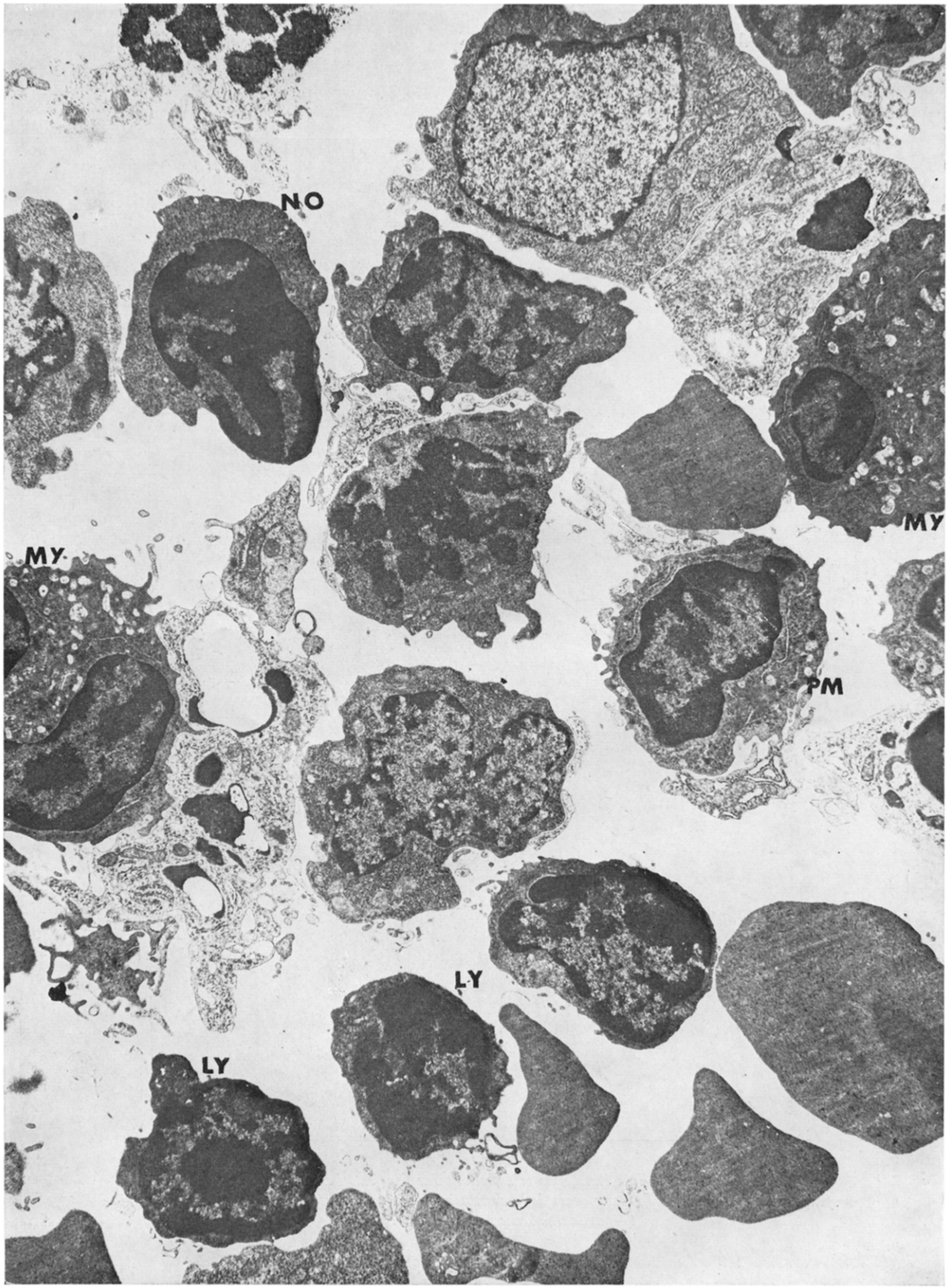


Fig. 3. Moelle en régénération 11 jours après une irradiation de 500 R. Présence de lymphocytes (LY), de normoblastes (NO), de métamyélocytes (MY). Notez l'existence de cellules myéloïdes de petite taille (PM) (× 5,200).

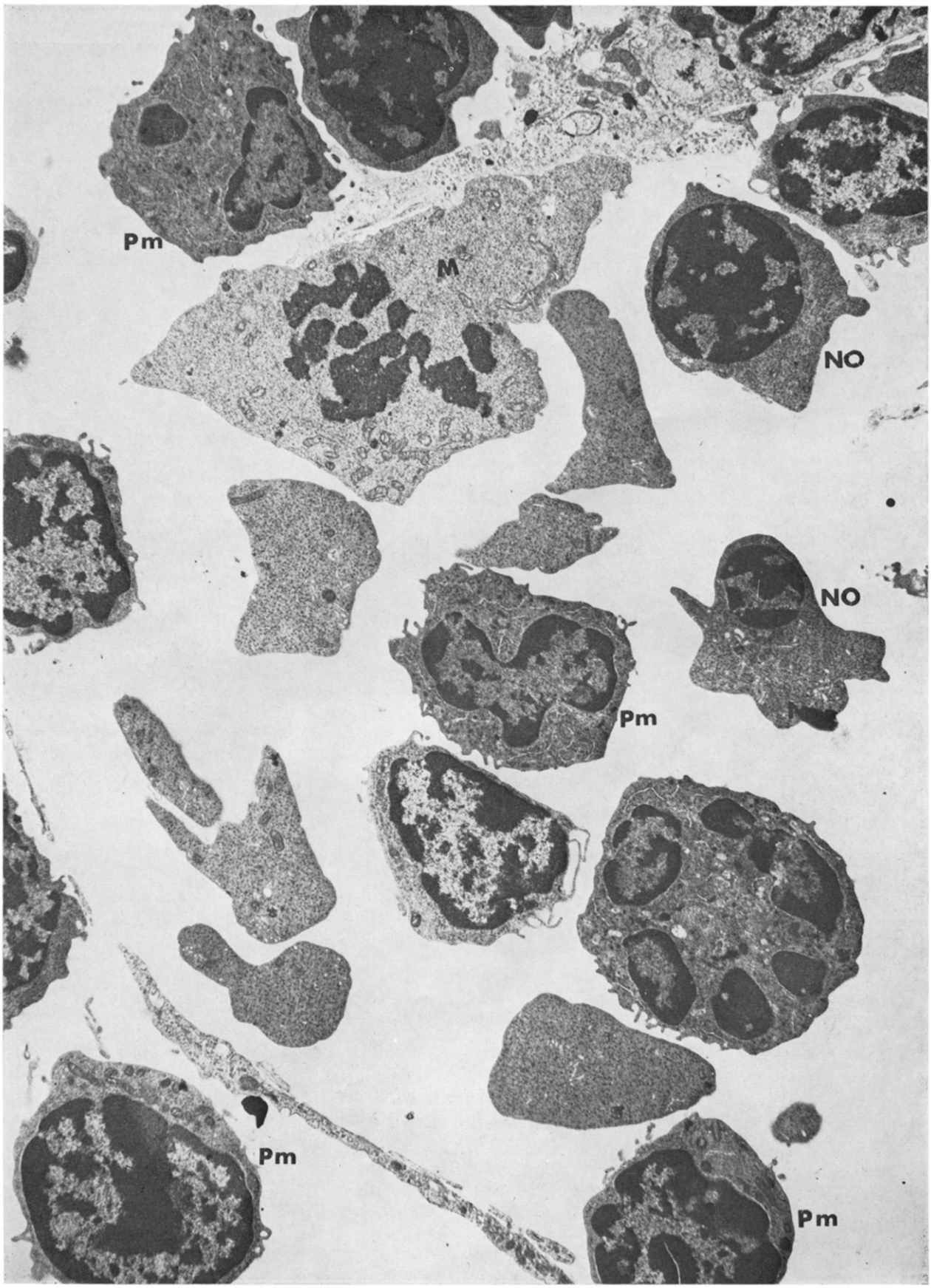


Fig. 4. *Moelle en régénération* 10 *jours après une irradiation de* 500 *R. Nombreuses cellules myéloïdes de petite taille* (*PM*). *Normoblastes* (*NO*). *Myéloblaste* (*M*) (× 4,800).

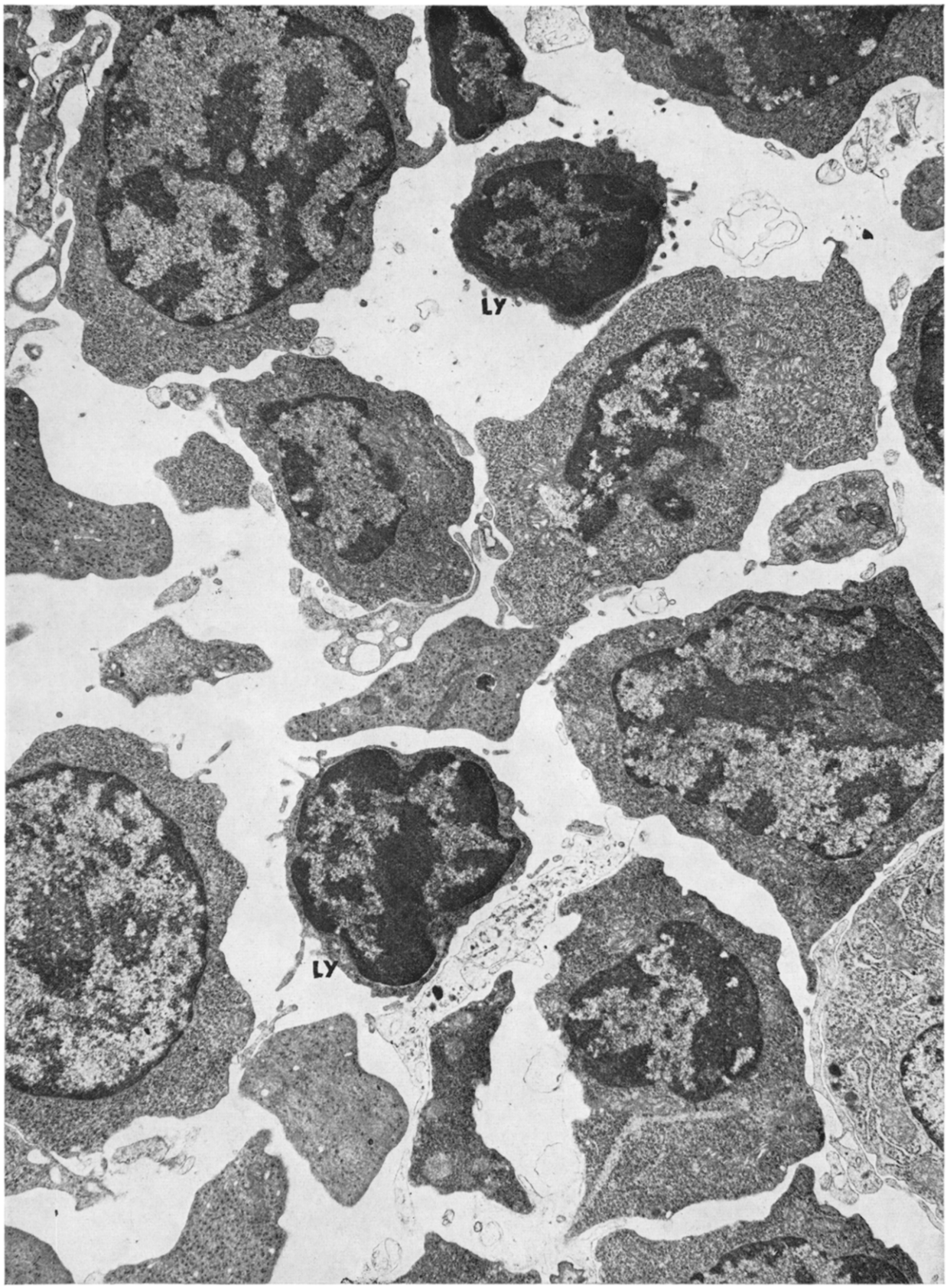


Fig. 5. *Moelle en régénération* 11 *jours après* 500 *R. Apparition de nombreuses cellules d'un type particulier* ('*cellules X*') (×5,850).



Fig. 6. *Détail du cytoplasme d'une 'cellule X'. Nombreux ribosomes groupés en rosettes* ($\times$ 42,500).

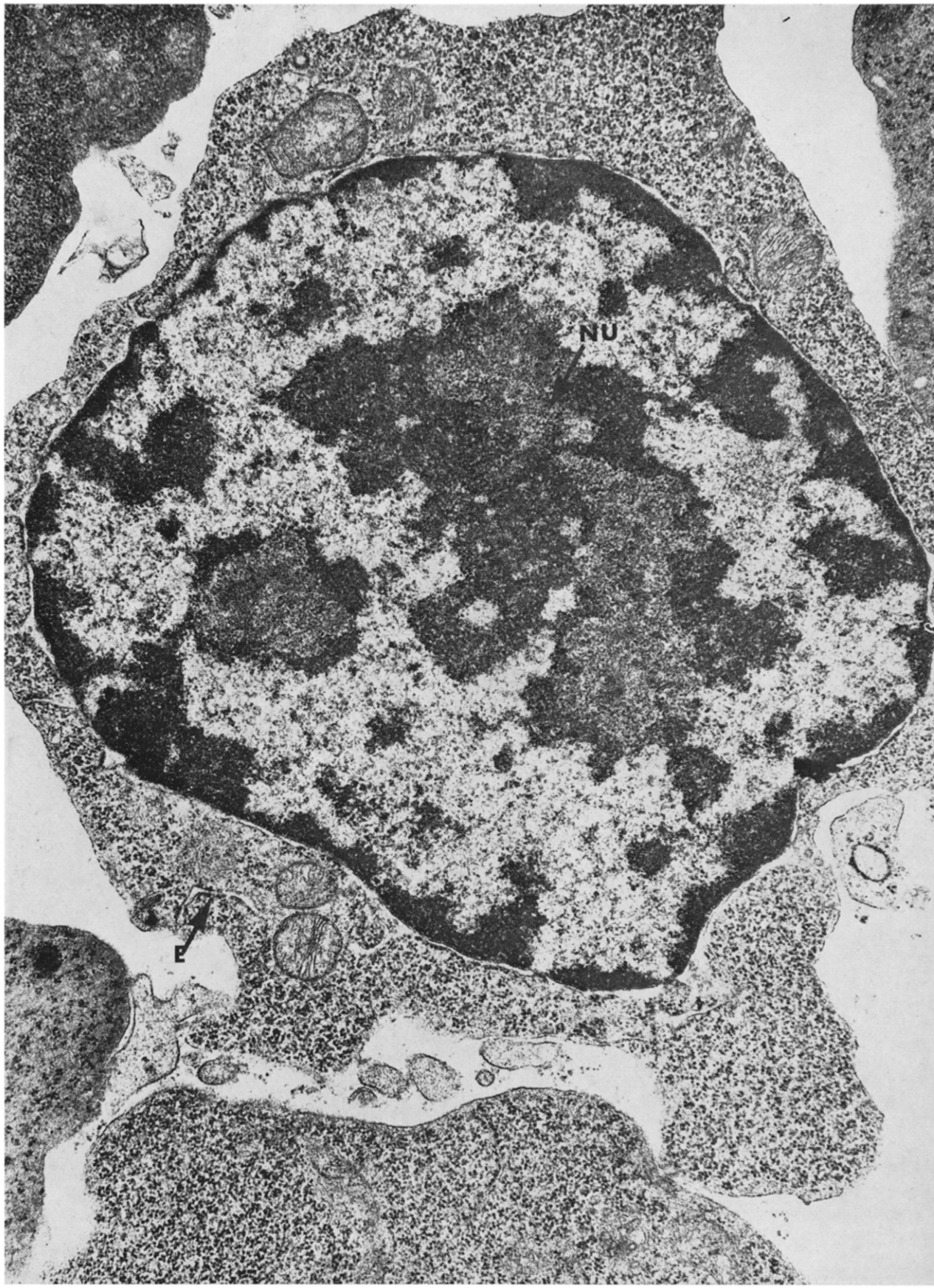


Fig. 7. 'Cellules X' dans une moelle–régénération. Chromatine condensée en blocs périphériques; volumineux nucléole (NU) avec séparation très marquée de la zone à grains fins et à gros grains; ergastoplasme (E) peu développé; nombreux ribosomes (× 22,750).



Fig. 8. *Lymphoblaste médullaire normal. Ribosomes groupés en rosettes. Quelques sacs ergastoplasmiques aplatis* (*E*). *Chromatine nucléaire dispersée. Nucléole* (*NU*) *généralement sphérique* (× 19,000).

Tableau 1. Pourcentage de différentes fractions, cellulaires mesurées au compteur électronique, des souris normales et des souris irradiées à 500 R. Les chiffres entre parenthèses indiquent le délai après l'irradiation

Animaux	Groupes cellulaires		
	100–120 μ3 (%)	120–180 μ3 (%)	200–300 μ3 (%)
Témoins			
A	10,00	12,73	38,18
B	11,45	12,70	35,55
C	11,78	16,84	41,17
Irradiés			
D (10J)	33,06	36,32	17,15
H (11J)	22,97	32,64	22,71
K (11J)	26,94	27,27	21,88
F (12J)	22,00	25,25	24,25

taille d'un petit lymphocyte (100–120 μ3) et celui des cellules plus volumineuses (120–180 μ3); les résultats sont réunis dans le Tableau 1. On voit que l'accroissement relatif de la population de petites cellules porte à la fois sur les deux sous-groupes envisagés, et ce, dans une proportion comparable.

Etude cytologique des frottis

L'examen des frottis confirme l'existence de nombreuses cellules de type 'lymphoïde'. Beaucoup d'entre elles sont pourtant différentes de petits lymphocytes caractéristiques. Elles en diffèrent par la taille légèrement supérieure, une basophilie intense du cytoplasme et un aspect plus dispersé de la chromatine. Cet aspect correspond à celui des 'transitional cells' décrites par différents auteurs.

Etude ultrastructurale

(1) *Moelle normale.* Nous ne décrirons pas l'ultrastructure des cellules des différentes lignées médullaires car la plupart de ces aspects sont bien connus. Nous voudrions simplement insister sur deux points.

La moelle de la souris normale renferme un certain nombre de cellules lymphocytaires dont la structure nucléaire et cytoplasmique est comparable à celle des petits lymphocytes, tels qu'on les voit dans les tissus lymphoïdes. Nous avons pourtant observé un aspect particulier de certains noyaux. Il est caractérisé par la présence de fentes fines, bordées par une membrane simple, ayant un trajet sinueux et pénétrant dans les masses denses de chromatine. Elles semblent constituées par des invaginations de l'espace périnucléaire (Fig. 2).

La lignée granulocytaire est constituée par des cellules à tous les stades de différenciation. Si le volume de ces éléments est nettement supérieur à celui des cellules lymphoïdes, il faut pourtant signaler la présence d'un petit nombre d'éléments myéloïdes dont la taille, plus petite, est semblable à celle des lymphocytes. Leur cytoplasme, qui contient les granulations typiques de la série myéloïde, est caractérisé par sa densité particulière. Celle-ci est due uniquement à l'existence d'un 'feutrage' cytoplasmique plus abondant, et non à une augmentation du nombre des ribosomes. Ces cellules se présentent comme des promyélocytes et des myélocytes de petite taille. Ces éléments constituent environ 1% de l'ensemble des cellules myéloïdes.

(2) *Moelle des souris irradiées.* Les cellules de type lymphocytaire sont nettement plus abondantes que dans la moelle des témoins, mais leur aspect ultrastructural n'est en rien différent; ici encore, on retrouve les fentes intranucléaires décrites plus haut (Fig. 2).

La lignée myéloïde présente une modification dans le rapport des différents types cellulaires. Les formes les plus jeunes sont nettement plus fréquentes que dans la moelle normale. Cette augmentation des stades jeunes porte également sur les promyélocytes et les myélocytes de petite taille (Figs. 3 et 4).

L'ultrastructure de tous ces éléments est semblable à celle des éléments médullaires de la souris normale. Les cellules jeunes de la lignée érythrocytaire sont en nombre inférieur à la normale.

A l'examen de la moelle des animaux irradiés, on est immédiatement frappé par l'abondance de cellules d'un type particulier. Il s'agit d'éléments généralement ovoïdes, dont le grand axe mesure environ 11 μ et dont les contours sont très irréguliers; ils sont souvent réunis en amas (Fig. 5). Ces cellules se caractérisent par certains aspects particuliers, tant du cytoplasme que du noyau. Leur cytoplasme, qui présente des prolongements de formes diverses, est très riche en ribosomes. Le plus souvent, ceux-ci sont groupés en

rosettes de 4 à 8 unités; toutefois, on rencontre des cellules à ribosomes isolés. Les mitochondries, généralement sphériques, sont assez peu nombreuses et n'ont pas de localisation particulière. L'ergastoplasme, très peu abondant, est le plus souvent réduit à quelques petites fentes bordées de rares ribosomes (Fig. 6). Quand elle est observée, la zone de Golgi est de petite taille. De façon inconstante, on retrouve des tubes allongés, non bordés de ribosomes, ne contenant aucun matériel visible et ayant un diamètre de 30 à 35 mμ. Lorsqu'il en existe plusieurs au sein d'un même cytoplasme, ils sont disposés parallèlement les uns aux autres. A côté de ces tubes assez larges, on rencontre également des faisceaux de microtubules. Le noyau de ces cellules particulières a un aspect également typique (Fig. 7). Il est allongé dans le grand axe du corps cellulaire; sa chromatine est condensée en une assez large bande périphérique et en quelques gros blocs épars. Il existe pratiquement toujours un ou deux nucléoles très volumineux, souvent accolés à la membrane. Ils ont un contour géographique et la séparation entre la zone à grains fins et celle à gros grains y est très marquée. Des figures de mitoses sont découvertes avec une grande fréquence. Dans la suite de ce travail, nous appellerons ces cellules 'cellules X' pour ne pas préjuger de leur nature. Nous avons recherché ce type de cellules dans les moelles normales et ne les avons pas retrouvées; si elles y existent, elles doivent être en proportion très faible. Nous avons tenté de compter les différents types cellulaires rencontrés dans les coupes examinées au microscope électronique. Pour chaque animal, nous avons examiné au minimum 6 fragments de moelle différents, choisis au hasard, et nous avons étudié au moins 100 cellules par fragment. Si l'examen au microscope électronique permet aisément de classer les cellules, il existe cependant un certain risque d'erreur dû au fait que, dans les moelles, les différents types cellulaires tendent à être groupés en foyers. Les résultats de ces numérations, réunis dans le Tableau 2, sont donc donnés à titre purement indicatif. On voit néanmoins qu'il y a une augmentation nette de la proportion de cellules lymphoïdes et des petites cellules myéloïdes. Quant aux 'cellules X', elles représentent une fraction importante de la population médullaire.

DISCUSSION

Les résultats rapportés dans le présent travail confirment les observations faites précédemment par différents auteurs, à savoir que la régénération médullaire après irradiation corporelle totale passe par un stade où la moelle est riche en cellules mononucléées de petite taille. Dans nos conditions d'expérience, cette population cellulaire est nettement augmentée au 10–12e jour après une irradiation de 500 R. Nos résultats montrent que cet accroissement porte à la fois sur des cellules qui ont la taille de petits lymphocytes et sur des cellules de taille généralement légèrement supérieure.

Le but de notre travail a été de déterminer de façon plus précise la composition de cette population. Pour cela, nous avons étudié l'aspect ultrastructural des éléments médullaires apparaissant après l'irradiation et l'avons comparé à la moelle normale.

Il faut remarquer d'abord que, dans la moelle des souris R3, il existe une proportion non négligeable de 'lymphocytes' dont la structure est proche de celle des petits lymphocytes dans les tissus lymphoïdes. Signalons cependant que, dans les 'lymphocytes' médullaires, on trouve avec une grande fréquence des invaginations de l'espace périnucléaire, créant des fentes allongées qui pénètrent

Tableau 2. Pourcentage des différents types cellulaires observés au microscope électronique chez des souris normales et des souris sacrifiées du 10e au 12e jour après une irradiation de 50 R. Les chiffres entre parenthèses indiquent que le pourcentage de cellules myéloïdes de petite taille est calculé par rapport à l'ensemble de la lignée myéloïde

	Témoins (%)	Souris D 10e jour (%)	Souris K 11e jour (%)	Souris H 11e jour (%)	Souris F 12e jour (%)
Lignée myéloïde	80,5	50	31	29	19
Lignée myéloïde de petite taille	(0,8)	(5,8)	(2,9)	(2,7)	(1,5)
Lignée lymphoïde	6	2,4	16	15	14
Lignée rouge	7	2,6	1	0	2,4
Cellules réticulées, macrophages	6,5	7	5	8	2,6
Cellules X	0	38	47	48	62

profondément dans la masse chromatinienne. Nous n'avons aucune interprétation de cet aspect; rappelons seulement que nous ne l'avons jamais trouvé dans les lymphocytes ganglionnaires. Signalons aussi que Harris et Kugler [14] ont trouvé des fentes nucléaires dans les 'transitional cells' de cobayes soumis à une irradiation de 160 R. Ils ne signalent pas d'aspect similaire dans les lymphocytes médullaires normaux.

Dans la moelle normale, il nous faut signaler aussi la présence de promyélocytes et de myélocytes dont la taille est à peine supérieure à celle des petits lymphocytes.

On peut raisonnablement admettre que la population des petites cellules de 100 à 180 μ^3, telle qu'on la mesure dans la moelle à l'aide d'un compteur électronique de particules, est constituée en majeure partie par les 'lymphocytes' auxquels viennent s'ajouter les cellules myéloïdes de petite taille et quelques éléments jeunes de la lignée érythrocytaire. Dans la moelle en régénération, l'augmentation des petites cellules mononucléées paraît due à l'accumulation de lymphocytes, de cellules myéloïdes de petite taille, et de cellules X. En revanche, la proportion de cellules érythrocytaires jeunes est diminuée.

La présence de cellules particulières que nous appelons 'cellules X', pour ne pas préjuger de leur nature, pose les problèmes de leur origine et de leur devenir. Disons tout de suite qu'il est impossible de répondre à ces questions sur la base des seuls critères morphologiques. Il est difficile de considérer cette cellule comme faisant partie de la lignée granulocytaire. En effet, tout stade postérieur à celui du promyélocyte peut être exclu par l'absence de sacs ergastoplasmiques développés ou de granulations spécifiques. L'hypothèse d'une relation entre la cellule X et le myéloblaste est difficile à retenir sur la base de l'ultrastructure du noyau. Dans le myéloblaste normal, la chromatine est disposée en une mince couche périphérique et le nucléole, volumineux, est homogène. Dans la cellule X, au contraire, la chromatine est concentrée en gros blocs et le nucléole est distribué en zones bien distinctes.

Il nous paraît qu'on peut éliminer également une relation entre les cellules X et les cellules jeunes de la lignée érythrocytaire. Dans le proérythroblaste, le noyau a une structure comparable à celle du myéloblaste. Le cytoplasme du proérythroblaste contient de longs sacs ergastoplasmiques minces, plus nombreux que dans la cellule X, et de petits granules de ferritine que nous n'avons jamais retrouvés dans ce dernier type de cellule.

Reste à envisager la nature 'lymphoïde' de cette cellule. On peut émettre deux hypothèses:

(a) la cellule X dérive du lymphoblaste et est éventuellement un stade intermédiaire entre celui-ci et le lymphocyte;

(b) la cellule X provient d'un lymphocyte qui s'est 'transformé'.

Certains arguments plaident en faveur d'une relation des cellules X avec la lignée lymphoïde. La disposition en rosettes des ribosomes se retrouve tant dans les lymphoblastes que dans les cellules X (à titre de comparaison nous donnons l'aspect ultrastructural d'un lymphoblaste normal (Fig. 8). Rappelons aussi le développement de rosettes dans les lymphocytes cultivés en présence de phytohémagglutinine [15]. Le nucléole, formé de deux zones distinctes, se rencontre quelquefois dans les petits lymphocytes ganglionnaires. Au stade actuel de nos connaissances, ces éléments paraissent insuffisants pour tirer une conclusion.

Nos observations posent le problème de la nature des 'transitional cells' décrites par différents auteurs [2–5, 10, 11]. Il s'agit là d'éléments présentant une certaine variabilité, tant de la taille que des caractéristiques cytologiques. Nos résultats suggèrent que cette population est probablement hétérogène. A côté de cellules ayant les principales caractéristiques des lymphocytes, il existe des cellules jeunes appartenant incontestablemen- à la lignée myéloïde. La présence de granut lations spécifiques révélées par l'examen au microscope électronique en témoigne. Ces promyélocytes et myélocytes de petite taille ne constituent pourtant qu'une faible proportion de l'ensemble des 'transitional cells'. La plupart de celles-ci semblent être des 'cellules X' dont des recherches ultérieures devraient préciser la nature.

En terminant, rappelons que Yoffey *et al.* [16] ont décrit l'aspect ultrastructural des 'transitional cells' dans la moelle du cobaye normal. Pour des raisons techniques, il est difficile d'interpréter les images; il semble cependant exister des différences notables entre ces éléments et nos cellules X.

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RESUME

Au cours de la régénération hématopoïétique qui suit une irradiation, on observe, dans la moelle fémorale de la souris R3, une augmentation importante et transitoire des petites cellules de type lymphoïde. Une étude ultrastructurale de cette population cellulaire a été réalisée sur des moelles sélectionnées en fonction des courbes obtenues au moyen d'un compteur électronique de particules. Ont été choisies dans ce but 4 moelles, prélevées 10 à 12 jours après une irradiation X de 500 R, moment où les petites cellules mononucléées représentent 50 à 60% de l'ensemble des éléments médullaires. Les moelles de 3 souris normales de mêmes souche, âge et sexe, ont servi de témoins pour ces observations.

Chez les souris normales, la population de petites cellules paraît constituée essentiellement par des éléments de type lymphocytaire. On y trouve, en outre, quelques cellules érythrocytaires jeunes et de rares cellules myéloïdes de petite taille.

Chez les souris irradiées, la population de petites cellules est extrêmement hétérogène; comme la moelle normale, la moelle irradiée contient des petits lymphocytes, des éléments myéloïdes de petite taille et des cellules érythroïdes. L'ensemble de ces cellules ne constitue cependant qu'une partie réduite des éléments médullaires de petite taille. La majeure partie de ces derniers appartiennent à un type cellulaire particulier qu'on ne trouve pas dans la moelle normale. L'origine et la signification de ces cellules ne sont pas encore précisées.

SUMMARY

During the recovery period which follows an X-rays irradiation, the bone marrow size distribution curves obtained with an electronic particle counter are characterized by an important increase of the small lymphoid-like cell population. An ultrastructural study of this population has been realized on four bone marrows selected by the particle counter from a group of R3 mice irradiated 10–12 days before at the dose of 500 R. In these marrows, the lymphoid-like cells ranged from 50 to 60% of the total amount of nucleated cells. The marrow of three normal mice of the same strain, age and sex, has been used as controls.

In the normal mice, the small cell population is composed mostly of small lymphocytes; it contains also small sized myeloid cells and a few cells from the erythrocytic series.

In the irradiated mice, the population of small cells is extremely heterogenous; it is partly constituted by small lymphocytes, small myeloid cells and rare erythrocytic cells. Besides these elements, the marrow is crowded with cells of a special kind with characteristic nuclear and cytoplasmic structures, the nature and function of these cells is still unknown; their possible relation with the so-called 'transitional cells' is discussed.

ZUSAMMENFASSUNG

Während der Erholungsperiode, die einer Strahlenschädigung folgt, lässt sich mit Hilfe eines elektronischen Volumenverteilungszählgerätes (Coulter Counter) eine starke, aber vorübergehende Zunahme der kleinen lymphozytenähnlichen Zellen in dem Schenkelbeinmark der R3-Maus feststellen. Diese Zellpopulation wurde mit dem Elektronenmikroskop untersucht. Dazu wurden Mäuse 10 bis 12 Tage nach einer Bestrahlung mit 500 R getötet, und das Mark mittels des Zählgerätes untersucht. Das Mark von vier Mäusen, deren Kleinzellpopulation 50 bis 60% der Gesamtzahl ausmachte, wurde für die ultrastrukturelle Untersuchung ausgewählt. Drei unbestrahlte Mäuse gleichen Stammes, Alters und Geschlechtes dienten als Kontrolle.

In bestrahlten Mäusen ist die Kleinzellpopulation sehr heterogen; wie in den Kontrolltieren enthält das bestrahlte Mark kleine Lymphozyten, kleine myeloische Zellen und seltene Zellen der Erythropoese. Die Gesamtheit dieser Elemente bildet aber nur einen geringen Teil im Vergleich zu den kleinen Markzellen; die Mehrheit gehört einem eigentümlichen Zelltypus an, den man in dem normalen Knochenmark nicht findet. Der Ursprung und die Bedeutung dieser Zellen sind noch nicht genau bekannt.

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The Sensitizing Carcinogenic Effect of Small Doses of Carcinogen

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INTRODUCTION

THE SIGNIFICANCE of the effect of small doses of chemical carcinogens has great theoretical and practical importance, and it has been studied by various methods. First it was shown that though a decrease in dosage is followed by a strikingly lower incidence of tumors and by a lengthened latent period, tumors can still be observed after very small doses of carcinogen [1, 2].

The study of carcinogenesis also yielded information about the effect of small doses of carcinogen. In the course of these experimentations it was shown that the specificity of neoplasms appeared very rapidly after the application of a small dose of carcinogen and that the carcinogen could then be substituted by a non-carcinogenic substance exhibiting only the so-called co-carcinogenic action [3–13].

The results of these investigations served as the basis for the theory of 2-stage development of cancer.

Since 1951 work has been carried out in our laboratory to determine whether intermittent applications of small doses of these carcinogens, which do not induce tumors themselves on one or different sites, has a facilitating effect on tumor induction, and whether the total dose of carcinogen effective for tumor induction is different from the total dose in continuous treatment.

The results of these experiments are the subject of this paper.

MATERIAL AND METHODS

The experimental animals were mice C57BL, CC57BR and non-inbred white mice, received from the animal farm of the USSR Academy of Medical Sciences. In each series the mice were of the same sex, usually male; they were fed a standard diet (white bread, oats, groats, milk, and roots) and were about 3 months of age at the beginning of the experiments.

The mice were first given a small dose of carcinogen, as a rule not sufficient to induce tumours. After this short contact, the mice were subjected to no other carcinogenic influences. After an interval, the application of carcinogen to these mice and simultaneously to the control ones of the same age, sex and origin (see scheme of experiments) was renewed. The phases of hair cycle were not taken into consideration because, in every experiment, the application of carcinogen took place many times independently of the cycle. In all series of experiments, the dates of the development of the first tumors in the sensitized and control mice was noted as the criterion of possible sensitizing effects of small doses of carcinogen.

Experiment I. CC57BR and non-inbred males received one drop of 0·5% solution of 20-methylcholanthrene in benzol (about 0·125 mg of carcinogen) on a hairless area of skin of the interscapular region once a week for 5 weeks. Subsequently, they had no contact with carcinogen for 8 or 14 weeks. After this interval, the application of the same dose of methylcholanthrene on the same site was repeated and simultaneously the application on an analogous place of the skin of the control mice (3 series) was begun [14–16].

Experiment II. This experiment was carried

out on non-inbred male mice, with the same method but with 0·6% solution of 3,4-benzpyrene in benzol (about 0·15 mg of carcinogen) instead of methylcholanthrene. The interval between sensitization and the renewal of applications was 8 weeks (3 series) [17].

Experiment III. Carried out on non-inbred male mice, with the application of 0·1% solution of 9,10-dimethyl-1,2-benzanthracene (DMBA) (about 0·025 mg of carcinogen). The interval was 8 weeks (3 series) [17].

Experiment IV was carried out with both C57BL mice and non-inbred ones. The mice were sensitized with the same dose of methylcholanthrene 5 times interscapularly. The same carcinogen was reapplied after an 8-week interval on the skin of the sacral region (2 series) [18].

Experiment V. 0·3% solution of methylcholanthrene in an anhydric lanolin was applied interscapularly 3 times a week during 14 weeks on the skin of non-inbred male mice. Two weeks after sensitization, 1 mg of methylcholanthrene in sunflower oil was injected to the sensitized and control mice subcutaneously in the groin region (4 series) [14–16].

Experiment VI. Non-inbred male mice were used for 5-time application of the DMBA solution (as in experiment III). After an 8-week interval, a benzpyrene solution was applied (3 series) [17].

Experiment VII. The sensitization was carried out by means of a benzpyrene solution and the application of DMBA was started after an 8-week interval (3 series) [17].

Experiment VIII. The mice were sensitized: (a) with 1 mg of orthoaminoazotoluene (OAAT) in 1% solution in sunflower oil orally 6 times a week during 2 months (the total dose about 50 mg of carcinogen); (b) by cutaneous applications of 3 drops of 1% solution of OAAT in benzol 3 times a week also during 2 months (0·75 mg); or (c) by 2 subcutaneous implantations of paraffin pellets containing 30 mg OAAT, separated by a monthly interval. Four months after the beginning of sensitization, application of the DMBA solution was started on the interscapular region (5 series) [17].

Experiment IX. The sensitization of mice in this experiment was carried out by oral administration of OAAT or by cutaneous application (as in experiment VIII) but the second phase of the experiment IX was carried out with benzpyrene (instead of DMBA) (4 series) [17].

RESULTS

Experiment I. In 3 series of control mice, the first papillomas developed after 15, 12, and 12 applications, whereas in mice sensitized with 5 applications of the same carcinogen 8 or 14 weeks earlier, they appeared after only 6, 3, and 5 repeated applications, respectively. In the control mice, the first carcinomas appeared

Table 1. *The latent period (weeks) of the development of the first papilloma and the first carcinoma in mice following the sensitizing of the skin by* 5 *interscapular applications of carcinogen and repeated applications of the carcinogen on the same or on another area of the skin after an interval of* 8–14 *weeks*

Experiments No.	Carcinogen	No. of applications after which the first papillomas (resp. carcinomas) appeared: Sensitized mice	Control mice	Difference (weeks)
		A. Renewal of application on the same place		
I	Methylcholanthrene	*P*– 6, 3, 5 (40)	15, 12, 12 (40)	9, 9, 7
	(3 series)	*C*–14, 10, 12 (24)	23, 20, 21 (19)	9, 10, 9
II	Benzpyrene	*P*– 3, 4, 5 (101)	7, 9, 12 (111)	4, 5, 7
	(3 series)	*C*–17, 19, 20 (75)	24, 28, 33 (54)	7, 9, 13
III	DMBA	*P*– 3, 2, 3 (98)	5, 6, 5 (98)	2, 4, 2
	(3 series)	*C*–17, 16, 17 (73)	20, 24, 21 (53)	3, 8, 4
		B. Renewal of application on another place (on the skin of the sacral region)		
IV	Methylcholanthrene *P**	– 4, 13 (40)	23, 21 (8)	19, 8
	*C**	–21, 20 (25)	36, 26 (8)	15, 6

P—papilloma, *C*—carcinoma.
*Tumours on the place of renewal of applications
In parentheses—number of mice with papillomas, resp. carcinomas.

after 23, 20 and 21 applications while in sensitized mice, they appeared after 14, 10, and 12 weeks only. Therefore sensitized animals developed the first papillomas 7–9 weeks earlier than the control mice and the first carcinomas 9–10 weeks earlier (Table 1). The latent periods of papilloma development in 40 control mice of the above 3 series were to 15–26, 12–23, and 12–20 weeks, while in 40 sensitized animals they were 6–19, 3–16, and 5–16 weeks, respectively. The latent periods of carcinomas were 23–26, 20–27, and 21–32 weeks in 19 control mice and 14–18, 10–23, and 12–27 weeks, respectively, in 24 sensitized animals.

Experiment II. Similar results were obtained in experiments using benzpyrene: 7, 9, and 12 applications of carcinogen were necessary for the development of the first papillomas in control mice and only 3, 4, and 5 in the mice which had been sensitized 8 weeks previously. The first carcinomas appeared after 24, 28 and 33 applications in the control mice but only after 17, 19, and 20 in the sensitized ones. The difference was 4–7 weeks for papillomas and 7–13 for carcinomas (Table 1). The latent period for papillomas in these 3 series of 111 control mice were 7–29, 9–32, and 12–33 weeks, and in 101 sensitized animals 3–19, 4–20, and 5–24 weeks, respectively. The latent periods of carcinomas were 24–48, 28–54, and 33–56 weeks in 54 control mice and 17–38, 19–41, and 20–43 weeks, respectively, in 75 sensitized animals.

Experiment III. The following results were received in experiment with DMBA: 5, 6, and 5 applications of carcinogen were necessary for the development of the first papillomas in control mice and only 3, 2, and 3 applications in sensitized ones. The first carcinomas appeared in control mice after 20, 24, and 21 applications but only after 17, 16 and 17 in sensitized ones. The difference was 2–4 weeks for papillomas and 3–8 weeks for carcinomas (Table 1). The latent periods for papillomas in these 3 series in 98 control mice were 5–19, 6–21, and 5–20 weeks, while in 98 sensitized animals they were 3–17, 2–19 and 3–20 weeks respectively. The latent periods of carcinomas were 20–39, 24–45 and 21–45 weeks in 53 control mice and 17–32, 16–36 and 17–38 respectively in 73 sensitized animals.

Experiment IV. It was found that, after resumption of methylcholanthrene applications on another place, the first papillomas appeared on the skin of the sacral region in two series of sensitized mice (C57BL females in the first series and non-inbred males in the second one) after 4 and 13 additional applications, while in control mice 23 and 21 additional applications were necessary. The first carcinomas appeared in sensitized animals after 21 and 20 applications but, in control mice they appeared after 36 and 26 applications (it should be noted, however, that tumors are known to develop later on the skin of the sacral region than on the interscapular one). The difference was 8–19 weeks for papillomas and 6–15 weeks for carcinomas (Table 1). The latent periods for papillomas were 23–28 and 21–23 weeks in 8 control mice of these 2 series and 4–40 and 13–28 weeks in 40 sensitized animals. The latent periods for carcinomas were 36–46 and 26–28 weeks in 8 control mice and 21–69 and 20–30 in 25 sensitized animals.

Seven mice among the sensitized animals of the first series developed papillomas by week 21 after the beginning of application of carcinogen on the skin of the sacral region; at that time the control mice had no papillomas. Malignant transformation of the papillomas occurred by week 29 in 5 sensitized mice and had not been observed in the controls by that time.

Among the sensitized mice of the second series 15 animals developed papillomas by week 19 after the beginning of application of carcinogen on the sacral skin and no control mouse had developed a papilloma by that time. After 24 weeks 6 sensitized mice had carcinomas which were not observed in the control animals.

It is interesting to note that papillomas developed interscapularly (on the place of sensitizing) in 16 mice. Five of these papillomas transformed into carcinomas. Several mice had two carcinomas: both in the sacral and interscapular regions. After sensitization without resumption of applications, the development of papillomas was noticed only in two mice after more than 500 days.

Experiment V. (Table 2). The first sarcomas were discovered in 4 series of control mice on the place where methylcholanthrene in oil had been injected after 115, 99, 92, and 64 days (the total of 19 sarcomas) and after 74, 71, 71, and 29 days, in mice which had been sensitized by skin painting with lanolin solution of methylcholanthrene (the total of 28 sarcomas).

All the sarcomas were discovered earlier among sensitized mice of the first and the second series than in the control groups. The sarcomas appeared in sensitized animals of the first series not later than day 99 but in control

Table 2. The latent period of the development of the first sarcoma (days) after sensitizing of the skin of mice with lanolin solution of methylcholanthrene and injection of methylcholanthrene subcutaneously (experiment V)

Series	Experiment	Control	Difference
I	74	115	41
II	71	99	28
III	71	92	21
IV	29	64	35

mice between days 115 and 131; in sensitized animals of the second series after 71–92 days, and in the controls between day 99 and 148. The sarcomas were found in sensitized mice of the fourth series between days 29 and 67, and in the controls between days 64 and 85. The first sarcomas in sensitized animals of the third series also appeared earlier than in controls, but subsequent development of tumours was delayed for a more prolonged period.

It is important to emphasize that skin papillomas developed on the place of sensitizing in 14 sensitized mice from experimental group but in only two control mice (without subsequent injection of methylcholanthrene subcutaneously).

Experiment VI. This was carried out with various carcinogens: the mice were sensitized with small doses of DMBA and, after an 8-week interval, the applications were repeated on the same place but with benzpyrene. In control groups (3 series without sensitizing) the first papillomas induced by benzpyrene appeared after 7, 9 and 12 applications, and the first carcinomas after 24, 28 and 33 applications. In mice sensitized with DMBA, the first papillomas appeared after 2, 3 and 2 applications, and the first carcinomas after 13, 17 and 14 applications, that is 5–10 weeks earlier for papillomas and 11–19 weeks earlier for carcinomas than in control mice (Table 3). The latent periods for papillomas in 110 control mice of these 3 series were 7–29, 9–32, and 12–33 weeks, while in 94 sensitized animals 2–15, 3–21, and 2–17 weeks respectively. The latent periods of carcinomas were 24–48, 28–54, and 33–56 weeks respectively in 63 control mice and 13–31, 17–36, and 14–35 weeks in 71 sensitized animals.

Experiment VII. The sensitizing was carried out with benzpyrene and the second course of application with DMBA. The first papillomas appeared in control mice (3 series) after 5, 6

Table 3. The latent period (weeks) of the development of the first papilloma and the first carcinoma in mice after sensitizing with one carcinogen and renewal of applications with different one, after an interval

Experiments No.	Carcinogens		Number of applications after which the first papillomas (resp. carcinomas) appeared: Sensitized mice	Control mice	Difference (weeks)
VI	DMBA–benzpyrene (3 series)	*P*	– 2, 3, 2 (94)	7, 9, 12 (110)	5, 6, 10
		C	–13, 17, 14 (71)	24, 28, 33 (63)	11, 11, 19
VII	Benzpyrene–DMBA (3 series)	*P*	– 1, 1, 2 (97)	5, 6, 5 (98)	4, 5, 3
		C	–13, 15, 18 (76)	20, 24, 21 (53)	7, 9, 3
VIII (a)	OAAT per os–DMBA (2 series)	*P*	– 2, 3 (78)	P–8, 10 (79)	6, 7
		C	–16, 17 (63)		12, 14
VIII (b)	OAAT on skin–DMBA (2 series)	*P*	– 4, 5 (68)	C–28, 31 (52)	4, 5
		C	–20, 22 (56)		8, 9
VIII (c)	OAAT (implantations)–DMBA (1 series)	*P*	– 4 (25)	9 (26)	5
		C	–18 (21)	25 (21)	7
IX(a)	OAAT per os–benzpyrene (1 series)	*P*	– 1 (30)	P–11 (29)	10
		C	–16 (27)		14
IX (b)	OAAT on skin–benzpyrene (1 series)	*P*	– 1 (24)	C–30 (16)	10
		C	–18 20		12

P—papilloma, *C*—carcinoma.

Table 4. Scheme of experiments

Weeks	1	2	3	4	5	6	7	8	9	10	11	12	13	14/1	15/2	16/3	17/4	18/5	19/6	.....
Experiment I	Mc	Mc	Mc	Mc	Mc	–	–	–	–	–	–	–	–	Mc	Mc	Mc	Mc	Mc	Mc	.....
Control	–	–	–	–	–	–	–	–	–	–	–	–	–	Mc	Mc	Mc	Mc	Mc	Mc	.....
Experiment II	Bp	Bp	Bp	Bp	Bp	–	–	–	–	–	–	–	–	Bp	Bp	Bp	Bp	Bp	Bp	.....
Control	–	–	–	–	–	–	–	–	–	–	–	–	–	Bp	Bp	Bp	Bp	Bp	Bp	.....
Experiment III	DMBA	DMBA	DMBA	DMBA	DMBA	–	–	–	–	–	–	–	–	DMBA	DMBA	DMBA	DMBA	DMBA	DMBA	.....
Control	–	–	–	–	–	–	–	–	–	–	–	–	–	DMBA	DMBA	DMBA	DMBA	DMBA	DMBA	.....
Experiment IV*	Mc	Mc	Mc	Mc	Mc	–	–	–	–	–	–	–	–	Mc	Mc	Mc	Mc	Mc	Mc	.....
Control	–	–	–	–	–	–	–	–	–	–	–	–	–	Mc	Mc	Mc	Mc	Mc	Mc	.....
Experiment V										see text										
Experiment VI	DMBA	DMBA	DMBA	DMBA	DMBA	–	–	–	–	–	–	–	–	Bp	Bp	Bp	Bp	Bp	Bp	.....
Control	–	–	–	–	–	–	–	–	–	–	–	–	–	Bp	Bp	Bp	Bp	Bp	Bp	.....
Experiment VII	Bp	Bp	Bp	Bp	Bp	–	–	–	–	–	–	–	–	DMBA	DMBA	DMBA	DMBA	DMBA	DMBA	.....
Control	–	–	–	–	–	–	–	–	–	–	–	–	–	DMBA	DMBA	DMBA	DMBA	DMBA	DMBA	.....
Experiment VIII										see text										
Experiment IX										see text										

Mc—20-methylcholanthrene.
Bp—3,4-benzpyrene.
DMBA—9,10-dimethyl-1,2-benzanthracene.
– – No applications of carcinogene.
Part of experiment IV was carried out with 14 week interval.
*Applications were renewed on another place.

and 5 applications of DMBA and the first carcinomas after 20, 24 and 21 applications, but in sensitized mice the first papillomas appeared after 1, 1, and 2 applications and the first carcinomas after 13, 15 and 18 applications, that is 3–5 weeks earlier for papillomas and 3–9 weeks for carcinomas than in control mice (Table 3). The latent periods for papillomas in 98 control mice of these 3 series were 5–19, 6–21 and 5–20 weeks, in 97 sensitized animals they were 1–12, 1–12 and 2–14 weeks. The latent periods of carcinomas were 20–39, 24–45 and 21–45 weeks in 53 control mice and 13–27, 15–29, and 18–30 weeks in 76 sensitized animals.

Experiment VIII, series (a). The mice received OAAT orally during 2 months, and after the 8-week interval we began to apply DMBA on their skin. The first papillomas appeared in 2 control series after 8 and 10 applications of DMBA, and the first carcinomas after 28 and 31 applications. In 2 series of sensitized mice, the first papillomas and carcinomas appeared after 2 and 3 applications and 16 and 17 applications respectively, that is 6–7 weeks earlier for papillomas and 12–14 weeks earlier for carcinomas (Table 3). The latent periods of papilloma development in 79 control mice were 8–25 and 10–26 weeks, while in 78 sensitized animals they were 2–16 and 3–16 respectively. The latent periods for carcinomas were 28–48 and 31–50 weeks in 52 control mice and 16–38 and 17–37 weeks in 63 sensitized animals.

Experiment VIII, series (b). The mice were sensitized with applications of OAAT on the skin, and after the 8-week interval we began to apply DMBA on the same area of their skin. In 2 series of control mice (the same as in series a), the first papillomas appeared after 8 and 10 applications of DMBA, and the first carcinomas after 28 and 31 applications, but sensitized mice developed the tumors after 4 and 5, and 20 and 22 applications respectively, that was 4–5 weeks earlier for papillomas and 8–9 week for carcinomas (Table 3). The latent periods for these papillomas were 8–25 and 10–26 weeks in 79 control mice, and 4–16 and 5–19 weeks in 68 sensitized animals. The latent periods for carcinomas proved to be 28–48 and 31–50 weeks in 52 controls, and 20–34 and 22–41 weeks in 56 sensitized animals.

Experiment VIII, series (c), (with implantation of the OAAT pellets). The first papillomas appeared in control mice after 9 weeks and in sensitized animals after 4 applications of DMBA. The first carcinomas appeared after 25 weeks in control mice and after 18 weeks in sensitized animals, that is 5 weeks earlier for papillomas and 7 weeks earlier for carcinomas. The latent periods for papillomas were 9–24 weeks in 26 control mice and 4–17 weeks in 25 sensitized animals. For carcinomas they were 25–46 weeks in 21 control mice and 18–40 in 21 sensitized ones.

Experiment IX. In mice which received OAAT per os (series (a)) and benzpyrene on the skin, the first papilloma appeared after only 1 application of benzpyrene (the last one after 14 applications) and the first carcinoma after 16 applications (the last one after 32 applications). In series (b), where OAAT was applied on the skin, the first papilloma also appeared after 1 application (the last papilloma after 13 applications) and the first carcinoma after 18 applications (the last one after 33 applications). In the control series the first papilloma appeared after 11 applications (the last one after 25 applications) and the first carcinoma after 30 applications (the last one after 42 applications). Thus, the first papillomas appeared 10 weeks earlier in 30 and 24 sensitized mice and the first carcinomas 14–12 weeks earlier in 27 and 20 animals respectively than in control mice (29 with papillomas and 16 with carcinomas).

DISCUSSION

The results of these experiments show that small doses of carcinogens which, under usual conditions, induce no tumours are nevertheless not biologically inert. These doses create a general and local predisposition of carcinogen sensitized animal tissues to the development of neoplasia and to malignant transformation after subsequent contact with the carcinogenic substances.

It is important that the difference between the number of applications in control and sensitized mice as a rule exceed the latter by more than five (see the last columns of Tables 1 and 3), especially for carcinomas. The mechanisms of the above mentioned phenomenon cannot be explained as a result of a simple addition of 5 sensitizing applications (before the interruption) and the ones after it: this total is smaller as a rule than in control mice [19, 20]. Obviously the initial small doses of carcinogen increase the susceptibility of tissues to the subsequent action of the same or other carcinogens. The single exception with

DMBA experiments (experiments III and VII) was probably due to the too strong carcinogenic action of this substance. It might be that smaller doses of DMBA than those which were used in our experiments should be used for sensitizing.*

The appearance of tumours on the place of sensitizing in experiments IV and V is of special interest because subsequent treatment with the carcinogen was applied on another place subcutaneously or on a distant area of the skin.

For a long time almost no attention was paid to this peculiar sensitizing action of small doses of carcinogens though it was the only way to understand the nature of their initiating effect.

The sensitizing effect of small doses of carcinogens is probably universal because it can be observed in non-inbred and inbred mice of several strains and with combinations of different carcinogens inducing not only the skin tumours, but also subcutaneous sarcomas (experiment V) and liver neoplasms (experiments VIII and IX). The results of these experiments cannot be explained by a simple additive effect of the combined carcinogens because their mechanisms of action differ.

Similar results were obtained using various methods for diminishing the doses of sensitizing carcinogen: such as shortening of the period of contact of the organism with the carcinogen or delaying its absorption by means of special solvents (carcinogens are badly absorbed out of lanolin).

The last method was proposed by J. Rosicky and R. Hatschek [21]. W. L. Simpson and W. Cramer [22] applied methylcholanthrene in benzol on the same place of the skin, which had been previously treated with the same carcinogen, but in lanolin solution. Tumors developed more rapidly and their incidence was higher in such mice than in controls. Similar effect was observed in our experiments [14–16] after the injection of oil solution of methylcholanthrene subcutaneously far from the place sensitized with lanolin solution of methylcholanthrene. M. B. Sulzberger *et al.* [23] obtained the same results.

U. Saffiotti [24] found that previous sensitizing of mice with small doses of DMBA resulted in an acceleration of tumour development after repeated contact of the animals with this carcinogen. E. Kojevnicova [25] noticed a similar effect in experiments with methylcholanthrene. She also injected 0·25 mg of methylcholanthrene into the kidney and after 2 months began to apply methylcholanthrene in benzol to the skin of some of these mice. In 5 months these animals had twice as many kidney tumours as the control mice which had not been subjected to the skin application of the carcinogen.

Our findings show that the interval between sensitizing of animals with small initial doses of carcinogens and the repeated contact with them considerably increases the effect of sensitizing. This fact emphasizes the significance of the time factor in carcinogenesis [26]. Our experiments on the interval time-response effect are still in progress and the results will be published later on.

Data presented here prove once again the impossibility of establishing any reliable permissible 'harmless' concentrations of carcinogens in the human environment. There is too much experimental evidence that the exposure to carcinogenic agents in concentrations insufficient for tumour induction increases the susceptibility of the organism to subsequent contact with the same or other carcinogens.

*As it was mentioned in the introduction, the time of appearance of the *first* papillomas and carcinomas was registered in order to detect the sensitizing action of small doses of carcinogens. Moreover, statistically significant differences in the average time of development of the total number of tumors ($p<0·001$) was found between the experimental and control series in all our experiments except experiment III where the difference was significant ($p<0·001$) only for carcinomas but not for papillomas. We have already mentioned that obviously the dose of DMBA used in experiments with this very active carcinogen was too large.

RESUME

La mise en contact peu prolongée de la peau de la souris avec des doses faibles de cancérigènes (20-méthylcholanthrène, 3,4-benzopyrène, 9,10-diméthyl-1,2-benzanthracène), qui sont incapables, en principe, de provoquer le développement de tumeurs, rend néanmoins l'organisme plus susceptible aux effets cancérigènes d'une de ces substances, si elle est appliquée 8 à 14 semaines après le début du traitement. Chez les animaux expérimentaux, les papillomes apparaissent plus tôt, et évoluent plus rapidement vers la malignité, après la seconde application du cancérigène, que chez les animauz de contrôle.

L'apparition accélérée des papillomes, et leur évolution maligne après le second badigeonnage, se produisent également dans des régions cutanées éloignées de l'endroit de la première

application de méthylcholanthrène. L'endroit de la première application, dans les conditions de l'expérience, ne présente pas de tumeur; cependant, le traitement secondaire, par un cancérigène appliqué à quelque autre endroit de la peau, fait apparaître des papillomes, et parfois même des carcinomes à l'endroit de la première application.

Des résultats analogues peuvent être observés au cours d'expériences avec l'application cutanée, suivie, après un intervalle, de l'injection sous-cutanée de méthylcholanthrène: des sarcomes apparaissent plus tôt chez les animaux faisant l'objet de l'expérience que chez les animaux témoins, et des néoplasmes apparaissent à l'endroit de la première application. L'administration, aux souris, de doses d'orthoaminoazotoluène, insuffisantes pour provoquer des hépatomes, suivie de badigeonnages par du 9,10-diméthyl-1,2-benzanthracène ou par du benzopyrène, détermine également un développement accéléré de papillomes et de carcinomes.

Ces expériences soulignent le fait que des doses de cancérigènes, insuffisantes pour provoquer des tumeurs, rendent cependant l'organisme plus susceptible à des effets cancérigènes ultérieurs.

SUMMARY

A short-term contact of the skin of mice with small doses of carcinogens such as 20-methylcholanthrene, 3,4-benzpyrene and 9,10-dimethyl-1,2-benzanthracene, which as a rule do not induce any tumours during the usual life span of the animals, sensitizes the organism to subsequent applications of one of these carcinogens after an 8–14-week interval. The skin papillomas appear on the site of the repeated applications earlier than in control mice and malignant transformation of these papillomas takes place earlier.

The development of papillomas and their malignant transformation also accelerated if the carcinogen is applied during the second course of treatment on areas of the skin far from the place of initial sensitizing (experiment with methylcholanthrene). On the place of sensitizing, where tumours as a rule do not appear, some mice develop papillomas, and even carcinomas, if methylcholanthrene had again been applied on another area of the skin.

Similar results were received when the sensitizing of the skin had been followed by subsequent subcutaneous injection of methylcholanthrene. In this case, sarcomas developed earlier than in control mice and tumours of the skin appeared in some animals on the place of sensitizing.

The acceleration of papilloma and carcinoma development is also found in experiments with 9,10-dimethyl-1,2-benzanthracene or benzpyrene when the mice have been previously sensitized with doses of orthoaminoazotoluene, insufficient for hepatomas induction.

All the data obtained show that the doses of carcinogens, which are insufficient to induce tumours, increase the susceptibility of the organism to subsequent carcinogenic influences.

ZUSAMMENFASSUNG

Eine kurzdauernde Behandlung der Mäusehaut mit kleinen Mengen einiger Cancerogene (20-Methylcholanthren, 3,4-Benzpyren, 9,10-Dimethyl-1,2-benzanthrazen) ist im allgemeinen nicht ausreichend zur Tumorentwicklung. Sie macht aber den Organismus empfindlicher für diese Substanzen, wenn sie nach einer Pause von 8-14 Wochen angewendet werden. Papillome bilden sich und malignisieren früher als bei Kontrollmäusen.

Den gleichen Effekt beobachtet man auch, wenn die spätere Behandlung der Haut entfernt vom Sensibilisierungsort erfolgt. Am Sensibilisierungsort, wo ohne weitere Behandlung keine Geschwülste entstehen, entwickeln sich bei einen Teil der Mäuse Papillome und manchmal sogar Carcinome, wenn man an einer anderen Stelle Methylcholanthren appliziert.

Ähnliche Resultate erhielt man in Versuchen, in denen als Zweitbehandlung Methylcholanthren subcutan injiziert wurde: Sarkome entstanden hier früher als bei den Kontrollen und an der Sensibilisierungsstelle bildeten sich Hautgeschwülste.

Ebenso wirkte eine Sensibilisierung mit einer für die Hepatomentwicklung unzureichenden Menge an ortho-Aminoazotoluol, wobei sich bei der Applikation von 9,10-Dimethyl-1,2-benzanthracen oder Benzpyren auf die Haut Papillome und Carcinome schneller entwickelten.

Nach diesen Versuchen bewirken unterschwellige Dosen an Cancerogenen eine Steigerung der Empfindlichkeit eines Organismus genen weitere Einwirkungen von Cancerogenen.

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Letter to the Editor

On the 'Human' Nature of Highly Malignant Heterotransplantable Tumors of Human Origin

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THE STUDIES reported in a recent issue of this journal by Haemmerli, Schmid and Lindemann on our GW-127 tumor system [1] present several points of conflict both with part of our own data and with our interpretation of certain phenomena pertaining to this tumor, so that we deem it necessary to present herewith our views on this subject.

The unique aggressive behavior of GW-127 in a foreign host [2] naturally prompted us to raise the question of an "adaptive" change having taken place in the tumor cell population after transplantation of the patient's ovarian carcinoma to the hamster cheek pouch. We were confident that GW-127's highly malignant character in the hamster made it in some way different from those tumor lines hitherto derived from man both in this laboratory and elsewhere (cf. reference 3). GW-127's resemblance to the hamster karyotype, as reported by Haemmerli, Schmid and Lindemann [1], and also by our own group [4], thus seems plausible, although, as a very aneuploid tumor, a complete absence of the human genome cannot be assumed on the basis of the evidence available.

Haemmerli's often-voiced claim that human tumors growing in *unconditioned* hosts cannot be a continuum of such xenogeneic sources, but, as suggested in the study cited here, are either spontaneous or induced hamster neoplasms, would then also be applicable to a number of other tumors of human origin reported as transplantable in unconditioned hamsters (cf. reference 3), for which evidence of their human origins is, however, available [3]. Thus, cultivation of human tumors in unconditioned animal hosts would not seem, by itself, to be sufficient cause to doubt the retainment of human qualities by these neoplasms.

The immunological experiments performed by Haemmerli, Schmid and Lindemann attempt to prove that GW-127 lacks human antigen. The fact that absorption of the anti-GW-127 serum with pooled hamster tissues resulted in a total absorption of antibodies to human tissues can of course be interpreted as meaning that GW-127 contains only hamster antigens, although the non-absorbed antiserum did yield precipitation lines with human tissue. It has been our experience that, at the most, two protein systems (albumin and an α_2/β-globulin fraction) are present in both man and hamster which crossreact [5]. With regard to the experiments cited above, we are compelled to question whether their method of preparing the GW-127 tumor extract for sensitization yielded sufficient species-specific antigen systems (*e.g.*, after deep-freezing) and if the mode of sensitization used produced sufficient GW-127 antibodies. Indeed, we are of the opinion that it is more advantageous to employ commercially-available, standardized, high-titer, species-specific antisera for the identification of species-specific antigens.

Upon complete absorption against both

antihuman and antihamster sera (in two separate runs) we have found, by means of the Ouchterlony test, that GW-127 has both hamster *and* human antigens, and that, when compared to the H.Ad. No. 1 tumor (since this tumor has been shown to have retained human antigen and the human karyotype in spite of it being propagable in unconditioned hamsters, see reference 6), no qualitative difference could be demonstrated with regard to the human antigen present in H.Ad. No. 1 and GW-127 [7]. Electrophoretic analysis of the "human" precipitation lines obtained has indicated, furthermore, that they are biochemically different from the species-specific protein components of the hamster [5].

On the basis of this evidence, and in view of the additional four tumors of human origin with similarly aggressive growth patterns recently successfully xenografted to unconditioned hamsters in our laboratory [8]—all derived from different neoplastic tissues and of somewhat different histopathology and chemotherapeutic sensitivity—we prefer at this time to entertain the working hypothesis that such tumor systems represent a situation similar to the interspecies hybridizations performed by others *in vitro* [9–11]. As we have discussed at length elsewhere [12], the *in vivo* fusion of tumor and normal cells, even when xenogeneic, might very well be a more common event than hitherto suspected. Whereas evidence for the retainment of human antigen and the human karyotype has been the standard set by many researchers in this field for designating such transplantable tumors as 'human', our own five tumor systems of human origin that are invasive and metastatic in their unconditioned hamster hosts present some new intriguing considerations, with apparent pertinence to the processes of tumor progression and foreign graft adaptation.

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Reticulum Cell Sarcoma and Lymphosarcoma

A Retrospective Study of Potential Survival in Loco-Regional Disease

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INTRODUCTION

THE PURPOSE of this paper is to make a study, in retrospect, of the course of disease in patients presenting with loco-regional glandular or upper respiratory–digestive-tract lesions due to lymphosarcoma and reticulum cell sarcoma, the specific intent being to obtain information that might suggest radiotherapeutic measures which could improve the prognosis.

MATERIAL AND METHODS

Between 1953 and 1963, 343 patients with lympho-reticulum cell sarcoma were treated at the Rotterdamsch Radio-Therapeutisch Instituut. One hundred and forty-two cases (40% of the total) are considered to have been loco-regional glandular or upper-respiratory–digestive tract (U.R.D.T.) lesions at the time of their first treatment.

The retrospective staging of all cases has been done according to Vera Peters' modified classification, recommended for international use at Paris and Rye in 1965.

Loco-Regional

- Stage I_1: Disease limited to one anatomical region.
- Stage I_2: Disease limited to two contiguous anatomical regions, on the same side of the diaphragm.
- Stage II: Disease in more than two anatomical regions or in two non-contiguous regions on the same side of the diaphragm.
- Stage III: Disease on both sides of the diaphragm, but not extending beyond the involvement of lymph nodes, spleen, and/or Waldeyer's ring.
- Stage IV: Involvement of the bone marrow, lung parenchyma, pleura, liver, bone, skin, kidneys, gastrointestinal tract, or any tissue or organ in addition to lymph nodes, spleen, or Waldeyer's ring.

In order to avoid error in our retrospective staging, we did not attempt any sub-division according to the presence or absence of systemic symptoms. Lymphography was not taken into account, as it was not performed as a routine prior to 1963. Primary visceral lesions, considered as separate clinical entities, were not included in this survey of loco-regional glandular and U.R.D.T. lesions.

In all cases studied, histological slides of biopsy specimens were reviewed, and only unequivocal lymphosarcoma and reticulum cell sarcoma have been included in this analysis. The two types of tumour have been considered together, because many of the older slides were not optimally stained and in consequence some uncertainty persisted in regard to distinguishing histological criteria.

All loco-regional cases were irradiated with conventional orthovoltage X-ray machines (200–250 kV, the half-value layer varying according to the location of the lesion). Large fields were used, covering the whole of the clinically involved area, with a large margin of healthy tissue, as indicated in Figs. 1–3 (I_a-IX_a) but

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Table 1. *Reticulum cell sarcoma and Lymphosarcoma R.R.T.I.* 1953–1963 (N=343). Survival acc. to stage and site

Stage or Site	N.	Survival		
		1 year (%)	3 year (%)	5 year (%)
Glandular + upper resp. dig. tract				
I_1	79	85	75	66
I_2	34	85	56	50
II	29	65	38	34
III	32	31	16	3
dubious III or IV	31	23	6	3
IV	45	13	2	2
? (Part of treatment else)	12	33	17	8
Skin or Parenchymal lesions				
Skin	19	79	58	31
Testis	11	45	27	18
Stomach + Intestines	18	45	33	28
Other visceral sites	33	36	27	18

no attempt was made to include prophylactically all the contiguous regional lymphatic areas. Tumour doses, given in six weekly fractions, varied from 2000 rad in 6 fractions, to about 5000 rad in 30 fractions, depending on the size and site of the treated area. Chemotherapy was resorted to in 17 cases after generalized disease became evident.

All the patients were seen at regular intervals during at least five years after the first treatment, none being lost to follow-up.

For purposes of comparison, some general data have been evaluated for all the cases, while the clinical course of loco-regional disease has been analysed in detail with special regard to the site of the second clinical manifestation of the tumour—local recurrence, regional extension, distant spread—and the relation which this

Table 2. *Reticulum cell sarcoma and Lymphosarcoma R.R.T.I.* 1953–1963
Stage I_1, I_2, II (N=142)
Course after 1st treatment

	I_1		I_2		II	
	U.R.D.T.	Gland.	U.R.D.T.	Gland.	U.R.D.T.	Gland.
N	36	43	22	12	16	13
No 2nd lesion during 5–10 yr*	19 53%	18 42%	6 27%	6 50%	4 25%	3 23%
Loc. rec.	1 3%	4 9%	2 9%	—	—	1 8%
Reg. ext.	2 5·5%	6 14%	4 18%	—	3 19%	4 31%
(Combined with loc. rec.)	1	2	1	—	—	1
Dist. spread	6 17%	12 28%	8 36%	4 33%	6 38%	4 31%
Late ly	5 16%	4 9%	1 4·5%	2 17%	—	—
Intercurr.†<5 y.	2 5·5%	1 2%	2 9%	—	3 19%	2 16%
>5 y.<10 y.	2	—	—	—	—	—
5 y. surv.	24 67%	28 65%	8 36%	9 75%	5 31%	5 39%
10 y. surv.	3/15	13/20	2/5	6/8	1/8	2/5
Lost sight of	0	0	0	0	0	0

*Intercurrent death excluded.
†Death.

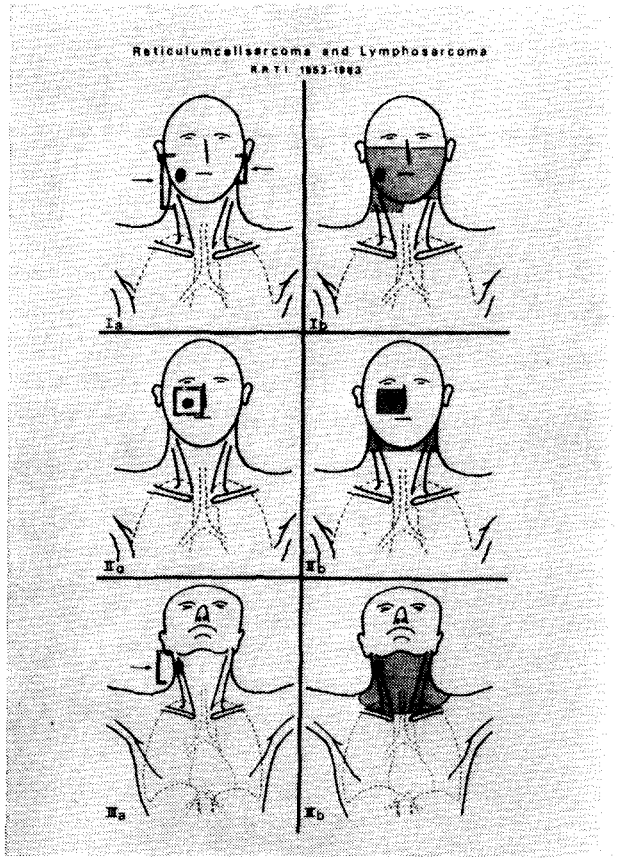


Fig. 1. In stages I_2 and II the relevant combinations of prophylactic irradiation areas are proposed.

● *Neoplasm.* □■□■□ *Actually employed irradiation fields.*
■ *Suggested prophylactic irradiation area.*

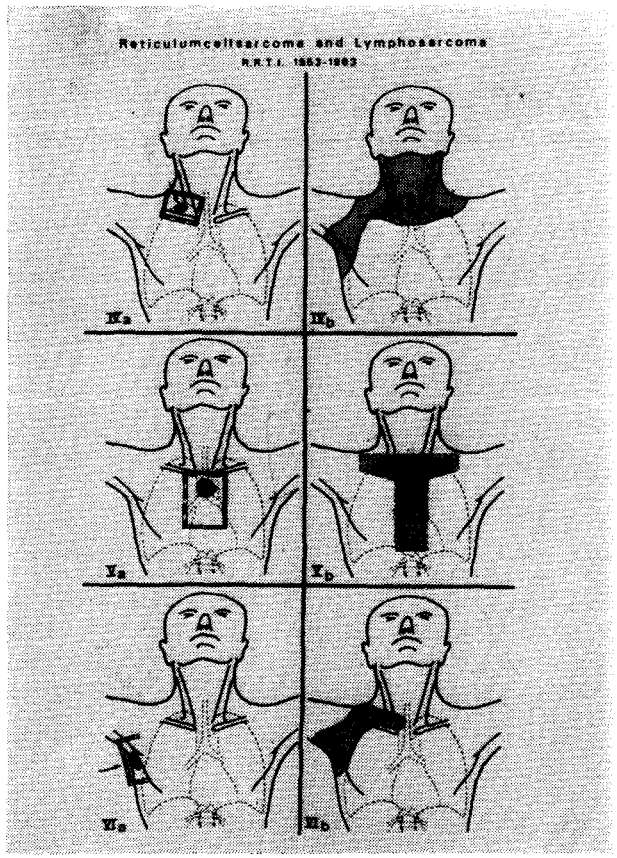


Fig. 2. In stages I_2 and II the relevant combinations of prophylactic irradiation areas are proposed.

● *Neoplasm.* □■□■□ *Actually employed irradiation fields.*
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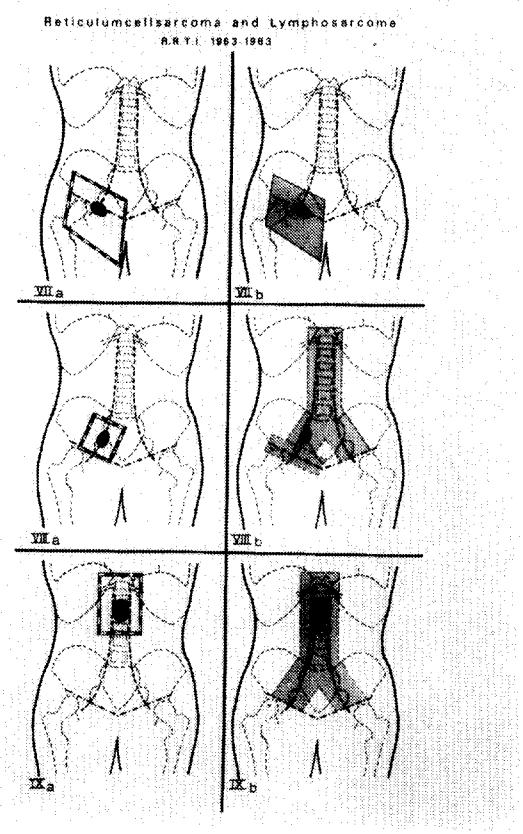


Fig. 3. In stages I_2 and II the relevant combinations of prophylactic irradiation areas are proposed.

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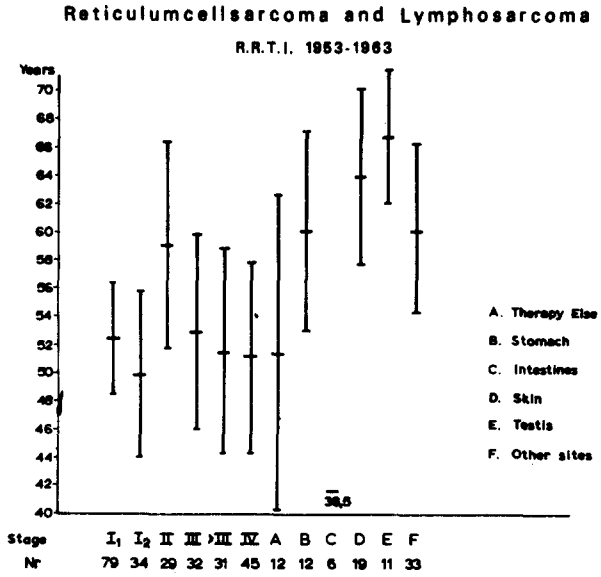


Fig. 4. Average age (with 95% confident limits) by stage and site.

bears to prognosis. On the assumption that prevention of such a 'second' lesion might promote ultimate 'cure', special attention has been paid to radiotherapeutic techniques which might be one step ahead of the disease.

RESULTS

Data relating to survival and mean age by stage and site for all cases are presented in Table 1 and Fig. 4. A survey of the course of loco-regional disease is presented in Table 2.

Local recurrence was observed eight times at the site of the primary (6%) and twice at the site of a second lesion; in both instances this became evident within two years of the first treatment (Fig. 5).

Prognosis after local recurrence was poor: only 1 out of 8 patients survived for 5 years after retreatment. No correlation was found to exist between stage and local recurrence (Table 2).

One hundred primaries and 8 secondary lesions remained cured for at least 2 years. Irradiation data obtained from these cases were used to calculate the regression line for 'local cure', depicted in Figs. 6 and 7.

Thirty-four primaries exhibited no local recurrence but were followed up for less than 2 years (owing to death) and have been excluded from our calculations as indeterminate cases.

Regional extension, diagnosed in 19 patients,

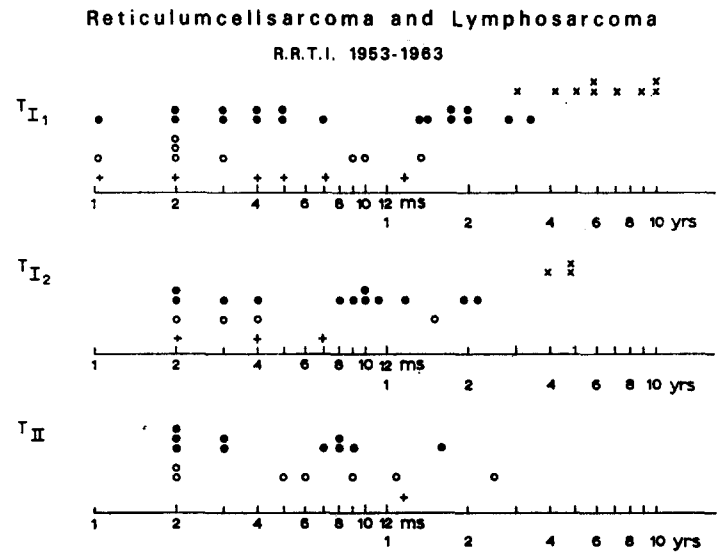


Fig. 5. Intervals between treatment of primary and second lesion in each stage.
\+ *Local recurrence* o *Regional extension*
● *Distant spread* × *Late lymphoma*

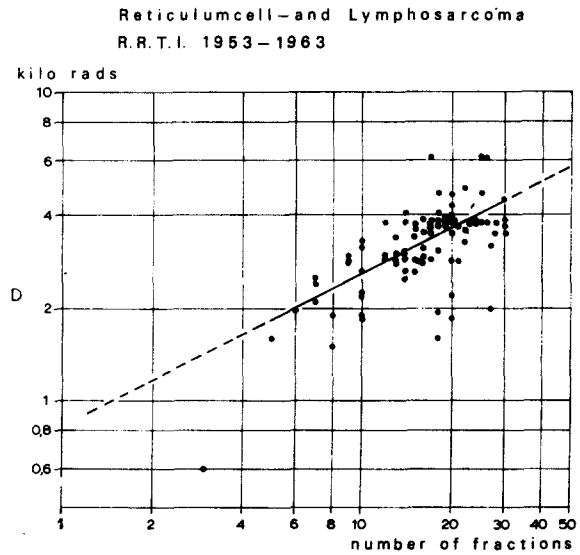


Fig. 6. Regression line for local cure.
● *Local cure*

(13·5%) became clinically evident within 2 years of the first treatment (Fig. 5) and in 5 cases coincided with local recurrence. Stage II lesions displayed the most pronounced tendency towards this type of progression (Table 2).

Prognosis for these second lesions was poor: only 2 out of 19 patients (10·5%) remained without evidence of disease for at least 5 years after a second course of radiotherapy.

Tables 3–5 show by stage for each primary site the area to which regional extension occurred. Some facts of clinical interest can be derived from these tables.

With the exception of lesions of the maxillary antrum, primary U.R.D.T. lesions stage I_1, showed no tendency to regional extension beyond the irradiated area. For technical reasons, part of the adjacent homo- and contralateral lymphatic areas had been included in all U.R.D.T. Irradiation fields (Fig. 1 I_a) with the exception of primaries of the maxillary antrum (Fig. 1 II_a). In the latter, regional extension was observed in two out of 3 instances.

In cases with lymph node involvement (U.R.D.T. stage I_2 and II, or glandular stage I_1, I_2, and II) above the diaphragm extension to adjacent unirradiated cervical and axillary regions was noted 13 times in 90 cases (14%).

In 32 cases with purely glandular primaries

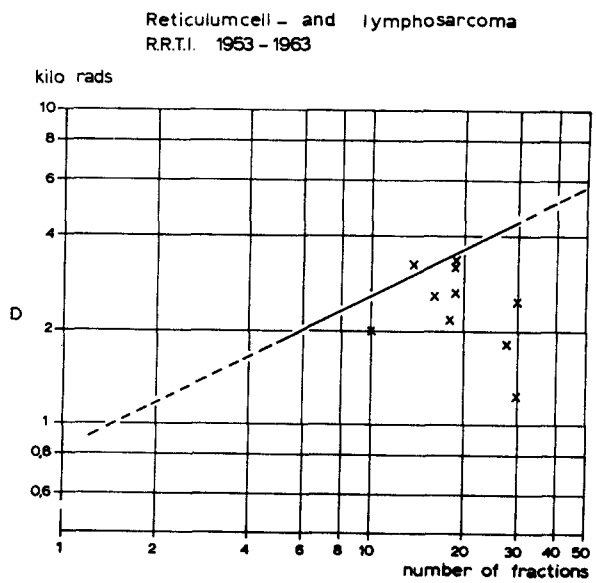


Fig. 7. Regression line for local cure as depicted in Fig. 6.
× *Local recurrence*

Table 3. *Reticulum cell sarcoma and Lymphosarcoma R.R.T.I.* 1953–1963

Stage I_1 Pattern of spread after treatment of primary

Primary		Second lesion					
		Regional ext.		Distant spread		Late lymphoma	
Site	N	N	Site	N	Site	N	Site
Tonsil	23	—	—	3	Epigastric	1	Stomach
				1	Breast + lung	1	Other tonsil*
						1	Epigastric
Base of tongue	4	—	—	1	Epigastric	1	Inguinal
Nasal cavity	3	—	—	1	Epigastric	—	—
Maxillary antrum	3	2	Bilat. cervic. (1 with loc. rec.)	—	—	—	—
Nasopharynx	3	—	—	—	—	1	Base of tongue*
High-mid cerv.	17	3	Supraclavic. (homo+ contra-lateral) (1 with loc. rec.)	1	Epigastric	1	Tonsil
				1	Breast		
				1	Lung		
				1	Liver + spleen		
				1	Axilla		
Supraclavic.	2	1	Axilla	—	—	1	Epigastric
Axilla	8	1	Supraclavic. with loc. rec.	1	Liver	—	—
				1	Epigastric		
				1	Bone		
				1	Tonsil		
Mediast.	1	1	Supraclavic.	—	—	—	—
Paraaortic	3	—	—	1	Liver	—	—
				1	Cervical		
Iliac	1	—	—	—	—	—	—
Inguinal	11	—	—	1	Hilar region	1	Other inguinal
						1	Mid cervical

*Previously irradiated with primary.

in which high cervical nodes were involved, the irradiation technique employed did not include Waldeyer's ring in the radiation field (Fig. 1, III_a), but in spite of this no regional extension to that area occurred. In cases presenting with tonsillar or lingual primaries, extension to the nasopharynx was noted only once in 65 cases (coinciding with a local recurrence), though the nasopharynx had not been included completely in the irradiation field (Fig. 1, I_a). However, it seems possible that the divergent beam of orthovoltage radiation and its scatter could have delivered sufficient dose to cover the whole ring of Waldeyer adequately.

Though chest X-rays and tomography were carried out at regular intervals, regional extension to the mediastinum was found only

Table 4. *Reticulum cell sarcoma and Lymphosarcoma R.R.T.I.* 1953–1963

Stage I_2 Pattern of spread after treatment of primary

Primary		Regional ext.		Distant spread		Late lymphoma	
Site	N	N	Site	N	Site	N	Site
Tonsil	16	1	Nasoparynx (with loc. rec.)	1	Skin	—	—
				1	liver		
		2	Mediast.	1	Epigastric		
		1	Supraclavic.+epigastric	1	Stomach		
				1	Inguinal		
Base of tongue	4	—	—	1	Kidney	—	—
				1	Stomach		
				1	Epigastric		
Maxillary antrum	2	—	—	—	—	1	Bone
Cervical glands	11	—	—	2	Liver+Spleen	1	Inguinal
				1	Stomach	1	Skin
				1	Contralat. axilla		
Iliac. aortic gl.	1	—	—	—	—	—	—

Table 5. Reticulum cell sarcoma and Lymphosarcoma R.R.T.I. 1953–1963

Stage II — Pattern of spread after treatment of primary

Primary		Second lesion			
		Regional Ext.		Distant spread	
Site	N	N	Site	N	Site
Tonsil	5	—	—	1	Spleen
Base of tongue	3	1	Axilla	1	Inguinal
Nasal cavity	1	1	Axilla	—	—
Maxillary antrum	1	1	Supraclavic.	—	—
Nasopharynx	6	—	—	2	Inguinal
				2	Liver
Bilat. cervical (+supraclavic.)	4	1	Hilar glands	—	—
Cervic. (+supraclavic)+Axill.	4	—	—	2	Inguinal
Mediast.+cervic. (+supraclavic.)	4	1	Axilla	1	Liver
		1	High cervic.	1	Epigastric
Mediast.+axill.	1	1	Supraclavic. (with loc. rec.)	—	—

3 times, although this region was adjacent to midcervical or supraclavicular lesions and consequently 'at risk' in 26 instances.

In cases presenting with inguinal primaries no extension to adjacent regions took place. Undetected extension to iliac glands (in the absence of lymphography) could be excluded because 10 out of 11 inguinal primaries (I_1) remained cured (2 after treatment of distant spread). The remaining case developed a midcervical lesion more than 6 years after treatment of the inguinal primary.

In 5 patients with abdominal para-aortic or iliac primary lesions no regional spread was found, although without lymphograms this could not be excluded except in one stage I_1 iliac-gland case which remained cured.

Distant spread, i.e. a second lesion occurring beyond the adjacent lymphatic regions of the primary, subsequent to treatment of the primary, was observed in 40 instances (28%). The majority of these (37 out of 40) became clinically evident within 2 years (Fig. 5). The incidence of distant spread did not vary significantly in the various stages (Table 2).

After retreatment by irradiation 5 out of these 40 patients remained free from evident disease for at least 5 years.

Tables 3–5 present the locations of distant lesions according to stage and site of primary.

In 126 cases with primary lesions above the diaphragm, distant spread occurred 37 times (29%). The liver–spleen–epigastric region (including the stomach), became involved in 23 instances (18%), and the inguinal region 6 times (5%). Apart from these apparent predilection sites, no pattern for distant spread could be detected.

A further group of second lesions appeared significantly later than the above-mentioned manifestations of malignant activity. The majority were diagnosed after a tumour-free interval of 5 years or more (Fig. 5). Although not coinciding with local recurrence, 2 of these involved a site not previously affected by the primary, but included in its irradiation fields. These late tumour manifestations did not occur in stage II. The incidence in stage I_1 and stage I_2 was about equal (Table 2). Prognosis was slightly better than for cases with other secondaries: 3 out of 12 (25%) remained free of disease at least 5 years after retreatment. As these lesions were considered to be clearly distinct from the other second tumour manifestations, by reason of the above mentioned characteristics, they have been called 'late lymphomas'.

From Tables 3 and 4 it is evident that no predilection sites for late lymphomas could be elicited from this material, and that no special primary localization predisposed their appearance.

DISCUSSION

From the foregoing analysis it would appear that the prognosis in loco-regional glandular and U.R.D.T. lesions of lympho- and reticulum cell sarcoma, treated by radiotherapy is apart from intercurrrent death predominantly influenced by the occurrence of local recurrence, regional extension, distant spread, and 'late lymphoma'.

If it be presumed that by precluding secondary lesions further malignancy can be forestalled, then potential 5-year survival rates can be computed for various radiotherapeutic measures which might prevent second lesions from occurring. The increase in survival rate and (in cases which are otherwise

Table 6. *Reticulum cell sarcoma and Lymphosarcoma*
Regional spread acc. to literature

	Ennuyer [3] 1963	Scheer [2] 1963	Tin Han [1] 1967	R.R.T.I. 1968
Upper resp. dig. tr. I_1 I_2 II	18/323 (8 with loc. rec.) 5%	—	—	9/74 (2 with loc. rec.) 8%
Glandular				
I_1 total	—	—	19/55* 34%	6/43* (2 with loc. rec.) 14%
I_2 Cervical only	—	13/51† 25%	14/36* 39%	4/19* (1 with loc. rec.) 21%
I_2 Total above diaphr.	—	—	8/19* 42%	0/11
I_2 Cervical only	—	—	55%	0/11

*to mediast.: 0×.
†id.: 1×(2%).
Dancot 1968: no reg. ext. to Mediastinum.

cured only after irradiation of a second lesion) the avoidance of a second course of radiotherapy, could be defined as a 'benefit'.

Prevention of local recurrence in cases of lympho-reticulum cell sarcoma ought to present no problem since this growth is usually radiosensitive. As local recurrence still accounts for a small percentage of the failures, the computed regression line for local cure (Fig. 6, 7) might contribute towards improvement of the prognosis. Most modern authors favour a tumour dose of 4000–5000 rad in 4 weeks. We were not able to find an iso-effect graph in the literature.

Only a few authors have contributed to our knowledge of regional extension. In early 'purely glandular' lesions, Tin Han [1] and Scheer [2], found a considerably smaller tendency towards regional extension in lympho-reticulum cell sarcoma as compared with Hodgkins lymphoma. The only relevant data pertaining to U.R.D.T.-lesions come from Ennuyer [3]. Figures published by these authors are in agreement with ours (Table 6).

In his retrospective study Tin Han [1] detected a certain 'reluctance' of early lympho-reticulum cell sarcoma to extend to the mediastinum. Our data confirm his findings and are in agreement with those of Scheer [2] and Dancot [4] (Table 6).

In our patients with a primary lesion above the diaphragm prophylactic irradiation of adjacent cervical and axillary areas, as indicated in Figs 1 and 2 (I_bVI_b), would theoretically have increased the 5-year survival rate by about 10% in stages I_1 and I_2 and by about

Table 7. *Reticulum cell sarcoma and Lymphosarcoma R.R.T.I.* 1953–1963
Theoretical benefit of various types of prophylactic irradiation
Stage I_1, I_2, II with primary above diaphragm

Actually applied technique			Suggested prophylactic regional irradiation		Prophylactic liver–spleen –epigastric irradiation		Prophylactic regional+ liver–spleen–sudbiaphr.– irradiation	
Stage	N	Actual 5–y. surviv.	Benefit	Pot. 5–y. surviv.	Benefit	Pot. 5–y. surviv.	Benefit	Pot. 5–y. surviv.
I_1	64	65%	8/64 (12%) *[1]	76%	9/64 (14%) *[3]	74%	17/64 (26%) *[4]	85%
I_2	33	48%	2/33 (6%)	54%	8/33 (24%) *[1]	69%	11/33 (33%) *[1]	78%
II	29	34%	6/29 (21%)	55%	5/29 (17%)	51%	16/29 (55%) *[1]	86%
			A		B		C	

*Survived 5 years after treatment of 2nd lesion.

20% in stage II (Table 7A). Such a 'restricted' prophylactic regional radiotherapy would have caused no harm or discomfort to the patient.

In cases having high cervical primaries, this analysis showed no regional extension to Waldeyer's ring, so this area has been excluded from prophylactic regional irradiation (Fig. 1 III_b).

In view of the tendency of the neoplasm to "mediastinal skip" and the hazard of mediastinal post-irradiation fibrosis, especially in elderly patients, prophylactic regional mediastinal irradiation is not proposed. Our findings suggest that it would have increased potential curability by 6% in stage I_2 and 4% in stage II, but careful tomography of the mediastinum at regular intervals would probably provide sufficient security.

In our material no regional extension could be detected in cases with inguinal primaries stage I_1. Consequently no regional prophylactic irradiation is advocated in such cases (Fig. 3, VII_b).

In the absence of lymphographic information in cases having iliac and para-aortic abdominal primaries it is probably advisable to employ regional prophylactic irradiation (Fig. 3, $VIII_b$, IX_b).

Vera Peters [5] found that no advantage appeared to be gained from prophylactic regional irradiation in two non-randomized series. Kaplan [6] is running a clinical trial in order to assess the value of prophylactic regional radiotherapy. Dancot [7] improved prognosis considerably by applying both prophylactic regional irradiation and higher dose. His 5-year survival rates after this new technique are in agreement with ours and with those of other authors who did not practice regional prophylaxis (Table 8).

No results of attempts to prevent regional extension by prophylactic chemotherapy have so far been published. Since irradiation is more effective on tumour tissue than chemotherapy and virtually without effect on healthy tissues beyond the irradiated area, radiotherapeutic prophylaxis would theoretically seem to be superior to chemotherapy.

Distant spread favoured the liver-epigastrium-spleen area. By prophylactic irradiation of this region in cases with primaries above the diaphragm, a potential cure-rate is achieved similar to that after "restricted" prophylactic regional irradiation (Table 7B). If irradiation dosage to the liver does not exceed 3000–3500 rad in 3–4 weeks minimal untoward effects may be anticipated [8].

The combination of these two prophylactic irradiation measures would theoretically double the benefit and the increment of 5-year survival rate. If this combined radiotherapy were extended to include the subdiaphragmatic and inguinal glandular areas, then potential survival rate would rise to about 80% (Table 7C). This would appear to be the theoretical upper limit of possible radiotherapeutic achievement. Such a super-radical prophylactic irradiation scheme has been judiciously practised by Kaplan [9] in Hodgkin's disease. From table 7C it is apparent that stage II cases will profit most from this irradiation modality. Possibly, being a stage II growth, i.e. one exhibiting contiguous spread, indicates a biological characteristic of the tumour to spread in an orderly manner, which fits into an irradiation scheme. Also, the absence of 'late lymphomas' in this stage contributes to the effectiveness of such super-radical irradiation.

The cause of 'late lymphomas' remains a matter for speculation. The very characteristics mentioned above, which would appear to distinguish them from other second lesions, bar them from prophylactic radiotherapy: for example their unpredictable siting and their possible appearance in previously irradiated areas. It is not known whether prophylactic chemotherapy can prevent their development, but in any case since late lymphomas tend to appear 5 years and more after the first treat-

Table 8. *Reticulum cell sarcoma and Lymphosarcoma*
5–y. survival acc. to literature

	Ennuyer [3] 1963		Scheer [2] 1963		Dancot [4] 1966 'Ancienne'		Dancot [4] 1966 'Nouvelle'		R.R.T.I.	1968
Upp. resp.–dig. tr.										
I_1	41/75	51%	15/20	75%	10/21	47%	17/26	65%	24/36	67%
I_2	47/122	38%	—		—		—		8/22	36%
I_2+II	60/198	30%	—		1/32	3%	7/34	21%	13/38	34%
Glandular										
I_1	—		52/78	67%	18/47	17%	8/10	80%	28/43	65%
I_2+II	—		—		3/40	7%	3/15	20%	14/25	56%

ment prophylactic chemotherapy would probably have to be maintained for a period longer than is generally considered advisable.

Meticulous follow-up during at least ten years will lead to early detection of these lesions, and adequate irradiation then might still provide a cure.

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RESUME

Dans une étude rétrospective de 142 *cas de formes leuco-régionales de lympho-réticulosarcomes, des données ont été recueillies concernant les récidives locales, l'extension régionale, les métastases à distance et les lymphomes tardifs. D'après ces données, quelques schémas thérapeutiques ont été considérés, et leur bénéfice théorique évalué.*

SUMMARY

In a retrospective study of 142 *cases of loco-regional lymphoreticulum cell sarcoma data have been collected concerning survival, local recurrence, regional extension, distant spread, and 'late lymphomas'. As the result of our findings some radiotherapeutic approaches have been envisaged and their theoretical advantage evaluated.*

ZUSAMMENFASSUNG

Hunderzweiundvierzig Fälle von leuko-regionalen Retikulumzell- und Lymphosarkomen wurden retrospektiv auf Prognose, 1. *R., r. A., F. und 'späte Lymphome' untersucht. Auf diesen Ergebnissen fussend, wurden einige Schemata der Strahlentherapie entwickelt und ihr Vorteil theoretisch erörtert.*

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Cellular Detachment by Purified Lysosomal Cathepsin B

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CELLULAR detachment from glass and other substrata is produced by different proteinases such as trypsin [1], pronase(s) [2] and collagenase(s) [3]. Short time incubation in weak trypsin solutions (0·05–0·2%) has become an established method for cell separation although this involves some temporary damage to cell surfaces by removing part of the protein coating rich in *N*-acetyl neuraminic acid [4]. It is also known that dead cells become rapidly digested by trypsin treatment [5], and that cells first treated by a chelating agent or a non-ionic detergent become more vulnerable to a subsequent trypsin treatment [6].

These various proteinases either do not occur in normal mammalian tissues and tumours or else thay are effectively inhibited, like trypsin, by specific inhibitors in blood and tissue fluid. It is therefore of interest to demonstrate that a normally occurring cellular hydrolase like cathepsin B, once in a free and active state outside the cytoplasm, will exert a marked capacity to detach cells. This enzyme has now been found to exert a cell surface effect qualitatively very similar to that of trypsin.

MATERIAL AND METHODS

For the study of cellular detachment the C13 hamster fibroblasts (Stoker line) were grown as still cultures in Eagle's basal medium with 10% fetal calf serum added. All controls and short-time incubations were performed in calcium- and magnesium-free Hank's solution (CMFH) at a carefully checked pH of 7·1 (no serum albumin added). Crystalline trypsin (Worthington) and salt-free lyophilized cathepsin B was used at concentrations mentioned below. This enzyme hydrolyses similar C–N bonds as trypsin does, and is activated [7, 8] by chelating agents like EDTA or lactic acid+ added -SH compounds like cysteine or glutathione (GSH), but is inhibited by added heavy metal ions (Mn^{2+} and Mg^{2+}). This enzyme has been isolated by Snellman [9] from lysosomal concentrate obtained from calf liver and was supplied in a highly purified state without admixture of cathepsin D, aminopeptidases or other enzymes. Proteinase activity was maximum at pH 5·8, falling to one third at pH 7. Cathepsin B could not be activated in the usual way by addition of 0·05 M EDTA+0·004 M cysteine since the cells would deteriorate. Hence, the environmental conditions in tissue fluids, particularly of solid tumours, were mimicked; instead of EDTA a weak chelation was applied by addition of 0·2% lactic acid to CMFH and cysteine was replaced by 0·16% GSH. The acid pH shift was corrected by added NaOH until full stability was reached for 1 hr. Only a fraction of the digestive capacity of cathepsin B was thus utilised at a stable pH of 7·1 as compared with the much higher proteinase activity of similar amounts of added trypsin.

RESULTS

C13 fibroblasts subcultured on coverslips for 24 hr in Petri dishes presented a rather dense

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felt-like monolayer with long pseudopodia and slender cell bodies; all cells were firmly attached to the glass. Such cultures were just washed in CMFH and transferred to cavity slides for enzyme treatment applying about 0·25 ml enzyme solution to each monolayer. The results were very obvious and qualitatively registered under the phase-microscope with green light at magnifications up to 500 times. The results summarized in Table 1 were similar using either trypsin or activated cathepsin B. Inside 3–10 min all pseudopodia were withdrawn, the cells rounded up, became short, plump, strongly refractile and most cells left their attachment to the coverslips. Cellular aggregation due to stickiness was less marked with cathepsin B than following trypsin treatment. After 20–30 min treatment no further changes were noticed. It was not established whether this was due to deterioration of enzyme activity (both enzymes are rather instable). An interesting fact was that the cathepsin B effect was reversible at an enzyme concentration up to 1·5 mg/ml. When returned to their original growth medium the cells attached to glass again, became elongated and fully resumed their original growth pattern in about 5 hours' time.

The actual percentage of cell death, as ascertained by the Lissamine green dye-exclusion test [10], was not increased after 30 min treatment with cathepsin B while trypsin treatment at a concentration of 1·5 mg/ml. caused both cell death and some cytolysis.

Another question was whether cathepsin B migh digest damaged and dead cells as observed following trypsin treatment. This was tested on suspensions of ELD tumour cells in CMFH recently killed by repeated freezing and thawing. Several series of cells counts were made at frequent intervals following the addition of trypsin and activated cathepsin B at the same concentrations. Cathepsin B was equally effective as trypsin; dead cells permeable to the dye were digested at a similar very rapid rate inside 10 min by added cathepsin B, but in addition cells less damaged and not yet permeable to the dye also became digested by the more powerful trypsin activity but not by added cathepsin B. Both suspensions were rich in ghosts and cell remnants. The effect of extraneous cathepsin B was most likely increased by activation of the lysosomal proteinase in damaged cells, since the rate of cytolysis became markedly increased also in cases where only lactic acid+GSH were added. Hence, the percentage of living cells, as determined by the dye-exclusion test was overestimated, owing to digestion of the dead cells by cathepsin in 30 min.

COMMENTS

The experiments demonstrate that a normal intracellular proteinase with an activity extending above pH 7 once released and activated will bring about both cellular detachment and rapid digestion of damaged cells. The digestive capacity of liver cathepsins during autolysis was studied by Sawant *et al.* [11]. Cellular

Table 1. *Cellular detachment at different concentrations of added Trypsin and activated Cathepsin B*

pH of incubation medium carefully maintained at 7·1

Test solutions	Changes of C13 fibroblasts
Controls	
In CMFH only	No changes in shape for 60 min
In CMFH+0·2% lactic acid added	Some rounding up, no detachment
In CMFH+L. acid+0·15% GSH added	Some retraction of pseudopodia, but no detachment
Crystalline trypsin added	
0·05% in CMFH	Slow and partial detachment of cells
0·1% and 0·15% in CMFH	Rapid pseudopodia retraction in a few minutes; most cells detached
Purified cathepsin B added	
In CMFH containing L. acid and GSH for activation+0·05% cathepsin B	Partial detachment of cells and general pseudopodia retraction
0·1% and 0·15% cathepsin B	Rapid and complete pseudopodia retraction in 3–10 min. Almost all cells lost attachment to glass

detachment was cursorily tested by Leon Weiss who used an impure and non-activated lysosomal concentrate [12]. Present results with pure cathepsin B thus open new and important avenues of approach into several fields of pathology notably with a view to the study of cell detachment associated with the invasiveness of tumours, the chemical mechanisms of their destructive activity, and possibly to metastasis formation (*cf.* references 13 and 14). The investigations are being continued in order to obtain more quantitative *in vitro* data [15] and in order to study the topical distribution in tumours of cathepsin B with histochemical [8, 16] and biochemical micromethods [17].

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RESUME

Ce travail décrit l'action in vitro *de la cathepsine B hautement purifiée, activée par l'acide lactique et le glutathion. L'enzyme active avait des effets très semblables à ceux de la trypsine; elle provoquait rapidement un détachement des cellules des surfaces de verre et une digestion des cellules mortes. La signification de ces propriétés est discutée en tenant compte du concept selon lequel le détachement des cellules tumorales serait un facteur essentiel dans la formation des métastases.*

SUMMARY

In vitro *studies are reported using highly purified cathepsin B which was activated by added lactic acid+glutathione. The active enzyme presented effects very similar to those of added trypsin; detachment of cells from glass surfaces as well as digestion of dead cells was rapidly achieved. The apparent implications are discussed with a view to the detachment of tumour cells forming an essential prerequisite to metastasis formation.*

ZUSAMMENFASSUNG

Hochgereinigtes Kathepsin B wurde durch Zugabe von Milchsäure und Glutathion aktiviert. Das aktive Enzym bewirkte in vitro *Effekte, die denen von Trypsin ähnlich sind. Loslösung von Zellen vom Glase konnte ebenso wie die Verdauung toter Zellen schnell erzielt werden. Die Bedeutung der Befunde wird im Hinblick auf die Loslösung von Tumorzellen diskutiert, die eine notwendige Voraussetzung der Metastasenbildung ist.*

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Induction de Tumeurs Mammaires par Injections d'Extraits Acellulaires de Leucémies PS à des Souriceaux d'autres Souches

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LA SOUCHE de souris PS, actuellement à la 34e génération de croisements consanguins, est caractérisée, chez les femelles, par l'apparition spontanée de tumeurs mammaires et de leucémies [1]. L'association, dans la même souche de souris, de ces deux types de malignité est exceptionnellement signalée [2].

On a cherché à préciser, par des expériences d'inoculations d'extraits acellulaires de tumeurs mammaires ou de leucémies, l'étiologie virale des manifestations malignes de la souche, et à isoler le ou les agents viraux oncogènes éventuels. Les résultats ont été contradictoires: les extraits acellulaires de tumeurs mammaires ne produisant que des leucémies chez les souris Swiss [3], tandis que les souris 'tout venant' présentaient des tumeurs mammaires et des leucémies avec le même extrait, quel que fût le type histologique de la tumeur ayant servi à sa préparation [4].

Le présent travail rapporte les résultats d'inoculations d'extraits acellulaires de tissus leucémiques de la souche PS à des nouveau-nés d'autres souches. Ici, c'est à l'apparition de tumeurs mammaires que l'on assiste. De ce fait, l'obtention d'un 'passage' régulièrement actif, tel que l'a réalisé Gross [5, 6], n'a pas été possible.

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Ces expériences soulèvent le problème des relations étiologiques entre les tumeurs mammaires et les leucémies de la souche PS.

MATERIEL ET METHODES

Les organes leucémiques de la souche PS proviennent:

de 3 leucémies spontanées,

de 4 radioleucoses,

de 3 premiers passages de leucémies sur souris Swiss, induits par extraits acellulaires de tumeurs mammaires de la souche PS,

de greffes de leucémies utilisées aux premier et deuxième passages sur souriceaux isologues. Les ganglions et rates leucémiques sont inoculés, au trocart, par voie souscutanée axillaire. Une leucémie généralisée s'observe de 10 jours à 3 semaines après l'inoculation, avec ou sans tumeur au point d'injection.

Les extraits acellulaires sont confectionnés selon les méthodes de Gross [6] ou de Moloney [7].

Les gradients de concentration, préformés de citrate de potassium, sont préparés par superposition de solutions de densité décroissante, puis laissés 12 heures à 4°C pour permettre la diffusion; 0,4 ml d'un extrait acellulaire de leucémies, préparé selon la technique de Moloney, sont déposés au sommet du gradient. L'ultracentrifugation (Spinco L, rotor SW 40) est effectuée, pendant 3 heures, à 35.000 *t*/min, selon la technique de O'Connor et Zeigel [8]. Les fractions du gradient corres-

pondant aux zones visibles (Fig. 1) sont collectées par ponction du fond du tube, puis centrifugées à 28.000 *t*/min., pendant 15 min. Le culot obtenu est homogénéisé dans 1 ml de soluté isotonique (NaCl 0,16 M). C'est la zone dense de densité de flottaison 1,19 qui sert à pratiquer les tests biologiques; dans un cas cependant, cette zone a été mélangée à la zone opalescente susjacente.

Les animaux injectés: les extraits acellulaires de tissus leucémiques et les zones de gradients, obtenues après ultracentrifugation de certains de ces mêmes extraits, sont inoculés à 186 souriceaux: Swiss/Gif, BALB/c/Gif, C3H/eB/Gif et PS. L'injection est faite par voie intrapéritonéale, à raison de 0,2 ml environ par souriceau, dans les heures suivant la naissance, jusqu'à 5 jours au plus tard.

Les témoins sont constitués par des souris de souche:

Swiss/Gif: 41 femelles et 17 mâles non inoculés,

BALB/c/Gif: 46 femelles et 27 mâles non inoculés,

C3H/eB/Gif: 4o femelles et 20 mâles non inoculés,

PS:—pour les femelles, un relevé des tumeurs spontanées a été établi sur 234 femelles de la 25e génération, contemporaine des animaux PS injectés. Il été calculé d'une part globalement, d'autre part en fonction de leur parité.

—pour les mâles, 39 mâles des 21e et 25e générations ont été observés jusqu'à 15 mois.

Les souris sont placées par cage de 10, sans séparation des sexes. Elles sont alimentées suivant le régime de Haddow. Eau ad libitum.

Tous ces animaux sont maintenus en observation jusqu'à l'apparition de tumeurs, ou jusqu'à l'altération de l'état général. Les souris sont alors tuées. Une autopsie est ensuite pratiquée, s'accompagnant du prélèvement systématique des foie, rate, reins et organes tumoraux. La fixation est faite au Bouin-Hollande, la coloration à l'hématoxyline, phloxine, safran.

RESULTATS

Sur les 186 souriceaux inoculés, il ne sera fait état ici que de 102 animaux ayant survécu 9 mois. Il s'agit de 24 souris BALB/c/gif, 6 C3H/eB/Gif, 28 Swiss/Gif et 44 PS.

1. Le Tableau 1 montre globalement, en fonction du sexe, les types de malignité observés chez les souris BALB/c/Gif, C3H/eB/Gif et Swiss/Gif, inoculées avec les extraits acellulaires de la souche PS, ou avec leurs fractions partiellement purifiées après ultracentrifugation sur gradients de citrate de potassium.

Tableau 1. Tumeurs induites par extraits accellulaires de leucémies PS sur nouveaunes d'autres souches (résultats) globaux

Sexe	Nombre d'animaux	Tumeurs mammaires	Tumeurs pulmonaires	Tumeurs diverses	Negatifs	Malignité (%)
Femelles	39	19* AM=16 48,7%	5 AM=17,3	1 AM=18,8	14	64
Mâles	19	0	2† AM=20	2 AM=17	15	21

AM=âge moyen en mois.

*A trois reprises, la tumeur était associée à un autre type de malignité: tumeur pulmonaire, hépatome, leucémie.

†Un mâle était porteur d'une tumeur pulmonaire associée à une leucémie.

Dix-neuf femelles sur 39 ont présenté des *tumeurs mammaires*, soit 48,7%, à un âge moyen de 16 mois. Il s'agit de tumeurs siégeant au niveau d'une seule mammelle, sans métastases pulmonaires. Trois d'entre elles sont associées à un autre type de malignité: une tumeur pulmonaire, un hépatome, une leucémie.

Du point de vue histologique, elles revêtent les types A et B de Dunn, où l'on peut voir des formations acineuses et des boyaux de cellules glandulaires indifférenciées (Fig. 2). Parfois, la tumeur présente des dilatations kystiques (Fig. 3). Enfin, à deux reprises, des formations épithéliales en bulbes d'oignons (Fig. 4), caractéristiques de l'adéno-acanthome, ont permis de classer ces néoplasmes dans le groupe C de Dunn [9].

Les tumeurs diverses sont représentées par un hépatome, un fibrosarcome du flanc et une tumeur parotidienne. L'âge moyen d'apparition est de 17,5 mois. Les tumeurs pulmonaires, survenant tardivement (âge moyen: 18,5 mois),

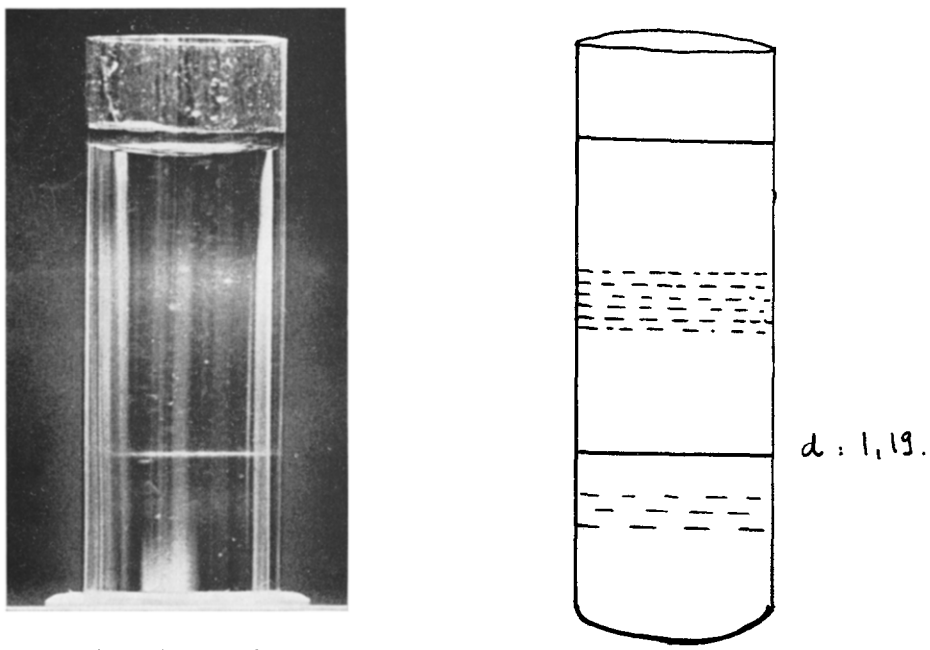
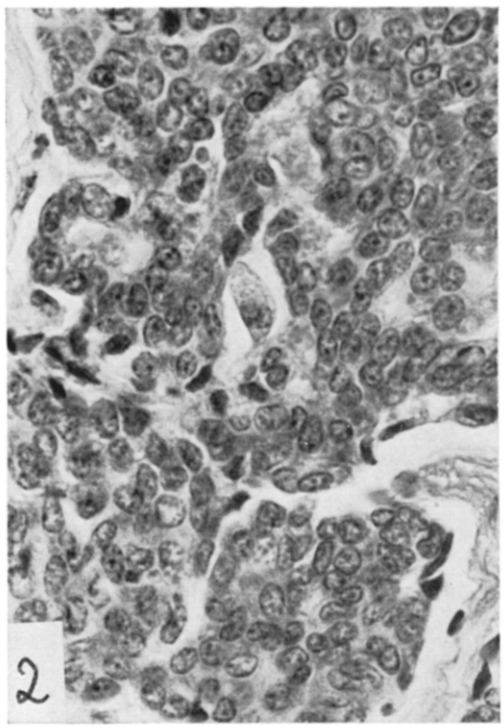
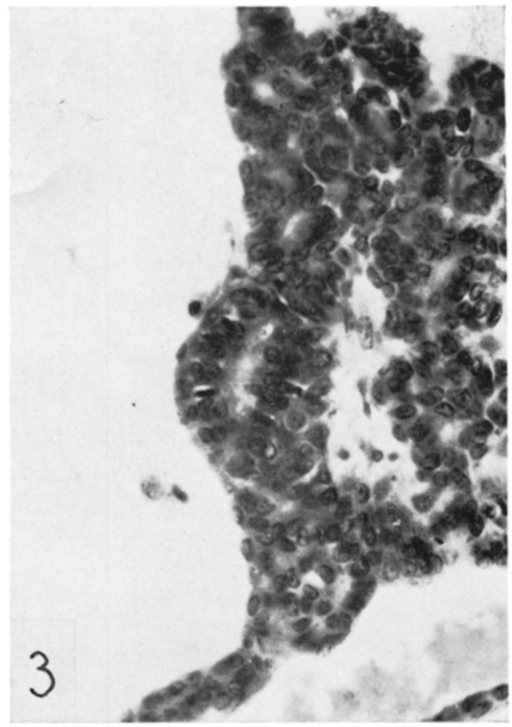
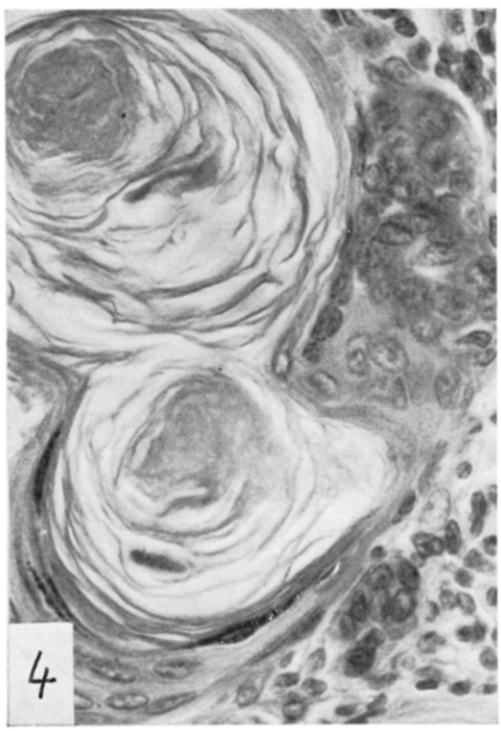


Fig. 1. *Aspect des zones observées après ultracentrifugation d'extrait acellulaire de leucémie sur gradients préformés de citrate de potassium.*

(to face p. 564)







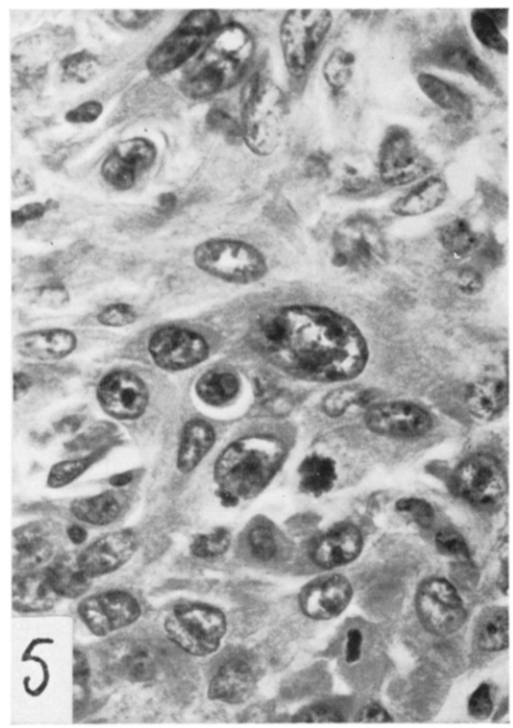


Fig. 2. *Adénocarcinome mammaire de type A de Dunn, survenu chez une femelle C3H/eB à l'âge de* 14 *mois, inoculée dans les* 24 *heures suivant la naissance avec un extrait acellulaire de leucémie PS.* (*Gr* × 500).

Fig. 3. *Adénocarcinome mammaire de type B de Dunn. Le tissu carcinomateux bien différencié est contigu à une vaste dilatation kystique. La femelle BALB/c, âgée de* 13 *mois, avait été inoculée, à la naissance, avec la zone de gradient obtenue après ultracentrifugation d'un extrait acellulaire leucémique sur gradient de potassium.* (*Gr* × 320).

Fig. 4. *Adénoacanthome mammaire, survenu, à l'âge de* 18, 5 *mois, chez une souris BALB/c, a inoculée à la naissance avec la zone de gradient obtenue après ultracentrifugation sur gradient de Ficoll d'un extrait acellulaire leucémique.* (*Gr.* × 320).

Fig. 5. *Sarcome mammaire survenu, à l'âge de* 17 *mois, chez une souris Swiss inoculée à la naissance avec un extrait acellulaire leucémique.* (*Gr* × 500).

Tableau 2. Tumeurs induites par extraits acellulaires de leucemies PS en fonction des souches inoculées

Souche	Tumeurs mammaires	Leucémies	Tumeurs pulmonaires	Tumeurs diverses	Négatifs	Malignité (%)
BALB/c 16 ♀, 8 ♂	10 AM=15, 3 62, 5%	—	—	2 AM=15, 2	12	50 AM=15,3
Témoins 42 ♀, 27 ♂	—	—	—	—	73	0
C3H/eB 6 ♀	4 AM=14, 3 66%	—	—	1 AM=17, 5	1	83,3 AM=14,9
Témoins 46 ♀	1 AM=32 2,1%	—	—	—	45	2,1 AM=32
Swiss 17 ♀, 11 ♂	— 4 AM=16, 5 23%	1+TM−AM 18* 1+TP−AM 20	8 AM=18, 7 28, 5%	1 AM=19	15	46 AM=16,5
Témoins 41 ♀, 17 ♂	3 AM=14 7, 3%	1 AM=20	4 AM=16,6 6,8%	1 AM=17	49	15,5 AM=15,8

AM=âge moyen en mois, TM=tumeurs mammaires, TP=tumeurs pulmonaires.

*Ces deux animaux ont été comptés respectivement dans le groupe des tumeurs mammaires et des tumeurs pulmonaires.

ne sont rencontrées que chez les souris Swiss.

Les leucémies sont exceptionnelles: elles ne sont observées que chez 2 souris Swiss, associées dans les deux cas à une autre forme de malignité: tumeur mammaire et tumeur pulmonaire. Leur âge d'apparition est de 19 et 20 mois.

Ces résultats sont exprimés en fonction des souches de souris injectées, au Tableau 2. Le pourcentage élevé de tumeurs mammaires domine celui des autres malignités. Remarquable pour les souches BALB/c/Gif (62,5% contre 0% chez les témoins, et les C3H/eB/Gif: 66% contre 2,1% chez les témoins), il est moins spectaculaire chez les souris Swiss où il passe de 7,3% chez les témoins à 23% chez les souris injectées, l'âge moyen d'apparition étant curieusement retardé.

Le Tableau 3 représente les malignités observées chez les animaux BALB/c/Gif et Swiss/Gif survivant à l'inoculation de fractions partiellement purifiées, obtenues après ultracentrigation des extraits leucémiques sur gradients. Ici encore, le pourcentage de tumeurs mammaires est élevé (63%) et les leucémies absentes. Il paraît intéressant de confronter les résultats obtenus avec un extrait acellulaire de leucémie et les effets de ce même extrait après ultracentrifugation sur gradients de citrate de potassium et inoculation de la zone de densité 1,19 obtenue (Tableau 4). Ces résultats ne sont que mentionnés ici, du fait du petit nombre d'animaux survivants. Ils ont le mérite de montrer l'activité tumorigène des extraits leucémiques partiellement purifiés

Tableau 3. Tumeurs induites par injection de 'zones' de gradients obtenues après ultracentrifugation d'extraits acellulaires de leucémie de la souche PS, à des souriceaux BALB/c et Swiss

Souche	Nombre d'animaux	Tumeurs mammaires	Leucémies	Tumeurs diverses	Tumeurs Pulmonaires	Negatifs	Malignite (%)
BALB/c et Swiss	16 (11 femelles) (5 mâles)	7 AM=15,1 63%	0	2 AM=15,2	1	6	62%

AM=âge moyen en mois.

Tableau 4. Comparison entre la malignité produite par un extrait acellulaire de leucémie et par la zone obtenue par ultracentrifugation du même extrait sur gradient de citrate de potassium

	Femelles			Mâles		
	Tumeurs mammaires	Tumeurs pulmonaires	Négatifs	Tumeurs pulmonaires	Tumeurs diverses	Négatifs
Extrait acellulaire	1	1	—	2	1	3
Gradient	4	1	1	—	—	2

par ultracentrifugation sur gradients. Malheureusement, il n'y a eu que 2 femelles parmi les animaux survivant à l'injection de l'extrait acellulaire ordinaire correspondant.

2. Les resultats obtenus, chez les 44 souriceaux PS inoculés avec des extraits acellulaires de leucémies isologues, sont consignés au Tableau 5. Trente-six d'entre eux ont été injectés avec des extraits acellulaires de greffes leucémiques. Encore une fois, ce sont les tumeurs mammaires qui retiennent l'attention. Si leur pourcentage s'est élevé de façon peu significative (72,4% contre 62,5%), l'âge moyen d'apparition s'est considérablement abaissé, 6,4 mois contre 10,3 mois.

L'augmentation du pourcentage de leucémie chez les femelles est faible (10,3% contre 3%). Elle est plus nette chez les mâles (20% contre 7,6%); mais ici encore, c'est l'abaissement de l'âge moyen d'apparition qui est significatif dans les deux sexes: 6,6 mois contre 10 mois et 7,6 mois contre 9 mois respectivement.

Les tumeurs diverses sont représentées par un sarcome du flanc et deux sarcomes rénaux.

DISCUSSION

La souche de souris PS, actuellement à la 34e génération de croisements entre frère et soeur, est caractérisée chez les femelles, par une incidence tumorale globale, sans tenir compte de la parité, de 64,5% à la 25e génération, répartie en 35,8% de tumeurs mammaires, 22,8% de leucémies, 4,2% de tumeurs mammaires associées à une leucémie et 1,7% de tumeurs diverses.*

La présente étude avait pour but d'établir un 'passage suractivé' PS, comme l'a obtenu Gross [5, 6], par injections d'extraits acellulaires de tissu leucémique PS ou des fractions obtenues après ultracentrifugation sur gradients de concentration, à des souriceaux de souches C3H/eB, BALB/c, Swiss et PS.

Le pourcentage de tumeurs mammaires, observé chez les souris BALB/c et C3H/eB

*Les chiffres, établis à l'occasion de ce travail, diffèrent de ceux de publications antérieures et portant sur la 13e génération [1]. Le taux de leucémies est passé de 33% à 22,8%. L'étude de cet abaissement très important fera l'objet d'un travail indépendant.

Tableau 5. Effets de l'injection d'extraits acellulaires d'isogreffes leucémiques PS sur nouveau-nés de la même souche

Souche PS	Tumeurs mammaires	Leucémies	Tumeurs diverses	Négatifs	Malignité (%)
Femelles Accouplées Injectées 29	21 AM=6,4 72,4%	3 AM=6,6 10,3%	3 AM=17,5	2 AM=10	86, 9 AM=6,6
Femelles Accouplées Témoins 56	35 AM=10,3 62, 5%	2 AM=10 3%	—	19 AM=10,3	65,5 AM=10,3
Mâles Injectés 15	—	3 AM=7,6 20%	—	12 AM=9,8	23 AM=7,6
Mâles Témoins 39	—	3 AM=9 7,6%	—	36 AM=14,7	7, 6 AM=9

AM=âge moyen en mois.

(respectivement 62,5% et 66%), après injection d'extraits acellulaires de leucémie, mérite de retenir l'attention. Leur âge d'apparition s'échelonne entre 10,5 et 19 mois, les plus précoces étant apparues après injection d'extraits purifiés sur gradients de concentration. Par leur nombre et leur âge d'apparition, elles se distinguent des tumeurs pulmonaires observées sur souche Swiss seule et des tumeurs diverses survenant à un âge plus avancé.

Le type histologique des tumeurs mammaires les classe, pour la majorité d'entre elles, dans les catégories A et B de Dunn [9]. Murray admet que ces deux groupes histologiques correspondent à des tumeurs où intervient le facteur lacté, alors que les fibrosarcomes, les hémangioendothéliomes, les adénoacanthomes, d'apparition plus tardive, relèveraient d'une autre étiologie [10].

L'inoculation d'extraits acellulaires de tissus leucémiques à des souriceaux PS a entraîné un raccourcissement très significatif du temps d'apparition des tumeurs mammaires (6,4 mois contre 10,3 mois), deux fibrosarcomes des reins ont également été constatés chez les femelles. Dans les deux sexes, l'incidence leucémique a augmenté tandis que l'âge d'apparition s'abaissait de façon significative.

Les résultats obtenus auraient pu dépendre de l'âge d'inoculation des nouveau-nés. On sait que le choix de la période de tolérance immunitaire a été décisive dans les résultats obtenus par Gross et que, passé 24 heures, le temps de latence est considérablement allongé [11]. Par contre, pour le facteur lacté, l'inoculation peut avoir lieu dans des délais beaucoup plus longs (14 jours ou plus). De fait, ici, aucune différence n'a été observée entre les animaux inoculés dans les 24 heures et ceux injectés plus tardivement, ce qui a autorisé à les grouper tous dans cette même étude.

L'induction de tumeurs mammaires par extraits acellulaires de leucémie n'est signalée qu'à propos du virus du Polyome. Elles ne sont qu'une des manifestations d'un polymorphisme tumoral où dominent les tumeurs parotidiennes, thymidiques, rénales etc. L'âge d'apparition est précoce. Le nouveau-né âgé de plus de 24 heures résiste à l'action du virus [12–14]. Les tumeurs diverses, peu nombreuses et d'apparition tardive, signalées ici, ne peuvent être retenues. Néanmoins, pour écarter l'intervention du virus du Polyome dans la génèse des tumeurs mammaires précoces observées, des extraits acellulaires ont été confectionnés à partir de ces dernières, puis inoculés sur fibroblastes d'embryons de souris. Aucun effet cytopathogène n'a été observé aux premier et deuxième passages, après 4 semaines de cultures. Les animaux inoculés avec les milieux de cultures sont restés indemnes de tumeurs [15].

Enfin, le virus de Polyome ne provoque pas l'apparition de leucémies [16]. Or, ici, le pourcentage de ces tumeurs a cependant augmenté, et l'âge moyen d'apparition diminué, aussi bein chez les femelles que chez les mâles PS inoculés avec des extraits acellulaires de leucémies.

L'identification du facteur lacté dans la souche PS repose sur l'observation de multiples particules virales de types A et B dans les cellules mammaires tumorales [17, 18]. Des tests biologiques d'allaitement croisé ont confirmé ces observations: 18 nouveau-nés C3H/eB, sans facteur lacté, ont été nourris par des mères PS. L'incidence de tumeurs mammaires a été de 37,5% à un âge moyen de 9,9 mois et de 85% à un âge moyen de 10 mois, à la génération suivante [15].

On connaît l'activité tumorigène du sang de souris possédant le facteur lacté. Signalée par Woolley, Law et Little [19], elle est confirmée par Bittner [20] qui observe une activité tumorigène dans le plasma de souris porteuses de tumeurs mammaires. Pour Hummel et Little [21], la présence du facteur lacté serait liée à la fraction figurée. Nandi, De Ome *et al.* [22, 23] précisent que c'est aux globules rouges qu'est liée l'activité tumorigène et que seules les femelles en lactation présentent le facteur lacté dans leur plasma. Mühlbock signale enfin que la rate est une importante source de virus [24].

L'intervention du facteur lacté véhiculé par le sang, et dès lors présent dans les organes hématopoïétiques leucémiques, pourrait expliquer les résultats rapportés ici. Il serait plus actif que l'agent présent dans les tumeurs mammaires dont les extraits injectés à des nouveau-nés isologues n'ont pas modifié l'incidence tumorale spontanée de la souche [25]. Ces constatations sont en discordance avec d'autres observations signalant que la production de tumeurs mammaires est plus importante par le lait et les tumeurs mammaires que par le sang [21, 22, 26].

Les différentes souches utilisées ont présenté une susceptibilité variable à l'agent inoculé, les souris PS étant les plus réceptives, suivies par les C3H/eB, les BALB/c et enfin les Swiss.

Ce raisonnement ne saurait s'appliquer à l'augmentation de l'incidence des leucémies et la diminution de leur temps de latence, observées chez des animaux isologues injectés avec des extraits acellulaires de greffes.

Le problème des relations étiologiques entre tumeurs mammaires et leucémies se trouve posé par les résultats présentés ici et par une expérience récente de greffes de tumeurs mammaires où, au 3e passage, tous les animaux inoculés ont présenté une leucémie généralisée 11 jours après l'injection de cellules tumorales mammaires [27].

Si ces résultats sont le fait de l'association, dans la souche PS, du facteur lacté et d'un virus leucémigène, il est surprenant que de pareilles observations n'aient pas été faites à propos d'autres souches de souris, puisque la coexistence de ces deux virus a été signalée par différents auteurs.

Squartini a montré que les deux virus sont présents dans la souche de souris R III, bien que par interférence virale, seul le facteur lacté se traduise par l'apparition de tumeurs mammaires [28].

A diverses reprises, des virus leucémigènes ont été isolés de tumeurs mammaires spontanées [29] induites par carcinogènes chimiques [30] et de tumeurs mammaires en cultures [31] sans que le virus leucémigène n'ait eu auparavent de traduction clinique. Par contre, mis à part le virus du Polyome, on ne trouve pas mention dans la littérature, à notre connaissance, d'induction de tumeurs mammaires par extraits acellulaires de leucémies: rappelons que Gross a effectué une partie de ses 'passages' sur souris C3H porteuses du facteur lacté.

En ce qui concerne la souche PS, si la microscopie électronique en objectivant des particules A, B et C suggère l'existence de deux agents étiologiques exceptionnellement associés chez la même souris, elle ne renseigne pas sur les facteurs qui déterminent l'expression pathologique d'un des agents plutôt que celle de l'autre. On peut se demander si les particules virales en question n'ont pas une parenté beaucoup plus étroite que ne laissent supposer les descriptions morphologiques et si, en particulier, la ou les particules A [32–34] ne seraient pas des précurseurs à potentialité multiple qui prendraient leur virulence spécifique en fonction de la cellule dont elles sont issue. Cette hypothèse est étayée par l'existence d'un antigène commun aux leucémies et au facteur lacté dans la souche DBA/2 [35].

Des recherches ultérieures tenteront d'étayer ces hypothèses de travail.

RESUME

Des extraits acellulaires de 10 *leucémies et de* 2 *passages d'isogreffes leucémiques PS ont été confectionnés selon les techniques de Gross ou de Moloney. Certains de ces extraits ont été en outre ultracentrifugés sur des gradients préformés de citrate de potassium. Les extraits acellulaires et les zones obtenues après ultracentrifugation ont été inoculés à des souriceaux d'autres souches:* 101 *souris BALB/c, C3H/eB, Swiss et PS ont survécu* 9 *mois.*

Ce sont essentiellement des tumeurs mammaires qui sont apparues chez ces animaux: 62,5% *chez les BALB/c,* 66% *chez les C3H/eB,* 23% *chez les Swiss. Chez les PS, l'âge moyen d'apparition des tumeurs mammaires a passé de* 10,3 *mois à* 6,4 *mois.*

Ces résultats sont discutés. La participation éventuelle du virus du Polyome est peu probable. L'activité du facteur lacté véhiculé par le sang, et dès lors présent dans les organes leucémiques, est discutée. Ces résultats diffèrent de ceux obtenus chez d'autres souches de souris où coexistent le facteur lacté et un virus leucémigène.

SUMMARY

Cell free extracts from 10 *leukaemias and* 2 *passages of leukaemic isografts PS were obtained following Gross's or Moloney's technique. Some of them were submitted to ultracentrifugation on preformed density gradients of potassium citrate. Cell free extracts and the zones collected after untralcentrifugation were injected into new-born and weanling mice of different strains:* 101 *BALB/c, C3H/eB, Swiss and PS mice survived* 9 *months.*

The occurrence of mammary tumours was the prominent feature of this study: 62,5 *per cent of the BALB/c,* 66% *of the C3H/eB,* 23% *of the Swiss developed mammary tumours. In the PS strain, the mean age of mammary tumours' development fell from* 10,3 *months in the controls to* 6,4 *months in the inoculated mice.*

These results are discussed. So is the action of polyoma virus and the blood-borne activity of mammary tumour agent.

These results are compared with those obtained with other strains of mice carrying both a mammary tumour agent and a leukaemia virus.

ZUSAMMENFASSUNG

Zellfreie Extrakte von 10*PS-Leukämien und von* 2 *Passagen von PS-leukämischen Isotransplantationen wurden nach der Gross und Moloneys Technik hergestellt. Einige dieser Extrakte wurden ausserdem im Kaliumcitrat-Dichtegradienten zentrifugiert. Die zellfreien Extrakte und die nach Dichtegradienten-Zentrifugation erhaltenen Fraktionen wurden jungen Mäusen anderer Stämme übertragen:* 101 *BALB/c, C3H/eB, Swiss und PS-Mäuse überlebten* 9 *Monate.*

Es waren hauptsächlich Mamma-Carcinome, die bei diesen Tieren auftraten: 62,5% *bei den BALB/c,* 66% *bei den C3H/eB,* 23% *bei den Swiss-Mäusen. Bei den PS-Mäusen sank das Durchschnittsalter des Mamma-Carcinom-Auftretens von* 10,3 *auf* 6,4 *Monate.*

Diese Befunde sowie eine eventuelle Teilnahme des Polyoma-Virus werden besprochen. Die Frage des durch das Blut beförderten und dadurch in allen leukämischen Organen vorhandenen Milchfaktors wird erörtert. Es ist darauf hinzuweisen, daß diese Befunde von denen zu unterscheiden sind, die mit anderen Mäusestämmen, wo Milchfaktor und leukämieerzeugendes Virus zugleich bestehen, erhalten wurden.

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Tolérance du 5-Fluoro-Uracile en Cures Intensives à Forte Dose: Perfusions de 4 Heures ou de 24 Heures?

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INTRODUCTION

LE 5-FLUORO-URACILE (FU) et son désoxyriboside, la 5-fluoro-2′-désoxyuridine (FUDR), possèdent un effet antitumoral utilisé en clinique, principalement pour le traitement des stades avancés des cancers du sein, de l'estomac, du côlon et du rectum. Le mode d'action biochimique de ces pyrimidines fluorées est connu: leur transformation *in vivo* en 5-fluoro-2′-désoxyuridine-5′-monophosphate (FUDRP) permet une inhibition de la thymidylate synthétase, ce qui bloque la synthèse d'acide désoxyribonucléique (ADN) [1].

A dose égale, le FU est plus toxique en injection intraveineuse rapide qu'en perfusion lente, alors qu'il se produit exactement le contraire pour le FUDR, dont la valeur thérapeutique et les effets toxiques hématologiques et muqueux sont pourtant identiques à ceux du FU [2]. Sur le plan de la thérapeutique, perfusion lente et injection directe permettent d'obtenir un effet antitumoral avec l'une comme avec l'autre drogue. Le pourcentage d'améliorations objectives habituellement obtenu plafonne à environ 30%, sans qu'on puisse affirmer que le mode d'administration le plus toxique soit plus efficace [3, 4].

Pour le FU, 15 mg/kg, par jour, par voie intraveineuse directe, entraînent, après 5 jours en moyenne, une toxicité muqueuse (stomatite, cheilite, inappétence, épigastralgies, nausées, vomissements, douleurs abdominales, diarrhées, hémorragies) et hématologique (leucopénie, thrombopénie, agranulocytose, infection grave, diathèse hémorragique) variant beaucoup d'un cas à l'autre, habituellement contrôlable et réversible, mais pouvant exceptionnellement mettre en danger la vie du malade. Sous forme de perfusions lentes de 4 heures, à même dose journalière, la tolérance reste longtemps très bonne, et les signes toxiques apparaissent assez progressivement pour être décelés à temps. Mais, du fait même de cette bonne tolérance, le traitement dure trop longtemps, 3 semaines ou plus, et doit parfois être interrompu avant la dose maximum tolérée, en raison de la fatigue du malade, des complications veineuses dues aux perfusions, ou de l'évolution de la tumeur.

A la recherche d'une posologie plus satisfaisante, nous avons choisi l'administration par perfusion d'une dose quotidienne nettement plus forte que la dose habituelle de 15 mg/kg, soit 25 mg/kg. Sous cette forme, la durée du traitement, déterminée par l'apparition des signes de toxicité sanguine ou muqueuse, peut être raccourcie, ce qui limite la période d'hospitalisation, de contrôle strict et de fatigue pour de malade. La comparaison entre l'injection directe et la perfusion suggérant que l'application lente diminue la toxicité sans réduire dans la même proportion l'effet thérapeutique, on peut émettre l'hypothèse que la *durée* de perfusion modifie également la tolérance du FU sans altérer sa valeur thérapeutique. C'est ainsi que le but de la présente étude est de déterminer la tolérance du FU en perfusions lentes à 25 mg/kg par jour, ainsi

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que les modifications de cette tolérance, apportées par le remplacement de la perfusion habituelle de 4 heures par une perfusion continue de 24 heures.

MATERIEL ET METHODES

Vingt-neuf malades atteints, à un stade avancé, de cancers du sein, de l'estomac, du côlon, du rectum ou de localisation primaire inconnue, histologiquement confirmés, ont reçu une cure de FU sous forme de doses journalières successives de 25 mg/kg de poids corporel, en perfusions veineuses. Tous les malades étaient hospitalisés pendant ce traitement. La cure a parfois été interrompue un jour par semaine, le dimanche. Le but de l'étude étant la tolérance au traitement, le choix des malades n'a pas porté uniquement sur ceux présentant une ou plusieurs localisations tumorales mesurables. De ce fait, l'évaluation objective quantitative de l'effet antitumoral n'est pas possible.

Après que l'indication au traitement a été posée, les malades ont été répartis en deux groupes, selon que leur jour de naissance fût pair ou impair. Quatorze malades ont reçu le FU, en perfusions de 4 heures, dans 500 ml d'une solution de glucose à 5%. Un malade diabétique, traité en 4 heures, a reçu le FU dans du NaCl 0,9%. Quatorze malades ont reçu le FU sous forme de perfusion continue dans 1.000 ml de glucose à 5%, par 24 heures. Pendant, et immédiatement après traitement, les contrôles effectués tous les jours ont porté sur l'examen clinique, les éventuelles tumeurs mesurables, les signes habituels de toxicité, le nombre de leucocytes et de thrombocytes par mm^3. En outre, les valeurs sanguines d'hémoglobine, de phosphatase alcaline, de transaminases, d'azote uréique et de créatinine ont été contrôlées au début et à la fin du traitement. La dose de FU n'a habituellement pas été modifiée en cours de cure, mais le traitement a été interrompu dès l'apparition de signes certains de toxicité sanguine ou digestive. Les caractéristiques des deux groupes ressortent du Tableau 1.

L'évaluation de la toxicité hématologique a été réalisée en comparant les valeurs de leucocytes et de thrombocytes obtenues immédiatement avant la première dose avec les valeurs minimum consécutives au traitement. Il s'est révélé difficile de chiffrer cette toxicité sanguine. L'interprétation d'une leuco- ou thrombopénie dépend des valeurs de départ, ici très inégales: leucocytose et thrombocytose élevées pour certaines tumeurs intestinales, leucothrombopénie au contraire dans certains cas, particulièrement dans un cancer du sein à localisations osseuses multiples, déjà traité par une autre chimiothérapie. En outre, le traitement peut avoir été interrompu pour intolérance digestive, sans toxicité sanguine. Nous avons donc établi les comparaisons selon plusieurs critères différents: valeur en % de la chute des leucocytes et thrombocytes dans chaque groupe complet, traité en 4 heures ou 24 heures (appelé par la suite "groupe complet"); mêmes valeurs en %, en se limitant, dans chaque groupe, aux cas ayant présenté des signes nets de toxicité sanguine, soit une chute de 30% ou plus du nombre de leucocytes ou de thrombocytes, et des valeurs minimum inférieures à $5.000/mm^3$ pour les leucocytes, et $180.000/mm^3$ pour les thrombocytes (groupe qualifié par la suite "avec toxicité sanguine"); nombre de cas de chaque groupe à valeur minimum inférieure à 3.000 ou 5.000 leucocytes/mm^3, et à 100.000 ou 180.000 thrombocytes/mm^3.

L'évaluation semi-quantitative de la toxicité digestive a été faite en tenant compte du nombre de manifestations d'intolérance, et de leur intensité, selon une classification en quatre catégories allant de 0 à +++.

RESULTATS

Les résultats obtenus dans les deux groupes, soit par perfusion de 4 heures, soit par perfusion continue de 24 heures sont résumés sur les Tableaux 2, 3, 4 et 5. Dans les deux groupes, la tolérance au traitement fut suffisante. Aucune toxicité grave, mettant la vie du malade en

Tableau 1. Répartition des malades traités par perfusions de 4 heures ou de 24 heures de 5-fluoro-uracile

		4 heures	24 heures
Nombre de malades		15	14
Hommes/femmes		8/7	9/6
Age moyen (extrêmes)		64,7 (43–78)	55,8 (28–74)
Tumeur:	sein	0	3
	estomac	1	2
	côlon	5	4
	recto-sigm.	8	4
	inconnue	1	1

Tableau 2. Toxicité du 5-fluoro-uracile en perfusions de 4 heures à 25 mg/kg. Résumé des cas

Malades (âge, sexe)	Tumeur*	Doses à 25 mg/kg	Chute leucocytes (%)	Chute thrombocytes (%)	Toxicité digestive	Remarques
1. 67/F	E	5	47	42	0	Interruption: toux irritative
2. 51/M	C	8	82	55	+++	Alopécie
3. 71/F	C	5,7	0	38	0	Interruption: ponctions veineuses impossibles
4. 69/F	C	9	38	11	++	Asthénie
5. 63/F	C	5	31	41	0	—
6. 70/M	C	5	3	31	+	Interruption: décès
7. 71/M	R	8	22	0	0	Interruption: décès
8. 60/F	R	6	48	18	+	—
9. 57/M	R	6	26	15	++	—
10. 63/F	R	5	53	22	+	—
11. 43/F	R	6	65	31	+	—
12. 78/M	R	3	63	41	0	—
13. 71/M	R	12	43	50	0	—
14. 59/M	R	14	0	0	0	Arrêt sans toxicité: traitement inefficace
15. 77/M	X	9	51	47	++	—

*S=sein; E=estomac; C=côlon; R=recto-sigmoïde; X=inconnue.

danger, ne fut observée. La dose totale moyenne par malade, exprimée par le nombre de doses journalières à 25 mg/kg situe pour les groupes complets à $7{,}1 \pm 1{,}6$* en 4 heures et à $6{,}5 \pm 1{,}2$* en 24 heures. Pour les groupes avec toxicité sanguine, les valeurs moyennes sont de $6{,}1 \pm 1{,}9$* en 4 heures, et de $6{,}0 \pm 1{,}0$* en 24 heures. Dans les deux groupes, la toxicité sanguine fut très variable, d'une part, parce que le traitement fut souvent interrompu par la toxicite digestive et non sanguine, et, d'autre part, parce que les traitements furent interrompus à des degrés inégaux de toxicité, modifiés par l'interprétation subjective du médecin et du malade. En outre, le traitement ne put être poursuivi malgré l'absence de toxicité, dans 6 cas, pour diverses raisons indiquées sur les Tableaux 2 et 3.

*Limites de confiance pour 95%

Tableau 3. Toxicité du 5-fluoro-uracile en perfusions de 24 heures à 25 mg/kg. Résumé des cas

Malades (âge, sexe)	Tumeur*	Doses à 25 mg/kg	Chute leucocytes (%)	Chute thrombocytes (%)	Toxicité digestive	Remarques
1. 61/F	S	5	23	46	+	—
2. 51/F	S	6	32	45	0	—
3. 57/F	S	5	36	34	0	Interruption: toux, fièvre
4. 72/F	E	5	13	27	+	—
5. 61/M	E	8	54	57	0	—
6. 45/F	R	9	43	64	+	—
7. 57/M	R	6	52	67	+++	—
8. 57/M	R	4,1	37	33	0	Interruption: phlébite du bras
9. 43/F	R	4,3	75	0	++	Phlébite du bras
10. 74/F	C	7	60	36	++	—
11. 68/M	C	11,5	0	0	0	Interruption: phlébite du bras
12. 28/F	C	7	11	17	0	Interruption: refus du traitement
13. 45/M	C	6	60	52	0	—
14. 63/F	X	7	64	57	+	—

*S=sein; E=estomac; C=côlon; R=recto-sigmoïde; X=inconnue.

Tableau 4. Comparaison de toxicité sanguine après traitement au 5-fluoro-uracile en perfusions de 4 heures ou de 24 heures

	Groupes complets		Groupes avec toxicité sanguine	
	4 heures	24 heures	4 heures	24 heures
Nombre de doses à 25 mg/kg	7,1±1,6	6,5±1,2	6,1±1,9	6,0±1,0
% de chute des leucocytes	38,3±13,9	40,7±12,1	54,2±15,7	50,3±11,0
% de chute des thrombocytes	29,5±9,8	38,2±12,4	38,4±12,8	50,6±9,3

La comparaison quantitative de la toxicité hématologique, faite selon les critères décrits plus haut, reste cependant possible. Les taux de chute leucocytaire sont pratiquement égaux dans les deux groupes complets, de même que dans les groupes avec toxicité sanguine sur la série blanche. Pour les taux de chute thrombocytaire, en revanche, il apparaît entre les groupes une différence montrant une toxicité plus forte après perfusion continue de 24 heures, valeurs cependant non significatives ($P>0,05$). La mesure de la durée de la leuco-thrombopénie toxique n'a pas été réalisable dans tous les cas. On peut constater toutefois que le retour à des valeurs sanguines normales s'est fait presque toujours en l'espace d'une semaine, la durée la plus longue ayant été de 3 semaines, et correspondant à une chute leucocytaire à 1900/mm³, soit 18% de la valeur de départ. Les valeurs minimum de leucocytes et de thrombocytes ont été notées au cours des 4 jours suivant l'arrêt du traitement dans tous les cas sauf trois, dont deux traités en 24 heures. Dans les quatre séries comparatives établies, le taux de chute des thrombocytes est plus faible que celui des leucocytes. Quant à la comparaison des valeurs leucocytaires et thrombocytaires minimum absolues après traitement, elle fait apparaître un nombre plus grand de leucopénies à moins de 3000/mm³ et de thrombopénies à moins de 100.000/mm³ dans le groupe traité en 24 heures.

La comparaison semi-quantitative de la toxicité digestive dans les deux groupes ne montre pas de différence. Dans le groupe traité en 4 heures, 7 cas n'ont pas présenté de signes de toxicité digestive, 4 cas ont été qualifiés +, 3++ et 1+++. Dans le groupe traité en 24 heures, on trouve 7 cas sans toxicité digestive, 4+, 2++, 1+++. Les signes de toxicité ont été de même nature dans les deux groupes. Leur durée a été courte, comparable à celle des signes toxiques hématologiques.

En outre, nous avons constaté, deux fois, comme autre signe de toxicité, des crises de toux irritative non hémoptoïque, accompagnées une fois de fièvre, dans les deux cas sans image radiologique pulmonaire ni auscultation anormales. Un cas d'alopécie complète, passagère, a été observé après traitement. Enfin, les causes de l'arrêt du traitement ont été, dans le groupe traité en 4 heures, digestives dans 6 cas, hématologiques dans 4 cas, ni digestives ni hématologiques dans 5 cas. Dans le groupe traité en 24 heures, elles ont été hématologiques dans 5 cas, digestives dans 5 cas, ni hématologiques ni digestives dans 4 cas. Trois traitements par perfusion continue ont provoqué une phlébite superficielle de la veine perfusée, ce qui ne fut jamais le cas dans le groupe perfusé en 4 heures.

DISCUSSION

Les différences considérables de toxicité des pyrimidines fluorées, en fonction de la vitesse d'injection, peuvent être expliquées par les particularités de leurs voies métaboliques

Tableau 5. Leucopénie et thrombopénie après traitement de 5-fluoro-uracile en perfusions de 4 heures ou de 24 heures

Nombre de cas avec valeurs minimum après FU de:	Groupes complets		Groupes avec toxicité sanguine	
	4 heures	24 heures	4 heures	24 heures
leucocytes < 3000/mm³	2	4	2	4
leucocytes de 3 à 5000/mm³	6	8	5	5
leucocytes > 5000/mm³	7	2	–	–
thrombocytes < 100.000/mm³	1	5	1	5
thrombocytes de 100 à 180.000/mm³	8	6	6	4
thrombocytes > 180.000/mm³	6	3	–	–

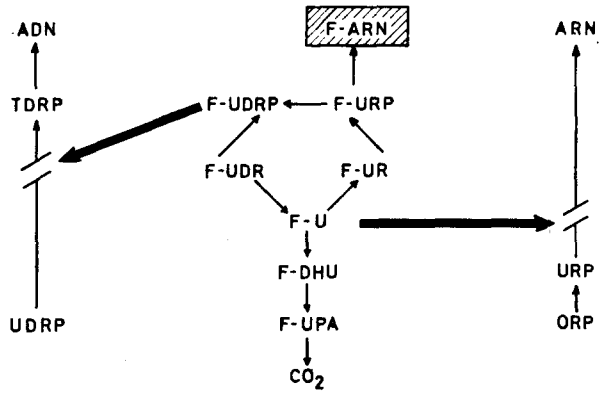


Fig. 1. Métabolisme et mode d'action biochimique des pyrimidines fluorées.

ADN:	*ac. désoxyribonucléique*	*F-UPA*	*ac. -fluoro-uréido- propionique*
ARN:	*ac. ribonucléique*	*F-UR:*	*5-fluoro-uridine*
F-ARN:	*ac. ribonucléique fluoré*	*F-URP:*	*5-fluoro-uridine-monophosphate*
F-DHU:	*dihydrofluoro-uracile*	*ORP:*	*ac. orotidylique*
F-UDR:	*5-fluoro-2'-désoxyuridine*	*TDRP:*	*ac. thymidylique*
F-UDRP:	*5-flouro-2'-désoxyuridine-monophosphate*	*UDRP:*	*ac. désoxyuridylique*
F-U:	*5-fluoro-uracile*	*URP:*	*ac. uridylique*

(Fig. 1) [1]. Le FU suit les voies anaboliques de l'uracile, avec toutefois une importante exception: la présence d'un atome de fluor en position 5 empêche la méthylation en 5 du noyau uracile de FUDRP pour aboutir à l'acide thymidylique (TDRP). De ce fait, le FU n'est pas incorporé dans l'ADN. En revanche, il est, comme l'uracile normal, incorporé dans l'acide ribonucléique (ARN). L'effet antitumoral du FU et du FUDR peut résulter de plusieurs mécanismes biochimiques. La formation de FUDRP bloque la transformation UDRP–TDRP par inhibition de la thymidylate-synthétase. Ce mécanisme est responsable de l'effet thérapeutique chez l'homme. En outre, le FU détériore la synthèse d'ARN, soit en inhibant la transformation uracile (U)—uridine-monophosphate (URP), soit par son incorporation dans un ARN pathologique fluoré. Ces altérations de l'ARN ne semblent pas jouer un rôle dans l'inhibition de croissance tumorale chez l'homme.

L'étude des taux sanguins et urinaires de FU, après administration par perfusion continue, ou par injection intraveineuse rapide, montre qu'injecté rapidement, le FU atteint un taux sanguin élevé qui décroît lentement, et qu'environ 20% du produit est éliminé dans l'urine sans modification, alors qu'en perfusion continue, un taux sanguin faible reste constant, avec une élimination urinaire presque nulle [5]. On peut donc en déduire que le FU est métabolisé plus complètement en perfusion lente qu'en injection rapide. On sait d'ailleurs qu'*in vivo* le FU est rapidement catabolisé en urée, ammoniaque et gaz carbonique, après transformationendihydrofluoro-uracile (FDHU), acide α-fluoro-β-uréidopropionique (FUPA), acide α-fluoro-β-guanidopropionique (FGPA) et α-fluoro-β-alanine, tous dépourvus d'effet antimitotique [1].

On postule ainsi que les différences de toxicité du FU en fonction de la vitesse d'injection est due au fait qu'en perfusion lente, la plus grande partie du FU peut être immédiatement catabolisée, alors qu'en injection rapide, la concentration élevée obtenue permet qu'une partie plus importante de la substance suive la voie anabolique aboutissant au FUDRP responsable de l'efficacité [6]. Cette explication n'est cependant pas satisfaisante: elle explique bien les différences de toxicité selon la vitesse d'application, mais non l'effet thérapeutique relativement peu modifié, donc assez indépendant des facteurs faisant varier la toxicité. Aucune autre explication ne résout actuellement cette question. On peut toutefois émettre l'hypothèse que le FU en injection rapide devrait théoriquement être plus efficace en raison de l'explication fournie plus haut, mais que, dans ces conditions, l'augmentation d'intensité et de vitesse d'apparition des signes toxiques rend la thérapeutique moins maniable, donc moins sûre et d'un moins bon rendement, ce qui égalise les résultats pratiques des modes d'administration rapide ou lent. On peut, d'autre part, supposer que la perfusion lente, en augmentant le temps de contact entre la drogue et la cellule tumorale en période de synthèse d'ADN, améliore l'effet d'inhibition de croissance de tumeurs solides dont on sait que le cycle mitotique est lent [7].

Nous avons choisi d'administrer le FU en perfusion lente, à la dose de 25 mg/kg par

jour, soit nettement plus que la dose habituelle de 15 mg/kg par jour en raison des considérations suivantes:

—le traitement "classique" par injections rapides à 15 mg/kg par jour [8] est trop toxique;

—le traitement par perfusions lentes à 15 mg/kg par jour [2, 4] est efficace, quoique nettement mieux toléré que le traitement par injections rapides, mais la longue durée de la période de perfusions est un handicap;

—l'étude expérimentale de l'effet des agents chimiques antitumoraux sur la croissance tumorale réalisée par Skipper et coll. sur la leucémie L 1210 [9], puis sur certaines tumeurs solides animales [10], suggère que l'effet thérapeutique optimum sera obtenu par un traitement intense de courte durée pendant lequel le temps de contact entre le cellule tumorale et l'agent thérapeutique sera le plus long possible.

Les résultats de cette étude montrent que le traitement de FU à 25 mg/kg par jour en perfusion lente remplit les conditions théoriques d'un traitement satisfaisant selon les considérations ci-dessus: concentration sur un temps court, durée de contact drogue-tumeur longue, toxicité tolérable et prévisible. Une évaluation des résultats, sous forme de taux de rémission objective, ne peut être faite ici: dans plusieurs cas, le diagnostic et l'extension du processus néoplasique résultait d'une constatation opératoire, sans localisations cliniquement ou radiologiquement mesurables avec assez de précision. L'efficacité est toutefois attestée par 5 diminutions objectives du volume tumoral: 1 régression de métastases cutanées de cancer du sein, 2 réductions de volume de foie métastatique de cancers recto-sigmoïdien et côlique, 1 diminution de volume des métastases hépatiques d'un cancer du côlon, constatée à l'occasion d'une deuxième laparotomie, 1 guérison de fistule périnéale sur récidive après amputation abdomino-périnéale pour cancer du rectum. En outre, un effet antalgique, sans diminution du volume tumoral, fut observé dans 3 cas, dont 2 de douleurs hépatiques et 1 de douleurs sacrées.

La durée de l'effet thérapeutique fut très variable: de quelques semaines à plus d'un an, le traitement de départ étant suivi, en cas de succès, par une cure d'entretien sous forme d'une injection intraveineuse rapide par semaine. Enfin, la comparaison de la tolérance, en fonction de la durée de perfusion, ne fait pas apparaître l'avantage de la perfusion continue sur la perfusion en 4 heures, malgré les arguments théoriques qui auraient pu le faire supposer. L'explication la plus plausible est que les concentrations sanguine ou tissulaire de FU, produites par ces deux modes de traitement, sont trop peu différentes pur que la répartition entre voie catabolique et voie anabolique du FU perfusé ne soit pas la même dans les deux cas. Dans ces conditions, la dose maximum tolérée restant la même, que ce soit en 4 heures ou en 24 heures, il est peu vraisemblable que la valeur thérapeutique soit différente. Comme le traitement par perfusions continues est plus astreignant pour le malade, et a été responsable, dans la présente série, de 3 complications veineuses locales, sous forme de phlébites superficielles de la veine perfusée, ce qui ne s'est jamais produit en 4 heures, nous avons décidé d'abandonner l'administration de FU en perfusions continues.

RESUME

Recherchant un mode d'administration du 5-fluoro-uracile associant une tolérance suffisante à une concentration de la dose totale sur une courte durée, nous avons choisi la perfusion lente, quotidienne, d'une dose forte, soit 25 mg/kg, pour le traitement de 29 malades atteints de cancers disséminés du sein (3), de l'estomac (3), du côlon (9), du recto-signoide (12) ou d'origine inconnue (2). Les cas ont été répartis, au hasard, en deux groupes, l'un perfusé en 4 heures, l'autre sous forme de perfusion continue.

Les résultats montrent que ce mode d'administration du FU est toléré pendant 6 à 7 jours, avec des variations individuelles importantes, et que le surdosage peut être évité en raison de l'apparition progressive des signes toxiques hématologiques et digestifs, qui sont rapidement réversibles. L'évaluation de l'effet thérapeutique objectif n'est pas l'objet de cette étude. Toutefois, plusieurs diminutions du volume tumoral ont été constatées. Enfin, l'administration en perfusion continue n'a pas amélioré la tolérance par rapport à la perfusion habituelle en 4 heures.

SUMMARY

In order to find a method of administration of 5-fluorouracil that would permit concentration of the total dose over a short period and with sufficient tolerance, we have chosen slow

daily infusions at high dosage (i.e. 25 mg/kg) in the treatment of 29 patients suffering from disseminated cancer of the breast (3), colon (9), rectosigmoid (12), and of unknown origin (2). The cases were randomly divided into two groups, one group treated by infusion over 4 hours, the other by continuous infusion.

The results showed that this method of administering FU is well tolerated over 6 to 7 days, with important individual variations, and that overdosage can be prevented, due to the slow and progressive appearance of the signs of hematologic and digestive toxicity, these being rapidly reversible. The evaluation of the therapeutic effect was not the object of this study. Nevertheless, tumor volume was seen to be diminished in many of the cases. Administration in continuous infusion did not ameliorate tolerance over that seen in 4 hour infusion.

ZUSAMMENFASSUNG

Es wurde nach einer Applikationsart des 5-Fluorouracils gesucht, der das Medikament bei guter Verträglichkeit in hoher Gesamtdosis und kurzem Zeitintervall gegeben werden kann. 29 Patienten mit disseminierten Tumoren, darunter 3 Fälle von Brustkrebs, 3 von Magenkarzinom, 9 Fälle von Colon- und 12 Fälle von Rectosigmoïdkrebs sowie 2 Tumoren unbekannter Genese wurden mit einer täglichen langsamen Perfusion von 25 mg/kg 5-Fluorouracil behandelt. Die Patienten wurden durch Los in zwei Gruppen aufgeteilt, deren eine mit einer Perfusion über 4 Stunden, die andere mit einer Dauerperfusion behandelt wurden.

Unsere Ergebnisse zeigen, dass 5-Fluorouracil bei dieser Applikationsart mit starken individuellen Schwankungen 6–7 Tage lang gut vetragen wird. Ueberdosierungen können vermieden werden, da die Zeichen von hämatologischer Toxizität oder im Bereich des Verdauungstraktes nur allmählich auftreten und sich schnell zurückbilden. Die Auswertung des therapeutischen Effektes war nicht Ziel dieser Untersuchung. Es wurden jedoch mehrere Tumorrückbildungen beobachtet. Die Dauerperfusion wurde nicht besser vertragen als die Perfusion über 4 Stunden.

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The Susceptibility of Various Strains of Neonatal Mice to the Carcinogenic Effects of 9,10-Dimethyl-1,2-Benzanthracene

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INTRODUCTION

PIETRA, Spencer and Shubik in 1959 [1] demonstrated an increased incidence and shorter latent period of malignant lymphoma in new born mice injected subcutaneously with 9,10-dimethyl-1,2-benzanthracene (DMBA) as compared to similarly treated adult mice. Since then new born mice of different strains have been exposed to a variety of chemical carcinogens. Toth [2] in an extensive review suggested that the new born mouse differed from the adult in a number of ways which might influence the incidence of carcinogen-induced tumours. The metabolism of chemical agents may be slower in the new born and consequently the exposure of the tissues to a carcinogen may be prolonged. In the immediate postnatal period the rate of development of organs is rapid, the sex and some other hormones are not yet fully functional and immunological competence has not fully developed. With these factors in mind, Toth was unable to agree that the new born mouse is necessarily more susceptible to the action of carcinogenic agents, although this has been suggested by other workers.

This paper presents the results of an investigation of the relative importance of genetic and environmental factors in carcinogenesis in new born mice. Neonatal mice of a number of strains and their F1 hybrids were challenged with a standard dose of DMBA.

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MATERIAL AND METHODS

Animals

Mice were raised in this laboratory and maintained on Oxoid 41B diet and water *ad libitum*. The A^F, C57B1, C3H and BALB/c strains were originally obtained from Chester Beatty Research Institute (London), the CBA strain was acquired from 'Animal Supplies' (England) and the IF strain from this laboratory. At the start of the present experiment, these strains had been maintained in Leeds for 7 or 8 generations, except for the IF strain which was in the 77th generation. The mice of these strains behaved as described in the literature [3], except that the A^F strain carried the dominant agouti gene, as its F1 hybrids with C57B1 were agouti rather than the expected black coat colour. A^F mice crossed with another C57B1 subline also gave agouti F1 hybrids. Otherwise, no differences were observed between A^F and the accepted normal A strain mice. In addition reciprocal F1 hybrid crosses of $A^F \times$ C57B1 and of CBA $\times$ C3H strains were used. As is the custom, the female is written first in naming the reciprocal hybrids.

Treatment with DMBA

A suspension of DMBA in 3% aqueous gelatine was prepared by the method of Pietra, *et al.* [1]. One subcutaneous injection of 15 µl gelatine solution containing 30 µg of DMBA was given in the interscapular region not later than 16 hours after birth. Control mice were similarly injected with 15 µl of the gelatine suspension. Groups consisting of DMBA injected mice, mice injected with gelatine suspension alone, and untreated controls were established for each strain and each hybrid. Each group of animals contained 60 mice, approximately half of which were males and half females. The mice were weaned at 4–5 weeks of age and housed up to 6 males or 6 females per cage.

The mice were inspected daily and any that became moribund were killed and autopsied. Dead mice that underwent autolysis were discarded and are not included in the results. The surviving mice were killed at the end of one year.

Pathological assessment

The nature of the neoplasms was assessed from two points of view, the extent of the disease and the histological appearance of the lesions. The extent of neoplastic development in the lungs or liver was defined as: +=up to 10 nodules, ++=multiple nodules and +++=confluent masses of nodules. The degree of leukaemia was graded as: I=slight localized lymphoid involvement, particularly in the thymus with direct extension to the lungs, II=extensive involvement of the lymphoid system with infiltration of other organs and III=gross involvement of all the lymphoid system with gross infiltration of other organs. All the major organs and any other tissue which appeared abnormal were taken for histological examination.

RESULTS

Within each strain of mouse, the gelatine treated and untreated mice produced similar yields of tumours and had similar mean survival times. Therefore, the two groups have been combined and referred to as control mice. In the DMBA injected mice, the response was the development of lung adenomata, leukaemia and hepatomata, with a low incidence of other types of tumour (Tables 1–4).

A^F, C57Bl *and reciprocal* F1 *hybrid mice*

Control A^F mice of either sex developed a moderate incidence of lung adenomata (18%) by twelve months, whereas the C57Bl mice were free from these tumours (Table 1). The reciprocal hybrids, $A^F \times$ C57Bl and C57Bl $\times A^F$, resembled the A^F mice with tumour yields of 15 and 11% respectively. No other tumours were seen in these control mice.

Lung adenomata were observed in all the A^F and in all but one of the hybrid mice injected with DMBA at birth. The extent of

Table 1. *Pulmonary tumours in control mice and in mice treated with DMBA within* 16 *hours of birth*

Strain and sex		Control mice Total No. of mice/No. with adenoma of degree +	Average latent period (months)	DMBA treated mice Total No. of mice/No. with adenoma	No. with adenoma of degree +	++	+++	No. with carcinoma	Average latent period (months)
A^F	F	54/7	12	26/26	3	8	15	11	10·5
	M	53/12	12	22/22	3	12	7	7	9·7
C57Bl	F	59/0		26/1	1	0	0	0	12
	M	52/0		22/0	0	0	0	0	
$A^F \times$ C57Bl	F	58/10	11	26/25	5	12	8	2	7·7
	M	58/7	12	22/22	12	4	6	3	6·6
C57Bl $\times A^F$	F	57/5	12	24/24	5	7	12	7	8·5
	M	54/7	12	25/25	11	9	5	4	6·3
CBA	F	54/0		25/16	13	3	0	2	10·3
	M	60/0		21/14	11	3	0	3	10·6
C3H	F	57/0		24/12	12	0	0	0	9·3
	M	56/0		22/14	14	0	0	1	8·7
CBA $\times$ C3H	F	57/0		24/15	14	1	0	0	10
	M	59/0		26/17	16	1	0	2	11·3
C3H $\times$ CBA	F	57/0		25/14	13	1	0	1	9·3
	M	58/0		22/5	4	1	0	1	8·2
BALB/c	F	57/3	11·7	23/23	20	3	0	2	6·8
	M	60/1	12	26/24	12	9	3	7	8·1
IF	F	50/2	12	24/18	10	6	2	0	8·7
	M	64/3	12	21/20	14	5	1	1	8·1

Table 2. Leukaemia in control mice and in neonatal mice treated with DMBA

Strain and sex		Control mice: Total No. of mice/No. with leukaemia of degree +	Control mice: Average latent period (months)	DMBA treated mice: Total No. of mice/No. with leukemia	DMBA treated mice: No. with leukaemia of degree I	II	III	DMBA treated mice: Average latent period (months)
A^F	F	54/0		26/0	0	0	0	
	M	53/0		22/0	0	0	0	
C57B1	F	59/0		26/4	4	0	0	8·3
	M	52/0		22/0	0	0	0	
$A^F \times$ C57B1	F	58/0		26/10	1	6	3	4·5
	M	58/0		22/13	3	5	5*	5
C57B1 $\times A^F$	F	57/0		24/10	2	3	5	4·6
	M	54/0		25/15	2	7	6	4·7
CBA	F	54/0		25/12	6	3	3	6·7
	M	60/0		21/4	4	0	0	7
C3H	F	57/0		24/5	4	1	0	9
	M	56/0		22/7	3	1	3	6·1
CBA × C3H	F	57/0		24/9	4	0	5	6
	M	59/0		26/6	3	2	1	7
C3H × CBA	F	57/0		25/6	1	3	2	5·7
	M	58/0		22/10	2	5	3	5·4
BALB/c	F	57/8	12	23/12	5	1	6	5·2
	M	60/0		26/9	3	4	2	6·6
IF	F	50/0		24/3	2	1	0	6·3
	M	64/0		21/6	2	4	0	7

*One lymphosarcoma.

the lesions was considerably more marked than in the controls. On the other hand, only one of the 48 treated C57B1 mice developed a lung adenoma.

There was no leukaemia (Table 2) in the DMBA injected A^F mice or male C57B1 mice and only 15% in female C57B1 mice. In the hybrids, the incidence of leukaemia was raised by the treatment to 48% in $A^F \times$ C57B1 and 51% in C57B1 $\times A^F$. The mean latent period was 5 months or less in each case. There was a small number of hepatomata, injection site tumours and granulosa cell tumours of the ovary (Tables 3 and 4).

Table 3. Liver tumours in control mice and in neonatal mice treated with DMBA

Strain and sex		Control mice: Total No. of mice/No. with hepatoma of degree+	Control mice: Average latent period (months)	DMBA treated mice: Total No. of mice/No. with hepatoma	DMBA treated mice: No. with hepatoma of degree +	++	+++	DMBA treated mice: No. with malignant hepatoma	DMBA treated mice: Average latent period (months)
A^F	F	54/0		26/3	3	0	0	0	11·3
	M	53/0		22/1	1	0	0	0	12
C57B1	F	59/0		26/1	1	0	0	0	11
	M	52/0		22/4	4	0	0	0	12
$A^F \times$ C57B1	F	58/0		26/1	1	0	0	0	10
	M	58/0		22/1	1	0	0	0	12
C57B1 $\times A^F$	F	57/0		24/1	1	0	0	0	12
	M	54/0		25/6	6	0	0	0	11·3
CBA	F	54/2	12	25/9	6	3	0	0	11·4
	M	60/9	12	21/11	1	2	8	5	11·5
C3H	F	57/0		24/8	7	1	0	0	8·3
	M	56/15	12	22/17	6	6	5	1	8·1
CBA × C3H	F	57/0		24/14	4	8	2	0	10·4
	M	59/14	12	26/18	1	3	14	6	11·7
C3H × CBA	F	57/5	12	25/9	7	2	0	0	10·7
	M	58/11	12	22/9	4	3	2	0	9·6
BALB/c	F	57/0		23/0	0	0	0	0	
	M	60/0		26/3	2	0	0	0	12
IF	F	50/0		24/2	2	0	0	0	12
	M	64/0		21/3	3	0	0	0	10·7

Table 4. Miscellaneous tumours in mice neonatally treated with DMBA

Strain and sex		Total No. of mice	Mammary carcinoma	Ovarian carcinoma	Tumours at site of injection fibrosarcoma	Other tumours	
A^F	F	26	0	1	2	0	
	M	22			2	0	
C57B1	F	26	0	0	1	0	
	M	22			1	0	
$A^F \times$ C57B1	F	26	0	2	1	0	
	M	22			2	0	
C57B1 $\times A^F$	F	24	0	2	2	0	
	M	25			2	0	
CBA	F	25	0	2	0	0	
	M	21			1	0	
C3H	F	24	0	1	8*	2	Secondary
	M	22			5	0	sarcomata in lungs
CBA $\times$ C3H	F	24	0	3	1	0	
	M	26			2	1	Kidney adenoma
C3H $\times$ CBA	F	25	2	2	2		1 gut carcinoma
	M	22			6	0	
BALB/c	F	23	0	0	4†	0	
	M	26			2‡	1	Salivary carcinoma
IF	F	24	0	2	2	0	
	M	21			1	0	

*2 rhabdomyosarcomata; †2 squamous cell carcinomata; ‡1 rhabdomyosarcoma.

CBA, C3H and *reciprocal* F1 *hybrid mice*

Control pure line and hybrid mice were free from lung adenomata and leukaemia. Hepatomata occurred in male (15%) and female (4%) CBA control mice and in male (27%) C3H controls. There were hepatomata in CBA $\times$ C3H male (24%) but none in female controls, while in the reciprocal hybrids the incidence was 19% in the males and 9% in the females.

In the DMBA injected pure line and hybrid mice pulmonary tumours have been observed. The lowest incidence (23%) was in C3H $\times$ CBA male hybrids and the highest (63%) in male CBA $\times$ C3H hybrids (Table 1).

Leukaemia was found in all chemically treated groups in approximately a third of the animals (Table 2). There was an increase of the incidence of the liver tumours in DMBA injected mice. In each case the males developed more extensive hepatomatous lesions than the females. The reciprocal hybrids, which had a C3H mother developed fewer tumours than those with a C3H father ($X^2=5{\cdot}4$; $0{\cdot}01<P<0{\cdot}05$; Table 3).

The C3H strain and the C3H $\times$ CBA hybrids had more injection site tumours than the other mice. In the former, two of the injection site sarcomata had metastasized to the lungs (Table 4).

BALB/c *and* IF mice

Untreated mice of both of these strains had a low spontaneous incidence of pulmonary adenomata and this was increased after the injection of DMBA (Table 1). Leukaemia was present in 14% of the control female BALB/c mice and the incidence rose to 52% in DMBA injected mice. Leukaemia did not occur in untreated male BALB/c and male and female IF mice but developed after the neonatal injection of DMBA (Table 2).

In both strains of mice the treated animals developed a few hepatomata (Table 3), a few injection site tumours and a salivary carcinoma (Table 4).

Histology

The histological appearance of lung tumours from treated and untreated mice was similar. Neoplasms of various sizes and stages of malignancy, from adenoma to carcinoma, were often present in the same lung. Some of the tumours showed local invasion, while others invaded along the bronchial tree and occasionally, into the pleural space and the thoracic wall. It appeared that DMBA induced two types of adenomata according to the strain of mouse. C3H and CBA mice and their hybrids developed mainly a papillary type of adenoma, usually arising near the bronchioles, while the

other strains and hybrids developed an acinar type, which tended to arise near the surface of the lung.

Leukaemia was of either thymic or splenic type or both. The architecture of the affected organs was well preserved, containing localized accumulations of lymphoid cells of variable size. In the advanced stages of the leukaemia the organs were heavily infiltrated with these lymphocytes.

Liver tumours varied from 'benign' hepatomata to malignant trabecular carcinomata in CBA and C3H mice and their hybrids. In many of the livers large confluent neoplastic masses were present. In other strains of mice only small well organized hepatomata were observed and they were few in number in each liver.

The tumours at the injection site were mainly fibrosarcomata, although a few rhabdomyosarcomata and squamous cell carcinomata were seen.

The ovarian neoplasms were carcinomata of the granulosa cell type. One intestinal, one salivary carcinoma and a kidney adenoma were the only other neoplasms found.

DISCUSSION

The injection of DMBA into new born mice led to the development of pulmonary and hepatic tumours, leukaemia and a small number of tumours at other sites. It was shown that the genotype of the mice played an important role in determining the principal sites of tumour formation elicited by a single dose of DMBA. The frequency, extent and latent period of the tumours indicated that new born mice were particularly sensitive to this carcinogen. Furthermore, an incidence of other neoplasms was recorded in treated mice while no such tumours were found in comparable controls.

The lungs, liver and lymphoid tissue are, in the new born, in a state of development and the presence of replicating cells may be one reason for this high susceptibility to DMBA. In the postnatal period the alveolar cells of the lung have a high rate of proliferative activity [4]. The liver is increasing in size and its enzymatic composition is different from that of the adult [5]. The thymus is the organ which shows the most rapid rate of growth in the postnatal period [6, 7]. The immaturity of the immunological system of the new born mouse may also contribute to its enhanced susceptibility to carcinogenic agents [8] and this in turn may impair the immunological competence of the animal throughout the latent period of tumour development [9].

The importance of genetic factors was clearly brought out by the distribution of pulmonary tumours in both control and treated mice. The A^{F} strain and its hybrids and to a lesser extent the BALB/c and IF strains developed lung adenomata without treatment. After chemical treatment these mice developed high incidences of adenomata of a shortened latent period and the number of adenomata per mouse was increased. The C57B1 mouse, which is well known for its resistance to carcinogenesis [10], failed to develop more than one of these tumours following the neonatal administration of DMBA. The CBA and C3H mice, which are recognized as low pulmonary tumour strains, gave an intermediate incidence of these tumours after chemical treatment.

A similar pattern was apparent in the formation of hepatomata. The CBA, C3H and reciprocal hybrid mice developed hepatomata without treatment and the effect of the chemical was to enhance the number of tumour-bearing animals and the degree of involvement of the liver. A small number of hepatomata arose in treated mice of all the other strains. This was probably a reflection of the immaturity of the neonatal mice.

Polycyclic aromatic hydrocarbons generally do not induce hepatomata in mice treated when they are adult [11]. Klein [12] and Roe and Walters [13] reported the induction of hepatomata by these agents but only in male suckling or neonatal mice. In the present experiment both male and female mice were affected, although in the males the liver neoplasms were more numerous.

With the leukaemias genetic influences appeared to play a relatively small part in the present experiments. Only the BALB/c mice had a moderate spontaneous incidence of leukaemia while all other mice were of low leukaemia strains. However, after administration of DMBA, all groups of mice developed leukaemia with the exception of A^{F} and male C57B1 mice. It is known that viruses are involved in murine leukaemogenesis and can be transmitted vertically from one generation to another [7, 14]. These viruses have been detected in thymic tissue of apparently healthy mice of all ages and many strains [15]. It is possible that in such mice the genesis of leukaemia depends on the action of extrinsic factors on dormant viruses. Kaplan [16] has shown that radiation in adult C57B1 mice is capable of eliciting a viral leukaemia. In the present

study, DMBA did not induce leukaemia in A^F mice and only to a small extent in C57Bl mice, but was highly effective in the reciprocal hybrids. It is well known that hybridization may produce mice more susceptible to tumourgenesis than either of the parents [17, 18].

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RESUME

Une dose unique de 30 μg *de DMBA est capable d'induire des tumeurs de divers tissus, lorsqu'elle est administrée, par voie sous-cutanée, à des souris nouveau-nés de plusieurs lignées, et à leurs hybrides F*1. *Les néoplasies les plus fréquemment observées furent les tumeurs pulmonaires et hépatiques, et les leucémies. Le génotype de la souris jouait un rôle important dans le processus de cancérisation du poumon et du foie, mais ce rôle était moins évident dans l'induction de la leucémie.*

SUMMARY

A single dose of 30 μg *of DMBA induced tumours of several tissues following its subcutaneous injection into neonatal mice of a number of strains and their F*1 *hybrids. Pulmonary and hepatic tumours and leukaemia were the most usual response. The genotype of the mouse played an important role in the pattern of lung and liver carcinogenesis but was less evident in leukaemogenesis.*

ZUSAMMENFASSUNG

*Neugeborene Mäuse verschiedener Stämme und ihrer F*1*-Hybriden wurden mit einer einmaligen subkutanen Injektion von* 30 μg *DMBA behandelt, die Tumoren verschiedener Gewebe erzeugte, hauptsächlich der Lunge und Leber neben Leukämien. Der Genotyp der Mäuse spielte eine wichtige Rolle für das Muster der Carcinogenese in Lunge und Leber, aber weniger für die Entstehung der Leukämien.*

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Control of Mitosis in Rabbit Vx2 Epidermal Tumours by means of the Epidermal Chalone

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INTRODUCTION

IN RECENT years it has been found that normal epidermis contains a substance, now called the epidermal chalone, which by inhibiting mitotic activity appears to play a central role in the homeostatic control of the balance between cell gain and cell loss within the epidermis [1–3]. In carcinogenesis the basic event is the disruption of this normal balance, and it is clear that this must depend on some change in the control mechanism of which the chalone forms a part. In this connexion it was considered important to discover, first, whether the cells of a fast growing epidermal tumour continue to produce the epidermal chalone, and second, whether they are still able to respond to its influence.

MATERIAL AND METHODS

The tumour

The Vx2 epidermal tumour was derived from papillomata induced in domestic rabbits by the Shope virus taken from wild cottontail rabbits [4–6]. Its history has been described by Tjernberg [7]:

> "Since 1938 it has been possible successfully to transplant the malignant tumour in series through domestic rabbits. During the first years of its propagation it elaborated an antigen showing an immunological resemblance to the virus of the Shope papilloma. At some point between the 5th and 8th years of continuous passage, however, a change occurred . . . and this antigen could no longer be demonstrated. Despite the fact that the virus has not since been recoverable from the carcinoma, the tumour has retained all its original characteristics."

These characteristics are as follows: it is a poorly differentiated, fast growing, squamous cell carcinoma; it metastasises early, first to the lymph nodes and then especially to the lungs, liver, and bones; and it has a marked tendency to central necrosis. In the present experiments one small inoculation killed a full grown rabbit in about 4 weeks.

It is important to emphasise that the original description [4] states that this tumour arose from the surface epidermis and that the hair follicles were not involved. It is only the surface epidermis that can respond to the epidermal chalone; the hair follicles, from which many so-called epidermal tumours are derived, have a separate mitotic control system [1].

The technique

The Vx2 tumour was inoculated intramuscularly in the thighs of adult domestic rabbits (weight about 2·5 kg), and all experiments were performed 2 weeks later.

The epidermal chalone was extracted from pig skin by N. V. Organon (Oss, The Netherlands) and was partly purified in the form of the 70–80% ethanol precipitate [8]. In a few experiments the epidermal chalone was also used in a highly purified form obtained by the

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Table 1. Mitotic rate and mitotic duration in Vx2 tumour cells

Average mitotic counts per 2500 cells				Mitotic rate: cells entering mitosis per hr	Mitotic duration $\frac{4M_1}{M_2}$
Without colcemid		After 4 hr colcemid			
N_1	$M_1 \pm$S.E.	N_2	$M_2 \pm$S.E.		
43	30·3±1·11	33	115·0±4.53	1·15%	1 hr approx.

N, number of tumours.

electrophoresis and dialysis of the ethanol precipitate [9]. Since the chemical nature of the chalone is not yet precisely known it is necessary to express dosage in units; one unit has been defined as the amount which, when dissolved in 4 ml saline culture medium, reduces the mitotic rate of mouse epidermis *in vitro* by 50% [10]. Both *in vivo* and *in vitro* the maximum doses given were as high as the limited chalone supply would allow.

It is now well known that the stress hormones, adrenalin and hydrocortisone, powerfully augment the antimitotic power of the epidermal chalone [10, 11], and consequently in some experiments the two hormones were given simultaneously with the chalone. In the *in vivo* experiments, the chalone and the two hormones were injected intraperitoneally in aqueous solution and were allowed to act for 4 hr. In these experiments 1 mg adrenalin was the maximum that each rabbit could tolerate; adrenalin bitartrate was, in fact, used but in calculating the dose only the adrenalin part of the molecule was considered. With hydrocortisone 5 mg was injected into each rabbit.

Also in the *in vivo* experiments, where relevant, 5 mg colcemid in aqueous solution was injected intraperitoneally into each rabbit to arrest mitoses in the metaphase, the rabbit being killed 4 hr later.

In the *in vitro* experiments small pieces (about 3 mm cube) of the growing tumour periphery were maintained at 37°C in phosphate-buffered saline with 0·02M glucose and an oxygen gas phase [12]. The chalone, and, where present, the stress hormones were already in the saline medium when the pieces of tumour were added. There was a lapse of 1 hr to allow all the mitoses originally present to pass the metaphase, and then colcemid (4μg/ml) was added and allowed to act for 4 hr.

The tumours were sectioned 7μ thick and the arrested metaphases were counted in unit section areas of 1·5 mm². Such an area contained approximately 2500 cells. The experiments were repeated as many times as the chalone supply allowed. The results were combined, and in all cases N is the number of tumours studied.

RESULTS

Mitotic rate and mitotic duration

For comparison with the situation in normal epidermis, the mitotic rate in the highly active outermost layers of the Vx2 tumours was first determined. The mitotic counts are shown in Table 1 and from them the mitotic rate and mitotic duration are derived. It must be emphasised that the calculated figure for mitotic duration is only approximate since it depends on the unwarranted assumption that the cell population was homogeneous.

Table 2. Effects of epidermal chalone and of extracts of Vx2 tumours on the mitotic activity of mouse ear epidermis in vitro

4 ml culture medium with:	Frst 4 hr *in vitro*			Second 4 hr *in vitro*		
	N	M±S.E.	Depression (%)	N	M±S.E.	Depression (%)
Control	20	7·7±0·24	—	10	7·8±0·34	—
0·1 unit epidermal chalone	10	5·0±0·17	35	10	7·9±0·32	0
1 unit epidermal chalone	20	4·3±0·16	44	10	7·9±0·42	0
0·1 unit tumour extract	10	4·6±0·41	40	10	8·1±0·35	0
1 unit tumour extract	20	4·1±0·22	47	10	8·3±0·33	0

Average numbers of mitoses arrested by colcemid in 4 hr in unit lengths of 1 cm epidermis sectioned 7μ thick. N, number of mice.

Table 3. Effect of adrenalin wash on ability of epidermal chalone and of extract of Vx2 tumours to inhibit epidermal mitotic activity in mouse ear epidermis after loss of endogenous adrenalin

4 ml culture medium with:	Second 4 hr *in vitro* N	M±S.E.	Mitotic depression (%)
Control	10	7·4±0·30	—
1 unit epidermal chalone	10	4·2±0·22	43
1 unit tumour extract	20	3·7±0·17	50

After 5 hr *in vitro* epidermis washed for 30 min in 0·25 μg adrenalin per ml. Average numbers of mitoses arrested by colcemid in 4 hr in unit lengths of 1 cm mouse ear epidermis sectioned 7μ thick. N, number of mice.

The conclusions are as follows:

(i) the mitotic rate (1·15%/hr) is much higher than in normal epidermis (in which the average in the basal mitotic layer is about 0·1–0·2%/hr) and indeed, allowing for the fact that the mitotic cycles are not synchronised in the tumour cells, it is probably as high, or higher, than that in wounded epidermis (about 4%/hr [13]) in which the cycles are synchronised;

(ii) the mitotic duration (about 1 hr) is short compared with normal epidermis (in which it ranges between 2·6 and 3·5 hr [13]) and again it more closely resembles that of wounded epidermis (about 1·3 hr);

(iii) if the tumour cell population is regarded as homogeneous then each cell must enter mitosis once every 87 hr, but if, as seems certain, not all cells have an equal mitotic potential then in the most active cells the intermitotic interval must be considerably shorter.

The chalone content of the Vx2 tumour

The Vx2 tumours were then tested for the presence within them of the epidermal chalone. The cells were macerated, extracted in water, and the clear supernatant was used as the chalone source. The water extract was always freshly made and used immediately; it is known that in such an extract the epidermal chalone is unstable at room temperature.

The extracts were tested on the mitotic activity of normal mouse ear epidermis *in vitro* (for technique, see [10]) and the results were compared with those obtained using a standard extract of epidermal chalone (70–80% ethanol precipitate). These results are in Table 2.

From this table it is clear that during the first 4 hr *in vitro* the tumour extracts exerted a strong antimitotic action, but it is not immediately clear that this was due to chalone action. The epidermal chalone is characterised by the fact that its full antimitotic activity is only developed in the presence of adrenalin. Fresh mouse ear epidermis contains some endogenous adrenalin when it is first placed *in vitro*, but after about 4–5 hr this is lost and even in the presence of excess chalone the epidermal mitotic rate then returns to normal. Table 2 also shows that this occurred both with the tumour extracts and with the standard epidermal chalone.

A further identity test for this chalone involves a brief adrenalin wash to re-sensitise the epidermal cells after 5 hr *in vitro* [10]; by itself this adrenalin wash has no effect. Table 3 records the effects of such a wash made just before the final 4 hr *in vitro*. Both with the tumour extracts and with the epidermal chalone the adrenalin wash restored the antimitotic activity.

It is known that, normally, some of the epidermal chalone is released from epidermis into the body space, and that consequently the chalone from one region of epidermis is capable of reducing the mitotic rate in a

Table 4. Mitotic rates of ear epidermis in normal rabbits and in rabbits with large Vx2 tumours

Normal rabbits N	M±S.E.	Rabbits with tumours N	M±S.E.	Mitotic depression
5	9·6±1·03	5	0·2±0·02	98%

Average numbers of mitoses arrested by colcemid *in vivo* in 4 hr in unit lengths of 1 cm ear epidermis sectioned 7μ thick.
N, number of rabbits.

Table 5. Effect of epidermal chalone, with and without adrenalin and hydrocortisone, on mitotic activity in Vx2 tumours in vivo

Treatment	Numbers of mitoses N	M±S.E.	Mitotic depression (%)
Control	33	115·0±4·53	—
500 units chalone	31	98·7±5·09	14
500 units chalone +1 mg adrenalin +5 mg hydrocortisone	34	97·5±3·81	15
5000 units chalone	6	43·8±9·63	62
5000 units chalone +1 mg adrenalin +5 mg hydrocortisone	13	27·5±4·99	76

Average numbers of mitoses arrested by colcemid in 4 hr in 1·5 mm² of tumour sectioned 7μ thick. N, number of tumours.

distant epidermal region [14]. If there is a similar release of chalone from the Vx2 cells then in the presence of a large tumour mass the body chalone content may rise to an abnormal level and the mitotic rate of the general epidermis may be depressed. To test this the mitotic rates of the ear epidermis were determined in normal and tumour-bearing rabbits and the results are in Table 4.

From this it is clear that the presence of a large Vx2 tumour leads to a deep depression of the general epidermal mitotic rate. This could be caused, at least in part, by an excess of adrenalin and of a glucocorticoid hormone due to the stress felt by an animal with a large tumour. However, against this, it has been found that mice with large Harding Passey melanomata show a relatively small epidermal mitotic depression [15].

The conclusions are as follows:

(i) the epidermal chalone is present in the Vx2 tumour cells, which presumably synthesise it;

(ii) the high mitotic activity of the tumour cells indicates that these cells do not respond to this chalone, in spite of the fact that the low mitotic activity of the normal epidermal cells may indicate an excess of chalone in a tumour-bearing rabbit;

(iii) the possibilities are that the Vx2 cells synthesise the epidermal chalone at a detectable but inadequate rate, or that the rate of chalone synthesis is adequate but the rate of chalone loss into the blood stream is unusually high, or that the chalone content is adequate and it is the ability of the cells to respond that has been lost.

The responsiveness of the Vx2 tumour to chalone

The ability of the Vx2 tumour cells to respond to the epidermal chalone was first examined *in vivo*. The experiments were severely limited in number because of the large amount of chalone extract required for injection into such a large animal as a rabbit. The chalone, injected subcutaneously, was given both alone and together with the two stress hormones, and the results are in Table 5.

In spite of the small numbers of rabbits and tumours the conclusion is clear that with the higher chalone dose a marked mitotic depression was obtained. The addition of adrenalin and hydrocortisone proved to be unnecessary, perhaps because all the tumour-bearing animals were already producing an excess of these two hormones. However, the possibility exists that the mitotic depression may be a non-specific reaction to the relatively large amounts of foreign proteins present in the partly purified chalone extracts. The experiments were therefore repeated using highly purified extracts of epidermal chalone [9]. Only 3 mg of this material was required for a dose of 5000 units, and in 5 rabbits the average mitotic depression induced in the Vx2 cells was 82%.

The next experiments were *in vitro*. It has been noted above that in their mitotic activity the Vx2 cells most closely resemble the cells of wounded epidermis, which may lack chalone because of a high rate of loss through the damaged cell surfaces [1]. It has been found that to inhibit the mitotic activity of wounded epidermis *in vitro* it is necessary to add adrenalin and hydrocortisone with the chalone [16]. Consequently the Vx2 cells were treated with epidermal chalone, adrenalin, and hydrocortisone alone, in pairs, and all together. The combined results of a large number of experiments are summarised in Table 6. It will be noted that the mitotic rate of the Vx2 cells *in vitro* was much lower than *in vivo*

Table 6. *Effects of epidermal chalone, adrenalin, and hydrocortisone on mitotic activity in Vx2 tumour cells* in vitro

Control tumour N	Control tumour M±S.E.	Tumour treated with the following per 4 ml medium	N	M±S.E.	Mitotic depression (%)
47	14·1±0·86	1 unit chalone	35	15·8±1·07	0
47	14·1±0·86	10 µg adrenalin	34	15·4±0·85	0
47	14·1±0·86	10 µg hydrocortisone	30	15·3±1·26	0
20	15·2±1·40	1 unit chalone + 10 µg adrenalin	25	15·4±1·48	0
20	20·1±0·80	1 unit chalone + 10µg hydrocortisone	36	21·1±0·75	0
28	9·9±0·55	10µg adrenalin + 10µg hydrocortisone	58	8·8±0·24	11
52	11·6±0·57	1 unit chalone + 10µg adrenalin + 10µg hydrocortisone	75	5·5±0·29	53

Average numbers of mitoses arrested by colemid in 4 hr in 1·5 mm² of tumour sectioned 7µ thick. N, number of tumours.

(compare Table 1); a similar though not so acute mitotic depression in the less favourable conditions of the saline medium is also seen in wounded epidermis [16].

From these results the importance of the two stress hormones is obvious. In the relatively high concentration used none of the three substances had any action alone, while of the pairs of substances only adrenalin with hydrocortisone produced a small depression. It was only when the three substances were present together in the medium that a considerable mitotic depression was obtained.

The conclusions are as follows:

(i) the Vx2 tumour cells are capable of responding by mitotic inhibition to the presence of the epidermal chalone both *in vivo* and *in vitro;*

(ii) the *in vitro* experiments show that this response depends on the presence of adequate amounts of adrenalin and hydrocortisone, and the slight mitotic depression obtained when these two hormones are used together may perhaps depend on their co-operation with the limited amounts of endogenous chalone which are evidently present in the Vx2 cells.

DISCUSSION

It is now clear that the high mitotic rate of the Vx2 tumour cells is not due to the absence of the epidermal chalone or to the complete failure of the cells to respond to this chalone. However, it is possible that the chalone concentration within the tumour is low and that the cell sensitivity is reduced.

The Vx2 tumour cells have been compared with cells adjacent to a wound in which the high mitotic activity may be related to a temporary increase in the rate of chalone loss [1, 16]. One obvious possibility is that the Vx2 cells show a similar, but chronic, increase in the rate of chalone loss. This could lead to an increased chalone concentration in the blood and so could explain the deep mitotic depression observed in the epidermis of tumour-bearing rabbits. In malignant metastasising tumours the cell walls are believed to be abnormal and this could be accompanied by some loss of control of molecular transport across the surface.

It is in fact possible to make a rough estimate of the chalone content both of epidermal cells adjacent to a wound and of Vx2 cells. It has been shown elsewhere [10, 11] that adrenalin and hydrocortisone are not themselves anti-mitotic agents and that they inhibit epidermal mitosis only in co-operation with the epidermal chalone. It follows that the degree of epidermal mitotic inhibition caused by these two hormones, administered together, gives a measure of the chalone content of the cells. Results are available showing the *in vitro* effects of adrenalin and hydrocortisone (concentration of each, 2·5 µg/ml): in normal epidermis the mitotic inhibition was 100%, in epidermis adjacent to a wound it was 42%, and in Vx2 cells it was 11% (Table 6). On the assumption that with no chalone in the cells there would be no inhibition, these figures suggest that in epidermis adjacent to a wound the chalone concentration is temporarily reduced to less than half normal, while in the Vx2 cells it is permanently reduced to about 1/10 normal.

Support for these conclusions is given by Rytömaa and Kiviniemi [17], who have studied a rat granulocytic chloroleukaemia. Conditions in this tumour are remarkably similar to those in the Vx2 tumour: the

leukaemic cells contain the granulocytic chalone and retain the ability to respond to it, and further, when large numbers of leukaemic cells are present the mitotic activity of the normal granulocyte precursor cells is inhibited. More important, however, are the quantitative observations which show that each chloroleukaemic cell has less than 1/10 the chalone content of a normal granulocyte, while conversely the leukaemic serum has a higher than normal chalone content. The implication is that the low chalone content of the leukaemic cells is at least partly due to an unusually high rate of chalone loss into the blood.

It has been suggested that the chalone concentration, and therefore the mitotic rate, within such major tissue systems as epidermis or liver must be determined by the balance that is established between the rate of chalone synthesis within the tissue cells and the rate of chalone loss into and from the body space [1, 18]. According to this theory, only when the tissue mass is large enough in relation to the total body space can the chalone within the tissue cells reach a high enough concentration to inhibit mitosis and so to prevent any further tissue growth.

If in a group of tumour cells the rate of chalone loss is increased, or the rate of chalone synthesis reduced, then the resulting high mitotic activity should not continue indefinitely. If the animal can survive long enough, the cells should multiply only until a new point of balance is reached between the tumour mass and the total body space [19]. At this point the chalone concentration in the body space would be sufficient to allow the chalone in the tumour cells to reach a mitosis-inhibiting concentration.

Thus, although the growth of a tumour may at first be rapid, it should not continue indefinitely, and "eventually a limiting size should be approached" [20]. Evidence that tumour growth does slow down as tumour mass increases has been assembled by Laird [21], who has concluded that this "is not simply due to failure of the host to support growth, but is a product of host-tumour interaction."

In fact the growth of a tumour appears to be closely similar to the growth of an organ remnant during regeneration. When, for instance, a large part of the liver is excised the remnant is too small by reference to the total body space, and evidently it is unable to synthesise sufficient chalone to prevent its own mitotic activity. Growth therefore continues until the normal liver mass is regained. At this point the chalone output is high enough to raise the chalone concentration in the body space to such a level that, in turn, the chalone concentration in the liver cells reaches a mitosis-inhibiting level [19].

Any tumour which continues to produce the chalone of its tissue or origin and to react to this chalone should continue to obey this general law. In support of this it has been found that when, due to metastasis, several tumours are present in the body, the removal of one may stimulate the growth of those remaining [22], exactly as occurs in liver autografts after partial hepatectomy [23].

The present results and conclusions raise two important questions. First, it is necessary to discover whether the generality of tumours are similar to the Vx2 and chloroleukaemia in continuing to produce and respond to the chalones of their tissues of origin. Second, it is necessary to discover whether tumour growth can be suppressed by means of appropriate chalone injections. A preliminary and optimistic answer to this second question comes from the experiments of Mohr *et al.* [24], who have treated mouse and hamster melanomata with skin extracts containing the melanocyte chalone [15].

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RESUME

Les expériences relatées dans ce travail montrent que les cellules de la tumeur épidermique Vx2 des lapins contiennent une chalone épidermique. Les cellules tumorales Vx2 réagissent à cette chalone par une inhibition mitotique.

Le fait que la tumeur Vx2 conserve un index mitotique élevé et une croissance très rapide, comparables à ceux observés au niveau de l'épiderme en régénération d'une plaie, suggère que, soit la concentration de la chalone, soit la sensibilité des cellules tumorales à cette chalone, doit être basse. Certaines observations semblent en effet indiquer une diminution de la concentration cellulaire de la chalone qui résulterait d'un transfert accéléré de celle-ci vers les liquides circulants, comme le suggère une réduction marquée

à distance de l'activité mitotique de l'épiderme normal chez les animaux porteurs de tumeurs Vx2 étendues.

On suggère que, si la survie des lapins était suffisante, la croissance de la tumeur Vx2 finirait par se ralentir ou même par s'arrêter, car l'augmentation de masse de la tumeur devrait s'accompagner d'une élévation croissante du taux de chalone dans tout l'organisme.

SUMMARY

A study of the Vx2 epidermal tumour of rabbits has shown that its cells contain the anti-mitotic epidermal chalone and that they are able to respond by mitotic inhibition to the presence of this chalone. Since the Vx2 tumour has a high mitotic rate and grows rapidly, it is evident that either the chalone concentration or the cell sensitivity to it must be lower than normal.

There is, in fact, evidence to suggest that the intracellular chalone concentration is reduced and that this is at least partly due to an unusually high rate of chalone loss into the blood. This in turn leads to a pronounced mitotic inhibition in the surface epidermis.

It is suggested that, if the rabbit could be kept alive long enough, the growth of a Vx2 tumour should ultimately slow down, and even stop, since increasing tumour mass should be accompanied by an increasing chalone concentration in the whole body. There is evidence that the growth of tumours does indeed become slower with increasing tumour mass.

ZUSAMMENFASSUNG

Das Epidermis-Chalon wurde im Vx2 Epidermistumor vom Kaninchen gefunden. Dieser Tumor reagierte auch auf den Epidermis-Chalon-Extrakt aus normaler Haut mit Mitosehemmung.

Da diese schnell wachsende Geschwulst einen hohen Mitose-Index zeigt, kann angenommen werden, dass die Chalonkonzentration herabgesetzt oder die Ansprechbarkeit der Zellen dem Chalon gegenüber geringer war.

Befunde deuten darauf hin, dass die intrazelluläre Chalonkonzentration in den Tumorzellen reduziert ist, und dass der Hauptgrund für diese Reduktion eine ungewöhnlich hohe Chalonabgabe an das Blut ist. Das Resultat ist eine Mitosehemmung in der Epidermis.

Offenbar bringt die erhöhte Tumormasse eine erhöhte Chalonkonzentration im ganzen Körper mit sich.

Es wird diskutiert, dass das Wachstum des Vx2 Tumors sich verlangsamen und sogar einstellen würde, falls das Kaninchen am Leben erhalten werden könnte. Es bestehen Anhaltspunkte dafür, dass das Tumorwachstum mit steigender Tumormasse sich verlangsamt.

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Control of DNA Duplication in Rat Chloroleukaemia by Means of the Granulocytic Chalone

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INTRODUCTION

IT HAS been obvious for at least thirty years that a break in the 'internal' or 'organismal' control of cell multiplication is the outstanding event in carcinogenesis [1–3], and it is, therefore, reasonable to search for the characteristic of malignancy directly at the level of those mechanisms that normally control cell division.

In the case of blood cells the rate of cell production is known to be regulated by humoral substances which influence the production and mitotic activity of the progenitor cells in the bone marrow. It is also known that the point of balance between cell gain and cell loss is determined not only in terms of tissue mass but also in terms of functional demand [4]. The first, and by far the best known, of these regulator substances is erythropoietin, a factor which specifically stimulates red cell production, apparently by means of converting resting stem cells into erythrocytic progenitor cells [5, 6]. So far the existence of a granulopoietin for the conversion of stem cells into granulocytic progenitor cells has not been satisfactorily proved.

Besides the poietins, it has also been found that both the erythrocytic and granulocytic cell populations are restrained from excessive cell multiplication by a second humoral mechanism based on specific feedback inhibitors, or chalones [4]. These inhibitory systems are apparently similar to those which are controlled by other known chalones, including that of the epidermis [7].

It is also known that a third control mechanism exists in the granulocytic system. In conditions of acute functional demand for granulocytes, a factor, termed the granulocytic antichalone, is capable of directing the non-proliferating progenitor cells into the generative cell cycle [4, 8, 9]. It thus appears that the control of cell multiplication may be more complex in the granulocytic cell system than it is, for instance, in epidermis [7, 10].

It has been pointed out that the essential event in carcinogenesis is a break in the normal balance between cell production and cell loss, and the question therefore arises whether in granulocytic leukaemia there is a distortion of one or more of the three control mechanisms mentioned above. In the present paper an account is given of the state of the chalone mechanism in rat chloroleukaemic cells.

MATERIAL AND METHODS

The chloroleukaemia tumour (CHL)

Shay's myelocytic chloroleukaemia was originally induced by gastric instillation of methylcholanthrene and was considered to be a malignant derivative of the granulocytic cell system [11, 12]. It has two forms: when transplanted subcutaneously a local tumour develops with a relatively slow growth rate, but when injected intraperitoneally the result is a generalized and typical leukaemia. In two-week-old rats of both sexes about one half develop tumours after subcutaneous trans-

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plantation while almost all develop leukaemia after intraperitoneal injection [13, 14].

Both a local CHL tumour and any organ infiltrated with leukaemic cells appear green in colour and show a bright red fluorescence in the u.v. light due to the presence of protoporphyrin IX. This substance is apparently similar to, if not identical with, the porphyrin component of myeloperoxidase (verdoperoxidase), which is an enzyme specific to the granulocytic cells [15–17]. The CHL cells appear to be immature myelocytes [12]. It may also be noted that, although chloroleukaemia is known to occur in man, it is rare.

The mean survival times of the recipient rats with generalized chloroleukaemia are shown in Table 1, and the conclusions are:

(1) that the degree of malignancy of the CHL cells has increased considerably during the 10-year period in our laboratory;

(ii) that the degree of malignancy of different donor tumours, as indicated by the wide ranges of the mean survival times, shows considerable variation from one case to another.

Table 1. *Survival times of Sprague-Dawley rats with Shay's myelocytic chloroleukaemia*

Year	Survival times of recipient rats (days after transfer) Overall mean or 37%	Ranges	References
1960	35·6	23·8–42·9	[13]
1963	29·6	21·0–40·0	[14]
1967	21·8	15·9–28·0	–

Leukaemic cells injected intraperitoneally into 2-week old rats of both sexes. Survival times expressed either as arithmetic means [13, 14] or as time when 37% of rats remained alive.

Extraction of chalones

The mitotic inhibitor obtained from the serum or cells of normal untreated rats is tentatively termed the normal chalone, while material obtained from the CHL animals is termed the chloroleukaemia chalone. However, it is not intended to imply that any difference exists between these two chalones other than their origin.

The chalones were obtained from Sprague-Dawley rats either from the serum or from the cells. Normal cells were collected from the blood, the bone marrow, or from an aseptic inflammatory exudate in the peritoneal cavity; CHL cells were obtained either from the bone marrow or from a 'free' tumour growing in the peritoneal cavity. The cells were concentrated by centrifuging, washed once or twice in Hanks' BSS, and resuspended in a small volume of Hanks' solution to give about 20×10^6 nucleated cells per ml. Homogenized or intact cell suspensions were then incubated at 37°C for 1 hr, the cells were centrifuged (>3000 g for 20 min) and the clear supernatant was used as the raw chalone source.

Purification of chalone

Purified chalone preparations were obtained from the sera and the cell extracts by means of gel filtration on Sephadex G-75 in bead form (Pharmacia, Uppsala). The wet gel was sterilized by autoclaving at 105°C before use and the bed was stabilized by the eluant. Void volume (V_0) of the gel was determined using 'blue dextran'. Hanks' BSS was chosen as the eluant so that the fractions collected could be added directly to the assay cultures.

Because the main objective of the gel filtration was to purify the chalone preparations without excessive dilution of the active material, the sample volumes added to the column (usually 3–6% of the total bed volume) represent a compromise between optimum yield and optimum purification.

Phenol red was present in the Hanks' solution of the chalone extracts but not in the elution medium. It proved to be a useful indicator during chalone elution because, by watching its passage through the column, it was possible to follow the filtration during the actual preparative run. In the conditions used used here phenol red is eluted shortly after the chalone peak; the relative elution volumes (Ve/Vo) for the two substances are $2 \cdot 73 \pm 0 \cdot 035$ (mean$\pm$S.E.; $N=12$) and 3·10, respectively.

The amount of chalone present in these extracts cannot be determined chemically: it may be emphasised that no correlation exists between the chalone activity and the amount of organic material present in the extracts as judged by the u.v.-absorbances. However, a crude estimate of the chalone concentration may be obtained in terms of nucleated cells extracted per ml solution.

The low amount of organic material which is usually present in the chalone preparations produced by gel filtration of the cell extracts is indicated by the very low u.v.-absorbances;

the peak absorbancy (occurring at about 270–275mμ) has been as low as 0·035–0·040 and yet a 1:2 dilution of these solutions has been found to be capable of producing 60% mitotic inhibition in the target cells.

Bioassay technique

The chalone activity of the extracts was studied *in vitro* using homologous normal bone marrow (BM) cells and CHL cells, respectively, as the target tissue. The target cells were obtained from untreated Sprague-Dawley rats shortly before the start of the assay cultures.

Details of the culture system have been described previously [4, 8, 9]. The essential feature of the system is that in each individual culture an equal number of target cells is grown as a monolayer on a cover glass in a Pyrex glass culture tube. The culture medium was Hanks' balanced salt solution (BSS) with serum. The serum content was 20% when fresh serum chalones were tested, and 10% of 'old' (chalone-free) serum [4, 9] when the test material consisted of cell extracts.

Radio-actively labelled precursor substances (^{3}H-thymidine, sp.ac.1·52 and 3·0 c/m-mole, to label newly forming DNA; and ^{14}C-formate sp.ac.13·4 and 42·8 mc/m-mole, to indicate *de novo* synthesis of DNA and RNA; from the Radiochemical Centre, Amersham) were added to the cultures at zero time, usually to give a concentration of 1·0 μc/ml. After from the 2–10 hr incubation time the cover glasses were harvested and prepared for analysis [4, 9]. The main analyses used in this study were the following:

(i) Determination of the total radio-activity incorporated in the cells; this was done in a liquid scintillation counter (earlier IDL, Isotope Developments Ltd., and later Packard Tri-Carb, Packard Instruments). Preparations labelled with the ^{14}C-formate were also subject to hydrolysis in 1N HCl at 60°C for 6½ min to remove all RNA-bound radioactivity [4, 18]. The incorporation of ^{14}C-formate in different cell components in rat bone marrow *in vitro*, and the reliability of the analytical methods, were checked using ^{3}H-TdR, ^{3}H-adenine, ^{3}H-uridine, ^{3}H-leucine and ^{3}H-tryptophan labelled cultures as the basis of comparison. These results indicated that 45–50% of the total formate activity was in RNA and was removed by HCl hydrolysis, that another 45–50% was in DNA and was removed by subsequent hydrolysis with hot perchloric acid, and that less than 8% was in protein and was not removed by either hydrolysis. Details of these results will be given elsewhere.

(ii) Determination of labelling indices and grain counts of the same preparations were used to complement the results obtained from total radio-activities. The autoradiograms were prepared by the stripping film technique using Kodak AR-10 plates.

(iii) Determination of cell numbers on each cover glass was made by a point counting technique or by line sampling across the glass. In some cases counts of different cell types were also made in order to detect possible alterations in the cellular composition of the cultures.

Part of the mathematical and statistical analyses was carried out with a digital computer (IBM 1620) at the Computing Centre of the University of Helsinki.

RESULTS

The rate of cell proliferation in CHL cells

The proliferative activity of normal BM and of CHL cells, respectively, was studied under similar *in vitro* conditions and the results are summarized in Table 2.

This shows (column 5) that the proliferation rate of the progenitor cells is evidently faster in normal bone marrow than it is in the chloroleukaemic cells. It is possible, however, that these estimates may be subject to some error. In particular, it is difficult, if not impossible, to classify the cells accurately into mitotic and non-mitotic categories [4, 19]. However, since obvious non-mitotic cells, such as polymorphonuclear granulocytes, are common in normal bone marrow cells and rare among leukaemic cells, it is probable that the difference between the normal BM and the CHL cells is even greater than is shown in the table.

Another difficulty involved in the estimation of the proliferation rates is the accuracy of the DNA synthesis times. However, the techniques used here are adequate to determine the relative durations of DNA synthesis in the normal BM cells and the CHL cells, and the conclusion is that they were evidently the same.

The values seen in columns 6 and 7 (Table 2) indicate that the normal BM cells have incorporated considerably more ^{3}H-TdR than the CHL cells on a per cell basis. If it is now assumed that both types of cells synthesize the same diploid amount of DNA, these results would suggest either that the rate of DNA synthesis is higher in normal BM cells than in CHL cells or that the CHL cells do not utilize exogeneous thymidine with the same efficiency as do the normal BM cells. The rate of DNA synthesis is inversely proportional to the duration of DNA synthesis and therefore the

Table 2. Rate of cell proliferation in normal bone marrow cells and in chloroleukaemic cells in vitro

Cell type	Labelling index (per cent)		Approximate duration of DNA synthesis*	Rate of cell proliferation (per cent/hour)		Rate of uptake of ^{3}H-TdR (CPS per 10^6 nucleated cells)		Relative rate per labelled cell
	All cells	Progenitor cells		All cells	Progenitor cells	All cells†	Labelled cells†	
Normal bone marrow	11·85	40–45	5 hr	2·4	8–9	54·5	460	100
	12·0			2·4		51·0	426	
Chloro-leukaemia	20·5	30–35	5–6 hr	4·1	5–7	54·0	263	68·3
	23·4			4·7		80·0	343	

All figures indicate means of at least six measurements, or are ratios of two such means.
Values given in the same horizontal row are taken from the same experiment.

*Estimated from the time of reaching maximal radioactivity (grain count) per labelled cell.

†All variables are standardized to a constant level; concentration of ^{3}H-TdR in the medium to 1μc/ml at $t=0$, sp.ac. to 1·52 c/m-mole, incorporation time to 6 hr, and counting efficiency to an arbitrary relative value.

former alternative is contrary to the conclusion reached above.

It thus appears that the CHL cells may not take exogenous thymidine so readily and may rely preferentially on synthesis from endogenous substrates. In support of this it was found that the rate of incorporation of exogenous formate was higher in the CHL cells than in the normal BM cells (see Tables 2 and 5). The conclusion is that the 'shunt' pathway utilizing thymidine in the DNA is weak in the CHL cells and that the *de novo* pathway is strong. These differences between the normal BM and the CHL cells make it necessary, when studying chalone action, to carry out the experiments using both labels.

The conclusions indicate that:

(i) the rate of cell proliferation, expressed as a percentage of all the potentially mitotic cells, is lower in chloroleukaemia than in the normal bone marrow, but since, due to delayed maturation, there are more progenitor cells in the CHL than in normal BM, the rate of cell production and the growth in population of the leukaemic cells is higher;

(ii) the chloroleukaemic cells utilize exogeneous formate more effectively than do the normal BM cells.

The chalone content of CHL serum and cells

It has previously been demonstrated by Rytömaa and Kiviniemi [4] that strong chalone activity is present in the serum of CHL animals, whether the leukaemia is generalized or has the form of a solid tumor, and that the CHL cells, like the normal BM cells, are able to respond to this chalone. Presence of strong chalone activity in the CHL serum is also demonstrated by the results shown in Fig. 1 and in Table 4.

The similarity between the normal and leukaemic chalones is indicated, among other things, by their behaviour in gel filtration. In the example shown in Fig. 1 the sera were collected at the same time, fractionated in the same column, and tested simultaneously on the same target tissue (normal BM cells). Although the comparison shown in Figs. 1 and 2 of the elution characteristics of chalones extracted from serum with those of chalones extracted from cells (see below) is not entirely accurate because of the exceptionally large sample volumes used (17%), the relative elution volumes of the serum chalones (Ve/Vo $=2·6$–$2·7$ in Fig. 1) were well within the limits obtained from cell extracts (mean$\pm$S.D.$=2·73\pm0·120$).

The results in Fig. 1 also strongly suggest that the chalone activity in the CHL serum was greater than that in the normal serum (see also Tables 3 and 4). However, this greater activity does not necessarily indicate that the chalone was produced by the leukaemic cells themselves. It is even possible that excess chalone is produced as a form of defence mechanism against the malignant growth. The next step was therefore to determine the chalone content of the CHL cells.

In order to obtain test material which could be considered free of serum contamination, the CHL cells were obtained from the peritoneal cavity of rats into which the leukaemic cells had been transplanted about 1 month before. The tumour cells were suspended in Hanks' BSS (250×10^6 leukaemia cells per ml), washed

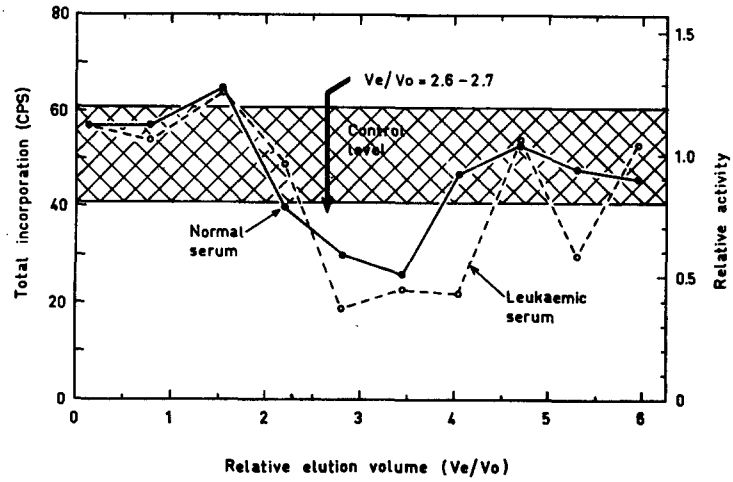


Fig. 1. *Purification of chalones from fresh normal and chloroleukaemic sera by gel filtration on Sephadex G-75. Activities of fractions were measured in terms of total incorporation of* ^{3}*H-TdR in target cells* in vitro (*normal bone marrow cells*). *Each point is the mean of duplicate cultures; the shaded area represents the mean* ± *S.D. of chalone-free control cultures* (N=4).

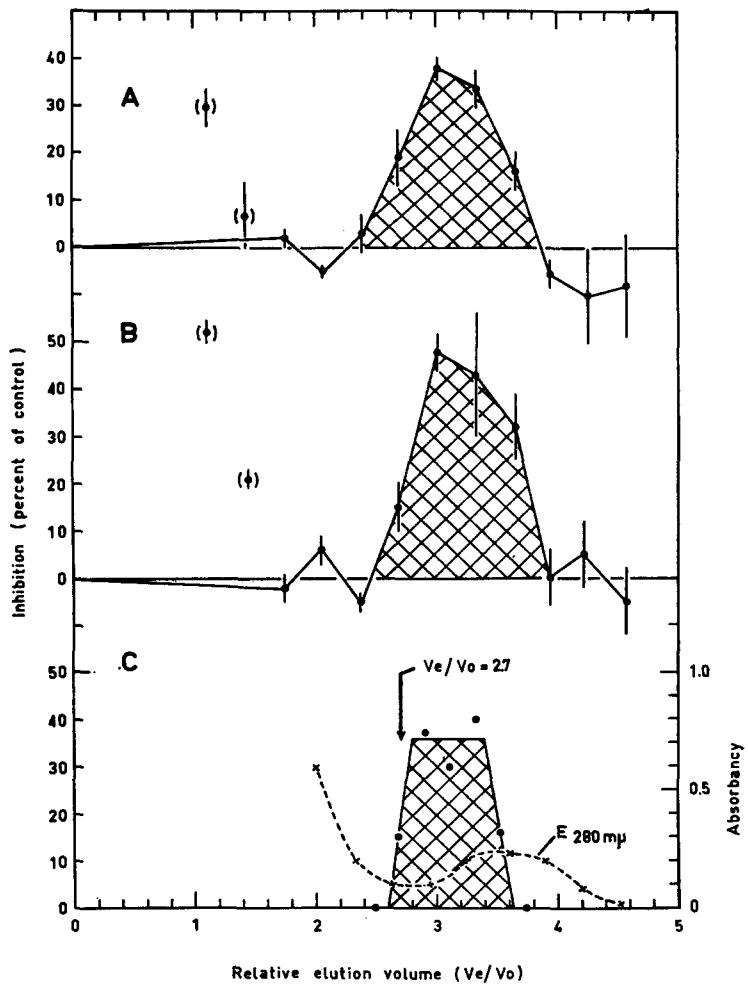


Fig. 2. *Purification of chalones from extracts of homogenized chloroleukaemic cells* (*A and B*) *and non-homogenized normal cells* (*C*) *by gel filtration on Sephadex G-75. Activities of fractions were measured in terms of total incorporation of* ^{3}*H-TdR in target cells* in vitro. *In A targets were CHL cells; in B and C targets were normal BM cells. In A and B each point represents the mean* ± *S.E. of* 4 *cultures; in C each point represents the mean of* 5 *experiments each of* 4 *cultures. The dots in parenthesis indicate fractions which were toxic to the target cells* (*see text*).

Table 3. Response of normal bone marrow cells and of chloroleukaemic cells to normal chalone

	Inhibition (per cent of control cultures) Normal bone marrow cells N	Mean±S.E.		Chloroleukaemic cells N	Mean±S.E.
TdR	5	6·3±7·3	TdR	5	−5·0± 9·9*
TdR	4	23·9±4·3	TdR	5	−3·5± 6·7*
TdR	3	24·0±5·0	TdR	4	−0·1± 2·3*
TdR	2	40·5(±3·5)	TdR	4	16·8±12·3
F, total	6	20·0±5·8	TdR	2	26·5(±4·5)
F, DNA	6	22·5±5·5	F, total	6	−7·7± 2·1*
			F, total	5	18·8± 8·6
			F, DNA	5	32·4± 6·7
	Overall mean 22·9			Overall mean 9·8	

Figures indicate mean ±S.E. values obtained from different experiments. N is the number of pairs of groups compared with each experiment. The control and experimental groups of each pair contain the same number (3–5) of individual cultures.
Symbols used: TdR=incorporation of ^{3}H-thymidine; F, total=total incorporation of ^{14}C-formate ; F, DNA=incorporation of ^{14}C-formate in DNA.
*Numerically an increase was observed in the presence of chalone.

twice, resuspended in the same volume, and homogenized. After extraction for 1 hr and subsequent purification on Sephadex G-75, all fractions were tested simultaneously using both normal BM cells and CHL cells as the target tissues. The results are shown in Fig. 2.

As a consequence of the high number of CHL cells needed to obtain sufficiently active chalone preparations, the protein content of these extracts was also high. The bulk of the proteins was concentrated in the first one or two fractions recovered from the gel and it was found in the tests that these fractions caused an excessive detachment of cells from the cover glasses. This fully explains the strong reduction in the total radioactivities caused by the first two fractions (see Fig. 2).

Also shown in Fig. 2 are results obtained using extracts of normal leucocytes. However, it was found that these extracts had such a high chalone content that only $10–20\times10^{6}$ non-homogenized cells were needed to give a similar mitotic depression to that obtained with the homogenized CHL cell extracts.

The conclusions are:

(i) chalone is present in excess in chloroleukaemic serum;

(ii) chalone is also present in the CHL cells, but a comparison with normal cell extracts suggests that, at a conservative

Table 4. Response of normal bone marrow cells and of chloroleukaemic cells to chloroleukaemic chalone

	Inhibition (per cent of control cultures) Normal bone marrow cells N	Mean±S.E.		Chloroleukaemic cells N	Mean±S.E.
TdR	4	30·0± 5·2	TdR	4	10·5±12·5
TdR	3	44·5± 7·3	TdR	4	48·0±7·8
TdR	3	52·3± 3·0	F, total	5	15·0±6·9
F, total	3	20·7±10·5	F, DNA	5	29·2±7·7
F, total	5	28·6± 5·7			
F, total	6	46·7± 4·4			
F, DNA	5	15·8± 3·8			
F, DNA	6	56·5± 3·4			
	Overall mean 36·9			Overall mean 25·7	

Figures indicate mean±S.E. values obtained from different experiments. N is the number of pairs of groups compared within each experiment. The control and experimental groups of each pair contain the same number (3–5) of individual cultures.
Symbols used: TdR=incorporation of ^{3}H-thymidine; F, total=total incorporation of ^{14}C-formate; F, DNA=incorporation of ^{14}C-formate in DNA.

estimate, each CHL cell contains only from 1/10–1/40 the amount present in a normal cell;

(iii) the chalones from normal and CHL cells are active on both normal BM and CHL cells;

(iv) judging from this and from the gel filtration characteristics, the chalone is evidently similar whether it comes from normal serum or cells or from leukaemic serum or cells.

The response of the CHL cells to chalone

A series of experiments was performed to test the activity of normal chalones on normal BM and CHL cells and the results are shown in Table 3.

Similar tests were then made using chalone from the chloroleukaemic serum and the results are in Table 4. The only methodological difference between the data in Tables 3 and 4 was in the origin of the test material: normal chalone was obtained partly from the serum and partly from cell extracts whereas all CHL chalone was obtained from the serum. CHL cells were excluded as a source of chalone because of the further purification needed to exclude toxic protein side effects (see above).

The conclusions drawn from these two sets of data are as follows:

(i) the inhibition of DNA synthesis in both types of target cells was stronger in the case of the CHL chalone than in the case of the normal chalone, and the most obvious explanation is that the CHL serum contains a greater chalone concentration;

(ii) normal BM cells are significantly more sensitive to the chalones than are the CHL cells.

Effects of endogenous chalone

The results so far obtained indicate the seemingly paradoxical situation that in the leukaemic rats the CHL cells contain much less chalone than do normal granulocytes while the serum contains much more. Such a situation could have several explanations, but the most probable may be that in this form of leukaemia there is an unusually high rate of chalone loss, so that the cells are unable to retain an effective intracellular chalone concentration. This could also explain why the response to chalone, whether endogenous or exogenous, is weaker in the CHL cells than in the normal BM cells.

It is also possible that the CHL cells may synthesize less chalone than do the normal BM cells, and the next experiments were planned in an attempt to detect any such difference. The basic assumption was made that in a mixed culture of CHL and normal BM cells one of the two populations is likely to liberate more chalone than the other. In these conditions the cell population which produces less chalone will probably be more strongly inhibited by comparison with a culture in which this population is present alone.

There were two important problems to be considered in the planning of these experiments. The first was the possible effect of different cell densities on the proliferation rates (the so-called 'gradient factor'; 4,20), and the second was the evident metabolic differences shown by the different rates of uptake of ^{3}H-TdR and ^{14}C-formate in the CHL and normal

Table 5. *Incorporation of ^{3}H-TdR and ^{14}C-formate in mixed cultures of chloroleukaemic (CHL) and normal bone marrow cells (BM)*

Number of cells present		Deviation: per cent of 'expected' incorporation ^{3}H-thymidine duration of incubation				^{14}C-formate duration of incubation			
CHL	BM	2 hr	4 hr	8 hr	22 hr	2 hr	4 hr	8 hr	22 hr
0·9	0·1	−18	−37	−16	+12	−27	+8	+10	+12
0·75	0·25	−17	−39	−11	+15	−18	+5	+35	−7
0·5	0·5	−9	−19	+11	+5	−11	−25	+16	+48
0·25	0·75	+2	−10	−6	+29	−9	−12	−7	−2
0·1	0·9	−5	−9	+5	+24	−11	+39	+45	+20
		Total incorporation in pure cultures (CPS)							
1·0	—	11·9	16·8	19·7	16·0	12·0	15·8	23·6	20·8
—	1·0	15·9	23·3	30·0	18·0	5·0	4·4	7·0	4·0

Values shown are expressed as per cent deviations of the observed means from the expected means, which were calculated from the mean values for pure cultures shown below. In each case N=4.

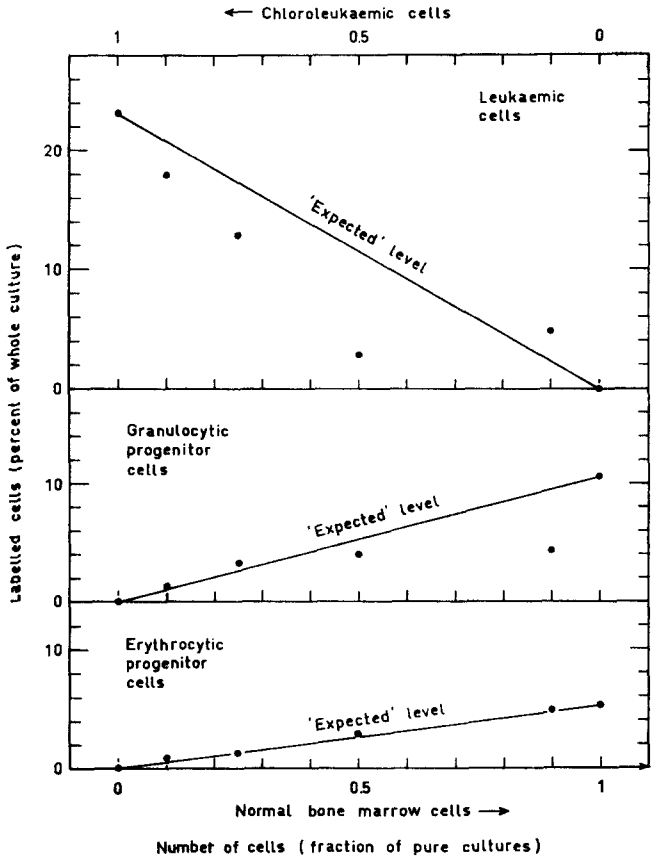


Fig. 3. Absolute numbers of ^{3}H-TdR labelled cells, expressed as percentages, in mixed cultures at 4 hours (see Table 5 and text).

BM cells (see p. 598). To avoid these difficulties the total number of cells per culture was kept constant throughout the whole range of cell mixtures and each experiment was duplicated using both labels.

The results are shown in Table 5 and Fig. 3. These show that in the short-term cultures deviations from the 'expected' radio-activities tend to become greater with increasing CHL cell content, and that the deviations in those cultures with the highest CHL cell content were sometimes even greater than would be expected from the complete inhibition of all the normal progenitor cells. These findings suggest strongly that the deviations were mainly, if not entirely, due to the inhibition of the CHL cells. The same conclusion is also suggested by the results of the autoradiographic analysis.

However, some of the results obtained by autoradiographic analysis may not be fully accurate because of the difficulty of distinguishing between the progenitor cells of the chloroleukaemic and of the normal granulocytic populations. The effect of this was particularly great in cultures consisting of 0·1 parts of CHL cells and 0·9 parts of normal BM cells, and it may explain the anomalous result in this experimental group, seen in Fig. 3, of the high labelling index in the leukaemic cells and the low labelling index in the granulocytic progenitor cells.

The conclusions drawn from the results in Table 5 and in Fig. 3 are as follows:

(i) with shorter incubation times it was clear that the rate of cell proliferation was inhibited in mixed cultures, that this inhibition was greatest in the CHL cells, and that it was evidently due to chalone liberated into the culture medium from the normal mature granulocytes;

(ii) this indicates that the chloroleukaemic cells probably synthesize less chalone than do the normal mature granulocytes;

(iii) with longer incubation times, the radio-activity of the cells was higher than the expected norm, and this indicates that a characteristic feature of the chalone inhibition is a delay rather than a permanent inhibition of the proliferation rate;

(iv) the data in Fig. 3 also show that the proliferation rate of the erythrocytic progenitor cells was not affected in the mixed cultures, and therefore that the chalone inhibition was tissue-specific.

DISCUSSION

One of the main results of this study has been the discovery that the chloroleukaemic cells contain, and presumably also produce, the granulocytic chalone, and, equally important, that they are capable of responding to this chalone by mitotic inhibition. However, it appears that each CHL cell contains only from 1/10–1/40 the amount of chalone present in a normal mature granulocyte.

It is interesting to note from the results obtained by Bullough and Laurence [21] that the Vx2 epidermal tumour cells in rabbits may also have their chalone content reduced to about $\frac{1}{10}$ normal.

These low chalone contents may have several causes but the results given here suggest that the most important of these may be a reduced rate of chalone production combined with an increased rate of chalone loss from the cells.

Although direct evidence is lacking the conclusion that the leukaemic cells lose chalone at an abnormally high rate is the necessary consequence of the discovery [4] that leukaemic serum contains an excess of chalone. The fact that a low rate of chalone production can yield a high chalone content in the serum must, of course, depend on the large mass of leukaemic cells that is present in the body.

These results and conclusions are clearly similar to those of Bullough and Laurence, who studied the Vx2 epidermal tumour in rabbits [21] and melanomata in mice and hamsters [22]. In all these widely different tumours the cells contain and respond to their normal tissue-specific chalones. It also appears that in all these cases the high rate of cell production is at least partly related to the reduced chalone concentration in the cells, and at least in the case of the chloroleukaemia it appears that the cells are less responsive to their chalone.

The results given here have also indicated certain abnormalities in the metabolism of the chloroleukaemic cells. It is well known that thymidine is incorporated in newly forming DNA, whereas formate is incorporated both in DNA and in RNA (see p. 597). An analysis of data given in Tables 3 and 4 indicates the differing actions of the granulocytic chalone on DNA and RNA synthesis in CHL and in normal BM cells, and these are summarised in Table 6.

Table 6

	Inhibition (% of control)			
	Normal BM		CHL	
	DNA	RNA	DNA	RNA
Normal chalone	22	18	32	5
CHL chalone	36	39	29	1

These figures show, first, that the chalone causes a strong inhibition of DNA synthesis in both CHL and normal BM cells, but, second, that an inhibition of RNA synthesis is only apparent in the normal BM cells. The failure of chalone to inhibit RNA synthesis in CHL cells indicates clearly that there is some marked difference in the basic metabolic pattern of the leukaemic cells.

From the present study the main metabolic differences between the CHL and normal BM cells seem to be: first, there is some difference in the quality of their respective RNAs or in the manner in which these RNAs are synthesized; second, since the chalone may be synthesized more slowly in CHL cells, there may be some abnormality in the mechanism by which it is synthesized; third, the high rate of chalone loss must indicate some change either in the chalone molecule or in the character of the cell wall, which is itself specified via the RNA mechanism; fourth, the different rates of uptake of ^{3}H-TdR and ^{14}C-formate must indicate some difference in the DNA replication system; and fifth, the slower duplication rate of CHL cells may indicate that there has been some damage to the mechanism of the mitotic cycle.

Damage to the mitotic cycle, leading to a slower proliferation rate, has been found in many other types of neoplastic cells [23–25], but it is not an essential characteristic of all malignant tumours [21].

It is commonly held that the essential damage leading to neoplasia must be genetic and it must involve damage to that mechanism which regulates cell division [3]. However, it appears from what is now known that in three very diverse and highly malignant tumours this mechanism is not destroyed; the cells are still able to recognize and react to their own tissue-specific chalones. This fact may prove to have considerable value in that it may provide the basis for the treatment of malignant tumours by chalone-containing extracts as has been demonstrated with melanomata by Mohr *et al.* [26]. However, in the case of granulocytic leukaemia prolonged treatment with chalone could be potentially dangerous in that there would also be a suppression of normal granulocyte production. Unless steps were taken to prevent it, this could result in fatal infection.

RESUME

Il est connu que l'activité mitotique des cellules précurseurs granulocytiques normales est réprimée par une chalone granulocytique dont l'action est spécifique à ce tissu (Ve/Vo= $2 \cdot 73 \pm 0 \cdot 035$ *sur Sephadex G-75). En employant la thymidine ^{3}H et le formate ^{14}C, un compteur opérant au liquide scintillant, ainsi que l'autoradiographie, on a démontré que cette même chalone existait aussi dans les cellules chloroleucémiques et qu'elle était capable de réprimer leur activité mitotique. Or, les cellules leucémiques ne sont pas aussi sensibles que les cellules précurseurs normales. On a remarqué que la concentration de la chalone dans une cellule chloroleucémique est de* 1/10 *à* 1/40 *de la concentration dans une cellule granulocytique mûre normale, et que les cellules leucémiques produisent probablement moins de chalone. La concentration de la chalone dans le sérum leucémique est plus élevée que dans un sérum normal. Il est évident que ceci est causé par le haut degré de perte de la chalone subie par les cellules leucémiques en conjonction avec le nombre élevé de ces cellules qui se trouvent dans l'organisme en question.*

En dépit de cette concentration réduite de la chalone dans les cellules, l'activité mitotique des cellules chloroleucémiques est moins élevée que cette activité dans les cellules précurseurs normales. Leur haut degré de production de cellules est causé uniquement par leur 'maturation ralentie'.

Les données citées, ainsi que d'autres observations, démontrent que la chalone granulocytique, dérivée soit des cellules normales, soit des cellules leucémiques, est spécifique au tissu, parce qu'elle n'a aucune influence sur l'activité mitotique des cellules précurseurs érythrocytiques. Son influence est entièrement réversible, parce qu'elle cause un retard sans provoquer de répression permanente de l'activité mitotique. Quoique la chalone cause une forte répression de la synthèse du DNA dans les cellules normales, ainsi que dans les cellules leucémiques, elle ne cause la répression de la synthèse du RNA que dans les cellules normales.

Les cellules chloroleucémiques utilisent la thymidine exogène avec un moins bon résultat et le formate exogène avec plus de succès que les cellules normales.

Les résultats principaux et les conclusions majeures présentés ci-dessus, en ce qui concerne les rats souffrant de la chloroleucémie, sont essentiellement pareils aux résultats et conclusions obtenus avec des lapins souffrant de la tumeur épidermique Vx2, ainsi qu'avec des souris et des hamsters atteints de mélanome.

SUMMARY

It is known that the mitotic activity of the normal granulocytic progenitor cells is inhibited by a tissue-specific granulocytic chalone which has Ve/Vo= $2 \cdot 73 \pm 0 \cdot 035$ *on Sephadex G-75. Using ^{3}H-thymidine and ^{14}C-formate and a liquid scintillation counter together with autoradiography, it has now been shown that this same chalone also exists in chloroleukaemic cells and that it is capable of inhibiting their mitotic activity. However, the leukaemic cells are not as sensitive as are the normal progenitor cells. It has been found that the chalone concentration in a chloroleukaemic cell is* 1/10–1/40 *of that in a normal mature granulocyte and that the leukaemic cells probably also synthesize less chalone. The chalone concentration in the leukaemic serum is higher than that in normal serum. This is evidently due to a high rate of chalone loss from the leukaemic cells combined with the large numbers of these cells that are present in the body.*

In spite of the reduced chalone concentration within the cells the mitotic rate of the chloroleukaemic cells is lower than that of the normal progenitor cells. Their high rate of cell production is due solely to their 'delayed maturation'.

From evidence given here, and from other evidence, it is clear that whether the granulocytic chalone is derived from normal or leukaemic cells, it is tissue-specific since it has no action on the mitotic activity of erythrocytic progenitor cells. Its action is fully reversible since it causes a delay rather than a permanent inhibition of the cell proliferation rate. Although the chalone causes a strong inhibition of DNA synthesis in both normal and leukaemic cells, it inhibits RNA synthesis only in normal cells.

The chloroleukaemic cells utilize exogenous thymidine less effectively and exogenous formate more effectively than do normal cells.

The main results and conclusions given here regarding chloroleukaemia in rats are

essentially similar to those obtained with the Vx2 epidermal tumour of rabbits and with melanomata of both mice and hamsters.

ZUSAMMENFASSUNG

Es ist bekannt, dass die Mitose-Aktivität der normalen granulozytischen unreifen Zellen durch ein gewebespezifisches granulozytisches Chalon gehemmt ist, welches einen Ve/Vo-Wert von 2·73±0·035 an Sephadex G-75 hat. Unter Verwendung von ^{3}H-Thymidin und ^{14}C-Formiat und einem Flüssigkeits-Szintillationszähler in Verbindung mit Autoradiographie wurde gezeigt, dass dasselbe Chalon auch in chloroleukämischen Zellen vorhanden ist, und dass es in der Lage ist die Mitose-Aktivität zu hemmen. Die leukämischen Zellen sind jedoch nicht so empfindlich wie die normalen unreifen Zellen. Es wurde festgestellt, dass die Chalonkonzentration in einer chloroleukämischen Zelle 1/10-1/40 der eines normalen reifen Granulozyten ist und dass die leukämischen Zellen wahrscheinlich ausserdem auch weniger Chalon herstellen. Die Chalonkonzentration im leukämischen Serum ist höher als im normalen Serum. Dies ist offenbar auf einen hohen Chalonverlust aus den leukämischen Zellen zurückzuführen, unter Berücksichtigung der grossen Anzahl dieser Zellen, die im Körper anwesend sind.

Trotz der reduzierten Chalonkonzentration innerhalb der Zellen, ist die Mitose-Aktivität der chloroleukämischen Zellen niedriger als die der normalen unreifen Zellen. Ihre hohe Zellproduktion ist auf ihr 'verzögertes Reifen' zurückzuführen.

Nach den hier aufgeführten und anderen Befunden ist es klar, dass das granulozytische Chalon, ob es von normalen oder leukämischen Zellen stammt, gewebespezifisch ist, da es keine Wirkung auf die mitotische Aktivität der erythrozytischen unreifen Zellen hat. Seine hemmende Wirkung führt nur zu einer Verzögerung und nicht zu einer dauerhaften Hemmung der Zellvermehrung. Obwohl das Chalon eine starke Verhinderung der DNS-Synthese verursacht, in normalen sowohl als auch in leukämischen Zellen, verhindert es die RNS-Synthese nur in normalen Zellen.

Die chloroleukämischen Zellen nutzen weniger exogenes Thymidin und mehr exogenes Formiat als normale Zellen.

Die hier aufgeführten Ergebnisse und Schlussfolgerungen in Bezug auf die Chloroleukämie in der Ratte sind im wesentlichen ähnlich denen, die mit Vx2 epidermalen Tumoren in Kaninchen und mit Melanomen in Mäusen und Hamstern gefunden wurden.

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Control of Mitosis in Mouse and Hamster Melanomata by Means of the Melanocyte Chalone

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INTRODUCTION

IN THE epidermal Vx2 carcinoma of rabbits [1] and in a granulocytic chloroleukaemia of rats [2] it has been found that the tumour cells continue to synthesise the antimitotic chalones of their tissues of origin, and furthermore that these tumour cells are still capable of responding by mitotic inhibition to the presence of these chalones. However, the chalone content of the tumour cells is abnormally low, partly due to an excessive chalone loss from the tumour into the blood, and it appears that this may provide some explanation for the high rate of cell production. The question arises whether a low intracellular concentration of the antimitotic tissue chalone is a common feature of fast-growing tumours and with this in mind Dr. U. Mohr (Deutsches Krebsforschungszentrum, Heidelberg) suggested that studies should be made on two transplantable melanomata to discover whether a similar situation exists in them.

The present paper deals with two questions: whether normal melanocytes of mammalian skin synthesise a tissue specific melanocyte chalone to control their own mitotic activity, and if so whether the melanoma cells still synthesise and are responsive to this chalone. This hypothetical chalone was sought in extracts of pig skin and of melanomata, and in a parallel investigation Mohr *et al.* [3] also studied the effects of repeated injections of these same extracts on the growth of the same two melanomata.

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MATERIAL AND METHODS

The melanocytes of mammalian skin are a population of dispersed cells derived originally from neural crest [4]. Their study involves two major practical problems: first, although it is well known that melanocytes undergo mitosis, it is difficult to measure their mitotic rate, and second, although it is possible to separate small numbers of melanocytes from the other skin cells, it is difficult to obtain the large number needed for the attempted extraction of an uncontaminated chalone.

To meet these difficulties the following assumptions were made: first, that melanomata may resemble the rabbit Vx2 tumour and the rat chloroleukaemia in that their cells may respond by mitotic inhibition to the presence of a melanocyte chalone, and therefore that these cells may be used as test objects in place of the normal skin melanocytes; and second, that the melanomata may also resemble the other two tumours in containing significant amounts of their own tissue chalone, and therefore that experiments with normal skin extracts may be supplemented by experiments using melanoma extracts.

The animals and the melanomata

Two tumours were used: the Harding–Passey melanoma, which was inoculated subcutaneously into adult NMRI and LAF 1 mice and which was used in the experiments when its diameter approached 1 cm; and an amelanotic melanoma (Green, Fortner) which was inoculated subcutaneously into adult Syrian hamsters and which was used in the experiments

when its diameter approached 2 cm. Both melanomata tended to become necrotic centrally and this led to a clear stratification with the mitotic zone externally, a non-mitotic zone medially (in the Harding–Passey melanoma the cells of this zone synthesised melanin), and the necrotic zone centrally (Fig. 1).

The reactions of the mitotic zone to extracts of skin and of melanomata were studied *in vivo* using the Harding–Passey melanoma and *in vitro* using the hamster amelanotic melanoma.

The chalone extracts

Aqueous extracts of pig skin, which were known to contain the epidermal chalone and which might also contain a melanocyte chalone, were used either partly purified as the 71–80% ethanol precipitate [5] or highly purified by additional electrophoresis and dialysis [6]. All the preparations were made and lyophilised by N. V. Organon (Oss, The Netherlands). Since the existence of a melanocyte chalone was at first only speculative it was necessary to express dosage in terms of the weight of the starting material, which was the water soluble conponent of the pig skin. A dose equivalent to 1 mg of this material was represented by 40 μg of the 71–80% ethanol fraction and by 0·6 μg of the highly purified fraction.

The extracts of the Harding–Passey melanomata and of the hamster amelanotic melanomata were also made and lyophilised by N. V. Organon, and again the dosage was expressed in terms of the weight of the original water soluble material. In the first experiments this material was itself tested, but in the later experiments partly purified ethanol fractions were used instead.

Experimental methods

In the *in vivo* experiments with Harding–Passey melanomata the NMRI mice were each injected subcutaneously with 0·5 ml of an aqueous solution of the 71–80% ethanol precipitate of skin extract or of the highly purified fraction, and immediately afterwards each mouse was also injected with 0·1 mg colcemid in 0·25 ml water. The injections were all given at 11·00 hr, early in a rest period, and the mice were then allowed to sleep for 4 hr before they were killed.

In the *in vitro* experiments small pieces (about 3 mm cube) of each hamster amelanotic melanoma were taken from the outer mitotic zone and one piece was placed in each of the experimental groups. Such a group contained a minimum of 5 tumour pieces from 5 different hamsters. Incubation was at 37°C in a phosphate buffered saline medium with 0·02M glucose and an oxygen gas phase. The skin extracts or melanoma extracts were added and the pieces of tumour were allowed to adjust to the culture conditions for 1 hr. Colcemid was then added (4 μg/ml culture medium) and allowed to act for a further 4 hr before the tumour pieces were fixed. Full details of this technique have been given by Bullough and Laurence [7].

Fixation was in Bouin's fluid and the pieces of tumour were sectioned 7μ thick and stained in Ehrlich's haematoxylin and eosin. The numbers of mitoses were counted in unit section areas of 1·5 mm², which contained approximately 3000 cells.

RESULTS

Effects of skin extracts on melanomata in vivo

In the first experiments the reactions of the mitotic zone of Harding–Passey melanomata to injections of the 71–80% alcohol fraction of skin and of the highly purified extract of epidermal chalone were determined. In all cases 300 mg equivalent of the original water

Table 1. *Effects of injection of pig skin extracts containing epidermal chalone into NMRI mice with large Harding–Passey melanomata*

Tissue	Uninjected controls	Injected 12 mg 71–80% ethanol fraction		Injected 180 μg highly purified fraction	
	M±S.E.	M±S.E.	Mitotic depression	M±S.E.	Mitotic depression
Ear epidermis	9·9±0·46 N=10	3·9±0·24 N=11	61%	3·9±0·33 N=4	61%
Melanoma	100·0±9·80 N=10	55·7±4·95 N=11	44%	110·1±9·72 N=4	0%

Average number of mitoses arrested by colcemid in 4 hr in unit lengths of 1 cm ear epidermis sectioned 7μ thick and in unit areas of 1·5 mm² of melanomata sectioned 7μ thick. N, number of mice.

soluble material (that is, 12 mg of the 71–80% ethanol fraction or 180 μg of the highly purified fraction) were injected subcutaneously into each mouse at a point distant from the tumour. After 4 hr colcemid treatment the mitotic rates of the melanoma cells were determined and so also, for comparison, were the mitotic rates of the ear epidermal cells. The results are in Table 1.

The conclusions are as follows:

(i) both the 71–80% ethanol fraction and the highly purified fraction of pig skin depressed the epidermal mitotic activity, and it is already known that in both cases the active agent is the epidermal chalone (5, 6).

(ii) the 71–80% ethanol fraction, but not the highly purified fraction, of pig skin also depressed the mitotic activity of the melanoma, and it follows that in this case the antimitotic factor could not have been the epidermal chalone.

Effects of skin extracts on melanomata in vitro

The problem of the identity of the anti-melanoma factor was next approached by means of *in vitro* experiments, using the outer mitotically active zones of hamster amelanotic melanomata. It was obviously possible that this factor might be a melanocyte chalone which, if it resembles the epidermal chalone, might be fully active only in the presence of adrenalin and a glucocorticoid hormone (8, 9). In preliminary experiments, therefore, the actions of the two stress hormones alone and together were determined, and the results are in Table 2. The hormone concentrations were similar to those used with the Vx2 epidermal tumour *in vitro* [1].

In the conditions of the *in vitro* experiments it was obvious that the mitotic rate of the control tumours was much below that seen *in vivo*, as has also been found with the Vx2 epidermal tumour and with certain normal tissues such as hair bulbs [1, 10], but by comparison with most normal tissues it still remained high.

The conclusion is that, although adrenalin and hydrocortisone used separately had no effect on the mitotic activity of the melanoma cells, when used together they induced a small but significant mitotic depression.

This result is similar to that obtained with the Vx2 epidermal tumour in which it was thought to be due to the cooperation of the two stress hormones with the limited amounts of epidermal chalone existing in the tumour cells [1]. Here, too, a similar explanation is possible if limited amounts of a melanocyte chalone exist in the melanoma cells.

In the next experiments the effects of the 71–80% ethanol fraction of pig skin (10 mg equivalent=400 μg of this fraction) and of the highly purified extract of epidermal chalone (10 mg equivalent=6 μg of this extract) were tested separately and together with one or both of the stress hormones in two concentrations. The results are in Tables 3 and 4.

The conclusions from these two tables are:

(i) the antimelanoma factor (Table 3), whether alone or in combination with either adrenalin or hydrocortisone, did not inhibit the mitotic activity of the melanoma cells, but when combined with both the stress hormones it induced a powerful mitotic depression;

Table 2. Effects of adrenalin and hydrocortisone on mitotic activity in hamster amelanotic melanoma in vitro

4 ml culture medium with:	M±S.E.	N	Percentage depression
control	32·6±1·60	36	
1 μg adrenalin	32·4±1·55	33	0
10 μg adrenalin	33·0±1·48	32	0
1 μg hydrocortisone	33·5±2·11	32	0
10 μg hydrocortisone	31·8±1·64	36	0
1 μg adrenalin + 1 μg hydrocortisone	27·6±1·62	33	15
10 μg adrenalin + 10 μg hydrocortisone	27·4±1·67	32	16

Average number of mitoses arrested by colcemid in 4 hr in unit areas of 1·5 mm² of melanoma sectioned 7μ thick. N, number of melanomata

Note: the percentage depression is recorded as zero if the difference from the control is not statistically significant.

Table 3. *Effects of* 71–80% *ethanol fraction of pig skin, with and without adrenalin and hydrocortisone, on the mitotic activity of hamster amelanotic melanoma* in vitro

4 ml culture medium with:	M±S.E.	N	Percentage depression
control	32·8±3·21	16	
400 µg ethanol fraction of skin	30·8±1·72	16	0
400 µg ethanol fraction of skin + 1 µg adrenalin	29·7±1·78	18	0
400 µg ethanol fraction of skin + 10 µg adrenalin	30·8±1·36	19	0
400 µg ethanol fraction of skin + 1 µg hydrocortisone	30·8±1·60	16	0
400 µg ethanol fraction of skin + 10 µg hydrocortisone	29·6±1·62	14	0
400 µg ethanol fraction of skin + 1 µg adrenalin + 1 µg hydrocortisone	12·6±0·59	15	62
400 µg ethanol fraction of skin + 10 µg adrenalin + 10 µg hydrocortisone	12·9±0·72	16	61

Average numbers of mitoses arrested by colcemid in 4 hr in unit areas of 1·5 mm² of melanoma sectioned 7 µ thick. N, number of melanomata.
Note: the percentage depression is recorded as zero if the difference from the control is not statistically significant.

(ii) the relatively small mitotic depression (Table 4) obtained when the highly purified extract of epidermal chalone was combined with adrenalin and hydrocortisone can be attributed solely to the combined action of the two hormones (see Table 2);

(iii) the antimelanoma factor was therefore only present in the 71–80% ethanol fraction, which confirms that this factor cannot be the epidermal chalone (see Table 1);

(iv) since the antimelanoma factor is only active in the presence of the two stress hormones, which is an outstanding characteristic of the epidermal chalone, the implication is that this factor may be the melanocyte chalone.

Table 4. *Effects of purified epidermal chalone from pig skin, with and without adrenalin and hydrocortisone, on the mitotic activity of hamster amelanotic melanoma* in vitro

4 ml culture medium with:	M±S.E.	N	Percentage depression
control	29·8±1·76	9	
6 µg epidermal chalone	29·3±1·68	9	0
6 µg epidermal chalone + 1 µg adrenalin	28·8±2·15	8	0
6 µg epidermal chalone + 10 µg adrenalin	28·2±1·70	9	0
6 µg epidermal chalone + 1 µg hydrocortisone	27·6±1·05	9	0
6 µg epidermal chalone + 10 µg hydrocortisone	27·2±1·67	7	0
6 µg epidermal chalone + 1 µg adrenalin + 1 µg hydrocortisone	23·7±0·71	8	20
6 µg epidermal chalone + 10 µg adrenalin + 10 µg hydrocortisone	22·2±1·15	9	25

Average numbers of mitoses arrested by colcemid in 4 hr in unit areas of 1·5 mm² of melanoma sectioned 7 µ thick. N, number of melanomata. Note: 10 mg of original skin extract is equivalent to 6 µg of purified epidermal chalone.
Note: the percentage depression is recorded as zero if the difference from the control is not statistically significant.

Table 5. *Effects of water extracts of hamster and mouse melanomata, with and without adrenalin and hydrocortisone, on the mitotic activity of hamster amelanotic melanoma* in vitro

4 ml culture medium with:	Extracts from hamster amelanotic melanoma			Extracts from Harding–Passey melanoma					
				in NMRI mice			in LAF1 mice		
	M±S.E.	N	Depression (%)	M±S.E.	N	Depression (%)	M±S.E.	N	Depression (%)
control	11·4±1·40	4							
10 mg extract	10·4±1·46	5	0	11·2±0·82	5	0	11·0±2·94	5	0
10 mg extract + 10 µg adrenalin	8·6±0·83	3	0	11·3±0·29	5	0	9·7±0·53	5	0
10 mg extract + 10 µg hydrocortisone	10·4±0·58	4	0	11·8±1·95	3	0	10·4±0·83	4	0
10 mg extract + 10 µg adrenalin + 10 µg hydrocortisone	5·5±0·80	5	52	6·2±0·90	3	46	2·7±0·39	4	76

Average numbers of mitoses arrested by colcemid in 4 hr in unit areas of 1·5 mm² of melanoma sectioned 7 µ thick. N, number of melanomata.
Note: the percentage depression is recorded as zero if the difference from the control is not statistically significant.

The fact that the antimelanoma factor, injected alone, was active against the mitotic activity of the melanoma cells *in vivo* (Table 1) was presumably due to the presence of adequate concentrations of the stress hormones in the body.

Effects of melanoma extracts on melanomata in vitro

If the melanocytes of the epidermis synthesise a melanocyte chalone, then this chalone may also be present in the cells of melanomata. Water extracts were therefore prepared from both hamster amelanotic melanoma and mouse Harding–Passey melanoma, and they were tested, without further purification, on amelanotic melanoma cells *in vitro*. The results of a preliminary series of experiments are in Table 5.

Although the number of melanomata used was only small the results are similar to those obtained with the 71–80% ethanol fraction of skin extract (Table 3). The conclusions are:

(i) water extracts of both the melanomata contained some substance which depressed mitotic activity in the amelanotic melanoma, but this substance was only active when adrenalin and hydrocortisone were also present;

(ii) this cooperation with the stress hormones suggests that the antimelanoma factors obtained from skin and from the melanomata were one and the same substance.

The reaction was then analysed further using larger numbers of amelanotic melanomata which were treated *in vitro* with a range of ethanol fractions obtained from water extracts of both hamster and mouse melanomata. In all cases adrenalin and hydrocotisone were also present, and the results are in Table 6.

These experiments confirm the results in Table 5 and show that the antimelanoma activity can be concentrated in the 80% ethanol supernatant.

The melanocyte chalone

The evidence therefore suggests that a melanocyte chalone is synthesised both by the melanocytes of normal pig skin and by the abnormal descendants of these cells which form the bulk of the melanomata.

If the epidermal chalone is taken as the type, then four of the characteristics of a chalone are that it is water soluble and heat labile; that it inhibits mitosis only in its tissue of origin, and that to be effective it requires the

Table 6. Effects of ethanol fractions of hamster and mouse melanomata, together with adrenalin and hydrocortisone, on the mitotic activity of hamster amelanotic melanoma in vitro

4 ml culture medium with:	Extracts from hamster amelanotic melanoma			Extracts from Harding–Passey melanoma					
				in NMRI mice			in LAF1 mice		
	M±S.E.	N	Depression (%)	M±S.E.	N	Depression (%)	M±S.E.	N	Depression (%)
control	42·4±5·13	19							
10 mg equiv. 0–50% ethanol precipitate	44·8±5·29	17	0	46·0±5·55	20	0	48·0±5·60	19	0
10 mg equiv. 51–70% ethanol precipitate	no ppt. obtained			46·6±5·65	18	0	51·3±5·60	17	0
10 mg equiv. 71–80% ethanol precipitate	no ppt. obtained			45·0±5·70	16	0	no ppt. obtained		
10 mg equiv. 80% ethanol supernatant	19·5±2·82	19	54	21·4±2·96	15	50	21·0±3·54	17	50

Average numbers of mitoses arrested by colcemid in 4 hr in unit areas of 1·5 mm² of melanoma sectioned 7μ thick. N, number of melanomata. Adrenalin and hydrocortisone both 2·5 μg/ml. The amounts of the various ethanol fractions used were equivalent to 10 mg of the original water soluble material.
Note: the percentage depression is recorded as zero if the difference from the control is not statistically significant.

presence of both adrenalin and a glucocorticoid hormone. Two of these characteristics, water solubility and cooperation with the stress hormones, have already been established for the melanocyte chalone. To test for heat lability, solutions of pig skin extracts were kept for 10 min at 100°C when it was found, using amelanotic melanoma *in vitro*, that all the antimitotic activity had been destroyed.

Tissue specificity was tested only in relation to epidermis. The first *in vivo* experiments (Table 1) showed that the melanoma cells do not respond to the epidermal chalone and later *in vitro* experiments (Table 4) confirmed this. The question of the response of the epidermal cells to the melanocyte chalone was approached both indirectly and directly.

It had previously been shown that in a rabbit with a large Vx2 epidermal tumour the body contains such an excess of epidermal chalone that mitotic activity in the normal epidermis is almost eliminated [1]. However, in NMRI mice with large Harding–Passey melanomata the epidermal mitotic count (after 4 hr colcemid) was as high as 9·9±0·46 (N=10), which compared with a count of 13·0±0·96 (N=15) from similar mice without melanomata. The difference between these figures is statistically significant but the mitotic depression associated with the melanomata, which were large (about 3 cm dia.), is much less than that associated with the presence of the epidermal Vx2 tumour [1]. It was in fact probably due to the stressed condition of the mice, and the fact that a melanoma has no direct inhibitory action on epidermal mitosis is emphasised in Fig. 2, which shows such activity in cells immediately overlying a large Harding–Passey melanoma.

The effects of extracts of melanomata on epidermal mitotic activity were next examined. It has been shown above that the melanocyte chalone was present in the 80% ethanol supernatant obtained from water extracts of both types of melanomata, and it was these extracts that were tested on mouse ear epidermis *in vitro*. The results are in Table 7, in which for comparison the action of the epidermal chalone is also included.

Clearly the melanoma extracts had no action on the epidermal mitotic activity and the conclusion must be that the melanocyte chalone, although presumably normally produced by cells which lie embedded in the

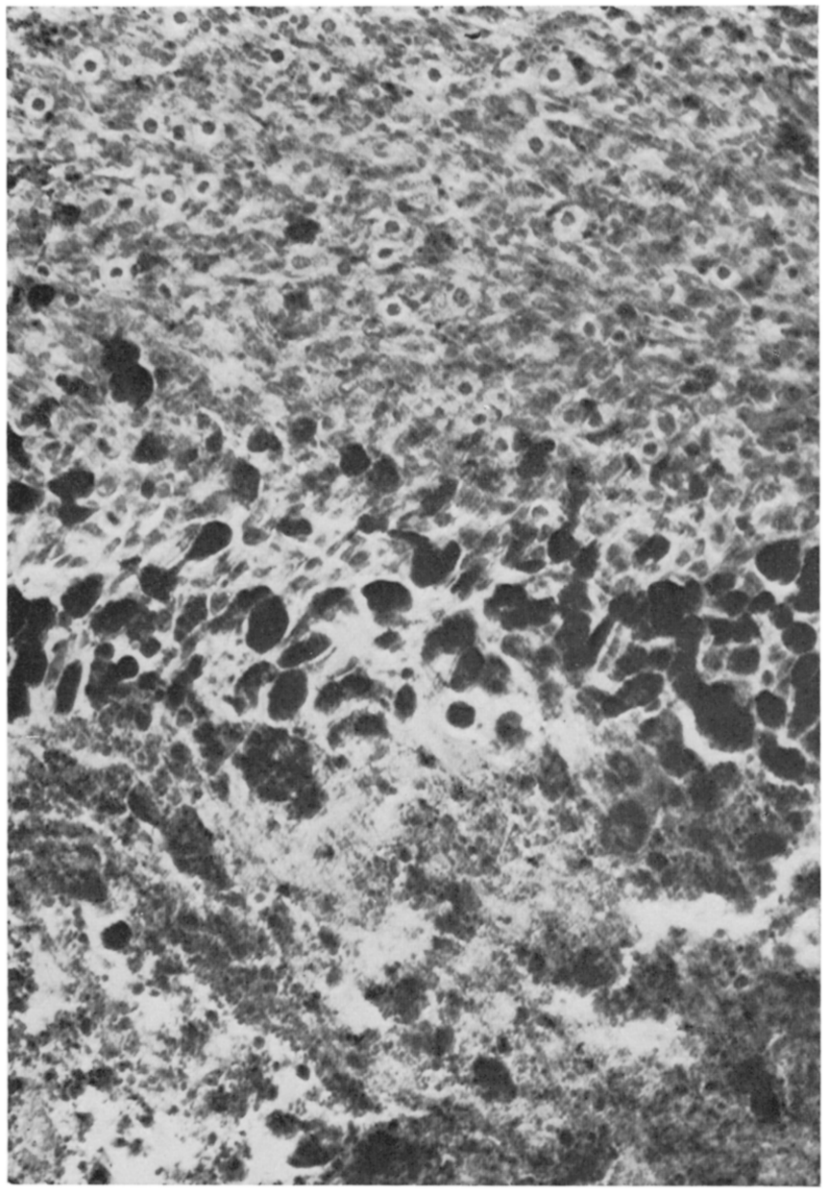


Fig. 1. *Section of a large Harding–Passey melanoma to show zonation: the mitotically active cells (above) are peripheral, the post-mitotic melanin synthesising cells are medial, and the dead cells (below) are central.*

(to face p. 612)

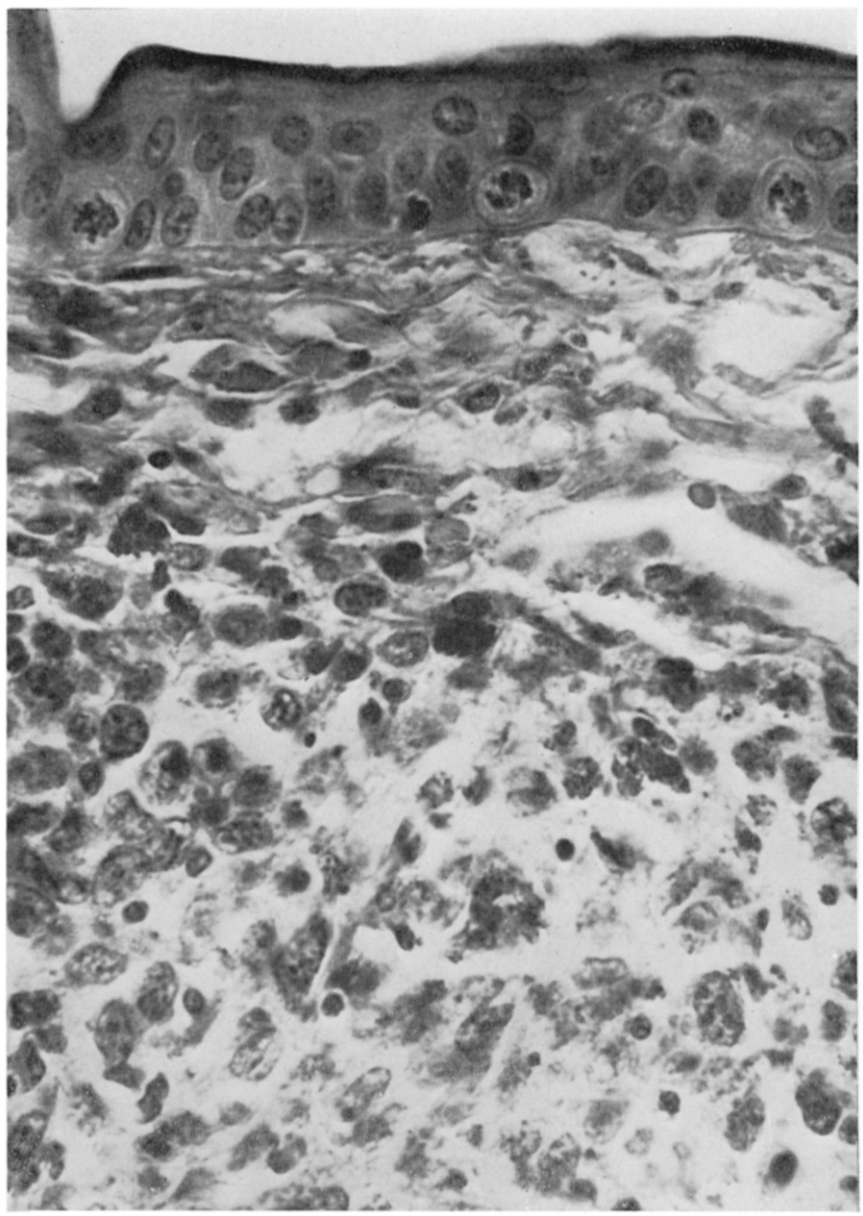


Fig. 2. Section of the outer edge of a large Harding–Passey melanoma and overlying it the thin dermis and the normal epidermis with mitoses.

Table 7. Effects of 80% *ethanol supernatant of extracts of mouse and hamster melanomata on the mitotic activity of mouse ear epidermis* in vitro

4 ml culture medium with:	M±S.E.	N	Percentage depression
control	8·4±0·27	5	
10 mg equiv. epidermal chalone	5·0±0·31	5	40
10 mg equiv. 80% ethanol supernatant Harding–Passey melanoma	8·5±0·31	5	0
10 mg equiv. 80% ethanol supernatant amelanotic melanoma	8·8±0·59	5	0

Average numbers of mitoses arrested by colcemid in 4 hr in 1 cm lengths of ear epidermis sectioned 7μ thick. In all cases the amount of extract used was that derived from 10 mg of the original water soluble material. N, number of mice.

epidermis, has no antimitotic action on the epidermal cells. Thus this substance is not a general mitotic inhibitor, and like the epidermal chalone it may prove to be strictly tissue specific in its action.

DISCUSSION

A chalone has been defined by Bullough [11] as, "an internal secretion produced by a tissue for the purpose of controlling by inhibition the mitotic activity of the cells of that same tissue", and the conclusion now is that the epidermal cells and the melanocytes lying among them are controlled by two quite independent chalones. These two substances share the characteristics of water solubility, heat lability, tissue specificity, and an inability to function fully except in the presence of adrenalin and a glucocorticoid hormone. This may support the suggestion which has already been made that chalones in general may prove to be a chemically related group of substances [12, 13]; regarding the epidermal chalone the evidence available at the moment suggests that it may be a glycoprotein with a molecular weight of about 30,000–40,000 [6].

The identification of the melanocyte chalone has only been possible because of the prediction from results obtained with the Vx2 tumour [1] and with chloroleukaemia [2] that the cells of a melanoma may contain and respond to the chalone of the normal melanocytes from which they were derived. Extending this prediction still further, it also seems probable that the melanoma cells have a subnormal chalone content, which may be the result partly of a reduced rate of synthesis and partly of an increased rate of loss into the body space.

In any normal tissue a post-mitotic cell becomes functional as it passes to its death along what has been called the ageing pathway [11]. The average life span of a post-mitotic melanocyte has not been determined, but it is certainly longer than that of an epidermal cell, which may be about 2 weeks [14].

In the two melanomata described here these basic rules are not broken; the normal pattern is merely distorted. Although in small tumours the relationships of the mitotic and post-mitotic cells may be confused, in the larger tumours the cells become stratified, with the zone of mitotic cells on the outside, the zone of post-mitotic cells medially, and the zone of dead cells in the centre (Fig. 1). The mitotic zone is wide (because an abnormally high proportion of the tumour cells are involved in mitosis) and the mitoses are abundant (evidently because the mitotic cycle is shortened). The post-mitotic cells traversing the ageing pathway also show certain abnormalities: although in the Harding–Passey melanoma these cells produce melanin in the normal manner (Fig. 1), in the amelanotic melanoma they do not; in both the melanomata the post-mitotic zone is only some 3 or 4 cells wide, and it therefore seems that the length of life of a post-mitotic cell is unusually short. It is in fact well known that in tissues which normally show a high mitotic rate the life of the post-mitotic cells is short. An example is stomach mucosa in which the post-mitotic cells pass to their death in about 2 days [11].

The implication may be that if the cells of the outer mitotic zone could be prevented from undergoing any further mitosis so that they were forced instead to enter the ageing pathway, their subsequent life might be short. The tumour might then shrink and even disappear completely. Since it is evidently a function of

the tissue chalone to determine the normal point of balance between cell gain and cell loss, and since the melanoma cells retain a capacity to respond to the normal melanocyte chalone, the obvious and natural way to attempt to force the cells from mitosis to death would be to raise the concentration of this chalone.

A series of experiments of this type have now been completed by Mohr *et al.* [3]. Using similar animals with similar tumours, and injecting the same extracts of pig skin and of melanoma as were used in the present work, they were able in their most successful experiments to destroy the mouse Harding–Passey melanoma completely. In all the other experiments, including all the experiments with the hamster amelanotic melanoma, the tumours were apparently destroyed but subsequently they recurred either in the original position or as metastases. Although it is not yet fully proved the probability is that in these experiments the antitumour principle was the melanocyte chalone. Since Rytömaa and Kiviniemi [15] have also found that the granulocytic chalone is active against a granulocytic leukaemia *in vivo*, the chalones may prove to be a new and natural group of carcinostatic agents, which moreover act in a tissue specific manner.

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RESUME

Après avoir prouvé que deux tumeurs malignes, l'une épidermique et l'autre granulocytique, continuent à synthétiser leurs chalones de tissu normal et à y réagir, nous avons tenté d'extraire une chalone mélanocytique de la peau de porc normale et de deux sortes de mélanomes, et de vérifier son action sur des cellules de mélanome in vivo *et* in vitro.

Nous avons trouvé que la peau de porc, le mélanome de Harding–Passey chez la souris, et un mélanome amélanique (Green, Fortner) de hamsters contiennent tous trois une substance qui a les caractéristiques d'une chalone mélanocytique et empêche les mitoses dans les mélanomes, tant in vivo *qu'* in vitro. *Cette substance n'empêche pas la mitose dans les cellules épidermiques, et, réciproquement, la chalone épidermique n'empêche pas la mitose dans les deux mélanomes.*

La chalone mélanocytique a les mêmes caractéristiques de solubilité dans l'eau, le même risque de destruction par la chaleur, la même spécificité de tissu et la même incapacité d'inhiber la mitose que la chalone épidermique, sauf lorsqu'elle est en présence de l'adrénaline et d'une hormone glucocorticoïde.

Nous suggérons que le grande activité mitotique et le taux de croissance rapide de ces deux mélanomes peuvent correspondre au contenu réduit en chalones de leurs cellules.

SUMMARY

Following earlier evidence that two malignant tumours, one epidermal and the other granulocytic, still synthesise and still respond to their normal tissue chalones, an attempt has been made to extract a melanocyte chalone from normal pig skin and from two kinds of melanoma and to test its action on melanoma cells in vivo *and* in vitro.

It has been found that the pig skin, the Harding–Passey melanoma of mice, and an amelanotic melanoma (Green, Fortner) of hamsters all contain a substance which has the characteristics of a melanocyte chalone, and which prevents mitosis in melanomata both in vivo *and* in vitro. *The substance does not prevent mitosis in epidermal cells, and conversely, the epidermal chalone does mot prevent mitosis in the two melanomata.*

The melanocyte chalone shares with the epidermal chalone the characteristics of water solubility, heat lability, tissue specificity, and an inability to inhibit mitosis except in the presence of adrenalin and a glucocorticoid hormone.

It is suggested that the high mitotic activity and fast growth rate of these two melanomata may be related to the reduced chalone content of their cells.

ZUSAMMENFASSUNG

Frühere Versuche zeigten, dass zwei schnellwachsende Geschwülste, eine epidermale und eine granulozytische, noch Chalone produzieren und noch auf ihr normales Gewebechalon

reagieren. Auf Grund dieser Befunde wurde ein Versuch gemacht, ein melanozytisches Chalon aus normaler Schweinehaut und aus zwei Melanomarten zu gewinnen und seine Wirkung auf Melanomzellen in vivo *und* in vitro *zu prüfen.*

Es wurde gefunden, dass die Schweinehaut, das Harding–Passey Melanom der Maus, und ein amelanotisches Melanom (Green, Fortner) des Hamsters alle eine Substanz enthalten, die die Eigenschaften eines melanozytischen Chalons hat, und die die mitotische Aktivität in Melanomen verhindert, sowohl in vivo *als auch* in vitro. *Diese Substanz hat keine Wirkung auf die mitotische Aktivität der Epidermiszellen und umgekehrt hat das epidermale Chalon keine Wirkung auf die mitotische Aktivität der Melanome.*

Das melanozytische Chalon hat mit dem epidermalen Chalon die folgenden Eigenschaften gemein: Wasserlöslichkeit, Hitzeunbeständigkeit, Gewebespezifität und die Unfähigkeit, Mitosen zu verhindern, wenn Adrenalin und ein Glucocorticoidhormon abwesend sind.

Es wird diskutiert, dass der hohe Mitose-Index und das schnelle Wachstum der Melanome mit dem verringerten Chalongehalt ihrer Zellen verbunden sind.

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Toxicity, Hæmatological Effects and Anti-tumour Activity of Epoxides derived from Disubstituted Hexitols. Mode of Action of Mannitol Myleran and Dibromomannitol

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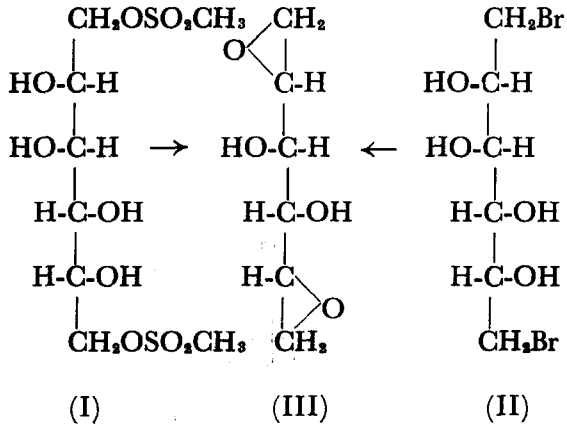
INTRODUCTION

1,6-DIMETHANESULPHONOXY-1,6-DIDEOXY-D-MANNITOL (I, mannitol myleran) and 1,6-dibromo-1,6-dideoxy-D-mannitol (II, DBM) are cytotoxic alkylating agents that exhibit characteristic effects on blood elements and also inhibit the growth of a number of experimental animal tumours. Davis and Ross [1] showed that when kept in near neutral aqueous solution mannitol myleran and DBM were converted into products which gave reactions associated with epoxide formation. They suggested that the *in vivo* effects of the two compounds might be mediated by the formation of 1,2:5,6-dianhydro-D-mannitol (III). This conclusion was criticised by Hungarian workers [2, 3] who failed to demonstrate the presence of the dianhydride in titrated solutions of DBM using NMR techniques. Proof that the dianhydride was formed in solutions maintained at pH 8 was obtained by Jarman and Ross [4] who were able to isolate the compound in crystalline form and confirm its structure by chemical and physical measurements.

This paper gives experimental details for the preparation of 1,2:5,6-dianhydro-D-mannitol from DBM and of the related 1,2:5,6-dianhydrogalactitol from 1,6-dibromo-1,6-dideoxygalactitol (also known as dibromodulcitol, DBD). The relative rates of chemical reaction of mannitol myleran and of DBM in aqueous solutions at controlled pH values have also been determined and the extent of epoxide formation under different conditions has been calculated.

The biological effects of 1,2:5,6-dianhydro-D-mannitol which are now described and compared with those of the disubstituted hexitol precursors provide evidence that the pharmacological action of mannitol myleran and of DBM is partly mediated through their conversion *in vivo* into 1,2:5,6-dianhydro-D-mannitol as shown.

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(I) (III) (II)

MATERIAL AND METHODS

Preparation of epoxides

1,2:5,6-*dianhydro*-D-*mannitol.* 1,6-Dibromo-1,6-dideoxy-D-mannitol (1·23g) suspended in water (5 ml) at 37° was continuously titrated with N aqueous sodium hydroxide using phenolphthalein as internal indicator and keeping the solution just pink, i.e. at pH ≈8, until 90% of the theoretical acidity had developed. The clear solution was then added dropwise to a stirred suspension of anhydrous sodium carbonate (26 g) in ethyl acetate (150 ml). After filtration, the ethyl acetate solution was concentrated under reduced pressure to 25 ml, redried with anhydrous magnesium sulphate, then evaporated to 2 ml and allowed to stand overnight at −15°. The yield of slender colourless needles was 0·187g (32%). The physical characteristics of the product were identical with those described for 1,2:5,6-dianhydro-D-mannitol prepared from 1,6-dimethanesulphonoxy-1,6-dideoxy-D-mannitol [4]. (Found: C, 49·45; H, 6·97%. $C_6H_{10}O_4$ (146·15) requires C, 49·28; H, 6·89%).

1,2:5,6-*Dianhydrogalactitol.* 1,6-Dibromo-1,6-dideoxygalactitol was converted into the dianhydride by a procedure similar to that described above except that the reaction was allowed to proceed until 80% of the theoretical acidity had developed. The product separated from ethyl acetate (5 ml) at room temperature. The yield of colourless platelets, m.p. 96·5–98·5°, $[\alpha]_D^{25} = +2°$ (c=1·25 in water) was 0·21 g (36%). The compound ran as a single spot in butanol saturated with water on Whatman No. 1 paper, Rf 0·39. It was detected by *cis*-diol reagents (borate-phenolphthalein and periodate-benzidine) and epoxide reagents (potassium iodide and sodium thiosulphate-phenolphthalein). (Found: C, 49·19; H, 6·79%. $C_6H_{10}O_4$ requires C, 49·28; H, 6·89%).

Determination of rate coefficients for the reaction of 1,6-*dimethanesulphonyl*-D-*mannitol and* 1,6-*Dibromo*-1,6-*dideoxy*-D-*mannitol*

Reactions were carried out at constant pH using a 'Radiometer' automatic recording titrator as previously described [1] except that 0·1 M aqueous $KClO_4$ was used intead of KCl to provide a solution of sufficient ionic strength to ensure accurate pH control. Using this salt of an anion of low nucleophilic capacity led to almost theoretical release of hydrogen ions in contrast to the results previously obtained using chloride ions.

In a typical run 6·5 mg. of 1,6-dibromo-1,6-dideoxy-D-mannitol was added to 10 ml of 0·1 M $KClO_4$ maintained at 60° and the apparatus set to keep the pH at the required value by the continuous addition of 0·1 N NaOH. The rate coefficients for the reaction at pH 7·5 and 8 were determined (Table 1)—the method of calculation has already been described [1]—and those at pH 6·5 and 7 were calculated (see below).

In one run, using the dibromide, reaction was allowed to proceed for 45 min at pH 7·5 when 90% of the theoretical amount of acidity had developed. Sodium thiosulphate (5g) in water (10 ml) was then added and the alkalinity produced was titrated with 0·1 N HCl. The titration, which was completed within 2 min, indicated that 80% of the initial reaction had led to epoxide formation.

The observed rate coefficient (k) is a composite of the rate coefficient (k_w) of the uncatalysed reaction with water and the rate coefficient (k_{OH^-}) of the hydroxyl ion catalysed reaction:

$$k = k_w[H_2O] + k_{OH^-}[OH^-]$$

Using the values of k in Table 1 one can calculate the rate coefficients k_w and k_{OH^-}. These coefficients, given in Table 2, were then used to calculate the rate coefficients for reaction at pH 6·5 and 7 (Table 1).

Toxicity, growth inhibitory and haematological effects

Rats of a Wistar albino colony were used and compounds were administered by intraperitoneal injection. 1,2:5,6-Dianhydro-D-mannitol and galactitol were dissolved in water and 1,6-dibromo-1,6-dideoxy-D-mannitol (DBM) was administered as a suspension in arachis oil. 1,6-Dimethanesulphonoxy-1,6-dideoxy-D-mannitol (mannitol myleran) was administered in aqueous solution to animals also treated with three i.p. doses of 5 g/kg of a 50% aqueous solution of glucose administered one hour

before, at the time of, and one hour following injection of the drug as described by Connors, Elson and Leese [5]. The glucose treatment greatly potentiates the pharmacological action of mannitol myleran by producing anuria which delays excretion of the drug.

Blood counts were carried out on blood taken from the tail vein direct into the pipettes in which it was diluted in the usual manner. Four rats were used for each dose of compound tested, counts were taken every 2 or 3 days, and individual response curves plotted as numbers of blood cells against time. A mean response curve was then compiled showing the percentage of normal count as a function of time after treatment with a single dose of the compound.

chemotherapeutic indices, LD_{50}/ED_{90}, where LD_{50} is the median lethal dose to tumour bearing animals and ED_{90} is the dose required to produce 90% inhibition of tumour growth. The T/C ratios shown for the L1210 assay (Table 8) equals [the average survival time of treated animals/the average survival time of controls] $\times$ 100. For comparison the effect of a standard inhibitor of the L1210 leukaemia, methotrexate, given as a single injection or on five consecutive days after inoculation is shown in Table 8.

RESULTS

Reaction kinetics

The rate coefficients for the reaction of 1,6-dimethanesulphonyl-D-mannitol and 1,6-dibromo-1,6-dideoxy-D-mannitol in aqueous solution at various pH's (Tables 1 and 2) show

Table 1. *Rate coefficients* (k) *for the reaction of* 1,6-*dimethanesulphonoxy- and* 1,6-*dibromo-*1,6-*dideoxy*-D-*mannitol in water at* 60°

	Rate coefficient $\times 10^{-5}$ sec^{-1}			
	pH 6·5 (calc.)	7·0 (calc.)	7·5	8·0
1,6-Dimethanesulphonoxy-1, 6-dideoxy-D-mannitol	44	48·5	66	119
1,6-Dibromo-1,6-dideoxy-D-mannitol	25·8	38·5	78	205

Table 2. *Rate coefficients* (k_w *and* k_{OH^-}) *for the reaction of* 1,6-*dimethanesulphonoxy- and* 1,6-*dibromo*-1,6-*dideoxy*-D-*mannitol in water at* 60°

	Rate coefficient sec^{-1}	
	k_w	k_{OH^-}
1,6-Dimethanesulphonoxy-1,6-dideoxy-D-mannitol	$7{\cdot}5\times10^{-6}$	775
1,6-Dibromo-1,6-dideoxy-D-mannitol	$3{\cdot}6\times10^{-6}$	1850

Anti-neoplastic assays

The protocols for testing the compounds as inhibitors of the growth of the transplanted Walker rat carcinoma 256 and the ADJ/PC6A mouse plasma cell tumour are described by Connors *et al.* [6] and Wade *et al.* [7] respectively. The method of assay against the mouse lymphoid leukaemia, L1210, is essentially that already described [8] except that the C_{57}/BDA2 hybrid strain of mouse was used as host. Compounds were administered as a single injection of an aqueous solution (except that for the sparingly soluble DBM arachis oil was used as vehicle) on the day following the implantation of the Walker tumour or inoculation of the L1210 leukaemia. In the case of the ADJ/PC6A tumour a single injection was given ten days after implantation. In Tables 6 and 7 the results of the Walker and ADJ/PC6A tumour tests are expressed as

that whereas the methanesulphonate reacts about twice as fast as the bromide in the uncatalysed reaction with water the coefficient for the hydroxyl ion catalysed reaction is about 2·4 times as great for the bromide. As a first approximation it could be assumed that the uncatalysed reaction leads to hydrolysis with glycol formation whilst the hydroxyl ion catalysed reaction leads to epoxide formation. If this assumption is correct then the reaction of 1,6-dibromo-1,6-dideoxy-D-mannitol at pH 7·5 should lead to $k_w[H_2O]/k$ $(3{\cdot}6\times10^{-6}\times55{\cdot}5/78\times10^{-5})\times100\%$ of hydrolysis (25·5%) and $k_{OH}[\overline{OH}^-]/k$ $(1850\times10^{-6{\cdot}5}/78\times10^{-5})\times100\%$ of epoxide formation (74·5%) The actual extent of epoxide formation under these conditions was found to be 80% which is in reasonable agreement with the predicted value. Similar calculations for the

Table 3. Extent of hydrolysis and epoxide formation from 1,6-dimethanesulphonoxy- and 1,6-dibromo-1,6-dideoxy-D-mannitol in water at 60°

	pH	7	7·5	8
1,6-Dimethanesulphonoxy-1,6-dideoxy-D-mannitol	% hydrolysis	85·5	63	35
	% epoxide	14·5	37	65
1,6-Dibromo-1,6-dideoxy-D-mannitol	% hydrolysis	52	25·5	10
	% epoxide	48	74·5	90

extent of hydrolysis and epoxide formation are given in Table 3.

Toxicity and body growth inhibition

Table 4 shows the toxicity to rats of single doses of mannitol myleran, DBM and 1,2:5,6-dianhydro-D-mannitol in relation to the time of death. Corresponding figures for myleran (1,4-dimethanesulphonoxybutane), which are also included in the Table show that even with a 100% lethal dose death does not occur until at least 10 days after treatment and is due to anaemia following thrombocytopenia. Rats treated with 1,2:5,6-dianhydro-D-mannitol die a 'nitrogen mustard type' death (see Elson [9]) showing gastrointestinal symptoms within 4 days. Similar early deaths and gastrointestinal effects occur in the animals treated with DBM and in those treated with mannitol myleran although in the latter case a few animals may show a delayed death resembling that caused by myleran.

The combination of 'mustard' and 'myleran' type response with mannitol myleran is again seen in the growth inhibitory response shown in Fig. 1. 1,2:5,6-Dianhydro-D-mannitol shows a typical 'mustard' type pattern of rapid weight loss (L_1) over 4–5 days followed by rapid recovery to normal growth rate, whilst myleran shows a very little initial (L_1) weight loss but a second prolonged weight loss period (L_2) commencing at 10 days. Mannitol myleran shows both L_1 and L_2 weight losses with the L_1 loss being more severe than the L_2 indicating that the 'mustard-like' action is

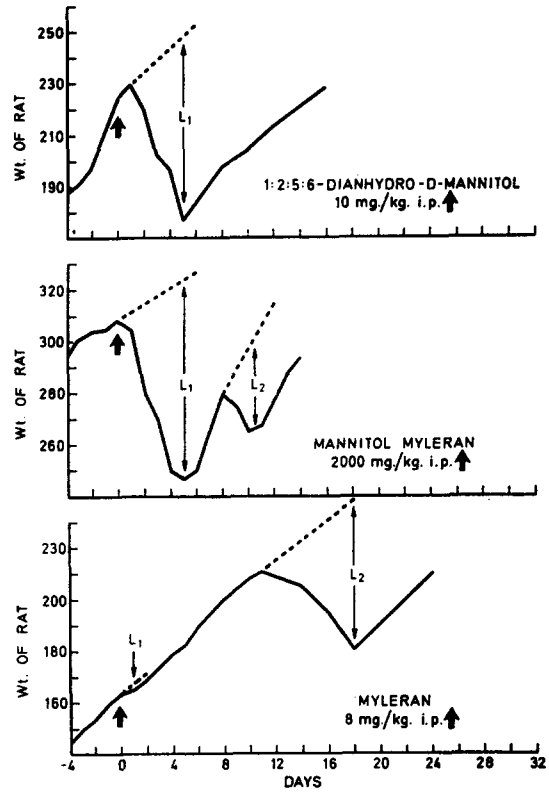


Fig. 1. Growth inhibitory response in the rat to 1,2:5,6-dianhydro-D-mannitol, mannitol myleran and myleran (typical curves, weights in g).

Table 4. Toxicity in rats in relation to the day of death

Compound	Dose (mg/kg)	No. of deaths	Days after treatment on which death occurred	Approx. LD_{50} (mg/kg)
Myleran*	20	5/5	10, 10, 12, 12, 12	18
1,2:5,6-Dianhydro-D-mannitol	5	0/2		14
	10	0/4		
	15	4/4	3, 4, 4, 5	
	25	2/2	3, 4	
1,6-Dimethanesulphonoxy-1,6-dideoxy-D-mannitol	1320	0/10		
	2000	7/15	4, 4, 4, 4, 6, 6, 8	2000
	2500	3/3	4, 4, 4	
1,6-Dibromo-1,6-dideoxy-D-mannitol	500	0/2		
	1000	4/6	3, 4, 4, 5	900

*Taken from Elson [10].

more pronounced than the 'myleran-like' effect.

Haematological effects

The differences between 'mustard' and 'myleran' type alkylating agents are clearly revealed in the peripheral blood response patterns to single doses of the drugs [9]. Figure 2 shows the blood response pattern to

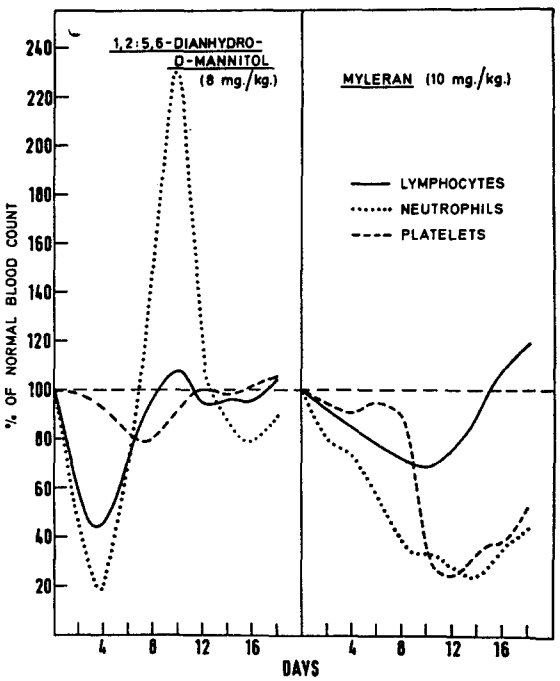


Fig. 2. Blood response patterns produced by 1,2:5,6-dianhydro-D-mannitol and myleran.

1,2:5,6-dianhydro-D-mannitol compared with that in response to myleran. The diepoxide produces a rapid fall in leucocytes, neutrophils being more depressed than lymphocytes. Platelets are not markedly affected. The leucocyte fall is followed by rapid recovery which, in the case of the neutrophils, leads to a marked neutrophilia in which the number of circulating granulocytes can be several times the normal value before returning to pre-treatment values. This 'mustard-type' pattern contrasts with that of myleran which shows relatively little depression of lymphocytes, a steady prolonged depression of neutrophils followed by progressive recovery without neutrophilia, and a delayed but severe fall in platelets.

From Fig. 3 it is seen that dibromomannitol gives a blood response pattern resembling more closely that of 1,2:5,6-dianhydro-D-mannitol than that of myleran although the reduced neutrophilia followed by a further depression of neutrophils and the delayed fall in platelets argue strongly that some myleran-like influence is still present. Mannitol myleran shows a similar complex blood response pattern with the myleran influence much more pronounced so that the partial rapid recovery of neutrophils does not raise the numbers to above normal before the delayed (myleran) effect sets in. Platelets also suffer a more severe delayed 'myleran' type fall.

These complex blood response patterns can be reproduced by treatment with a combination of myleran and 1,2:5,6-dianhydro-D-mannitol as is also shown in Fig. 3

1,2:5,6-Dianhydrogalactitol derived from 1,6 - dibromo - 1,6 - dideoxygalactitol shows a blood response pattern very similar to that of 1,2:5,6-dianhydro-D-mannitol (see Table 5). The response is characterised by a rapid fall in leucocytes which reach minimum values at about 4 days, neutrophils being affected more

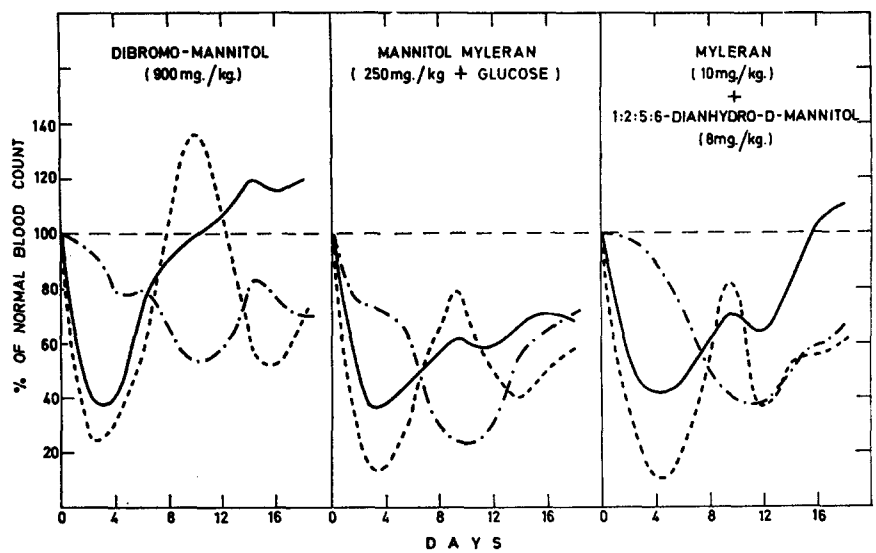


Fig. 3. Blood reponse patterns produced by DBM, mannitol myleran and a combinaton of myleran and 1,2:5,6-dianhydro-D-mannitol.

——— *lymphocytes* – – – – *neutrophils* –·–·–·–·– *platelets*

than lymphocytes, and then show rapid recovery with marked temporary neutrophilia. Again the relative lack of effect on platelets is notable. The galactitol derivative is quantitatively more active than the dianhydromannitol being about one and a half times more effective in lowering peripheral leucocyte counts.

Antineoplastic effects

Table 6 shows that the two dianhydrohexitols are effective and potent inhibitors of the growth of the transplanted Walker rat carcinoma. On a molecular basis 1,2:5,6-dianhydro-D-mannitol is about 16 times as potent as DBM and 28 times as potent as

Table 5. *Haematological action of* 1,2:5,6-*dianhydro*-D-*mannitol* (III) *and* 1,2:5,6-*dianhydrogalactitol* (IV)

Compound	Dose (d) (mg/kg)	% fall from normal Lymphocytes (L)	Neutrophils (N)	Platelets (P)	L/d	N/d	P/d	L/N
III	8	60	83	21	7·5	10·4	2·6	0·72
IV	6	65	93	19	11·0	15·5	3·2	0·70

Table 6. *Screening against the Walker* 256 *rat carcinoma*

Compound	LD_{50} (mg/kg)	LD_{50} (μmole/kg)	ED_{90} (mg/kg)	ED_{90} (μmole/kg)	Index LD_{50}/ED_{90}
1,2:3,4-Diepoxybutane	70	810	35	405	2
1,2:5,6-Diepoxyhexane	100	880	75	660	1·3
1,2:5,6-Dianhydro-D-mannitol	14	96	3	21	4·7
1,2:5,6-Dianhydro-galactitol	11	75	1·75	12	6·3
1,6-Dimethanesulphonoxy-1,6-dideoxy-D-mannitol	~2000	~5900	200	590	~10
1,6-Dibromo-1, 6-dideoxy-D-mannitol	900	2900	118	383	7·6

Table 7. *Screening against ADJ/PC6A mouse plasma cell tumour*

Compound	LD_{50} (mg/kg)	LD_{50} (μmole/kg)	ED_{90} (mg/kg)	ED_{90} (μmole/kg)	Index LD_{50}/ED_{90}
1,2:5,6-dianhydro-D-mannitol	22·5	154	0·7	4·8	32
1,6-Dimethanesulphonoxy-1,6-dideoxy-D-mannitol	2700	8000	86	254	31

Table 8. *Screening against mouse leukaemia* L1210

Compound	Dose (mg/kg)	T/C ratio
1,2:5,6-Dianhydro-D-mannitol	22	157
	11	115
	5·5	102
	30	132
	25	152
	20	136
	15	137
	10	124
	5	120
1,6-Dibromo-1,6-dideoxy-D-mannitol	1200	128
	600	102
	300	98
1,6-Dimethanesulphonoxy-1,6-dideoxy-D-mannitol	1600	126
	800	108
	400	102
Methotrexate	3	120
	3 (5 daily)	150
	3 (5 daily)	173
	3 (5 daily)	165

mannitol myleran as a tumour growth inhibitor. It is also interesting to note that the dianhydromannitol is about 19 times as potent and the dianhydrogalactitol about 34 times as potent as the simple 1,2:3,4-diepoxybutane. The galactitol derivative is over 50 times as potent as the non-hydroxylic analogue, 1,2:5,6-diepoxyhexane.

The chemotherapeutic indices of the dianhydrohexitols are significantly higher than those of diepoxybutane or diepoxyhexane. Despite some uncertainty regarding the LD_{50} values of the disubstituted hexitols—possibly due to rapid and variable elimination of the compounds—it does appear that the chemotherapeutic indices of these compounds are somewhat higher than those of the anhydrides.

In a comparative assay against the ADJ/PC6A mouse plasma cell tumour 1,2:5,6-dianhydro-D-mannitol and mannitol myleran showed similar chemotherapeutic indices (Table 7). The dianhydride was about 50 times as potent as mannitol myleran on a molecular basis.

1,2:5,6-dianhydro-D-mannitol showed appreciable activity against the L1210 mouse leukaemia producing a 50–60% increase in survival time at well tolerated doses (Table 8). Moderate activity against this leukaemia was also shown by mannitol myleran and DBM.

DISCUSSION

The formation of 1,2:5,6-anhydroderivatives from 1,6-disubstituted hexitols in aqueous solution at physiological pH can be taken as established. It remains to decide whether there is sufficient evidence to support the view that the biological effects of the substituted hexitols can be due to partial or complete conversion into these dianhydrides.

The body-growth inhibitory effects (Fig. 1) and the toxic effects (Table 4) show that mannitol myleran and DBM differ considerably from myleran in their mode of action and invoke a response much closer to that obtained with 1,2:5,6-dianhydro-D-mannitol. Mannitol myleran, however, does show some evidence of myleran-like effects, and when we come to consider the haematological responses (Figs. 2 and 3) it is seen that the complex blood response patterns shown by both mannitol myleran and DBM can be reproduced by a combined treatment with relatively low doses of myleran and 1,2:5,6-dianhydro-D-mannitol. It is clear therefore that the biological effects of mannitol myleran and DBM are consistent with their partial conversion *in vivo* into the dianhydride. These results are consistent with a greater degree of conversion into the dianhydride in the case of DBM—a finding that would have been anticipated from the kinetic data (Table 3).

The blood response is a reflection of previous events occurring in the haematopoetic organs, particularly in the bone marrow, and preliminary results of the effects of DBM and 1,2:5,6-dianhydro-D-mannitol on rat bone marrow support the above conclusions [11].

The nature of the blood response to 1,2:5,6-dianhydro-D-mannitol and 1,2:5,6-dianhydrogalactitol (Table 5) merits some consideration. Both compounds are highly active in depressing the number of circulating leucocytes and show rather surprisingly little effect in depressing platelet counts. The drugs show a preferential effect in depressing blood neutrophil levels indicating a predominant inhibition of myelopoesis. The L/N ratio indicating % depression of lymphocytes/% depression of neutrophils is about 0·7. This compares with a value of 1·14 for chlorambucil [12] and a value of 0·35 for myleran. The times to reach maximum depression are very different being 4 days for the epoxides compared with 14 days for myleran (see Fig. 1).

The fact that the two anhydrides are very potent inhibitors of the growth of the transplanted tumours and that the dianhydromannitol exerts an appreciable effect on the L1210 mouse leukaemia is also consistent with the view that the biological effects of mannitol myleran and DBM are mediated to a considerable extent through epoxide formation. It can be calculated that about a 3·5% conversion of mannitol myleran into the dianhydride *in vivo* would account for all the effect on the rat tumour and about a 2% conversion would be responsible for the whole effect on the mouse tumour. The somewhat higher potency (ED_{90}) of DBM as compared with mannitol myleran in the Walker tumour assay is again in accord with the greater extent of anhydride formation to be expected from this compound.

The identity of the chemotherapeutic indices of 1,2:5,6-dianhydro-D-mannitol and mannitol myleran when assayed against the ADL/PC6A tumour is also suggestive. The somewhat higher indices of the hexitol derivatives in the Walker tumour assays may be an expression of a differential transport characteristic in this system as compared with the dianhydrides.

1,2:3,4-Diepoxybutane and 1,2:5,6-diepoxyhexane have been known as carcinostatic agents for many years [13, 14] but their clinical use has been limited by the small

difference between the effective and toxic doses. This is clearly seen in the chemotherapeutic indices in the Walker tumour assay system recorded for the first time in this paper. The placement of hydroxyl groups on C_3 and C_4 in the hexane derivatives to give 1,2:5,6-dianhydro-mannitol and -galactitol results in a considerable enhancement of potency and chemotherapeutic index. These new diepoxides would seem to merit clinical investigation especially in view of the significant activity against the L1210 leukaemia which is resistant to all but the most effective alkylating agents. 1,2:5,6-dianhydro-D-mannitol exerts a greater effect in the L1210 mouse leukaemia assays than does DBM, a compound that has given encouraging results in the treatment of human myelogenous leukaemia [15, 16]. The relatively small effect in depressing platelets exhibited by the dianhydride may prove clinically advantageous.

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RESUME

Les auteurs décrivent la préparation des 1,2:5,6-*dianhydro*-D-*mannitol et -galactitol. La toxicité, les effets hématologiques et l'action inhibitrice sur la croissance tumorale de ces dianhydrides ont été comparés à ceux du* 1,6-*diméthanosulphonoxy*-1,6-*dideoxy*-D-*mannitol* (*mannitol myleran*) *et du* 1,6-*dibromo*-1,6-*dideoxy*-D-*mannitol* (*DBM*). *Les résultats obtenus montrent que les effets biologiques des dérivés hexitol peuvent être expliqués par la transformation de ceux-ci,* in vivo, *en des quantités appréciables de dianhydride.*

SUMMARY

The preparation of 1,2:5,6-*dianhydro*-D-*mannitol and -galactitol is described. A study has been made of the toxic, haematological and tumour growth inhibitory effects of these dianhydrides for comparison with those of* 1,6-*dimethanesulphonoxy*-1,6-*dideoxy*-D-*mannitol* (*mannitol myleran*) *and* 1,6-*dibromo*-1,6-*dideoxy*-D-*mannitol* (*DBM*). *The results are consistent with the view that the biological effects of the hexitol derivatives are mediated by their conversion into significant amounts of the dianhydrides* in vivo.

ZUSAMMENFASSUNG

Die Herstellung von 1,2:5,6-*Dianhydro*-D-*Mannitol und -galaktitol wird beschrieben. Die toxischen, hämatologischen und tumorhemmenden Effekte dieser Dianhydride werden bestimmt und mit denen des* 1,6-*Dimethansulfonsäure-Esters des* D-*Mannitols* (*Mannitol-Myleran*) *und des* 1,6-*Dibrom*-1,6-*didesoxy*-D-*Mannitols verglichen. Die Resultate sprechen dafür, dass die biologischen Wirkungen der Hexit-Derivate durch die* in vivo-*Umwandlung in die Dianhydride vermittelt werden.*

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Aryl-2-halogenoalkylamines

XXV. Derivatives of Sulphanilamide Designed for Selective Deposition in Neoplastic Tissue

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A LARGE number of aryl-2-halogenoalkylamines (aromatic nitrogen mustards) have been prepared and shown to possess significant tumour growth inhibitory activity [1]. Various attempts have been made to localise the action of such compounds within tumours based on presumed or established biochemical differences between normal and neoplastic tissues [2].

A fundamental metabolic property of all cancer cells, even those of the lowest degree of malignancy [3], is the production of lactic acid from glucose under aerobic conditions. As a consequence the pH of neoplastic tissues has a lower value than that of most normal tissues and this differential can be accentuated by the administration of glucose to the host. This finding has been repeatedly demonstrated in animal tumours [4, 5] and more recently in human tumours [6]. The measurement of tissue pH was carried out using small glass electrodes and must perforce relate to extracellular pH: it is possible that the intracellular concentration of lactic acid is even higher than that in the external fluid [7] but see also [8].

There are a number of ways in which this lower pH of tumour tissue could be exploited to produce a more selectively carcinostatic agent (see reference 2, p. 144). The approach considered in the present study was suggested by the observation of Stevens *et al.* [9] that sulphapyrazine is selectively deposited in the tumour areas of glucose treated rats bearing a transplanted Walker carcinoma. This observation can be rationalised by a consideration of the pH differences between normal and neoplastic tissues since suitably substituted sulphonamides are sparingly soluble in the undissociated form but yield very soluble anions. If, for the sake of calculation, one considers cancer tissue as having a pH of 6·5 and normal tissue a pH of 7·5 then relative solubility curves for compounds with different pK_a values can be plotted (Fig. 1). Figure 2 shows the percentage of the drug that is insoluble in cancer tissue divided by the percentage insoluble in normal tissue (C/N). This expression reaches a maximum for a compound with pK_a about 5·5 and then levels out at a value of 10 (dotted line, Fig. 2). However for a compound of low pK_a the percentage insoluble is too low for much deposition to occur. The optimum situation for the selective deposition of an appreciable amount of drug will be when the expression C/N × the percentage insoluble at a given pH reaches a maximum (full line, Fig. 2). This optimum is reached when the pK_a of the drug is about 6·6. Selective deposition of sulphadiazine (pK_a 6·5) and to a somewhat lesser extent sulphathiazole (pK_a 7·1) and sulphapyrazine (pK_a 6·0, all in water) should occur.

It has now been established that selective

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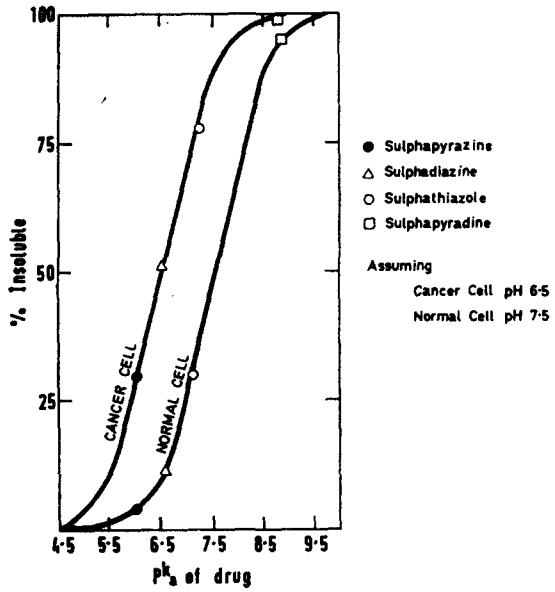


Fig. 1. Solubility of sulphonamides in normal and cancer tissue as a function of pK_a.

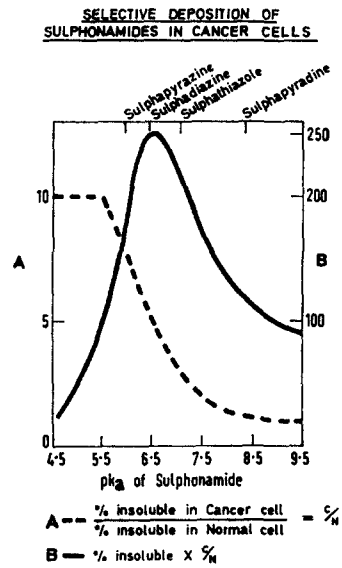


Fig. 2. Selective deposition of sulphonamides in cancer tissue as a function of pK_a.

concentration of sulphadiazine in the tumour regions of rats bearing the solid Yoshida sarcoma does occur. In contrast, a typical aromatic nitrogen mustard gas derivative, melphalan, which is highly effective against this tumour, does not concentrate selectively in the neoplastic tissue. It was reasoned that if the cytotoxic group present in melphalan could be attached to a structure that selectively deposited in tumour tissue then an even more effective agent should result.

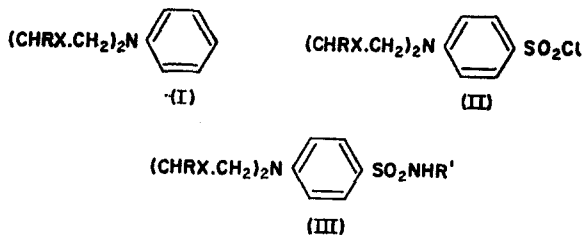
The present work has therefore involved the preparation of various sulphonamides to which have been attached cytotoxic halogenoalkylamine groups. An examination of the antitumour activity of derivatives having different pK_a values should indicate whether the expected selective deposition is occurring.

It has previously been shown that substitution of the hydrogen atoms in the amino group in sulphathiazole (pK_a 8·3 in 50% aqueous ethanol) by 2-chloroethyl groups has little effect on the dissociation constant—the nitrogen mustard gas derivative having pK_a 8·1 in the same medium.

It is known that a certain level of chemical reactivity of the halogenoalkylamine group is necessary for biological activity [2] and since the presence of the sulphonamido substituent, by virtue of its electron withdrawing potential, reduces the reactivity of such groups modifications have been made which should increase this reactivity. These include replacing the chlorine atoms by bromine atoms, inserting a 2-methyl substituent in the alkyl chain and incorporating substituents in the benzene ring in the position ortho to the halogenoalkylamine group.

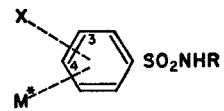
MATERIAL AND METHODS

Sulphanilamide derivatives of general formula (III) were prepared by first converting the aryldi-2-halogenoalkylamine (I) into the chlorosulphonyl derivative (II) by the method of Benn *et al.* [10] and then condensing this with the appropriate amine.



In this way representative compounds with R=H or Me, X=Cl or Br, and R'=H, 2-pyridinyl, 2-pyrazinyl, 2-pyrimidinyl and 2-thiazolyl were obtained (Table 1). Similar chlorosulphonation of *NN*-di-2-chloroethyl-*o*-toluidine and -*o*-anisidine (IV, R=Me or MeO) was shown to give products (V) in which the chlorosulphonyl group had entered the ring in a position *meta* to the substituted amino group. These sulphonyl chlorides were con-

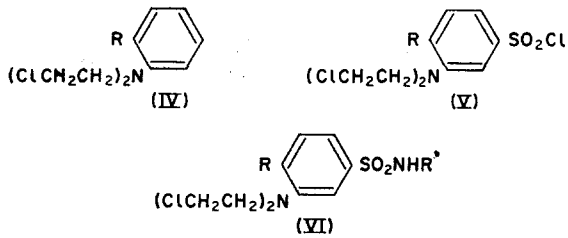
Table 1. *Di-(2-halogenoalkyl)aminobenzenesulphonamides*



(VII)

Compound (VII)					Found (%)						Formula						Hydrolysis
M*	X	R	m.p.(deg.)	form	S	C	H	Cl	N	S		C	H	Cl	N	S	%†
4-M	H	H															2‡
4-M	H	Ph															4‡
4-M	H	2′-pyrimidinyl	215	prisms	A	44·9	4·3	19·1	15·1	8·6	$C_{14}H_{16}Cl_2N_4O_2S$	44·8	4·3	18·9	14·9	8·5	7
4-M	H	2′-pyridinyl															3‡
4-M	H	2′-pyrazinyl	182–184	prisms	B	45·0	4·0	18·8	14·7	9·0	$C_{14}H_{16}Cl_2N_4O_2S$	44·8	4·3	18·9	14·9	8·5	8
4-M	H	2′-thiazolyl															4‡
4-M″	H	H	147–148	prisms	C	44·0	5·8	22·2	8·5	9·9	$C_{12}H_{18}Cl_2N_2O_2S$	44·3	5·5	21·8	8·6	9·9	10
4-M″	H	2′-pyrimidinyl	203–204	prisms	A	47·5	5·4	17·6	14·2	7·9	$C_{16}H_{20}Cl_2N_4O_2S$	47·6	5·0	17·6	13·9	7·9	15
4-M″	H	2′-thiazolyl	190–192	prisms	C-D	44·3	4·8	17·9	9·9	15·9	$C_{15}H_{19}Cl_2N_3O_2S_2$	44·1	4·7	17·4	10·1	15·6	12
4-M″	H	2′-pyridinyl	167–168	prisms	E	50·2	5·0	17·4	10·4	8·0	$C_{17}H_{21}Cl_2N_3O_2S$	50·7	5·2	17·6	10·5	7·9	11
4-M′	H	H															8‡
4-M′	H	2′-pyridinyl															6‡
4-M′	H	2′-pyrimidinyl	194–197	prisms	C	38·4	3·6	33·1 (Br)	11·3	6·5	$C_{14}H_{16}Br_2N_4O_2S$	38·5	3·6	33·0 (Br)	11·6	6·6	12
3-M	4-Me	H	107–108	prisms	C	42·8	5·3	22·2	9·0	9·9	$C_{11}H_{16}Cl_2N_2O_2S$	42·5	5·2	22·8	9·0	10·3	46
3-M	4-Me 1	2′-thiazolyl	151–152	needles	C-D	42·8	4·5	17·5	10·8	15·8	$C_{14}H_{17}C_2N_3O_2S_2$	42·7	4·3	18·0	10·7	16·2	42
3-M	4-Me	2′-pyrimidinyl	177–178	needles	B	46·2	4·7	18·1	14·7	8·6	$C_{15}H_{18}Cl_2N_4O_2S$	46·3	4·6	18·3	14·4	8·2	46
3-M	4-Me	2′-pyridinyl	145–146	prisms	B	49·5	4·6	18·2	10·7	7·9	$C_{16}H_{19}Cl_2N_3O_2S$	49·5	5·0	18·3	10·8	8·3	41
3-M	4-MeO	H	115	needles	C	40·9	5·0	22·4	8·6	10·9	$C_{11}H_{16}Cl_2N_2O_3S$	41·6	5·0	22·4	5·8	10·1	58
3-M	4-MeO	2′-thiazolyl	146–147	prisms	B	41·3	4·4	17·7	10·0	15·7	$C_{14}H_{17}Cl_2N_3O_3S_2$	41·0	4·2	17·3	10·3	15·6	54
3-M	4-MeO	2′-pyrimidinyl	140	needles	B	44·5	4·7	17·1	7·6	13·8	$C_{15}H_{18}Cl_2N_4O_3S$	44·4	4·4	17·5	7·9	13·8	57

M* M=N(CH_2CH_2Cl)$_2$, M′=N(CH_2CH_2Br)$_2$, and M′=N($CH_2CHMeCl$)$_2$.
S Solvents: A, ethyl acetate, B, ethanol, C, benzene, D, light petroleum (b.p. 60–80°), E, isopropyl alcohol.
†Extent of hydrolysis after 30 min in boiling 50% aqueous acetone (see reference 13) by titration of developed acidity (methyl red indicator).
‡Benn *et al.* [10].



verted into the sulphanilamides (VI) (Table 1).

The configuration of the products (VI) was established by unambiguous synthesis. 2-Aminotoluene-4-sulphonamide [11] was converted by successive treatment with ethylene oxide and phosphoryl chloride into the sulphonamide (VI, R=Me, R'=H) which was identical with the product obtained by condensing the sulphonyl chloride (V, R=Me), derived from (IV, R=Me), with ammonia. Similarly 2-nitroanisole-4-sulphonamide [12] was reduced to give the 2-amino derivative which was converted into the same sulphonamide (VI, R=MeO, R'=H) that was obtained from (IV, R=MeO).

Halogenoalkylaminobenzenesulphonyl chlorides (II) and (V)

The following were prepared by the action of chlorosulphonic acid on the amines (I) and (IV) essentially by the method of Benn *et al.* [10].

1-(Di-2-chloroethylamino)benzene-4-sulphonyl chloride, yellow needles, m.p. 101°, from benzene-light petroleum (b.p. 60–80°): lit. [10] gives m.p. 102°.

1-(*Di*-2-*chloro*-n-*propylamino*)*benzene*-4-*sulphonyl chloride*, yellow prisms, m.p. 87–89°, from light petroleum (b.p. 60–80°) (Found: C, 41·7; H, 4·5; Cl, 30·7; N, 4·1; S, 9·1% $C_{12}H_{16}Cl_3NO_2S$ requires C, 41·8; H, 4·6; Cl, 30·9; N, 4·1; S, 9·3%).

1-(Di-2-bromoethylamino)benzene-4-sulphonyl chloride, prisms, m.p. 106°, from chloroform–light petroleum (b.p. 60–80°): lit. [10] gives m.p. 106°.

2-(Di-2-chloroethylamino) toluene-4-sulphonyl chloride, obtained as an oil which was characterised by conversion into the amide (Table 1).

2-(*Di*-2-*chloroethylamino*)*anisole*-4-*sulphonyl chloride*, needles, m.p. 55–56°, from light petroleum (b.p. 40–60°) (Found: C, 38·1; H, 4·3: Cl, 30·6; N, 4·1; S, 9·2%. $C_{11}H_{14}Cl_3NO_2S$ requires C, 38·1; H, 4·2; Cl, 30·8; N, 4·1, S, 9·2%).

General methods for preparing sulphonamides

(a) The sulphonyl chloride (5 g) in acetone (20 ml) was added to aqueous ammonia (15 ml, d 0·88). After stirring for ½ hr the mixture was diluted with water and the product collected and crystallised from the appropriate solvent.

(b) The sulphonyl chloride (0·025 mole) in pyridine (20 ml) was added to a stirred solution of the appropriate amine (0·025 mole) in the minimum quantity of pyridine. Next day the mixture was poured on to ice cooled 2N HCl and the sulphonamide collected by filtration.

2-(*Di*-2-*hydroxyethylamino*)*toluene*-4-*sulphonamide*

Ethylene oxide (12 ml) was added to a solution of 2-amino-toluene-4-sulphonamide (5g) in water (3 ml) and acetic acid (3 ml). After keeping for 3 days at room temperature the solution was evaporated and the product crystallised from water. The *hydroxyethyl* derivative formed pale yellow prisms, m.p. 123° (Found: C, 48·2; H, 6·4; N, 9·9; S, 11·2%; $C_{11}H_{18}N_2O_4S$ requires C, 48·2; H, 6·6; N, 10·2; S, 11·6%).

2-(*Di*-2-*chloroethylamino*)*toluene*-4-*sulphonamide*

The hydroxyethyl derivative (1 g) and phosphoryl chloride) (1 ml) were heated on a steam bath for 1 hr. After adding concentrated hydrochloric acid (5 ml) the mixture was heated for 1 hr, then cooled and poured on to ice. The *chloroethyl* derivative was collected and crystallised from benzene; yield 800 mg., m.p. 107–108° undepressed by admixture with the product obtained by the action of ammonia on the corresponding sulphonyl chloride described above.

2-*Aminoanisole*-4-*sulphonamide*

2-Nitroanisole-4-sulphonamide (4 g) in ethanol (200 ml) containing palladium charcoal (5% Pd) was shaken in an atmosphere of hydrogen until the theoretical amount of gas had been taken up. Evaporation of the filtered solution gave the 2-*amino derivative* which formed needles, m.p. 143–144°, from water (Found: C, 41·4; H, 5·0; N, 13·8; S, 15·6%. $C_7H_{10}N_2O_3S$ requires C, 41·6; H, 5·0; N, 13·9; S, 15·8%).

2-(*Di*-2-*hydroxyethylamino*)*anisole*-4-*sulphonamide*, prisms, m.p. 117–118°, from ethyl acetate (Found: C, 45·8; H, 6·3; N, 9·5; S, 11·0%.

$C_{11}H_{18}N_2O_6S$ requires C, 45·5; H, 6·3; N, 9·7; S, 11·0%) and 2-(*Di-2-chloroethylamino*) *anisole-4-sulphonamide*, needles, m.p. 112°, from benzene, were prepared from the 2-amino-anisole-sulphonamide by procedures already described. The chloroethyl derivative was identical with that obtained by the action of ammonia on the sulphonyl chloride described above.

Determination of the tissue concentration of sulphadiazine and melphalan

The sulphadiazine content of tissues was determined essentially by the procedure of Bratton and Marshall [14]. Animals bearing seven day old solid Yoshida sarcomas were killed at intervals after the intraperitoneal injection of 500 mg/kg of sulphadiazine in arachis oil. One gram of tissue (tumour or liver) was homogenised in 5 ml water. A one ml sample of this homogenate was taken and evaporated to dryness to obtain a value for the dry weight of tissue assayed. To the remaining 4 ml aqueous sodium hydroxide (1 ml, 0·4N) was added and the mixture was incubated at 100° for 1 hr. Water was then added to bring the volume to 5 ml and protein was precipitated by the addition of 15% aqueous trichloroacetic acid (2 ml). Hydrochloric acid (1 ml, 4N) was added to 1 ml of the filtered solution followed by incubation for 1 hr at 100°. The volume of the solution was then made up to 10 ml by the addition of 15% trichloroacetic acid (2 ml) and water. After diazotisation by the method of Bratton and Marshall [14] the concentration of sulphadiazine was estimated colorimetrically.

The concentration of melphalan in liver and tumour tissue was determined by the use of tritiated drug as described by Ball *et al.* [15].

Antineoplastic assays

Yoshida sarcoma. Solid Yoshida sarcomas were obtained by subcutaneous implantation of 2×10^6 ascitic cells in the inguinal region of Wistar rats of the Chester Beatty strain. Seven days after implantation, when the tumours weighed 1·5–2·5 g, the compounds under test were administered by intraperitoneal injection of arachis oil solutions. After a further seven days the animals were killed and the tumours weighed. The percentage inhibition recorded in Table 2 was calculated from the expression:

$$100-100\left[\frac{\text{average weight of tumours in treated animals}}{\text{average weight of tumours in control animals}}\right]$$

The dose (†) shown in Table 2 is that dose which produces the maximum obtainable inhibitory effect — where more than one dose gives >90% inhibition this is recorded. In this system growth inhibitions of <50% are not regarded as significant. Table 3 gives the results of a comparative assay of melphalan and the pyrimidinyl derivative, CB 1932.

Walker rat carcinoma 256

The protocol for testing compounds as inhibitors of the growth of this tumour is described by Connors *et al.* [16]. Compounds were administered by intraperitoneal injection of an arachis oil solution on the day following implantation. The percentage inhibition recorded in Table 4 was derived as described above. In Table 5 the LD_{50} is the median lethal dose for tumour bearing animals, ED_{90} is the dose which will produce 90% inhibition of tumour growth and the chemotherapeutic index (C.I.) is LD_{50}/ED_{90}.

Blood counts (Professor L. A. Elson)

A single dose of CB 1932 (400 mg/kg) or chlorambucil (12·5 mg/kg) in arachis oil was administered by intraperitoneal injection into Wistar rats. Blood was taken from a tail vein. Four rats were used for each compound. A mean curve giving the percentage of the normal count (before injection) against time was plotted (Fig. 5).

RESULTS

Figure 3 shows that selective deposition of sulphadiazine in the Yoshida sarcoma does occur. From two to eight hours after intraperitoneal administration of sulphadiazine (500 mg/kg) the concentration of drug in the tumour remains twice as high as in the liver.

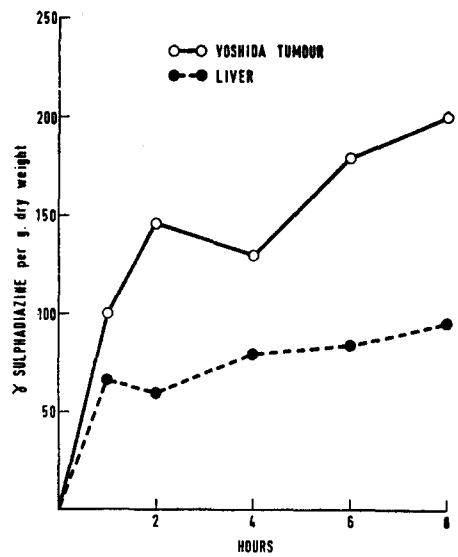


Fig. 3. Concentration of drug in liver and tumour in rats bearing a solid Yoshida sarcoma after i.p. injection of sulphadiazine (500 mg/kg).

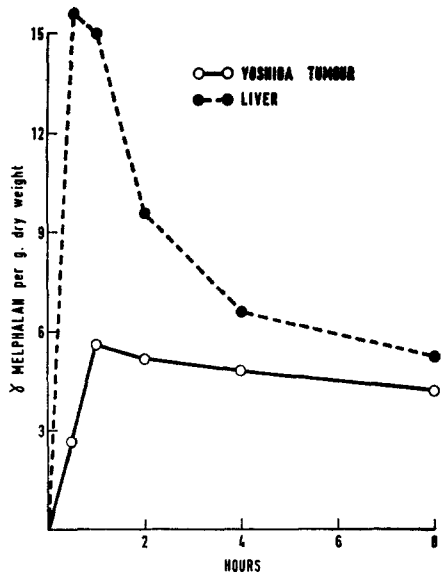


Fig. 4. Concentration of drug in liver and tumour in rats bearing a solid Yoshida sarcoma after i.p. injection of melphalan (2 mg/kg).

This contrasts with the distribution of melphalan — a compound that is very effective in causing regression of an established Yoshida sarcoma. One hour after intraperitoneal administration of melphalan (2 mg/kg) the liver concentration is twice as high as that of the tumour (Fig. 4). At no time does the concentration of the drug in the tumour exceed that found in the liver.

The new compounds prepared are listed in Table 1. Related compounds prepared by Benn *et al.* [10] are also included in this Table so that effects of structural modifications on the chemical reactivity may be compared. The extent of hydrolysis under standard conditions is recorded in the final column; a more detailed study of the relative chemical reactivities is given by Calvert [17].

Replacing a di-2-chloroethylamino group in (VII) by a 2-chloro-*n*-propylamino group has the predicted effect on reactivity. The increases in rates of hydrolysis are as follows: (where R=H, 2→10%; R=2-pyridinyl, 3→11%; R=2-pyrimidinyl, 7→15%; and R=2-thiazolyl, 4→12%). Replacement of a chlorine atom by a bromine atom also causes the expected increase: (R=H, 2→10%; R=2-pyridinyl, 3→6%; and R=2-thiazolyl, 4→12%). The sulphonamides with 3-di-(2-chloroethylamino) and 4-methyl or 4-methoxy substituents all have high reactivity. This is partly due to the lesser deactivating effect of the sulphonamido group in the *meta* position and partly to the rate enhancing effect of the *ortho* substituent which hinders coplanarity of the substituted amino group with the aromatic ring system. The more electron releasing 4-methoxy group increases the reaction rate more than the 4-methyl group.

As would be expected the more reactive compounds tend to be the more toxic though there are minor variations probably due to solubility and transport factors.

Preliminary tumour growth inhibitory assays against the Yoshida sarcoma indicated that the 2-pyrimidinyl compounds (VII where M=

Table 2. Screening against the Yoshida sarcoma

M*	Compound (VII) X	R	Inhibition (%)	Dose (†) (mg/kg)	LD_{50} (mg/kg)
4-M	H	Ph	none		1400
4-M	H	2′-pyridinyl	none		>1500
4-M	H	2′-thiazolyl	52	240	>1500
4-M	H	2′-pyrimidinyl	100	800	895
4-M	H	2′-pyrazinyl	none		1800
4-M′	H	2′-pyridinyl	20	600	>1500
4-M′	H	2′-pyrimidinyl	82	640	717
4-M″	H	H	93	40	56·5
4-M″	H	2′-pyridinyl	none		>625
4-M″	H	2′-pyrimidinyl	100	100	560
		(CB 1932)	100	200	
			100	400	
3-M	4-Me	H	69	4	5·7
3-M	4-Me	2′-pyridinyl	96	50	70
3-M	4-Me	2′-pyrimidinyl	63	20	26
3-M	4-MeO	H	100	4	4
3-M	4-MeO	2′-thiazolyl	61	50	71
3-M	4-MeO	2′-pyrimidinyl	85	20	29

M* as in Table 1.

Table 3. Comparative assay of melphalan and CB 1932 against the Yoshida sarcoma

Dose (mg/kg)	CB 1932 Survivors	Inhibition (%)	Dose (mg/kg)	Melphalan Survivors	Inhibition (%)
12·5	4/4	15·3	0·031	4/4	–25
25	4/4	97·7	0·062	4/4	4
50	4/4	100	0·125	4/4	70
100	4/4	100	0·25	4/4	100
200	4/4	100	0·5	4/4	100
400	4/4	100	1·0	4/4	100
800	0/4	—	2·0	4/4	100
			4·0	0/4	—
	LD_{50} 560 mg/kg ED_{90} 22 mg/kg C.I. 25·4			LD_{50} 2·8 mg/kg ED_{90} 0·21 mg/kg C.I. 13·3	

Table 4. Inhibition of the growth of the Walker carcinoma by CB 1932

Dose (mg/kg)	Survivors	Inhibition (%)
1·5	3/3	none
3·1	3/3	none
6·2	3/3	none
12·5	3/3	23
25	3/3	29
50	3/3	98·7
100	3/3	100
200	3/3	100
400	3/3	100
800	3/3	100
1600	0/3	—

Table 5. Comparison of CB 1932 with other carcinostatic alkylating agents in the Walker tumour assay

Compound	LD_{50} (μmole/kg)	ED_{90} (μmole/kg)	C.I.
CB 1932	2806	115	24·5
PhM	645	60	10·8
$PhN(CH_2CHMeCl)_2$	500	48	10·4
β-naphthylM	4059	363	11
MC_6H_4-*p*-$(CH_2)_3COOH$ (chlorambucil)	58·2	8·6	7
MC_6H_4-*o*-$CH_2CH(NH_2)COOH$(merophan)	11·5	0·79	14
MC_6H_4-*p*-$CH_2CH(NH_2)COOH$(Melphalan)	14·7	1·64	9
Triethylenemelamine $M{=}N(CH_2CH_2Cl)_2$	5·5	0·58	9·5

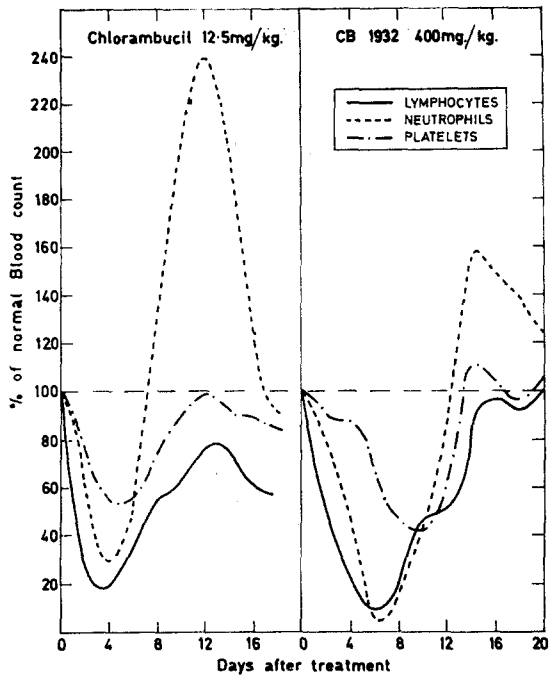


Fig. 5. Blood response pattern in the rat to chlorambucil (12·5 mg/kg, i.p.) and to CB 1932 (400 mg/kg, i.p.).

$N(CH_2CH_2Cl)_2$, $N(CH_2CH_2Br)_2$ and $N(CH_2CHMeCl)_2$ and X=H) were the most effective (Table 2). Compounds with high chemical reactivities all show anti-tumour growth effects which are not so dependent on the nature of the group R. One compound (III, R=Me, X=Cl, R′=2-pyrimidinyl; CB 1932) of relatively low chemical reactivity showed outstanding activity throughout an appreciable dose range. From the results of a comparative assay of melphalan and CB 1932 against the Yoshida sarcoma (Table 3) it can be seen that the chemotherapeutic index (25·4) of the pyrimidinyl derivative is about twice that (13·3) of the phenylalanine derivative.

CB 1932 was also assayed against the Walker rat carcinoma 256 for comparison with some alkylating agents which have found clinical usefulness. The results are shown in Table 4. Table 5 gives the chemotherapeutic indices of CB 1932 and some representative carcinostatic drugs; doses in this Table are given in μmoles/kg, since this facilitates comparisons of molar effectiveness of different structures. It can be seen that CB 1932 has a significantly higher index than other agents which would be consistent with its selective deposition within the tumour tissue.

Figure 5 shows that CB 1932 and chlorambucil have essentially similar effects on the peripheral blood elements. However, while chlorambucil produces a maximum depression of cell count at 3–4 days CB 1932 produces a maximum effect at 6–7 days which is somewhat more prolonged.

DISCUSSION

The improvement in chemotherapeutic index achieved in the Yoshida and Walker tumour assays when the cytotoxic di-2-chloro-*n*-propylaminophenyl group is attached to a sulphonamide structure which has been shown to be selectively deposited in tumour tissue fully justifies the present approach to the design of more specific carcinostatic agents.

The deposition of a cytotoxic alkylating agent of moderate chemical reactivity is probably advantageous for several reasons. There is evidence [18] that cells are more sensitive towards alkylating agents when exposed during the later G_1 and S phase of the mitotic cycle and this is a limited period of the whole cell cycle time. In a mixed cell population such as exists in the average tumour mass the cells will be passing through the sensitive phase at different times and it would clearly be an advantage to have an agent which would be localised in the tissue and then be reactive over a considerable period. In this way an optimum effect on the whole mass would be achieved.

A similar situation has recently been demonstrated by Skipper *et al.* [19] in the effect of cytosine arabinoside on the L1210 leukaemia. This drug acts specifically on the S-phase. By adjusting the dosage schedule so that every cell is exposed to a lethal concentration of the drug during the S-phase the malignant cells may be completely eradicated. Alternative dosage schedules employing even maximum tolerated doses are much less effective.

If the alkylating agent is too reactive chemically it would probably not be present in an effective form for long enough to affect all the cells. This may be why the sulphadiazine derivatives of higher reactivity are not so effective as CB 1932.

The delay and lengthening of the effect of CB 1932 as compared with chlorambucil on peripheral blood count could be due to the slow release of the less soluble compound from the site of injection or to a more selective effect on stem cells. Alternatively, deposition of the sulphonamide in blood forming tissue and a longer period of exposure to a less reactive compound would account for the results shown in Fig. 5.

Any procedure which further enhances the pH differential between normal and cancer tissue should increase the degree of selective deposition of the substituted sulphonamides.

Prior administration of glucose is known to accentuate this differential [4, 5] and the effect of such pretreatment on the effectiveness of CB1932 is now being studied.

Acknowledgements—Carcinostatic assays against the Walker rat carcinoma were carried out by Mr. B. C. V. Mitchley and those against the Yoshida sarcoma by Mr. M. Jones. This investigation has been supported by grants to the Chester Beatty Research Institute (Institute of Cancer Research: Royal Cancer Hospital) from the Medical Research Council and the British Empire Cancer Campaign and by the Public Health Service Grant No. CA-03188-09 from the National Cancer Institute, U.S. Public Health Service.

RESUME

On a montré que la sulphadiazine était concentrée dans la région tumorale de rats porteurs d'un sarcome de Yoshida sous forme solide. Les auteurs décrivent la préparation de plusieurs sulfamidés di-2-halogenoalkylaminobenzène qui seraient déposés sélectivement dans les tissus néoplasiques. L'un de ces nouveaux dérivés, la N^4-di-2-chloro-n-propyl-sulphadiazine, ou CB 1932, *a, sur la croissance de la tumeur de Yoshida et du carcinome de Walker* 256, *une activité inhibitrice particulièrement élevée.*

SUMMARY

Sulphadiazine has been shown to concentrate in the tumour region of rats bearing a solid Yoshida sarcoma. The preparation is described of a number of di-2-halogenoalkylamino-benzenesulphonamides which should be selectively deposited in neoplastic tissue. One of these new derivatives, N^4-di-2-chloro-n-propylsulphadiazine, CB 1932, *has shown exceptionally high activity as an inhibitor of the growth of the Yoshida tumour and of the Walker rat carcinoma* 256.

ZUSAMMENFASSUNG

Sulfadiazin reichert sich in der Tumorregion von Ratten an, welche das feste Yoshida-Sarkom tragen. Es wird die Darstellung einer Reihe von Di-2-Halogenalkylamino-benzolsulfonamiden beschrieben, von denen eine selektive Anreicherung in neoplastischen Geweben erwartet werden kann. Eins dieser neuen Derivate, das N^4-Di-2-Chlor-n-Propylsulfadiazin, CB 1932, *zeigte eine ungewöhnlich hohe Aktivität als Hemmstoff des Wachstums des Yoshida-Tumors und des Walker-Ratten-Carcinoms* 256.

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Chromosome Markers in Cultured Cells from Burkitt's Lymphoma

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IT IS commonplace to find that cells within a single tumour or cell line, all have a characteristic abnormal chromosome complement. On the other hand there is only one well documented instance of the same specific chromosome abnormality being present in the cells derived from a number of different patients all with the same neoplasm. This abnormality, the Philadelphia chromosome, which occurs in the myeloid cells of a high proportion of cases of chronic myeloid leukaemia seems to be a unique finding in malignant cells from man. There are now, however, a number of reports which suggest that specific chromosome abnormalities occur in the cells of Burkitt's tumour or of cell lines derived from such tumours. In particular the presence of a specific chromosome marker, a near terminal constriction in the long arms of a C group chromosome, has been demonstrated in four cell lines derived from different cases of Burkitt's lymphoma [1, 2]. The present study was carried out to try to confirm the presence of this marker chromosome in some of the cell lines in which it had previously been reported and to determine whether it is present in additional cell lines of similar origin.

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MATERIAL AND METHODS

Cell lines

The present paper records the cytogenetic status of the lines EB1 and EB1(H), EB2, EB3, EB4, EB5, RAJI and GOR in August 1967. EB3 was also examined in January 1968. The origin and history of these lines are indicated in Table 1.

Culture technique

The cells were maintained in Eagles Basal Medium or supplemented Eagles Minimal Essential Medium by the tissue culture methods already described [3].

Chromosome preparations

Forty-two hours after a medium change, 'Colcemid' was added to the culture. Three hours later the cells were treated with 0·075 M KCl for 10 min [4] and fixed in a mixture of three parts absolute ethyl alcohol and one part glacial acetic acid for 30 min. Preparations were made by allowing drops of cell suspension in fixative to dry on a slide at room temperature. The slides were then stained with acetic orcein and mounted in euparal.

Cytogenetic observations

The following observations were carried out on each of the seven cell lines.

(1) Full or partial analysis of the chromo-

Table 1

Line	Established	Reference	History	Months in culture at date of cytogenetic observations
EB1	December 1963	21	Same laboratory	44
EB1(H)	December 1963	21	To Henle, Philadelphia, October 1964 Returned to Epstein, December 1965	44
EB2	May 1964	22	Same laboratory	39
EB3	November 1964	23	Same laboratory	(a) 33 (b) 38
EB4	December 1965	24	Same laboratory	20
EB5	January 1966	24	Same laboratory	19
RAJI	October 1963	25, 26	To Epstein, January, 1965	46
GOR	November 1966	27	To Epstein, January, 1967	9

somes was carried out on at least 50 cells.

(2) Chromosome counts were carried out on 100 cells which in most cases were cells suitable for full analysis. These cells were scored for:

(i) Presence or absence of a near terminal constriction in the long arms of a number 10 chromosome.

(ii) Any other gaps, breaks or constrictions, particular attention being given to those involving both chromatids of a chromosome.

(iii) Presence of dicentric chromosomes, ring chromosomes or fragments.

(3) One thousand mitotic cells were scored for the degree of ploidy, endoreduplication and pulverisation.

RESULTS

There was a great deal of variation between the different cell lines some having a near diploid mode and some a near tetraploid mode. In some cases there was great variation within a single cell line and on several occasions the analysis of the 50 cells proved each cell to be different although the line as a whole conformed to some recognisable pattern. For this reason it is difficult to tabulate all the results. The main features of the cytogenetic observations are however presented in Table 2 and the special features of the individual cell lines discussed in the text. A summary of previous cytogenetic observations on these lines is presented in Table 3.

*EB*1 *and EB*1*(H)*

EBI

	Chromosome count distribution							
Total	62	75	77	80	81	82	83	84
100	1	2	1	2	8	6	13	16
	85	86	87	88	89	95	96	98
	19	15	9	4	1	1	1	1

There was considerable variation from one cell to another in detailed chromosome analysis. In the 15 cells fully analysed many structurally abnormal chromosomes were present and the distribution of the chromosomes within the recognised groups was also abnormal. In the 50 cells that were partially analysed evolution within the cell line was demonstrable. There were two easily recognisable abnormal chromosomes (Fig. 1), one larger than any in the normal complement with short arms similar in length to the arms of a number 1 and long arms longer than the long arms of a number 2 (marker A); the other more or less acrocentric, with long arms greater in length than the long arms of a number 2 (marker B). The distribution of these two chromosomes in the 50 cells was as follows:

Total	Marker A only	Marker B only
50	19	21
Marker A+ Marker B	Neither	No decision
6	3	1

The most likely explanation of the distribution of the two marker chromosomes is that a cell type containing both marker chromosomes has at one time given rise to cell types containing only one or the other. The Type A marker is similar to the chromosome described as a 'recurrent marker' in the lines OGUN, JIJOYE and SL1 [1].

Such chromosomes were also observed in direct preparations of three Burkitt's tumours [5]. Although this is of considerable interest it does not necessarily imply a common origin for these chromosomes. Neither of these markers has been described in previous observations on EB1.

Table 2

Line	100 Cells						1000 Cells. Degree of ploidy						
	Cells with No. 10 Marker	Chromatid aberrations				Cells with dicentrics	Approx. 2N	Approx. 3N	Approx. 4N	>4N	Endo.	Comment	Pulv. Cells
		No. of cells	Total aberrations	Isochro-matid	Comment								
EB1	0	18	31	7	No two identical	8	—	—	971	18	11	Many of 18 cells>4N contain dicentric chromosomes	
EB1 (H)	0	24	31	8	No two identical	6	—	—	970	18	12	Many of 18 cells>4N contain dicentric chromosomes	—
EB2	2	10	12	2	Not identical	4	—	—	993	6	1		—
EB3 (a)	0	10	11	2	Not	0	968	—	29	—	3	Majority of 4N's are true	—
(b)	10	12	12	1	identical	2	499	20	469	12	—	polyploids of 2N line.	
EB4	0	10	13	8	No two identical	0	964	—	36	—	—	Majority of 4N's are true polyploids of 2N line.	1
EB5	Many simple and complicated chromatid aberrations											See text	Present
RAJI	0	16	18	10	Two in same locus No. 16. No two others identical	5	—	—	995	5	—		—
GOR	10	48	52	34	32 identical involving abnorm. chromosome similar to No. 16	8	990	—	8	—	2	Majority of polyploids contain dicentric chromosomes	—

Table 3. Summary of previous cytogenetic observations on the lines studied

Line	Reference	Approx. time culture (months)	Ploidy	Analysis	No. 10 Marker	Other comments
EB1	2	12–18 (serially)	Diploid	Normal	Yes % not known	
		26	Diploid Hyperdiploid 4% polyploid	Abnormal (majority–3+ abnormal sub metacentric)	Yes 49% cells	
	7	16	Mainly hypodiploid some polyploids	All abnormal	Not mentioned	
	1	25	Hypodiploid 8/200 polyploid	All abnormal (+3–21) and variation	Not present	10/200 pulverised
EB2	2	4	Diploid	Normal	Yes 9/30 cells	Serial observations 4–17 months.
		17	Diploid Hypderdiploid 5% polyploid	Hyperdiploid (+1.C group)	Yes 38% cells	Increase in cells with 47+1C group
	6	<7	Diploid	Normal	Not mentioned	High % chromatid aberrations
	7	11	Mainly hypotetraploid Few diploid and triploid	All abnormal	Not mentioned	
	1	20	Diploid 32/200 polyploid	Abnormal	Yes 5–10% cells	2/200 pulverised
EB3	7	5–7	Diploid Few polyploids	Mainly abnormal Few normal	Not mentioned	
	1	14	Diploid 7/200 polyploid	Normal	Yes 2–3% cells	1/200 pulverised
	2	16	Diploid 0/200 polyploid	Normal	Yes 30% cells	
EB4	8	14–16 (serially)	Diploid 9–10% polyploid	Abnormal	Not present	
RAJI	1	25	Diploid 7/200 polyploid	Normal	Not present	0/200 pulverised

*EB*1 (*H*)

This sub-line was maintained in another laboratory for fourteen months but was returned to the original laboratory twenty months before the observations were made.

Chromosome count distribution

Total	79	80	84	85	86	87
100	1	1	1	4	10	21

	88	89	90	91	92	94
	18	16	14	9	3	1

There was a relatively stable but abnormal distribution of chromosomes within the recognised groups, e.g. 30 out of 50 cells had only two number 16 chromosomes. Either one or two abnormal chromosomes similar in proportions to a B chromosome but as long as a number 1 was present in all of the 50 cells fully or partially analysed. Several other abnormal chromosomes were present in individual cells. A representative cell is illustrated in Fig. 2. Small similar paired fragments were present in 24 of the 100 cells counted. The secondary constriction in a number 9 chromosome which occurs frequently in the normal karyotype was unusually pronounced in this line. The abnormal chromosomes (A and B) which were observed singly or together in 92% on the main EB1 line were not seen in any of the cell of EB1 (H).

Comparing the results on these two related sub-lines with previous observations on EB1 it is obvious that the cell line has changed considerably in culture, and moreover, that the two sub-lines have diverged considerably. At the time of the present observations there is no evidence of the diploid or hypodiploid lines previously observed, all the cells being hypotetraploid or having a greater degree of ploidy. Kohn *et al.* [2] have recorded the presence of the number 10 marker chromosome on repeated observations on this line and in February 1966 they found 49% of the cells, which were then pseudodiploid, to contain the marker. On the other hand Miles and O'Neill [1] found no evidence of the marker although their study was carried out at roughly the same time as that of Kohn *et al.* [2]. The marker chromosome was not seen in either substrain in the present study and there was no direct evidence of the marker being involved in a translocation.

*EB*2

It is clear that this cell line has also changed considerably in culture. Kohn *et al.* [2] on repeated examination from September 1964 to October 1965 report it as diploid or near diploid with the marker 10 chromosome in up to 38% of the cells. Stewart *et al.* [6] report it as normal female with a high percentage of chromatid aberrations. Observations made in

Chromosome count distribution

Total	74	79	80	81	83	84	86
100	1	2	1	2	3	1	10

	87	88	89	90	91	104	117
	15	29	19	8	1	1	1

April 1965 by Cooper *et al.* [7] showed a subculture of the line to be mainly hypotetraploid with a few abnormal diploid and triploid cells present. Miles and O'Neill [1] in January 1966 examined the cell line and reported on the chromosomes as being pseudodiploid or near diploid with the number 10 marker in 5–10% of the cells.

In the present observations this line was found to be hypotetraploid with few recognisably abnormal chromosomes. The distribution of chromosomes within the normal groups was relatively stable, but one or more small paired fragments was present in 21 of the 50 cells analysed (Fig. 3). A small ring chromosome was also present in 5 of the 50 cells. Two out of the 100 cells examined contained the number 10 chromosome marker.

*EB*3

Chromosome count distribution

Date	Total	43	44	45	46	47	48
Aug. 1967	100	—	1	9	88	1	—
Jan. 1968	100	1	3	7	45	—	—

Date	49	79	83	84	85	86
Aug. 1967	1	—	—	—	—	—
Jan. 1968	—	1	1	1	—	1

Date	87	88	89	90	91
Aug. 1967	—	—	—	—	—
Jan. 1968	2	1	5	5	7

Date	92	93	94
Aug. 1967	—	—	—
Jan. 968	14	3	2

At the time of the first observations this line was entirely pseudodiploid. The majority of the cells with 46 chromosomes were lacking one 17 and two C group chromosomes and had two additional chromosomes similar to a 16 chromosome, and one resembling a D chromosome but with more prominent short arms. The remainder of the cells were all clearly

derived from this stem line. The cells with 45 chromosomes showed random loss of chromosomes. Cooper *et al.* [7] reported this cell line as being mainly pseudodiploid but having a few cells with normal karyotypes. They mention specifically the stability of this cell line but do not give details of the abnormal karyotypes. The other two groups of workers who have examined the chromosomes of this line [1, 2] both report it as being diploid with a percentage of the cells having the number 10 chromosome marker. In August 1967 this marker was not seen in any cells but the fixation was considered to be less satisfactory than usual. It was felt that the marker might possibly have been present but not observed and so a repeat observation was made in January 1968. On this occasion the number 10 marker was quite clearly seen in 10 cells out of 100 examined (Fig. 3). At the same time it was found that the count distribution was now bimodal with a tetraploid mode which, with some slight variation, was a true tetraploid of the previously observed pseudodiploid karyotype. The number 10 marker was seen in both pseudodiploid and in tetraploid cells. Once again therefore we have evidence of a rapid evolution of the karyotype within a cell line.

*EB*4

Chromosome count distribution								
Total	40	41	42	43	44	45	46	47
100	1	3	1	4	9	16	63	3

None of the cells with 46 chromosomes had a normal karyotype. The line was therefore predominantly pseudodiploid. Virtually all of the cells had 4 marker chromosomes, one short acrocentric, one long acrocentric, one chromosome the size of a number 2 but with a more terminal centrometre and one chromosome larger than a number 1 but with the centromere slightly off centre (Fig. 5). This latter is like the 'recurrent marker' of Miles and O'Neill [1] and the long B chromosome like those found in RAJ1 and EB1(H) in the present study. None of the cells with 46 chromosomes had a number 1 chromosome and all had 5 G's. Although the actual number of chromosomes in the other groups varied there was a clearly defined, relatively stable, line in which the commonest cell type had 1(0), 2(3), 3(2), B(4), D(4), 16(3), E(3), F(4), G(5)+the four abnormals already described. There was a high proportion of hypodiploid cells and analysis of the cells with less than 46 chromosomes suggested that they were derived from the same stem line by loss of a single chromosome or by breakage during preparation of the slide. The polyploid cells are true polyploids of the pseudodiploid line. The number 10 marker was not observed in any cell.

Bishun and Sutton [8] have previously reported on this line but do not give full details of their analysis. They also found a large number of hypodiploid cells and a pseudodiploid karyotype. They record a long acrocentric marker which could be the same as the one reported here. They looked for but did not observe the number 10 chromosome marker.

*EB*5

We have not been able to get fully satisfactory chromosome preparations on this line of cells. The original observations were made in August 1967. The number of dividing cells was small, and the chromosomes appeared to be poorly fixed but this may be a feature of the cell line. Accurate counts were difficult because of very large numbers of chromatid aberrations. Approximate counts on 15 cells ranged from 50 to 160 chromosomes but 10 of these were between 82 and 89. It seems safe to conclude that the line is predominantly hypotetraploid. Attempts at analysis revealed many abnormal chromosomes as well as many chromatid aberrations some of which are complicated interchanges. Some cells showed pulverisation of the chromosomes (Fig. 6) while others showed despiralisation of the chromosomes. It was not possible to say whether the number 10 marker chromosome was present or not. Attempts to obtain better results were made in January 1968 and March 1968 but no good preparations were obtained.

There have been no previous cytogenetic observations on this line of cells.

RAJI

Chromosome count distribution								
Total	72	76	79	81	82	83	84	85
100	2	1	1	2	2	3	4	3
	86	87	88	89	90	91		
	7	19	24	24	6	2		

This cell line was entirely hypotetraploid and the distribution of the chromosomes within the groups was abnormal. Virtually every cell was different yet they were clearly all derived from a common stem line because the majority of the 50 cells fully and partially analysed contained three marker chromosomes, one a

All the figures are micrographs of the chromosomes of single cells from the cultured lines of Burkitt lymphoma cells. All are stained with orcein and the approximate magnifications are indicated separately.

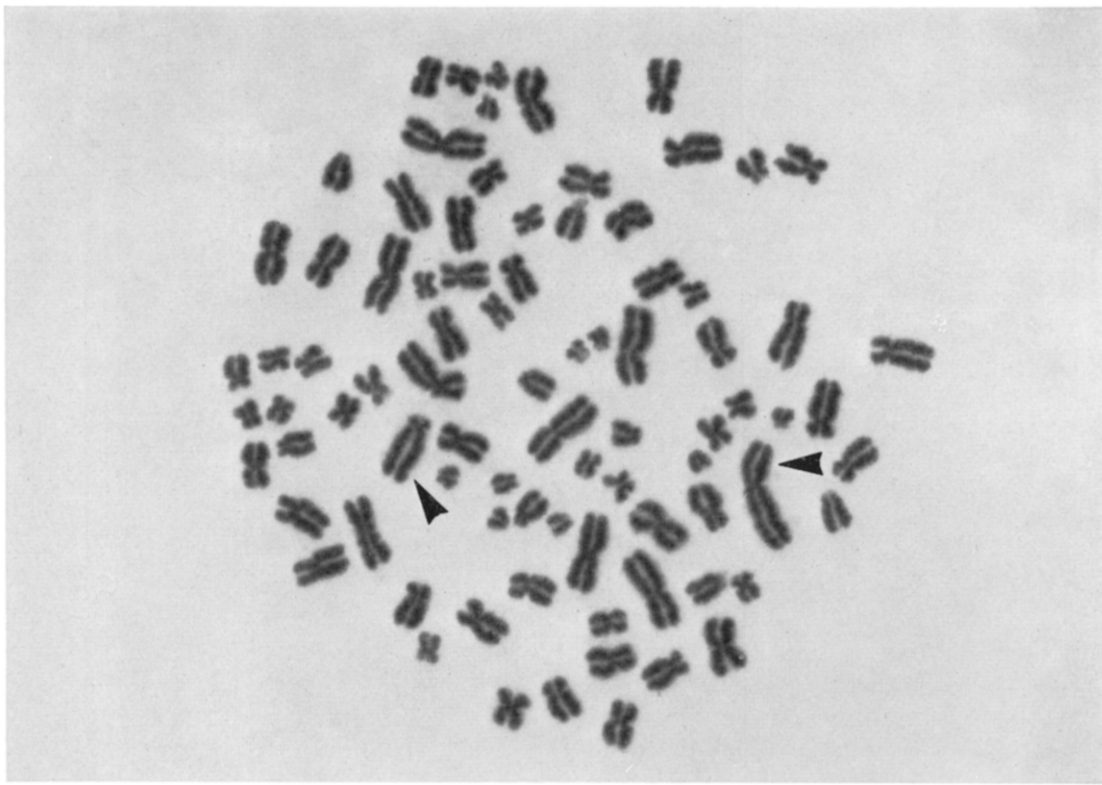


Fig. 1. *EB*1 *Cell showing two abnormal chromosomes A and B (arrowed)* (×2000).

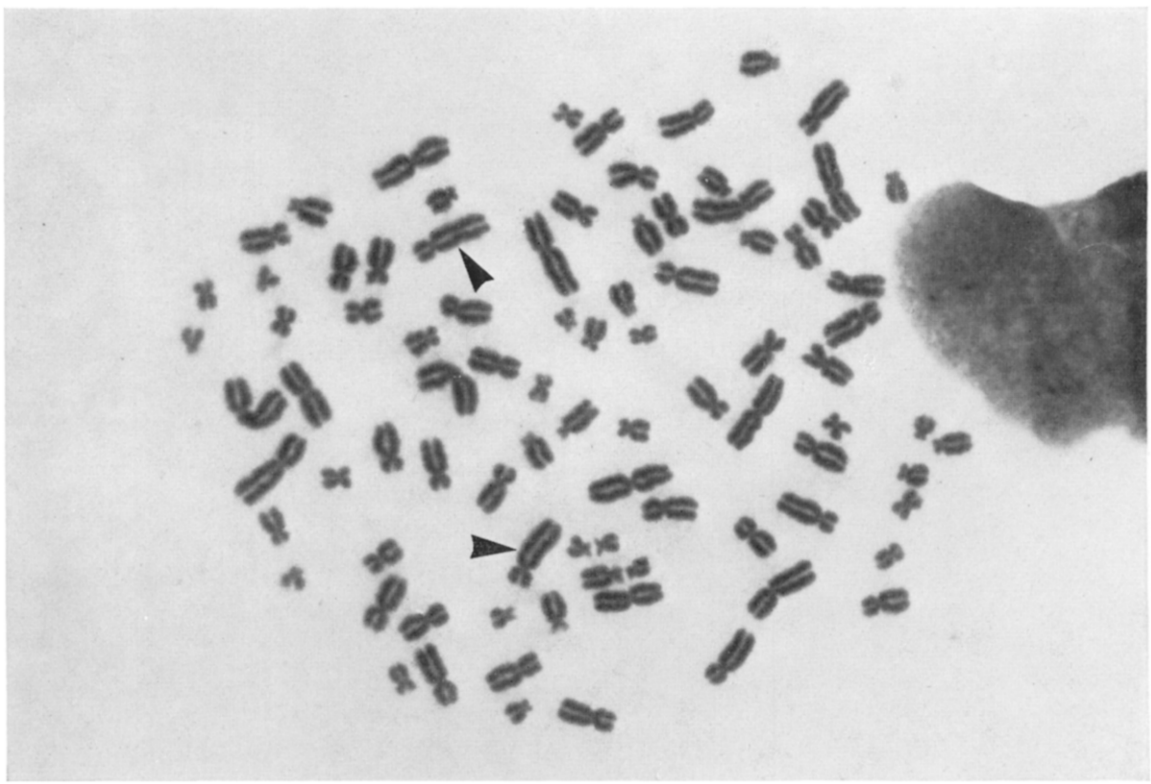


Fig. 2. *EB*1(*H*) *Representative cell showing the two B-like marker chromosomes* (×1500).

(to face p. 642)

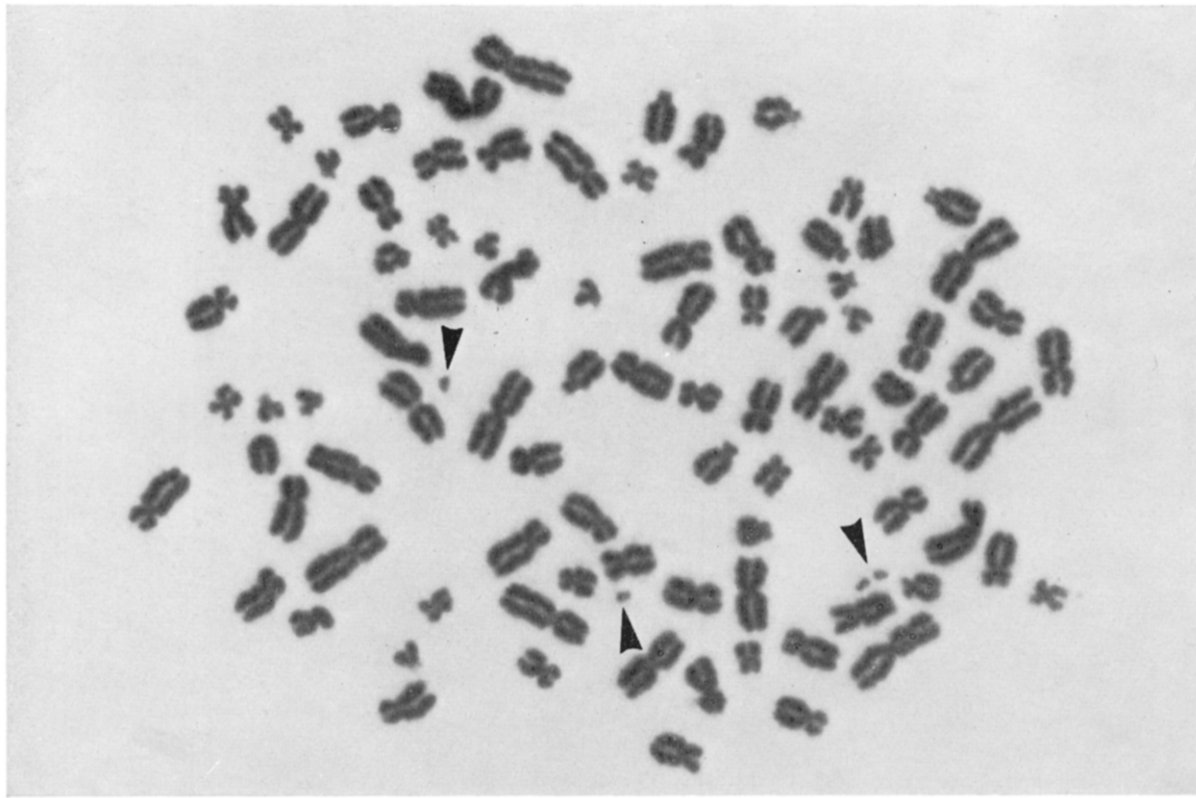


Fig. 3. *EB*2 *Representative cell showing small fragments* (×2500).

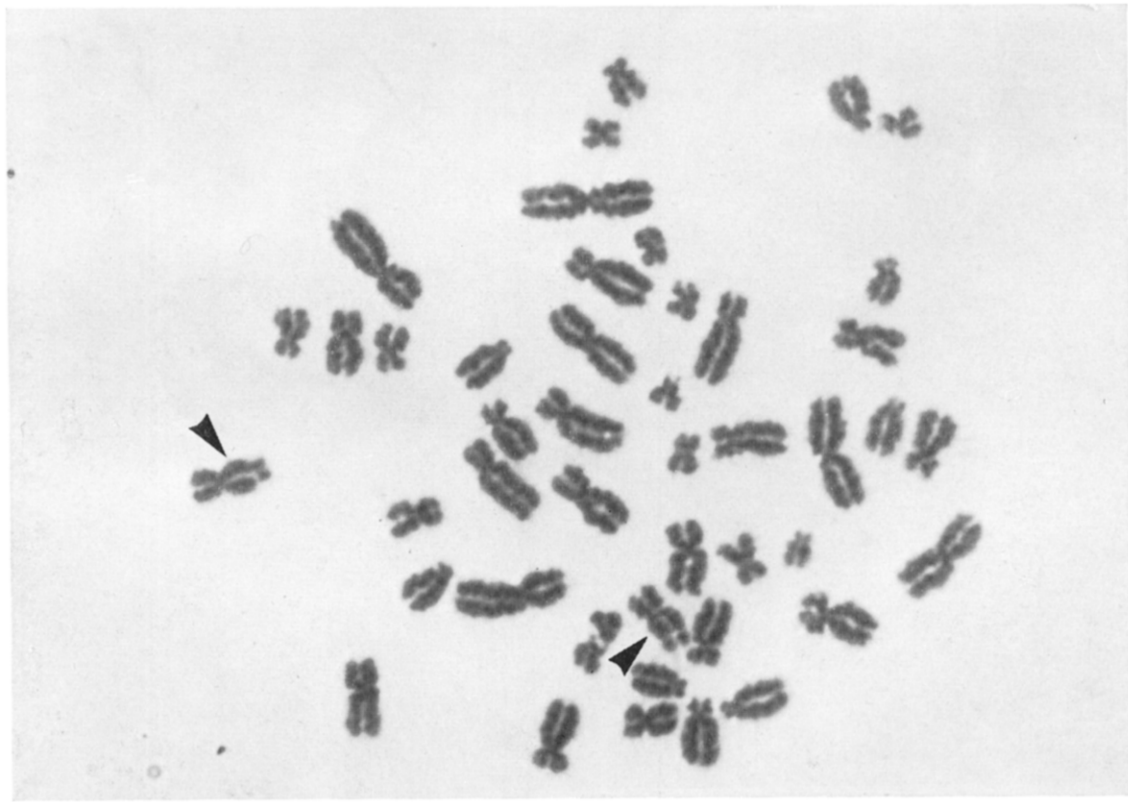


Fig. 4. *EB*3 *Pseudodiploid cell showing the number* 10 *marker chromosome* (×2250).

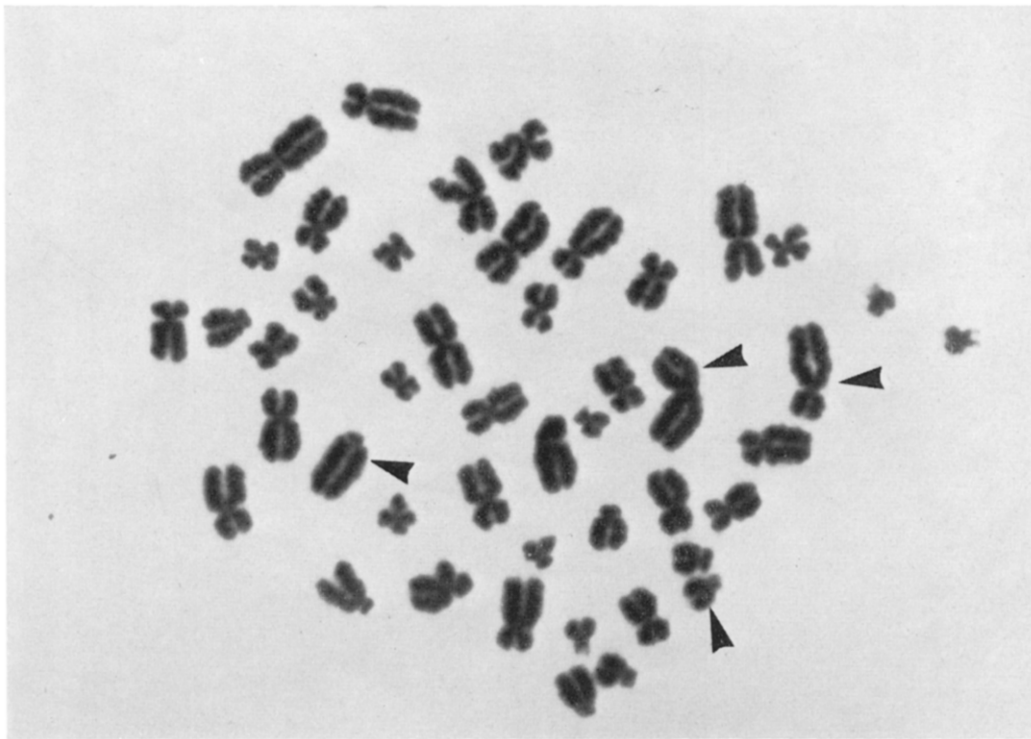


Fig. 5. *EB*4 *Pseudodiploid cell with four abnormal chromosomes* (×2250).

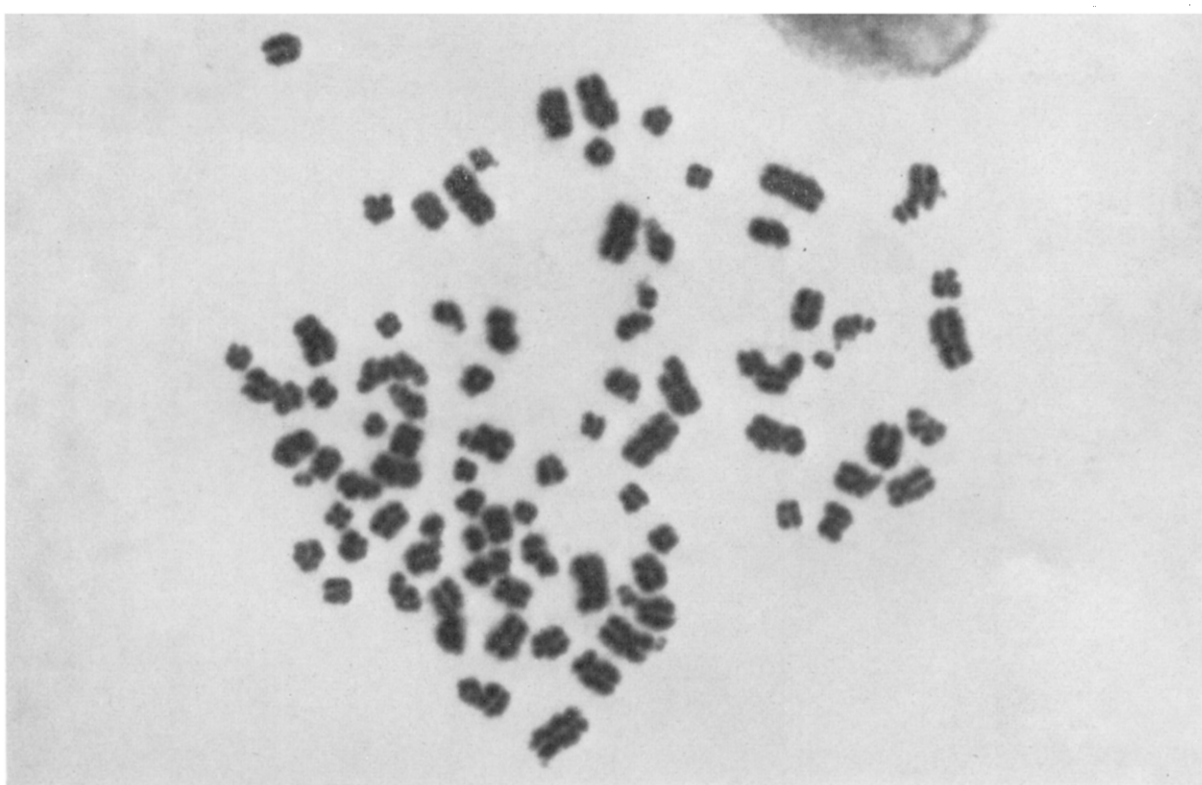


Fig. 6. *EB*5 *Cell with multiple chromatid aberrations* (×1750).

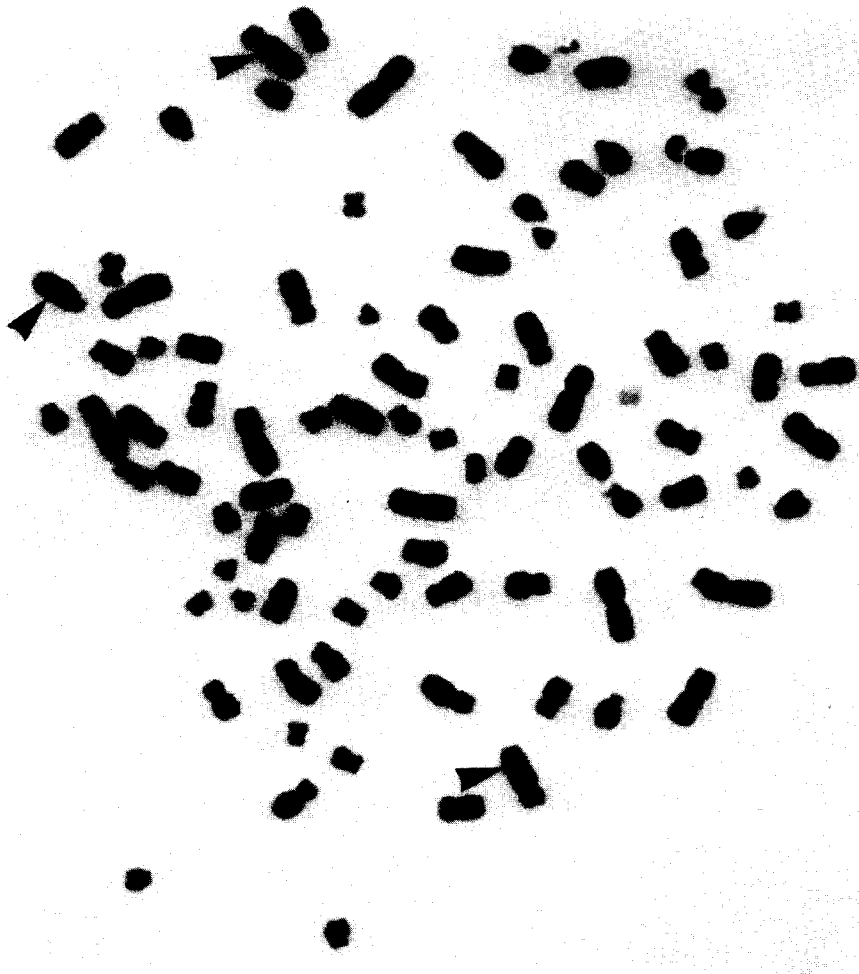


Fig. 7. RAJI Hypotetraploid cell with three marker chromosomes ($\times$ 1500).

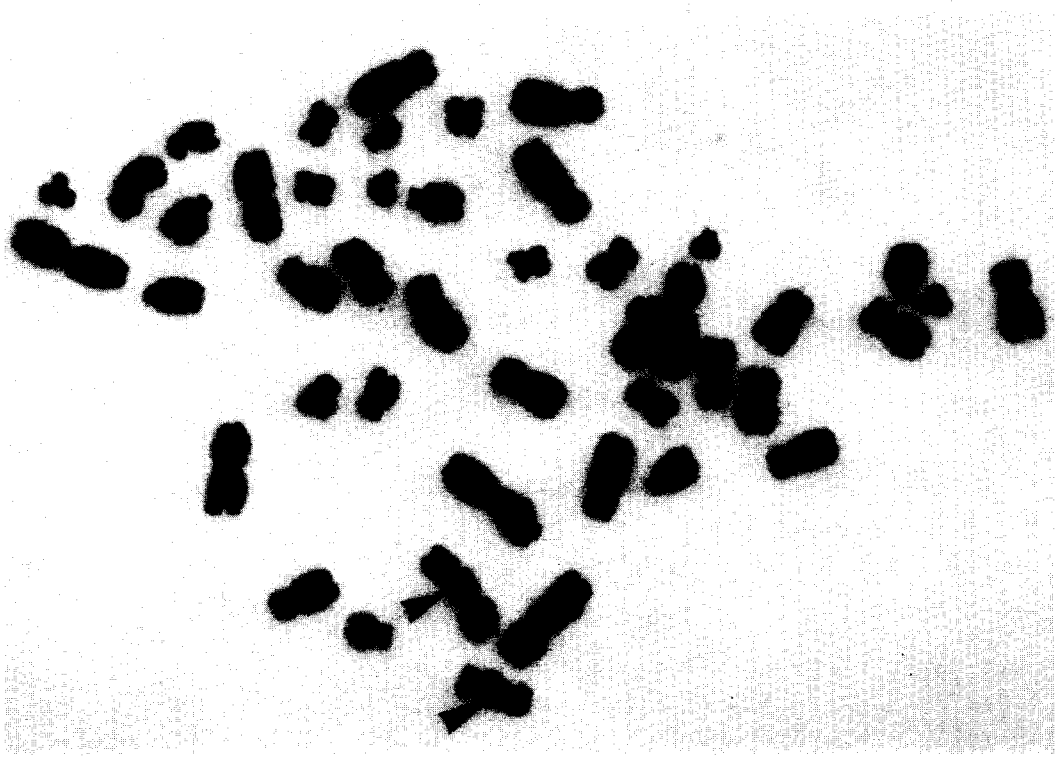


Fig. 8. GOR Pseudodiploid cell with number 10 *marker chromosome and dicentric chromosome* ($\times$ 2500).



Fig. 9. *GOR Hypodiploid cell with abnormal* 16 *chromosomes showing near terminal secondary constriction* (×2750).

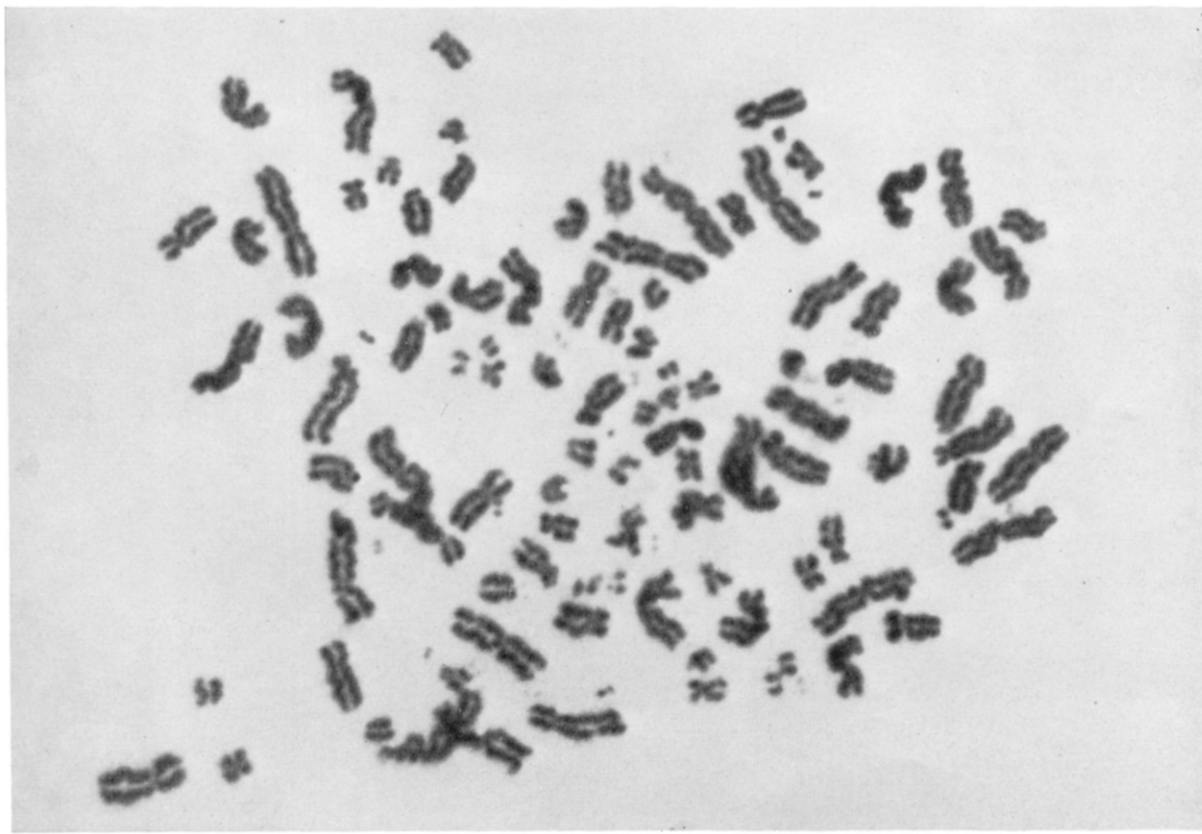


Fig. 10. *GOR Cell with multiple chromatid aberrations* (×2250).

long acrocentric chromosome and two chromosomes similar in proportions to a B chromosome but longer overall (Fig. 7). The number 10 chromosome marker was not seen in any cell.

Previous observations on this cell line were carried out by Miles and O'Neill [1] in January 1966 when they found it to have a normal male chromosome constitution. Again, therefore, there has been a major evolution of the karyotype of this line *in vitro*.

GOR

Chromosome count distribution

	Total	<45	45	46	47	48	49	50
Type A	42	2	8	28	4	—	—	—
Type B	58	—	2	1	4	9	40	2
Total	100	2	10	29	8	9	40	2

There was a clearly bimodal count distribution indicating two clearly distinct stem lines. Type A were diploid or near diploid. The chromosome complement of a small number of these was that of a normal male and the remainder varied from this by only relatively minor modifications. Ten of these 42 cells had the characteristic near terminal constriction in the long arms of one chromosome number 10 (Fig. 8).

The type B cells were hyperdiploid. The cells with 49 chromosomes were mostly 49,XY 2+4−C+D−16+E+G+ and the remainder of this cell type were clearly variants from this stem line. Of these 58 cells 32 had a near terminal isochromatid constriction in an abnormal chromosome which closely resembled a chromosome 16 (Fig. 9). The number 10 marker chromosome was not seen in any of these cells. It seems probable that this abnormal number 16 chromosome was derived from a number 10 by translocation and that this marker was in fact the same as the number 10 marker. If this is so the marker was present in 42% of the cells.

The frequency of chromatid aberrations (Fig. 10) and of dicentric chromosomes (Fig. 8) was high suggesting that the karyotype of this cell line is relatively unstable.

Cells with a polyploid chromosome constitution were selected for further examination. A number of these had constrictions in either two number 10 chromosomes or two number 16 chromosomes. It was also noted that the majority of polyploids contained dicentric chromosomes.

Cytogenetic observations on this line have not previously been reported.

DISCUSSION

Cytogenetic studies have previously been carried out on twelve different cell lines established from Burkitt tumours, EB1, EB2, EB3, EB4, RAJI, OGUN, KUDI, SL1, JIJOYE, AL2, AL1, and B35M [1, 2, 6–11]. SL1 and AL1 are however independently derived from the same tumour. The number 10 marker has been found in four of these lines EB1, EB2, EB3 and JIJOYE, but such a marker is not always easily detected especially if it is present in only a small proportion of the cells. It has been specifically searched for in nine of the twelve lines [1, 2, 8].

In the present series there was evidence for the presence of the chromosome marker in three of the seven cell lines examined. The finding of the marker in EB3 confirms previous reports [1, 2]. In EB2, the marker was found in only two cells out of one hundred, but since it has previously been reported in this cell line [1] this observation confirms its presence. In GOR, the cytogenetics of which has not previously been reported, a constriction in a number 10 chromosome was present in 10% of the cells but a similar constriction was also identified in an abnormal chromosome, presumed to have originated from a number 10 chromosome involved in a translocation, in a further 32% of the cells. In the other three lines in which a definite decision could be made, EB1 and EB1(H), EB4 and RAJI, there was no evidence of the marker chromosome although it had previously been reported to be present in EB1. In the last line, EB5, the preparations were not considered good enough on any occasion to give a firm decision.

This marker has now therefore been reported in five out of the fourteen cell lines that have been examined but it has not been found on every occasion on which these five lines have been studied cytogenetically. Kohn *et al.* [2] have found that there appeared to be an increase in the percentage of cells with the marker, with increasing time in culture. This cannot however be generally true since in the present study the marker was not seen in a line in which it had previously been found and further, the line GOR in which it was clearly identified is the line which has been most recently established in culture. In EB1 it is possible that the marker chromosome was lost during the process of change from pseudodiploid to hypotetraploid but this is not a necessary consequence of such a change since the marker was seen in hypotetraploid cells in EB3 and GOR. Alternatively it can be supposed that the marker is still present but not

observed for physiological or possibly even for technical reasons.

There are also reports of a similar marker in cell lines established from leukaemic cells [1, 12, 13]. Miles *et al.* [13] report it to be present in thirteen out of fourteen lines of cells examined. One of these lines is of non-leukaemic origin.

A herpes-like virus [14] has been detected in many of these lines whether of Burkitt tumour, leukaemic or normal origin. It is an attractive hypothesis that the specific chromosome marker is in some way associated with the presence of a virus. Henle *et al.* have carried out a series of experiments with a culture system of normal lymphocytes grown together with X-irradiated cells obtained from cell lines established from Burkitt's lymphoma (JIJOYE) and report the induction of the number 10 chromosome marker in the five lines of cells which, using the sex chromosomes as a marker, are almost certainly derived from the normal lymphocytes. Similar experiments using a cell line (RAJI) which had not been shown to contain the herpes-type virus failed to induce either cell growth or the chromosome marker.

It is now generally accepted that viruses can cause chromosome damage, but most reports show that the chromosome damage is not at a specific location on the chromosomes. There are however, a few reports suggesting that the breakage of chromosomes by viruses may not be random [16–18]. Wald *et al.* [19] describe a specific chromosome marker in a radiation induced leukaemia of the mouse and claim further that this leukaemia with its specific marker may be transferable by cell free passage.

In addition to the number 10 marker seen in Burkitt tumour cells, others have been described on several occasions in cell lines and in direct preparations from solid tumours. A chromosome similar in shape to a number 2 but larger than a number 1 has been reported in three direct preparations of solid tumours [5], in OGUN, SL1 and JIJOYE [1] and in the present study in EB1 and EB4. A long acrocentric was seen in five cases of primary tumours [5] in two independently established lines, AL1 and SL1, from the same tumour [1, 20], and in the present study in EB4 and RAJI. Markers like a long B chromosome were seen in the present study in EB1(H), EB4 and RAJI. Since human chromosomes are so varied in morphology there are only a limited number of abnormal chromosome types which would stand out as different and it may be that those described here fall into these categories, and are only a small selection from a very much larger, but unrecognised number of abnormal chromosomes present. For this reason we do not wish to put too much weight on this point but further observations on these and other cell lines may help to establish whether or not these aberrations also represent specific chromosome damage.

The present observations also show clearly that these lines of cells are continuing to evolve in culture. Direct preparations from the solid tumours have shown that the tumour cells *in vivo* are basically diploid although chromosome abnormalities might be present [5, 6, 9]. During the early culture period it has been shown that these cells have a propensity to remain diploid, however, chromosome preparations made directly from a tumour followed by observations on established cells have only been made on two occasions. In one instance the cytogenetic observations were similar in both the direct and the cultured preparations [6] whereas in the other the majority of the cells in the direct preparation were abnormal while those from the established line were normal in chromosome constitution [9].

The present observations show that the chromosome constitution of some of the cell lines differ considerably from those in the previous reports. Not only is the marker chromosome absent in one line but also five of the seven lines examined are near tetraploid with no evidence of diploid lines. Four of these are known to have been diploid at an earlier stage in their history. These changes may not only be a reflection of the length of time the cells have been in culture but may also depend on the varying conditions of culture in different laboratories. It is possible that other features of these cells which are not as readily detected as chromosome changes may also be altered during this evolution in culture.

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RESUME

Les chromosomes de sept lignées cellulaires dérivées de tumeurs de Burkitt ont été examinés. Le présence d'un chromosome marqueur spécifique a été confirmée dans deux de ces lignées. Un marqueur semblable a été observé dans une lignée qui n'avait pas été étudiée précédemment. Le marqueur n'a pas été mis en évidence dans une lignée dans laquelle il avait été observé précédemment.

SUMMARY

The chromosomes of seven lines of cells derived from Burkitt's tumour have been examined. The presence of a specific marker chromosome has been confirmed in two of these lines. A similar marker has been found in one line not previously examined. The marker was not found in one line in which its presence had been previously reported.

ZUSAMMENFASSUNG

Die Chromosomen von sieben, von Burkitt's Tumoren abstammenden Zell-Linien wurden bestimmt. Das Vorhandensein eines spezifischen Marker—Chromosoms konnte bei zwei dieser Linien nachgewiesen werden. Ein ähnliches Marker-Chromosom wurde bei früheren Studien in einer Linie festgestellt. In einer andern Linie, bei der zuvor von der Existenz eines Markerchromosoms berichtet worden war, konnte keines gefunden werden.

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