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Essai Clinique du Phénol bis (2-Chloroéthyl) Carbamate d'Oestradiol dans le Cancer Mammaire en Phase Avancée

GROUPE EUROPEEN DU CANCER DU SEIN*

INTRODUCTION

UNE PROPRIETE d'accumulation de l'oestradiol semblable à celle des organes qui sont les cibles normales de cette hormone a été mise en évidence dans certaines tumeurs mammaires du rat, induites par le 9,10-diméthyl-1,2-benzanthracène, et régressant après l'ovariectomie [1], ainsi que dans les tumeurs malignes du sein chez la femme [2]. La présence, dans le néoplasme, de "récepteurs" d'oestradiol pourrait permettre la localisation préférentielle, dans la tumeur, d'une autre molécule, par exemple un agent cytotoxique, préalablement liée à l'hormone. Grâce à cette captation privilégiée, la concentration du néoplasme en agent cytotoxique pourrait être supérieure à celle des tissus normaux les plus sensibles à son action, ce qui entraînerait une diminution ou même l'abolition des manifestations toxiques de ce type de traitement.

Comme des rémissions objectives de cancers mammaires en phase avancée peuvent être induites chez la femme par les agents alkylants [3], la liaison chimique de la nor-moutarde azotée à l'oestradiol, par le lien d'une fonction carbamate, a été réalisée afin de pourvoir l'agent alkylant d'un véhicule capable de le diriger vers les métastases. Ce composé, nommé LEO 299†, et dont la formule est indiquée à la Fig. 1, a un effet oestrogénique 24 fois inférieur à celui de l'oestradiol sur le frottis vaginal et le poids de l'utérus de la souris impubère.

Une étude de ce composé de type I, selon la classification du GECA [4], a été réalisée dans le cancer mammaire en phase avancée de la femme.

METHODES

Les critères utilisés pour la sélection des malades et l'évaluation des résultats sont ceux

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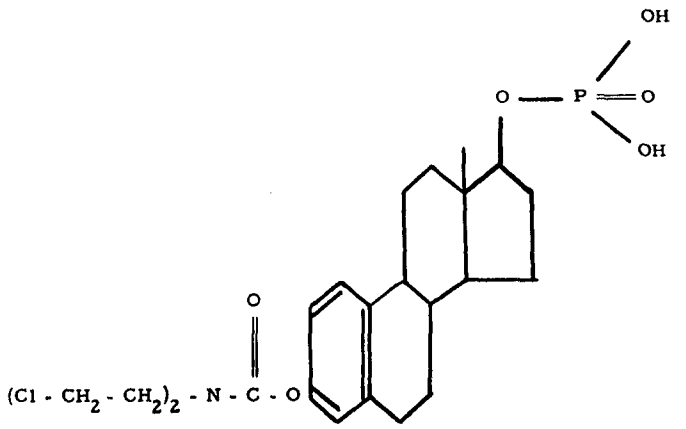

Fig. 1. *Formule du LEO* 299. *Phénol bis* (2-*Chloroéthyl*) *Carbamate d'Oestradiol.*

décrits dans le protocole des essais cliniques du Groupe [4, 5], à l'exception des points suivants:

(a) sont admises, dans la présente étude, les malades traitées antérieurement par des hormones, à condition que celles-ci n'aient pas été administrées sous une forme à action prolongée, que le traitement précédent soit terminé depuis 3 mois, et que l'affection néoplasique ne soit pas actuellement en rémission;

(b) l'évolutivité des lésions, avant le début du traitement par le LEO 299, n'est pas un critère obligatoire; cependant, une période d'observation de 3 semaines au moins doit précéder le début du traitement, afin de s'assurer qu'il n'y a pas de rémission objective des lésions, due à un éventuel traitement antérieur, ou à son arrêt.

Le LEO 299 est administré en solution glucosée, à la concentration de 1%, par voie intraveineuse, à la dose quotidienne de 160 mg. L'administration du composé n'est pas poursuivie au-delà de 6 semaines, sauf si une rémission objective est observée.

RESULTATS

Le Tableau 1 montre le nombre de malades traitées, leur répartition, et le nombre de rémissions objectives observées, sur un total de 34 malades traitées et évaluées. Deux rémissions objectives ont été notées lors de l'administration de LEO 299, chez des malades postménopausiques depuis plus de 5 ans, et porteuses de lésions cutanées. Dans ce même talbeau, les chiffres entre parenthèses indiquent le nombre de malades décédées pendant l'administration de LEO 299. Ces 7 cas décédés avaient une entreprise néoplasique des viscères.

Autres effets

Aucun effet sur le taux des globules blancs et des globules rouges n'a été noté; dans un cas, une chute du taux des plaquettes de 256.000 à 68.000 par mm^3 a été observée, sans que les radiographies du squelette ne montrent de lésions.

Chez 6 patientes, le dosage des 17 hydroxycorticoïdes plasmatiques a été pratiqué, avant et pendant le traitement, en utilisant la méthode de De Moor [6]. Comme le montre le Tableau 2, une élévation significative des valeurs a été observée au cours de l'administration de LEO 299. Bien que cette modification puisse être due à l'état de stress, il est probable qu'elle produite par les propriétés oestrogéniques de la molécule utilisée [7].

Tableau 1. *Groupe Européen du Cancer du Sein Etude No. V. Répartition des malades et résultats*

Lésion dominante	Nombre d'années par rapport à la ménopause				
	Moins de 1	1–5	6–10	Plus de 10	Total
Locale	0/1	0/2	1/2	1/4	2/9
Osseuse	—	—	0/2	0/5	0/7
Viscérale	0/2(1)	0/1(1)	0/2(2)	0/13(3)	0/18(7)
Total	0/3(1)	0/3(1)	1/6(2)	1/22(3)	2/34(7)

Composé administré: LEO 299, 160 milligrammes par jour, par voie intraveineuse.
Régressions objectives/nombre de malades.
Les chiffres entre parenthèses indiquent le nombre de malades décédées en cours du traitement.

Tableau 2. Concentration des 17-hydroxycorticoïdes plasmatiques chez 6 malades, avant et pendant l'administration de LEO 299

Catégorie des lésions métastatiques	Avant (γ/100 ml)	Pendant (γ/100 ml)
Locale	10	40
Locale	17	58
Viscérale	16	87
Viscérale	24	61
Viscérale	34	50
Viscérale	36	50

Les dosages ont été pratiqués selon la méthode de De Moor [6].
Les valeurs normales sont de 21, g γ±4, 7γ

Chez 8 malades, les frottis vaginaux ont été étudiés. Trois de ces frottis montraient une image d'imprégnation oestrogénique nette; trois frottis montraient une image de stimulation hormonale non spécifique; deux frottis ne permettraient pas l'évaluation (présence de champignons dans l'un, de trichomonas dans l'autre).

Des études de l'excrétion urinaire des oestrogènes et des gonadotrophines ont été pratiquées; elles feront l'objet de publications séparées par certains membres du Groupe.

Des thrombophlébites superficielles sont apparues, aux endroits d'injection, chez toutes les malades, après environ 15 jours de traitement, ce qui a nécessité l'utilisation de l'héparine et le placement de cathéters intraveineux à demeure, afin de pouvoir poursuivre l'administration du LEO 299.

DISCUSSION

Les quantités respectives d'oestrogène et d'agent alkylant, contenues dans les doses administrées de LEO 299, sont au moins égales aux doses de chacun de ces agents utilisé isolement dans le traitement du cancer du sein généralisé. L'absence d'effet hématologique indique que tout plus de très petites quantités d'agent alkylant ont pénétré dans la moelle osseuse. Ceci peut être dû, soit à la scission d'un très petit pourcentage de molécules de LEO 299, soit à l'apparition, lors de cette scission d'un dérivé non alkylant de la nor-moutarde azotée, soit à une accumulation intratumorale des molécules de LEO 299, avec libération uniquement locale de l'agent alkylant. Cependant, dans ce dernier cas, un nombre plus élevé de réponses favorables aurait été vraisemblablement observé. En fait, il n'y a aucune preuve d'une scission *in vivo* des molécules injectées. L'existence d'effet oestrogénique chez certaines malades, ne peut en être considérée comme le témoin, la molécule entière ayant un pouvoir oestrogénique net sur les organes génitaux de la souris impubère, bien que beaucoup plus faible que celui de l'oestradiol.

Le nombre des rémissions objectives induites dans cet essai paraît aussi très faible, si l'on considère que 28 des 34 malades étaient, depuis plus de 5 ans, postménopausiques, et constituaient dès lors un groupe où la sensibilité aux oestrogènes est la plus grande [8]. Il est donc possible que la molécule injectée dans l'organisme ne s'y scinde pas, et que la présence du carbamate de bis (2-chloroéthyl) l'empêche de jouer son rôle d'oestrogène au niveau de la tumeur.

Sur le plan pratique du traitement du cancer mammaire en phase avancée, le LEO 299 ne paraît donc pas utile. Cependant, les propriétés d'accumulation de l'oestradiol des tumeurs mammaires justifient la synthèse et l'étude de nouveaux composés, basés sur le même principe que le LEO 299.

RESUME

Trente-quatre malades atteintes d'un cancer mammaire en phase avancée ont reçu une substance obtenue par couplage de la nor-moutarde azotée, au phosphate d'oestradiol. La dose quotidienne administrée, par voie intraveineuse, était de 160 *mg.*

Deux rémissions ont été obtenues.

Certaines hypothèses sont formulées pour expliquer ce résultat, ainsi que certains autres effets observés pendant l'administration de ce composé.

SUMMARY

A compound resulting from the chemical binding of nor-nitrogen mustard to estradiol phosphate was given to 34 *patients with advanced breast cancer. A daily dose of* 160 *mg was given using the intravenous route.*

Two objective regressions were observed.

The small number of favorable responses and some biological effects noted during the administration of the compound are discussed with regard to the property of accumulating estradiol, attributed to mammary tumors.

ZUSAMMENFASSUNG

Eine Substanz, in der Nor-N-Lost an Oestradiolphosphat chemisch gebunden ist, wurde 34 *Patientinnen mit Brustkrebs gegeben. Die tägliche Dosis betrug* 160 *mg und wurde intravenös appliziert. Zwei objektive Rückbildungen wurden beobachtet.*

Die geringe Erfolgsziffer und einige unter der Substanzeinwirkung auftretende biologische Effekte werden diskutiert, auch im Hinblick auf die Auswirkungen der Akkumulation von Oestradiol, die Mammatumoren zugeschrieben wird.

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Murine Ascites Tumour Cells and Lymphocytes

Effect of Lysolecithin, Organic Mercurials and an Anti-Tumoral Agent on the Cell Membrane Permeability

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INTRODUCTION

CHEMOTHERAPEUTIC approaches to malignant disease have relied mainly on exploiting abnormalities in DNA production and cell replication. However, many of the distinctive properties of neoplasms, such as local invasion, metastasis and loss of contact inhibition, may depend on changes in the tumour cell membrane [1]. It is therefore important to examine the effect of surface active drugs on tumour cells, both from the theoretical aspect of clarifying membrane differences and from the therapeutic aspect of exploiting the differences in drug sensitivities.

Lysolecithin (LL) has been studied as a surface active haemolytic agent [2, 3] and has been implicated in many biological mechanisms, including immune haemolysis [4], hypersensitivity reactions [5, 6], inflammation [7, 8], control of oxidative phosphorylation [9, 10] and the phagocytic activity of polymorphonuclear leucocytes [11].

Fischer [4] using a cell–antibody–complement system, and assuming from previous work [12] that LL was produced in such a system, found that the respiratory rate of macro- and microphages was increased, while that of ascites tumour cells was reduced. Butterworth and Cater [13], using pure LL and different techniques, found that the respiratory rates of all the cell types studied were decreased. These authors found that ascites tumour cells were more sensitive to LL in terms of surface area than lymphocytes or phagocytes. They concluded that LL had initially a surface effect, followed by an intracellular effect on oxygen metabolism, possibly by derangement of mitochondrial lipids.

In recent years the Freiburg group of workers (Fischer *et al.*) have extensively studied the metabolic pathways of LL in erythrocytes and macrophages. It appears from their studies that generation of LL and its eventual metabolism might play an important role in the regulation of cell activity and permeability [14]. The aim of the present work was to examine further the initial surface effect of LL on BP8 ascites tumour cells and lymphocytes by studying changes in cell membrane permeability to potassium and phase-contrast microscopic appearance as criteria of cell damage.

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MATERIAL AND METHODS

(a) *Solutions*

Cell suspension and drugs were prepared in 0·145 M Analar NaCl, made 10^{-4} M with respect to $NaHCO_3$: pH 7·2±0·2 (standard saline) [15]. The water used for preparing these saline solutions was distilled twice. The solutions contained no detectable potassium over the range studied.

Lysolecithin emulsions were prepared by grinding it with saline in an agate motar [16], followed by treatment for 10 min in a Headland H60 ultrasonicator. In this instrument vibrations pass through the containing vessel and there is no direct contact of metal with the suspension.

Calagualine (kindly supplied by Dr. Horvath) dissolved readily in saline. Parachloromercuribenzoate, sodium salt (PCMBNa) and parachloromercuribenzenesulphonate, sodium salt (PCMBS) supplied by Koch Light Laboratories were used. The two chemicals dissolved with difficulty in saline, and were subjected to ultrasonic vibrations for 10 min.

(b) *Cell suspensions*

(i) *BP8 ascites tumour cells.* This sarcoma, initially induced by subcutaneous injection of benzpyrene into C3H/Bi mice and subsequently converted to the ascites form [17], was used because of its high degree of compatibility with C3H mice: immunological effects at the cell surface were therefore minimal. 1×10^5 cells, suspended in Hanks' solution containing heparin 3 mg% w/v, were injected i.p. into C3H mice. The mice were killed 7 to 13 days later by cervical dislocation, and the ascitic fluid was withdrawn into saline. This suspension was centrifuged at 70**g**, the supernatant sucked off and the cells resuspended. At this **g** value erythrocytes remained in the supernatant and were removed. The process was repeated 2–3 times, to minimise contamination with potassium from the ascitic fluid. Examination of the cells by phase contrast microscopy showed that about 90% were intact. Cell counts of at least 500 cells were made and the suspension was diluted to a final concentration of $1\cdot6\times10^7$ cells/ml.

(ii) *Lymphocytes.* For each experiment 6 Tuck No. 1 TT-tested mice were killed. Their inguinal, axillary, subscapular, thymic and para-aortic lymph nodes were dissected free of fat, washed free of blood and minced in saline in a plastic petri dish. The resulting suspension was strained through potassium free muslin to remove lymph node debris. Fat cells were removed by differential centrifugation at 170**g**, but it proved impossible to remove all erythrocytes and macrophages. Contamination was less than 15%. 'Viability', as judged by phase contrast microscopy, was about 80%. Suspensions were diluted to $1\cdot6\times10^7$ cells/ml.

(c) *Incubation procedure*

Time t_0 within 60–90 min of killing the mice, was reckoned from the instant that 1 ml samples of the final suspension were mixed with saline and/or the drug under investigation and the final volume made up to 5 ml. The samples were incubated for 30 min at 37°C, the sample tubes being inverted every 10 min to ensure an even suspension. At the end of the incubation period the suspensions were centrifuged at 260**g** to remove most of the cells: the supernatants were centrifuged again at 770**g** to remove any debris.

(d) *Mechanical disruption of cells*

Several methods of disrupting the cells and allowing escape of all the intracelluar potassium were tested. Osmotic lysis proved ineffective. Disintegration in a MSE homogeniser at 14,000 rev/min gave variable and inconsistent results. Chemical methods were not attempted, for fear of contaminating the samples with potassium. The method that disrupted the cells most effectively and gave highest and most consistent results was treatment with ultrasonic vibrations for at least 2 hr.

(e) *Potassium ion measurement*

Potassium determinations were carried out by flame photometry using both absorption (Unicam SP90) and emission (Unicam SP900) spectrophotometers. The error in the measurements of potassium concentration was small. Fluctuations caused by the 'noise' level of these flame photometers introduced an error of ±1%: and since it was the difference between samples that was important, this caused an error of ±5% in the 'effect'.

Summary of the experimental technique

(a) C3H (male/female) mice killed for BP8 tumour cells, 7–8–13 days after implantation of the tumour: ascitic fluid removed and poured into standard saline (0·145M NaCl, made 10^{-4}M with respect to $NaHCO_3$; pH: 7·2±0·2).

(b) Tuck TT-tested (male/female) mice killed, for lymph node cells, lymph nodes excised quickly, minced with saline and strained through muslin.

Samples from each mouse centrifuged for 5 min: 70**g** for BP8 ascites tumour cells; 170**g** for lymph node cells; supernatant discarded and cells washed in saline 1:50 (vol. ratio), thrice, centrifugation following each wash. Suspensions of BP8 ascites tumour cells or lymph node cells were then pooled and the concentration of cells adjusted to $1\cdot6\times10^7$/ml.

At time t_0 1 ml aliquots of above stock cell suspension withdrawn, mixed with saline for controls or with chemical (dissolved in saline), final vol. being made up to 5 ml to be incubated at 37°C for 30 min. Preparation of the material for incubation was completed within 60–90 min of killing the animal.

t_{30} samples with drawn from the incubating material, 30 min later and centrifuged at 260**g** for 5 min to obtain supernatant which in turn centrifuged at 770**g** for 5 min to remove cell debris. Final supernatant was examined for K^+ ion content by flame photometry.

RESULTS

All the data were programmed for a digital computer (Titan).

(*a*) *Control samples*

The potassium content of the supernatants after 30 min incubation of control samples, denoted t_{30} controls, was observed to be high and variable, both for BP8 cells and for lymphocytes and amounted to 28–52% of the total potassium content of the cells. Three possible causes are suggested:

(i) inhibition of the active transport process by the lack of potassium in the external medium, allowing diffusion of potassium from the cells along the chemical gradient,

(ii) membrane damage to control cells during the preparation of the suspensions, and

(iii) failure to remove all the potassium in the original external medium.

Addition of potassium to the external medium to give a final concentration of 0·8 mEq/l caused no significant reduction in the potassium loss from control samples, after corrections had been made for added potassium. Greater amounts than this could not be added, since the high final concentration would mask the loss from the cells. However, 0·8 mEq.K^+/l is about one quarter of the concentration in extracellular fluid and might be expected to reactivate the potassium transport system. It seems likely, therefore, that factor (i) played no part in the loss from control samples.

The loss of potassium from incubated control samples during a kinetic experiment was observed to rise rapidly after about 20 min. Accurate timing in centrifuging the incubated suspensions was therefore important. The use of siliconed glassware did not reduce the loss of potassium from control samples.

Examination by phase contrast microscopy of control samples showed the following characteristics.

(i) *BP8 cells.* Ninety per cent were 'viable', with regular, round cell margins, bright cytoplasm and sluggish amoeboid movements. Nuclei were either invisible or very blurred in appearance. The remaining 10% showed varying degrees of damage, ranging from demarcation of a clear nucleus to marked changes of intracellular refraction—the cells appeared black—with jagged and irregular cell margins.

(ii) *Lymphocytes.* Fewer cells were 'viable', probably because of the method of extraction, and at least 20% showed clear nuclei or membrane damage.

(b) *Total potassium*

The values of potassium concentration obtained by the ultrasonic treatment described above were a measure of the total potassium in the suspension; that is, of the intra-cellular potassium plus any contamination from the extracellular fluid. The amount of potassium available for release by drug action on the cell

Table 1

Cells	Type of mice	No. of pooled samples	Total intracellular K^+ ions mEq/l cell water	Chemical treatment	D_{50}^{K+} Dose required to cause 50% loss of K^+ ions		D_{50}^{O} Dose required to cause 50% inhibition of oxygen uptake [13]
					(μg/10^7 cells)	(μg/$\mu^2 \times 10^{-9}$)	(μg/10^7 cells)
BP8 ascites tumour	C3H (36)*	12	95 (22)†	None, saline control (47)†			
	C3H (36)*	12		lysolecithin (88)†	11·5	1·35	64
	C3H (15)*	6		LL with PCMBSNa or PCMBNa (62)†			
Lymphocytes	Normal tuck (54)*	9	97 (13)†	None saline control (18)†			
	(54)*	9		LL (54)†	3·8	3·72	45
	(36)*	6		LL with PCMBSNa or PCMBNa (40)†			
BP8 ascites tumour	C3H (6)*			Calagualine (11)†			

*Number of mice (2–3 months old, male/female) used.
†Number of samples (in duplicate) analysed.

membrane was the amount remaining in the control suspension after incubation; that is, the total potassium value minus the t_{30} control value. This is expressed as the net potassium content available for drug action and was found to have mean values of 68·5 mEq/l cell water for BP8 cells and 97 mEq/l cell water for lymphocytes. The amount of potassium actually released by the drug was the sample value minus the t_{30} control value and, for each experiment, was expressed as a percentage of the net potassium ion content.

Reasonable estimates of the total intracellular potassium were made by subtracting from the total potassium value that of the t_0 control. In this way damage during incubation, but not that during preparation of the cell suspension, could be eliminated. The mean value of 10 experiments was 96·0 mEq/l cell water for BP8 cells: the highest value was 108 mEq/l cell water.

(c) *Lysolecithin effect: BP8 cells*

Figure 1 shows the effect of increasing doses of lysolecithin, from 3·2 to 45 μg/10^7 cells, on BP8 cells. The dose required to cause 50% loss of potassium (hereafter called the $D_{51}{}^{K+}$) was 11·5μg. LL/10^7 cells. This dose is less than a quarter of that required to cause 50% inhibition of oxygen uptake ($D_{50}{}^{O}$) [13].

Cells from 12 and 13-day tumours proved significantly more sensitive to lysolecithin in doses of 9·6 μg/10^7 cells ($P<0·01$) and 12·8 μg/10^7 cells ($P<0·0005$).

Microscopic examination of the treated cells showed changes with all doses of LL. With 9·6 μg/10^7 cells a high proportion (about 75%) remained undamaged. With increasing doses, however, more cells showed changes of intracellular refraction, appearance of clearly marked nuclei with bright vacuoles, raggedness of membranes and cell swelling. Cell populations were variable in appearance, and even at the highest dose of LL (64 μg/10^7 cells) there remained some cells apparently undamaged. Gross clumping of cells was not observed.

(d) *Lysolecithin effect: lymphocytes*

Figure 2 shows the effect of increasing doses of LL, from 1·3 to 13 μg/10^7 cells, on potassium loss from lymphocytes.

The $D_{50}{}^{K+}$ was 3·8 μg/10^7 cells.

Microscopy of treated cells showed characteristic changes. With very low doses (3 μg/10^7 cells) the cell margins became more distinct, the nuclei appeared dark and in some cases the cytoplasm showed a finely reticulated pattern. With higher doses (up to 20 μg/10^7 cells) the cells appeared very dark, in some cases with high vacuoles. Cell swelling was observed, with the cell membrane becoming ragged

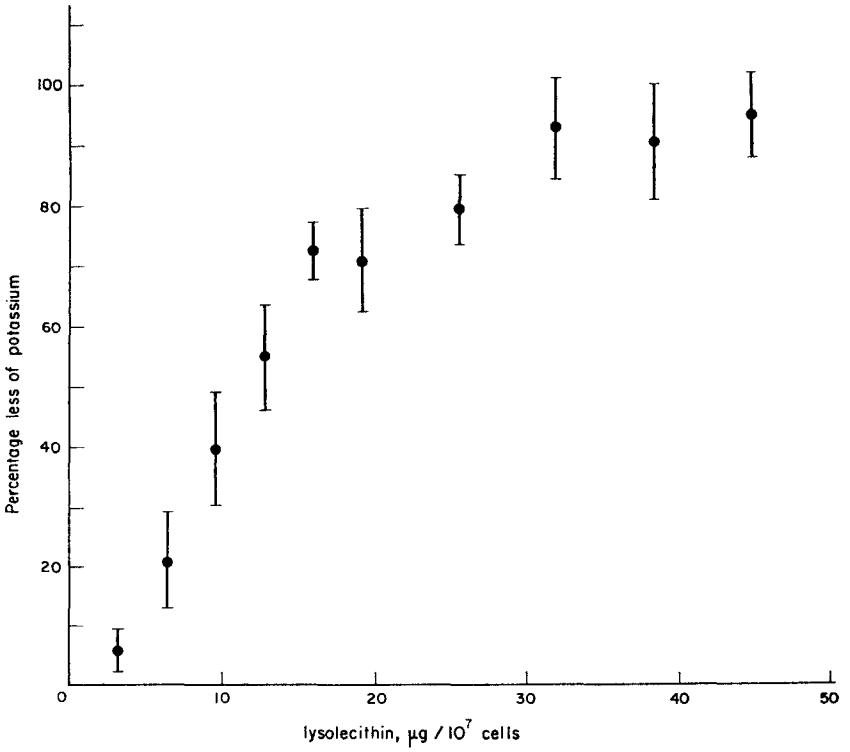

Fig. 1. Loss of potassium during ½-hr incubation from lysolecithin-treated cells. Mean and standard deviation of the mean worked out by a digital computer (Titan) are shown.

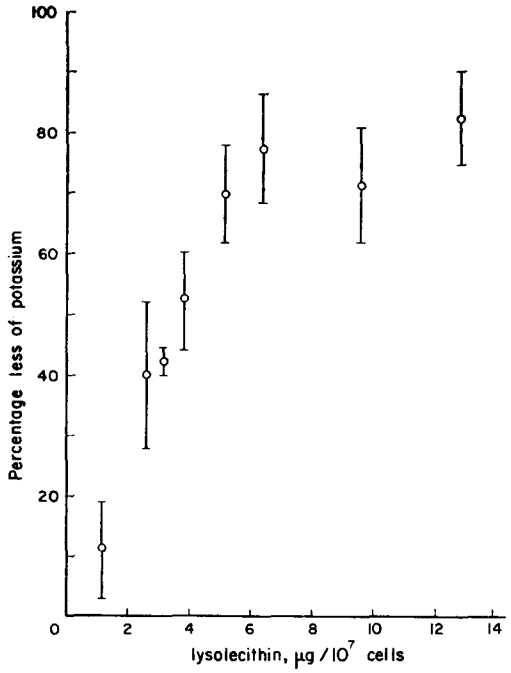

Fig. 2. Loss of potassium during ½-hr incubation from lysolecithin-treated lymphocytes.
Mean and standard deviation of the mean worked out by a digital computer (Titan) are shown.

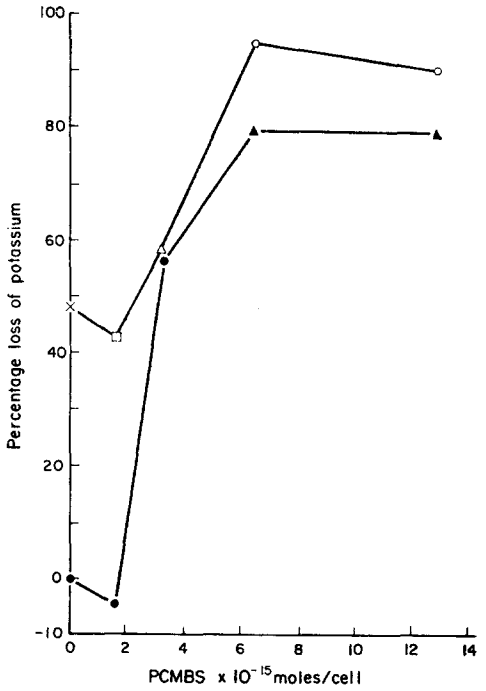

Fig. 3. Loss of potassium from BP8 cells treated with PCMBS alone (closed symbols) and with PCMBS + 9·6 µg lysolecithin/ 10^7 cells (open symbols).

Symbols represent number of observations:
×=7, □=6, △=4, ○=2.

or bulbous. At the highest concentration (20 µg/10^7 cells) much debris was seen. As in the case of BP8 cells even at these concentrations may cells appeared to be undamaged.

(e) *PCMBNa and PCMBS effects*

Munder, Ferber and Fischer [18] have demonstrated the presence of LL-destroying enzymes—an acyl transferase and a phospholipase B—in erythrocyte membranes, and have suggested that the different behaviour of cells to LL-induced lysis may depend upon the relative activities in their membrane. They have also shown that the SH-blocking reagent PCMB inhibits these enzymes, thereby enhancing the effect of LL on the cells. It seemed important to examine the effect of PCMB alone and in combination with LL on BP8 cells and lymphocytes, to discover whether the observed difference in sensitivity to LL was due to lesser activities of LL-destroying enzymes in the tumour cell membrane. The results are plotted in Figs. 3 and 4.

(*i*) *BP8* cells. Figure 3 shows the loss of potassium caused by PCMBS only (filled in symbols). The $D_{50}{}^{K+}$ is approximately $3{\cdot}0\times 10^{-15}$ moles/cell. Figure 3 also shows the effect on BP8 cells of PCMBS and LL combined. It appears that there is no enhancement of the LL effect except with high doses of PCMBS (6·4 and $12{\cdot}8\times10^{-15}$ moles/cell);

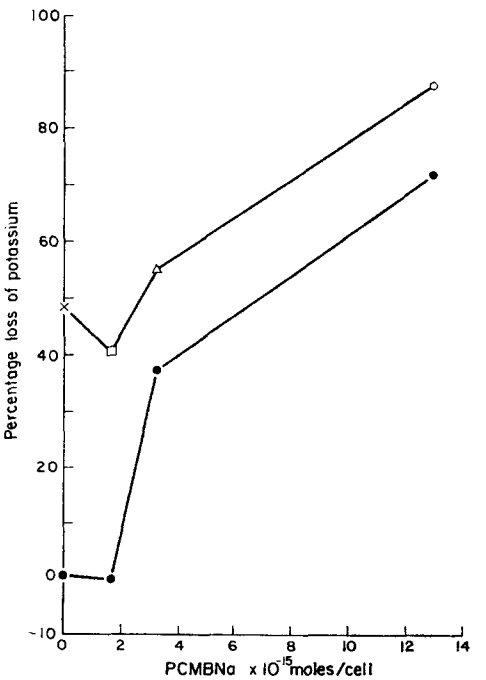

Fig. 4. Loss of potassium from BP8 cells treated with PCMBNa alone (closed symbols) and with PCMBNa+ 9·6 µg lysolecithin/10^7 cells (open symbols)

Symbols represent number of observations:
×=7, □=6, △=4, ○=2.

Table 2. Enhancement of lysolecithin-induced loss of potassium from lymphocytes by PCMBNa and PCMBS. Numbers in brackets represent numbers of observations

Dose of lysolecithin ($\mu g/10^7$ cells)	Percentage loss of potassium with Lysolecithin alone	PCMBS ($0{\cdot}64\times10^{-15}$M/cell)	PCMBNa ($0{\cdot}64\times10^{-15}$M/cell)
0	=	10·0 (8)	11·4 (8)
1·28	9·2 (6)	35·7 (7)	30·6 (9)
3·84	54·8 (7)	72·1 (7)	66·0 (2)

that is, those doses of PCMBS which themselves cause >70% loss of potassium. In these experiments, PCMBS was incubated alone with the cells for 5 min and LL was then added for a further 25 min. Figure 4 shows that similar results were obtained with PCMBNa. The D_{50}^{K+} was more than $3{\cdot}0\times10^{-15}$ moles/cell. It is interesting to note that this D_{50}^{K+} value is similar to that for PCMBS. Figure 4 also shows the results of combining LL with PCMBNa. Again, no additive loss of potassium was observed.

(ii) *Lymphocytes.* A single dose ($0{\cdot}64\times10^{-15}$ moles/cell) was used, both for PCMBS and for PCMBNa, and was found to cause 10% loss of potassium in both cases. But when combined with lysolecithin, a definite and *more* than additive enhancement of potassium loss was observed (Table 2).

(f) *Calagualine effect*

Calagualine has recently been reported to be an antitumoral agent thought to have surface active properties [19]. It significantly increased the loss of potassium from BP8 cells ($P<0{\cdot}01$) at concentrations of 0·25 and 0·5 mg/ml. Microscopic examination of treated cell showed no marked changes from control samples.

DISCUSSION

(a) *Potassium content*

The intracellular potassium content of Ehrlich ascites carcinoma cells have been measured by Ibsen and McKee [20]. These authors found a progressive increase from 78·1 mEq/l in 5 day tumours to 100·1 mEq/l in 9 day tumours. They suggested that in the younger tumours, which had a higher anabolic rate, there was insufficient allotment of energy for ion transport. Other workers, using the same tumours in different strains of mice, have obtained values of 134, 79 to 111 and 76·7 to 89·5 mEq/l [21–23]. From the present results, it appears that the potassium content of BP8 cells—a mean value of 94·6 and a maximum of 108 mEq/l of cell water—is of the same order of magnitude as the EAT cells. The results also suggest that there is a lower total intracellular potassium in older tumours. This agrees with Hempling's [21] results, but may also reflect the increased fragility of the older cell (see p. 8c) and the loss of more intracellular potassium by damage during preparation (Table 1).

(b) *Lysolecithin effect*

Bergenhem and Fahracus observed haemolysis of erythrocytes following treatment with lysolecithin [24], while later workers [25–28] found that concentrations of approximately 0·3 $\mu g/10^6$ cells were required to produce this effect. If the area of the erythrocyte is taken to be 140 μ^2 [29], this gives a value of $2{\cdot}15\times10^{-9}$ µg. lysolecithin/μ^2 cell surface area for haemolysis. Using Hughes' [30] figure of 108Å^2 for the area of lysolecithin, this is equivalent to a unimolecular film covering the erythrocyte.

Butterworth and Cater [13] found that the amount of LL required to inhibit oxygen uptake by tumour cells and leucocytes was considerably more, in terms of equivalent surface area, than that required to produce haemolysis. They suggested that two processes were involved; first, a membrane effect allowing entry of LL into the cell, and secondly, a mitochondrial effect with disruption of particles involved in oxidative phosphorylation.

The present results show that much lower doses of lysolecithin were required to cause potassium loss than to inhibit oxygen uptake. Values of D_{50}^{K+} were 11·5 $\mu g/10^7$ cells for BP8 cells, and 3·8 $\mu g/10_0$ cells for lymphocytes (as compared with D_{50}^{O} values of 64 and 45 $\mu g/10^7$ cells [13]). This study supports the suggestions outlined above: potassium release will commence after slight membrane damage, whereas inhibition of oxygen uptake will not occur until after the mitochondria have been badly disrupted.

In terms of dose of LL per cell, lymphocytes are more sensitive than BP8 cells. However, assuming that the effect is on the cell membrane, the cell surface area must be taken into account. Recent electron micro-

scopic observations on BP8 cells have confirmed these views. This means that in terms of the true surface area BP8 cells would probably be more sensitive than lymphocytes.

Using the previous figures for the radius of each cell types [13] D_{50}^{K+} values of $3 \cdot 72 \times 10^{-9}$ $\mu g/\mu^2$ for lymphocytes and $1 \cdot 35 \times 10^{-9}$ $\mu g/\mu^2$ for BP8 cells are obtained. Wilkinson and Cater have pointed out to us that the electron micrographs of BP8 cells and lymphocytes show that the BP8 cells have many microvilli on their cell surface whereas the lymphocytes usually have none (Table 1).

In other words, the BP8 cell is more than twice as sensitive to LL as the lymphocyte. The results expressed in this way can be compared with the dose of $2 \cdot 15 \times 10^{-9}$ $\mu g/\mu^2$ required for haemolysis (quoted above).

The mechanisms of LL-induced permeability changes has not yet been fully explained. LL inhibits active potassium transport and the Na^+/K^+—dependent ATPase in erythrocytes [31], while Schön and Menke [32] have demonstrated two ATPases in Ehrlich ascites tumour cell membranes, one of which is Na^+/K^+—dependent, and Hokin and Hokin [33] have shown that phospholipids may be important in the active transport process as carrier molecules. However, Hempling [21] found that in Ehrlich ascites tumour cells at equilibrium the potassium flux was $0 \cdot 8$M$/10^7$ cells/hr. In the present experiments on BP8 cells, the comparable figure for the loss of potassium was 0·003 mg/100 ml in half an hour, assuming that BP8 cells are similar to Ehrlich ascites cells and that active transport is completely inhibited. This loss is very small compared with the values that were observed (for instance 0·35 mg/100 ml for total potassium). Moreover, it has already been noted that active transport was inhibited anyway by the lack of potassium in the external medium. Therefore, the percentage of potassium loss caused by inhibition of active processes would appear to be small and constant.

Turning to passive diffusion, several possible mechanisms for the action of lysolecithin on membranes have been invoked. Collier's [34] experiments on erythrocytes suggested that lysolecithin–cholesterol complexes were formed. Saunders [35] showed that LL changed the micellar shape of lecithin from discs to spheres, and Klibansky and de Vries [3] suggested that this might account for the sphering and subsequent lysis of erythrocytes. The situation however, is probably not as simple as this, and would not account for the observed differences in the sensitivity of BP8 cells and lymphocytes.

There is little evidence to suggest that the membrane structure is precisely the same in all cells. Thus many workers (e.g. Ashworth and Green [36]) have pointed out differences in the qualitative and quantitative composition of cell membranes. Gray [37] has shown that tumours have a less specific organisation of fatty acids and a high proportion of breakdown products, and has suggested that this may represent a tendency to general breakdown in tumour lipids, perhaps by release of enzymes. Also cell membranes are not in a static state. There is a continuous breakdown and resynthesis of components, including lipids, and the rates of these processes must be considered as well as the quantities involved. For instance, Elsbach, Van den Berg, Van den Bosch and Van Deenen [38] have isolated a system of enzymes from rabbit polymorphonuclear leucocytes involved in the cycle lecithin–lysolecithin glycerylphosphoryl–choline. They have suggested that these enzymes may play a part in the breakdown and resynthesis of the cell membrane, and could therefore be important in phagocytic activity. Hence the observed increased sensitivity of tumour cells to LL may depend not only on static differences in the cell membrane, but also on different rates of metabolism of the added LL. Butterworth and Cater [13] noted that the rate of oxygen uptake after treatment was decreased but constant. Observations of phase contrast appearance and of electron microscopic appearance [39] have demonstrated great variability of cells after treatment. These findings suggest that LL is bound to the cells, and is then not available to affect other cells. This binding could involve LL destruction.

To test these ideas, we made use of the observation of Munder, Ferber and Fischer [18] that the sulphydryl-blocking reagent PCMB inhibits the enzymes responsible for LL destruction in the erythrocyte membrane. Our preliminary experiments have shown that PCMB enhances the effect of lysolecithin on lymphocytes (Table 2) but not BP8 cells (Figs. 3 and 4). This would suggest that LL is normally broken down more rapidly by lymphocytes than by BP8 cells: that is, that BP8 cells appear to have smaller amounts of lysolecithin-destroying enzymes. This may be an oversimplification, since it does not take into account the property of PCMB to alter permeability in its own right. This property could possibly be due to inhibition of destruction of endogenous LL. More likely,

however, the effect is caused by alteration in lipoprotein by masking of protein sulphydryl groups, as suggested by Robinson [40]. Whatever the mechanism, it is interesting to note that PCMBSNa and PCMBNa have equal effects in a relatively short time (Fig. 4), implying that the affected part is on the external surface of the cell membrane. Since Sutherland, Rothstein and Weed [41] have shown for erythrocytes that PCMBNa diffuses through the cell membrane and PCMBS enters the cells only very slowly [41, 42], the -SH groups involved in K^+ permeability are principally on the outside of the cell.

Finally, it must be reiterated that *in vitro* studies are themselves an oversimplification of *in vivo* conditions. The increased sensitivity of 12 and 13 day tumours, both in terms of potassium loss (this paper) and oxygen uptake [13], may represent changes *in vivo* associated with agglutination, haemorrhage and incipient death. Ponder and Ponder [43] have postulated the presence *in vivo* of a lysolecithin inhibitor, more strongly bound to normal tissues than to tumours. This may be equivalent to the enzymes observed *in vitro*. Changes in vascular permeability, recently investigated by Cater and Taylor [44], may complicate the issue. These *in vitro* studies, however, are useful in elucidating the mechanism of LL action of the cell membrane, and further work by one of us is being reported elsewhere [45, 46].

Preliminary data on the effect of calagualine on the permeability of the tumour cell membrane is interesting and further investigations will be reported.

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RESUME

On a étudié la perte de potassium des lymphocytes murins, ainsi que les cellules tumorales d'ascite BP8. La contenance totale en potassium était respectivement de l'ordre de 95 *et* 97 *mEq/l de cellules. La lysolécithine a provoqué un relâchement de potassium dans les deux types de cellules. La dose nécessaire pour provoquer* 50% *de perte* (D_{50}^{K+}) *était* 11,5 $\mu g/10^7$ *cellules pour les cellules BP8* ($1{,}35 \times 10^{-9} \mu g/\mu^2$), *et* 3,8 $\mu g/10^7$ *cellules pour les lymphocytes* ($3{,}72 \times 10^{-9} \mu g/\mu^2$). *Au point de vue de la* superficie, *les cellules BP8 étaient plus sensibles à lysolécithine que les lymphocytes. Dans quelques experiences, le PCMBS (acide para-chloro-mercuro-benzène sulphonique) et le PCMBNa (benzoate para-chloro-mercuro de sodium) intensifiaient l'effet de la lysolécithine sur les lymphocytes, mais ils n'avaient aucune action sur les cellules BP8. La calagualine, qui est un agent anti-tumoral, augmentait considérablement la perte en potassium des cellules BP8.*

SUMMARY

The loss of potassium from murine lymphocytes and BP8 ascites tumour cells has been studied. The total potassium content was of the order of 95 *and* 97 *mEq/l cells respectively. Lysolecithin caused a release of potassium from both types of cell. The dose required to cause* 50% *loss* (D_{50}^{K+}) *was* 11·5 $\mu g/10^7$ *cells* ($1{\cdot}35 \times 10^{-9}$ $\mu g/\mu^2$) *for BP8 ascites tumour cells and* 3·8 $\mu g/10^7$ *cells* ($3{\cdot}72 \times 10^{-9} \mu g/\mu^2$) *for lymphocytes. In terms of* surface area, *therefore, the BP8 cells were more sensitive to lysolecithin than the lymphocytes. PCMBS and PCMBNa in a few experiments enhanced the effect of lysolecithin on lymphocytes, but not on BP8 cells. Calagualine, an anti-tumoral agent, significantly increased the potassium loss from BP8 cells.*

ZUSAMMENFASSUNG

In der vorliegenden Arbeit wurde der Kalium-Verlust von Mäuse-Lymphozyten und BP8-Aszites tumor-Zellen untersucht. Der Gesamkaliumgehalt war 95 *m-equiv/l Lymphozyten und* 97 *m-equiv/l Tumorzellen. Lysolezithin hewirkte eine Freisetzung von Kalium aus beiden Zellarten. Die Dosis welche zu einem fünfzig-prozentigen*

Kaliumverlust führte (K_{50}^{K+}) *war* 11,5 μg/10^7 *Zellen im Falle der BP8-Zellen* (1,35 × 10^{-9} μg/μ²) *und* 3,8 μg/10^7 *Zellen für Lymphozyten* (3,72 × 10^{-9} μg/μ²). *Wurde dagegen die Gesamtzelloberfläche in Rechnung gesetzt, dann waren die BP8-Zellen empfindliche gegenüber Lysolezithin. In einigen Versuchen führten PCMBS und PCMBNa zu einer Verstärkung der Lysolezithin—Wirkung auf die Lymphozyten, jedoch nicht auf die BP8-Zellen. Der Antitumor-Wirkstoff Calagualine bewirkte eine signifikante Zunahme des Kalium-Verlustes von BP8-Zellen.*

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Chemotherapieversuche an der Virusinduzierten Rauscher-Leukämie

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EINLEITUNG

NACH der Inokulation von Mäusen geeigneter Stämme mit dem Rauscher-Virus [1] sterben die Tiere innerhalb von 4–7 Wochen an einem durch Splenomegalie, Thrombopenie und Ausschwemmung kernhaltiger Zellen gekennzeichneten Krankheitsbild [2, 3]. In einer vorausgehenden Arbeit haben wir die Morphogenese der Rauscher-Leukämie dargestellt [4]. Es handelt sich um ein recht komplexes Krankheitsbild, das in seinen Endstadien als Erythroleukämie imponiert. Mehr als 2 Wochen nach der Infektion treten im peripheren Blut massenhaft Erythroblasten auf. Ausserdem werden vermehrt kernhaltige Zellen gefunden, die keine Erythroblasten sind und von Hopkins und Siegel [5] zum Teil als 'Virozyten' bezeichnet wurden. Man findet sie auch bei Mäusen, die nach der Infektion mit dem Virus keine Leukämie entwickeln [6]. Die Vergrösserung der Milz ist auf eine starke, sofort nach der Infektion beginnende Proliferation erythropoetischer Zellnester zurückzuführen, deren Reifung gestört ist [7, 8]. Viele Beobachtungen sprechen für eine neoplastische Natur dieser Zellproliferation [8, 4]. Eine reaktive Erythroblastenwucherung als Folge einer hämolytischen Anämie [3] kann das Krankheitsbild nicht ausreichend erklären. Lymphatische Leukämien — ursprünglich von Rauscher selbst beobachtet — sind nach Verwendung neuerer Viruschargen nie mehr aufgetreten [9].

Über Chemotherapieversuche mit der Rauscher-Leukämie haben Chirigos und Mitarb. [10, 11] berichtet. Sie sahen Verlängerungen der mittleren Überlebenszeit auf etwa 200% mit 6-Mercaptopurin, 5-Fluorouracil und Triäthylenmelamin. Behandelt wurde jeweils in einer einmaligen Serie an 5 aufeinanderfolgenden Tagen. Ziel unserer Versuche war es zunächst, die Brauchbarkeit der Rauscher-Leukämie als Testmodell mit weiteren Substanzen zu prüfen. Wir haben dabei regelmässig 3-mal behandelt und dann neben der Verlängerung der Überlebenszeit die Ausschwemmung kernhaltiger Zellen bzw. deren Verzögerung durch Chemotherapeutika zur Beurteilung mit herangezogen. In einer zweiten Versuchsserie unterzogen wir die Tiere wiederholten Behandlungen. Der Zeitpunkt der Therapie richtet sich dabei nach der Zellzahl im peripheren Blut und dem Allgemeinzustand der Tiere. Diese 'klinisch' orientierte Behandlungsverfahren gestattet es, die Leukämie-Entwicklung und evtl. auftretende Remissionen an Einzeltieren über einen längeren Zeitraum zu verfolgen.

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MATERIAL UND METHODE

17–19g schwere BALB/c-Inzuchtmäuse beiderlei Geschlechts (Züchter: C. W. Friis, Dänemark) erhielten das Rauscher-Virus in 0,2 ml eines zellfreien Filtrates leukämischer Milzen intraperitoneal. Zehn bzw. 11 Tage nach der Infektion wurden die Tiere in gleichartige Gruppen von 15–20 Tieren eingeteilt. Die Therapie erfolgte mit folgenden Substanzen (in Klammern die Lösungsart):

Busulfan (5 Teile Äthanol, 5 Tiele Cremophor EL (BASF Ludwigshafen), 90 Teile 0.85%ige NaCl Lösung), Cactinomycin

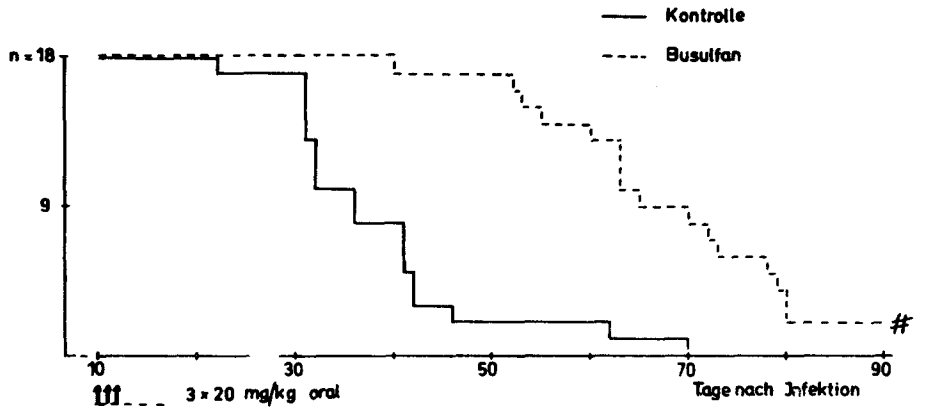

Abb. 1. Absterbekurve nach Behandlung mit Busulfan.

(Actinomycin C, 0,85%ige NaCl Lösung), Cyclophosphamid (0,85%ige NaCl Lösung), 6-Mercaptopurin (Suspension wie Busulfan), Triaziquon (0,85%ige NaCl Lösung), Vincristin (0,85%ige NaCl Lösung).

Alle Substanzen wurden jeweils an 3 aufeinanderfolgenden Tagen in konstantem Volumen von 0,01 ml/g Körpergewicht subcutan verabreicht; lediglich Busulfan wurde mit der Schlundsonde gegeben.

Die Zahl der kernhaltigen Zellen im peripheren Blut wurde in der Regel eine Woche nach Therapiebeginn bei behandelten und Kontrolltieren durch Punktion des retroorbitalen Sinus bestimmt. Die Zählung erfolgte mit dem Coulter Counter.

Versuchsgruppe A

Die Tiere wurden nur in einer einmaligen Serie behandelt und ihre Absterbefolge registriert. Alle Tiere wurden seziert. Die Angabe der Wirkung erfolgte durch die Errechnung eines Index aus den mittleren Überlebenszeiten ($\dot{U}LZ_{50}$=Anzahl der Tage nach Versuchsbeginn, wenn 50% der eingesetzten Tiere gestorben sind) der behandelten und der Kontrollgruppe. Für die Kontrollgruppe wird der Wert 100% festgesetzt; 200% bedeuten dann eine Verdoppelung der $\dot{U}LZ_{50}$.

Versuchsgruppe B

Mit Busulfan, 6-Mercaptopurin und Triaziquon haben wir ausserdem Versuche durchgeführt, bei denen die Zellzahl im peripheren Blut in wöchentlichen Abständen bestimmt wurde. Danach wurden die Tiere mit Zellzahlen über 10.000/mm³ einer erneuten Therapie interzogen. Im übrigen wurde wie bei A verfahren.

ERGEBNISSE

Versuchsgruppe A

Die Absterbekurven der Abb. 1–6 geben die optimalen Versuche für jeweils eine Substanz wieder. Die Tabelle 1 enthält die zu verschiedenen Zeiten bestimmten Zahlen der kernhaltigen Zellen im peripheren Blut am Beispiel der Behandlung mit Triaziquon. In Tabelle 2

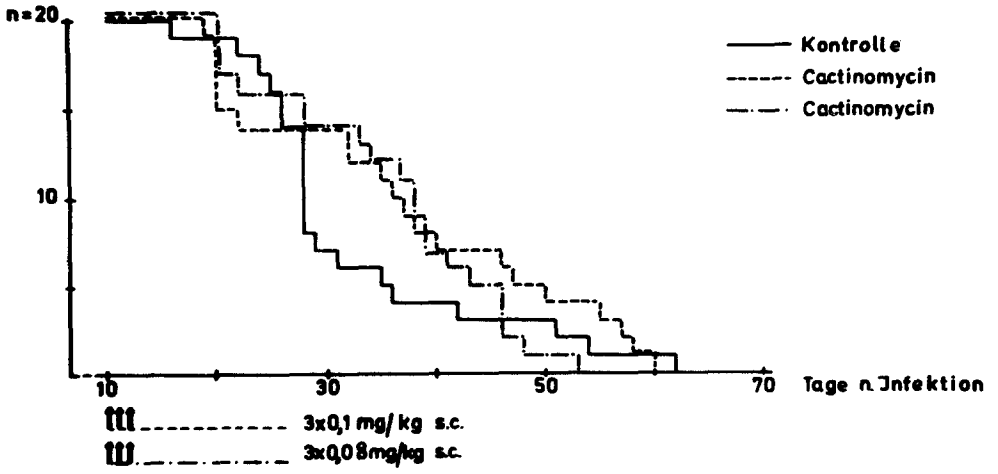

Abb. 2. Absterbekurve nach Behandlung mit Cactinomycin.

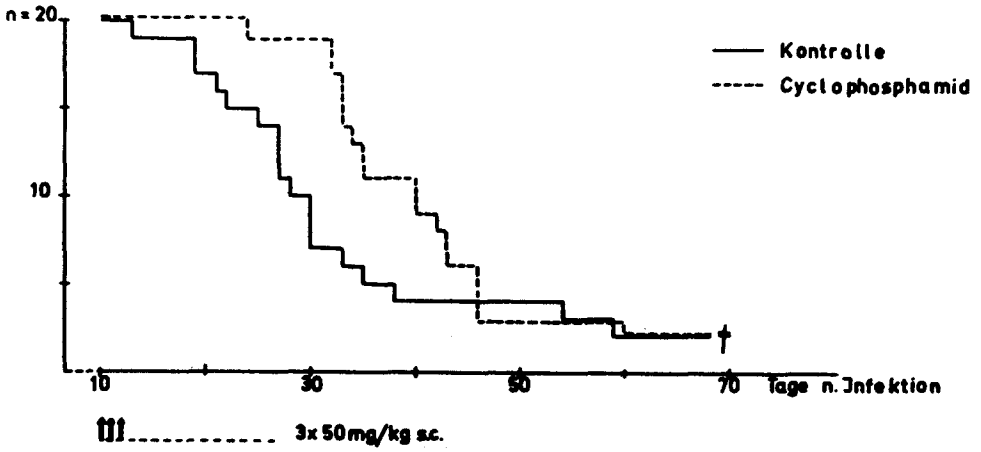

Abb. 3. Absterbekurve nach Behandlung mit Cyclophosphamid.

Tabelle 1

Tage nach Infektion	17		24	
	Kontrolle	Triaziquon	Kontrolle	Triaziquon
Tier 1	49.700	7.200	41.100	47.200
Tier 2	35.400	7.100	34.800	41.300
Tier 3	33.400	7.000	47.500	36.500
Tier 4	28.500	6.800	35.400	28.400
Tier 5	27.200	6.400	43.000	43.800
Tier 6	26.400	6.200	41.000	31.400
Tier 7	23.300	6.100	34.000	35.900
Tier 8	21.400	6.000	42.000	32.000
Tier 9	20.000	5.800	38.000	43.500
Tier 10	19.600	5.600	46.300	50.700
Tier 11	18.600	5.300	42.000	23.700
Tier 12	17.400	5.200	53.500	41.600
Tier 13	17.400	4.900	37.000	36.600
Tier 14	13.500	4.200	19.800	61.700
Tier 15	6.100	4.000	32.000	31.600
Mittelwert	23.860	5.900	39.200	39.100

Behandlung mit Triaziquon
je 0,1 mg/kg s.c. vom 10. — 12. Tag
Zellzahl/mm³ 17 bzw. 24 Tage nach Infektion.

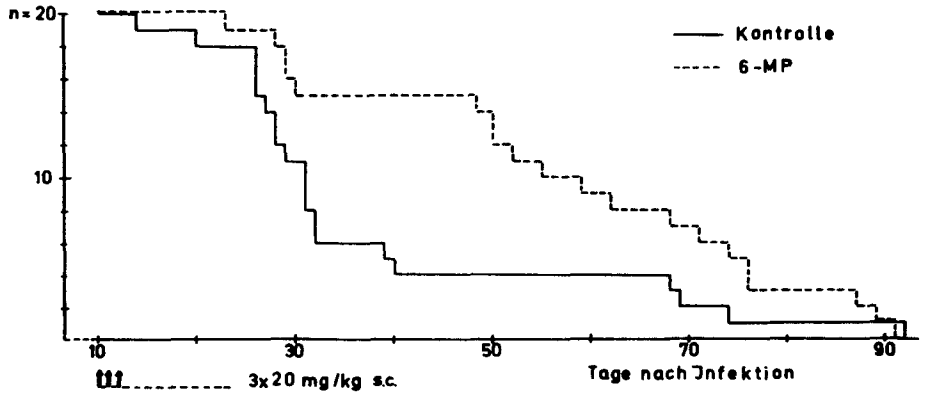

Abb. 4. Absterbekurve nach Behandlung mit 6-Mercaptopurin.

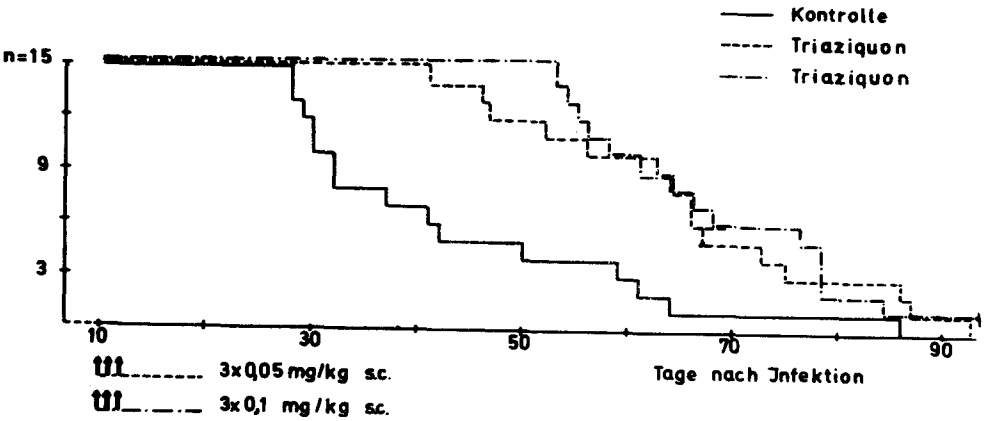

Abb. 5. Absterbekurve nach Behandlung mit Triaziquon.

Tabelle 2

Präparat	Dosis mg/kg	mittlere Überlebenszeit Tage Kontr.—behandelt	%
Busulfan	3×30 oral	30–59	198
	3×20 oral	36–68	190
	3×10 oral	32–47	145
Cactinomycin	3×0,1 s.c.	29–36	124
	3×0,08 s.c.	29–38	131
	3×0,05 s.c.	29–37	128
Cyclophosphamid	3×50 s.c.	29–40	138
	3×40 s.c.	31–36	116
	3×20 s.c.	33–44	133
6-Mercaptopurin	3×20 s.c.	31–57	184
Triaziquon	3×0,15 s.c.	31–69	223
	3×0,10 s.c.	37–66	178
	3×0,05 s.c.	37–66	178
Vincristin	3×0,5 s.c.	29–41	141
	3×0,3 s.c.	29–37	128

Verlängerung der mittleren Überlebenszeit in Tagen und in %. (Kontrolle=100%).

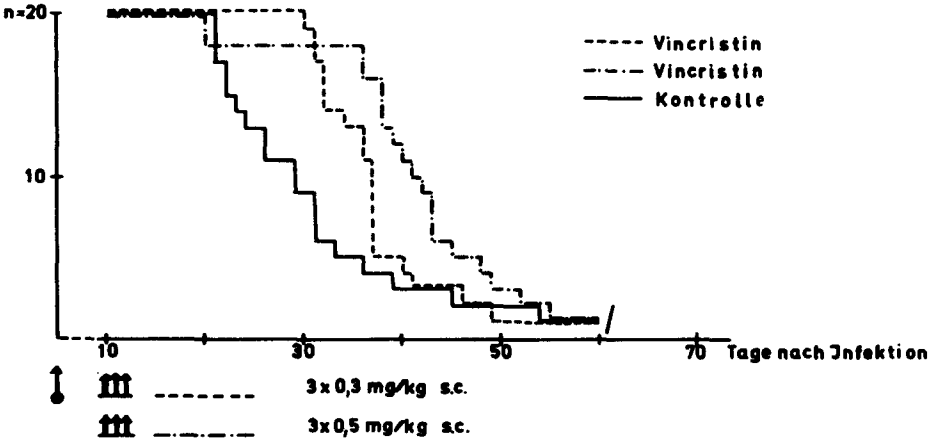

Abb. 6. Absterbekurve nach Behandlung mit Vincristin.

sind dann die in allen Versuchen mit verschiedenen Dosen erzielten Verlängerungen der mittleren Überlebenszeit ($\ddot{U}LZ_{50}$) zusammengestellt.

Busulfan führt bei oraler Gabe zu einer Verlängerung der $\ddot{U}LZ_{50}$ bis zu 190% (Abb. 1), bei Anwendung der allerdings schon toxischen Dosis von 3×30 mg/kg bis 200% (4 Tiere starben etwa 10 Tage nach Therapiebeginn ohne makroskopische Zeichen einer voll entwickelten Rauscher-Leukämie). Im peripheren Blut sind nach 3×20 mg/kg nur bei einem Drittel der Tiere pathologische Zellzahlen zu finden. Der Mittelwert beträgt mit 9000 Zellen/mm³ etwa ein Viertel von dem der Kontrolle.

Cactinomycin (Abb. 2). hat nur eine geringe Wirkung bei der virusinduzierten Rauscher-Leukämie, maximal werden Überlebenszeiten von 130% erreicht.

Bei allen verwandten Dosen sterben einige Tiere ungefähr 10 Tage nach Therapiebeginn an Infektionen; bei der Sektion hatten sie nur eine angedeutete Milzvergrösserung. Die Bestimmung der Zellzahl im peripheren Blut ergab eine dosisabhängige Ausschwemmungsverzögerung 1 Woche nach Therapiebeginn: die Mittelwerte der behandelten Tiere betrugen mit 14,500 nach $3\times0{,}1$ mg/kg und 26,800 nach $3\times0{,}05$ mg/kg etwa ein Drittel bzw. die Hälfte der entsprechenden Kontrollen.

Cyclophosphamid. Mit Cyclophosphamid wird in dem Dosisbereich von 3×20 bis 3×50 mg/kg s.c. nur ein mäßiger Effekt erzielt, die maximale Verlängerung der $\ddot{U}LZ_{50}$ beträgt 138% (Abb. 3). Die Ausschwemmungsverzögerung ist ebenfalls nur gering.

6-*Mercaptopurin* hat mit einer Verlängerung der $\ddot{U}LZ_{50}$ auf 184% eine gute Wirkung (Abb. 4). Der Kurvenverlauf und die Sektion der gestorbenen Tiere zeigen jedoch, daß bei der angewandten Dosis schon 5 von 20 Tieren ohne Leukämie an der Toxizität starben. Die gute Überlebenszeit der Resttiere ergibt somit ein unvollständiges Bild von der Wirkung der Substanz.

Triaziquon haben wir in 3 verschiedenen Dosen geprüft (Abb. 5). Schon bei $3\times0{,}05$ mg/kg werden 178% erreicht, ebenso nach $3\times0{,}1$ mg/kg. Bei der höheren Dosis beginnen die Tiere erst 10 Tage später zu sterben. Zum Zeitpunkt der Ablesung verlaufen die Absterbekurven jedoch gleich. Dreimal 0,15 mg/kg Triaziquon sind für ein Viertel der Versuchstiere bereits toxisch, sie sterben zusammen mit den Kontrollen, allerdings ohne Milztumor. Die restlichen Tiere leben wesentlich länger; daraus resultiert dann eine mittlere Überlebenszeit von 223%. Eine Woche nach Therapiebeginn mit $3\times0{,}1$ mg/kg (Tabelle 1) findet sich bei keinem behandelten Tier eine pathologische Erhöhung der Zellzahl im peripheren Blut. Dagegen haben alle Kontrollen (mit einer Ausnahme) ausgeschwemmt. Nach einer weiteren Woche besteht zwischen den beiden Gruppen kein Unterschied mehr. Die toxische Dosis von $3\times0{,}15$ mg/kg bringt einen Teil der Tiere zunächst in eine Leukopenie, und auch 2 Wochen nach der Therapie hat erst ein Drittel der Tiere pathologisch erhöhte Zellzahlwerte.

Vincristin ist mit 128% nach $3\times0{,}3$ mg/kg s.c. und 141% nach $3\times0{,}5$ mg/kg s.c. ebenfalls wirksam (Abb. 6). Die höhere Dosis ist schon mit 2 Frühtodesfällen ohne markoskopische Leukämiezeichen belastet. Achtzehn und 22 Tage nach der Infektion zeigen beide Versuchsgruppen eine dosisabhängige Ausschwemmungsverzögerung.

Versuchsgruppe B

Durch wiederholte Behandlungen in Abhängigkeit von der Zellzahl im Blut ('klinischer Behandlungsversuch') wurden mit *6-Mercaptopurin*, *Triaziquon* und *Busulfan* die folgenden Ergebnisse erzeilt:

6-*Mercaptopurin.* Nach insgesamt 13 Dosen von 20 mg/kg (s. Methodik) war die mittlere Überlebenszeit auf 200% verlängert (Abb. 7). In der Tabelle 3 sind die Verhältnisse im peripheren Blut bei 5 Tieren beispielhaft aufgeführt: die Substanz führt zu Remissionen; auch zu späten Zeitpunkten werden erhöhte Zellzahlen durch erneute Behandlung wieder auf Normwerte gesenkt. Auffällig ist das rasche Wiederansteigen nach einem behandlungsfreien Intervall. So werden bei Tier 138 Tage nach der Infektion 7000 Zellen/mm³, 1 Woche später 117,000 Zellen/mm³ im peripheren Blut gefunden. Wegen der Gefahr einer Leukopenie war eine Therapie in diesem Zeitraum unterlassen worden.

Triaziquon. Die mittlere Überlebenszeit wird durch insgesamt 18 Gaben von 0,05 mg/kg auf 274% verlängert (Abb. 7). Die Zellzahlen im peripheren Blut verhalten sich ähnlich wie nach 6-Mercaptopurin (Tabelle 4). In den Behandlungspausen kann sie innerhalb einer Woche von Normalwerten auf 111,000/mm³ ansteigen (Tier 2) und dann nach Therapie wieder zur Norm zurückkehren.

Busulfan. Insgesamt 17 Dosen von 20 mg/kg Busulfan führen zu einer Verlängerung der $\ddot{U}LZ_{50}$ auf 194% (Abb. 8). In Tabelle 5 sind die Zellzahlen bei allen Tieren zusammengestellt. Nur in wenigen Fällen war es erforder-

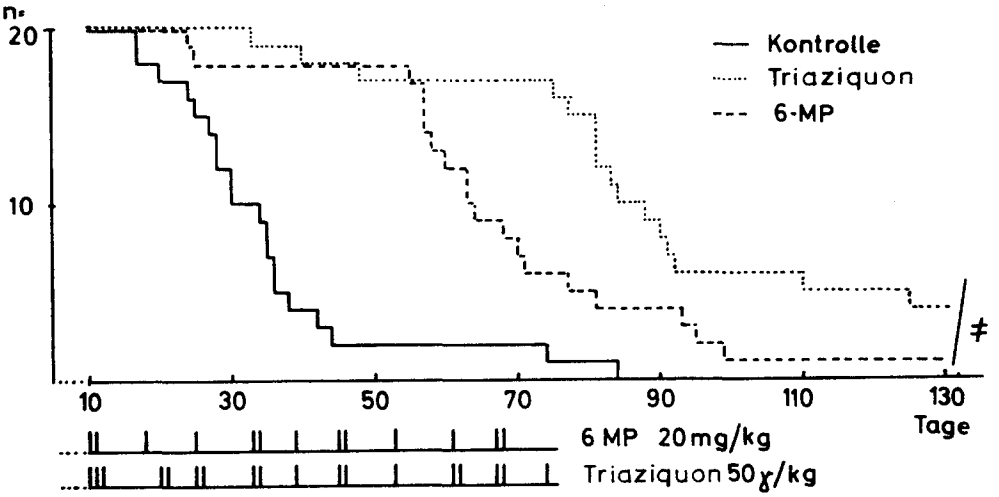

Abb. 7. Absterbekurve nach wiederholter Behandlung mit Triaziquon bzw. mit 6-Mercaptopurin.

Tabelle 3. Die Zahl der kernhaltigen Zellen im peripheren Blut während der Behandlung mit 6-Mercaptopurin bei 5 Tieren mit Rauscher-Leukämie

Tage nach Infektion		10	17	24	33	38	45	53	60
Zellzahl in tausend	1.	21xx	35x	11x	13xx	7	117xx	52x	33
	2.	13xx	8x	7x	31xx	6	17xx	8	24
(5 Tiere)	3.	11xx	7x	3	82xx	5	23xx	5	+
	4.	11xx	7x	4	45xx	9	36xx	5	+
	5.	10xx	16x	15x	23xx	8	12xx	9	72

Jedes x entspricht einer Behandlung mit 20 mg/kg 6-MP

lich, das gleiche Tier an zwei aufeinanderfolgenden Wochen zu behandeln, da die erzielten Remissionen länger anhielten. Derart drastische Ausschwemmungen wie nach den beiden anderen Präparaten wurden nicht beobachtet.

DISKUSSION

Aus versuchstechnischen Gründen mußten die Testungen in verschiedenen Ansätzen durchgeführt werden. Die spontane Absterbekurve der Kontrollgruppen variiert von Versuch zu Versuch aber nur wenig; die mittlere Überlebenszeit streut zwischen 29 und 36 Tagen. Berücksichtigt man, dass es sich um einen jeweils im einzelnen Tier induzierten Tumor handelt, so kann man auf eine bemerkenswerte Konstanz der Leukämogenese schliessen. Eine Gegenüberstellung der Resultate aus mehreren Versuchen erscheint daher zulässig.

Therapiebeginn war bei unseren Versuchen der 10. bzw. 11. Tag nach der Virusinfektion. Zu diesem Zeitpunkt sind die Erythropo-

Tabelle 4. Die Zahl der kernhaltigen Zellen im peripheren Blut während der Behandlung mit Triaziquon bei 5 Tieren mit Rauscher-Leukämie

Tage nach Infektion		10	17	24	33	38	45	53	60
Zellzahl in tausend	1.	14xxx	13xx	4	60xx	20	19xx	33xx	83
	2.	15xxx	12xx	5	111xx	10	25xx	16xx	26
(5 Tiere)	3.	9xxx	26xx	7	67xx	15	29xx	8	17
	4.	9xxx	7	20xx	11	42x	15xx	18xx	43
	5.	5xxx	11xx	3	55xx	115x	30xx	44xx	53

Jedes x entspricht einer Behandlung mit 0,05 mg/kg Triaziquon

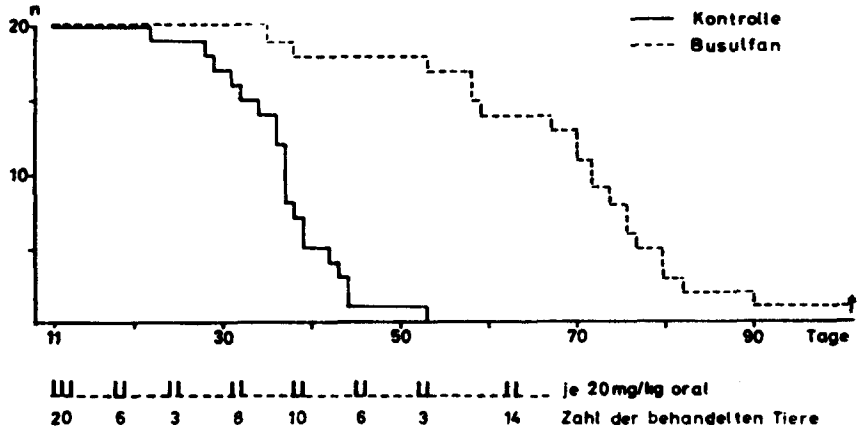

Abb. 8. *Absterbekurve nach wiederholter Behandlung mit Busulfan.*

esenester der Milz miteinander konfluiert, das mittlere Milzgewicht beträgt nahezu 1 g. Im peripheren Blut sind noch keine Erythroblasten nachweisbar. In der darauffolgenden Woche kommt es dann zu einer starken Erhöhung der Zellzahl im peripheren Blut, wobei die Erythroblasten zunächst nicht überwiegen; später bestimmen sie jedoch zunehmend das Differentialblutbild [4]. Durch die Bestimmung der Zellzahl vom 17. Tag an konnten wir diese Ausschwemmung bzw. ihre Verzögerung durch Cytostatica erfassen.

Nachdem Chirigos und Mitarb. [10, 11] den guten Effekt von 5-Fluorouracil, Triäthylenmelamin, 6-Mercaptopurin und auch Melphalan auf die Rauscher-Leukämie mitgeteilt hatten, haben wir mit Busulfan und Triaziquon zwei weitere hochwirksame Substanzen gefunden. Beide waren bisher nicht an virusinduzierten Leukämien geprüft worden.

Tabelle 5. *Die Zahl der kernhaltigen Zellen im peripheren Blut von Mäusen mit Rauscher-Leukämie während der Behandlung mit Busulfan. Nur Tiere mit mehr als* 10.000 *Zellen/mm³ wurden behandelt*

Tier-Nr.	Tage nach Infektion 17	24	31	38	45	52
1	18.900++	7.600	11.000++	9.700	15.700++	5.800
2	16.400++	5.900	7.400	11.800++	10.300++	6.600++
3	11.400++	4.600	6.300	5.400	13.600++	13.400++
4	11.400++	3.400	3.600	—	—	—
5	10.500++	3.900	6.600	6.100	11.000++	4.500
6	10.000++	6.300	7.000	11.500++	5.200	4.800
7	9.300	8.700	8.400	32.100++	9.600	6.500
8	9.000	5.000	5.000	8.800	12.500++	11.400++
9	8.500	18.700++	9.200	32.400++	8.200	9.900
10	8.300	4.400	12.800++	13.500++	7.100	2.400
11	8.200	4.000	19.400++	5.400	8.300	6.200
12	8.100	16.700++	12.400++	7.300	6.200	6.900
13	7.700	2.800	5.300	16.300++	7.600	5.900
14	7.600	4.300	11.200++	10.600++	8.200	6.600
15	7.400	12.700++	9.000	17.400++	7.000	9.000
16	7.300	3.000	10.200++	10.200++	3.100	1.500
17	7.300	4.800	9.000	21.700++	9.000	9.300
18	5.300	5.700	12.500++	7.800	7.300	10.400++
19	5.100	2.500	14.400++	4.300	10.200++	3.100
20	4.100	2.000	1.300	—	—	—
Zahl der beh. Tiere	6	3	8	10	6	3

Zellzahl/mm³ im peripheren Blut
Behandlung mit Busulfan oral je 20 mg/kg v. 10.–12. Tag, danach je nach Ausschwemmung (durch+dargest.)

Cactinomycin, Cyclophosphamid und Vincristin sind bei dem gewählten Behandlungsschema mit einer maximalen Verlängerung der $ÜLZ_{50}$ auf 140% weniger wirksam.

Die Bestimmung der Zahl der kernhaltigen Zellen im peripheren Blut gibt eine zusätzliche Information. Die Verzögerung der leukämischen Ausschwemmung eine Woche nach Therapiebeginn und die resultierende mittlere Überlebenszeit einer Versuchsgruppe entsprechen einander weitgehend. Bei der höchsten Dosis von Triaziquon (3×0.15 mg/kg), die in unseren Versuchen am besten wirkte, hat auch nach zwei Wochen erst die Hälfte der Tiere pathologische Zellzahlen. Da aber diese Dosis für einen Teil der Tiere bereits toxisch war, kann dieser Versuch nur mit Vorbehalt mit diskutiert werden. Gleiches gilt für die hohen Dosen von Busulfan und Vincristin. Bei Cactinomycin starben bei allen Versuchen einige Tiere an Infektionen. Die prognostische Bedeutung der Zellzählungen zu späteren Zeitpunkten erscheint jedoch beschränkt. So weisen Kontrollen und mit Triaziquon behandelte Versuchstiere (vgl. Tabelle 1) zwei Wochen nach Therapiebeginn (=24 Tage nach der Infektion) gleich hohe Werte auf. Die Absterbekurven (Abb. 5) zeigen aber die wesentlich längere Überlebenszeit der behandelten Tiere an.

Die Versuche mit wiederholter Therapie nach wöchentlicher Kontrolle des peripheren Blutes haben nur bei Triaziquon zu einer deutlichen weiteren Verbesserung der Überlebenszeit von 180% auf 274% geführt. Bei 6-Mercaptopurin und Busulfan ist der Effekt jeweils nur gering. Dies ist umso bemerkenswerter, als die Tabellen 3 und 4 zeigen, dass zumindest die Zellen des peripheren Blutes auch zu späten Versuchszeiten noch auf die Substanzen voll ansprechen. Die rasche Ausschwemmung in den Behandlungspausen bei 6-Mercaptopurin und Triaziquon deutet auf ein grosses Zellreservoir hin, das von den Substanzen offenbar weit weniger beeinflusst wird. Nach unseren histologischen Untersuchungen kommt hierfür vor allem die Milz infrage, deren Struktur im Verlaufe der Entwicklung der Rauscher-Leukämie völlig zerstört wird und die voll erythropoetischer Zellen ist [4]. Dies erklärt den geringen prognostischen Wert der Zellzahlen im peripheren Blut bei behandelten Tieren. In den Behandlungspausen nach Busulfan haben wir keine solch plötzliche Ausschwemmung beobachtet. Vielleicht werden von dieser Substanz die Zellen der Milz in vermehrtem Ausmass mitbetroffen.

Die vergleichsweise geringe Besserung der Wirkung durch wiederholte Therapie bleibt unbefriedigend. Wir möchten neben einer möglichen Summation toxischer Effekte, die wir nicht erfassen konnten, noch einen anderen Prozess bei der Rauscher-Leukämie in Erwägung ziehen: Die mit dem Rauscher-Virus infizierten Tiere entwickeln in fortgeschrittenen Krankheitsstadien eine schwere Anämie und Thrombocytopenie. Diese werden durch Cytostatica evtl. noch verstärkt und tragen zum Tod der Tiere mit bei. Findet man doch bei der Sektion häufig Blutungen in Milz und Darm.

Die neoplastische Natur der erythropoetischen Zellproliferation bei den mit dem Rauscher-Virus infizierten Mäusen wurde angezweifelt [7, 3]. Wir haben uns — wie andere auch [2, 8] — auf Grund histologischer Befunde für eine Erythroleukämie ausgesprochen [4] und glauben nicht, dass die sofort einsetzende Proliferation von unreifen Zellen der Erythropoese lediglich Antwort auf eine Hämolyse ist. Für letztere haben weder Dunn und Green [7] noch wir [4] nach morphologischen Kriterien Hinweise gefunden. Zu betonen ist auch, dass die Erythropoese in der Milz nach Rauscher-Virus inokulation eine Reifungshemmung aufweist [7, 8].

Auf die Abgrenzung einer cytoziden und einer antiviralen Wirkung, wie es Chirigos und Mitarb. [12] versucht haben, haben wir in der vorliegenden Arbeit verzichtet. Heilungen erscheinen freilich erst dann denkbar, wenn durch antivirale Substanzen die Virusreduplikation auch in den normalen Geweben, z.B. den Megakaryozyten [13], unterdrückt wird.

Es kann nicht Aufgabe der experimentellen Chemotherapieforschung sein, optimale Subtanzen für bestimmte Mäuseleukämieformen zu finden. Der Wert dieser Testmodelle für die Klinik muss immer wieder geprüft werden. Entsprechend ihrem Typ als Erythroleukämie kann die Rauscher-Leukämie als Modell für diese Form beim Menschen betrachtet werden, nach ihrem Ansprechen auf die von Chirigos und Mitarb. [10, 11] und von uns getesteten Cytostatica evtl. auch für myeloische Formen. Das Studium des gesamten Verlaufs und der Zellzahlen im peripheren Blut gibt aber Aufschlüsse über Intensität und Dauer der Präparatwirkung.

RESUME

Le busulfan, l'actinomycine C, la cyclophosphamide, la 6-mercaptopurine, le triaziquon et la vincristine ont été essayés dans la leucémie induite par le virus de Rauscher. Le busulfan, la 6-mercaptopurine et le triaziquon, lorsqu'ils sont administrés à partir du dixième ou du douzième jour suivant l'infection par le virus, doublent la durée de vie médiane; les autres substances sont moins actives. L'apparition de cellules leucémiques dans le sang périphérique, une semaine après le début du traitement, est inhibée ou retardée par rapport aux contrôles, d'une façon correspondant à l'effet sur la survie médiane.

Dans d'autres expériences avec le busulfan, la 6-mercaptopurine et le triaziquon, les traitements furent répétés en fonction des données fournies par des numérations hebdomadaires des cellules nucléées. L'amélioration du temps de survie par cette méthode de traitement fut très faible; les raisons en sont discutées.

SUMMARY

Chemotherapeutic assays on Rauscher virus-induced leukemia using busulfan, cactinomycin, cyclophosphamide, 6-mercaptopurine, triaziquon, and vincristine are described. Treatment from the 10 to 12 day after virus infection with busulfan, 6-mercaptopurine, and triaziquon almost doubles the median survival time; the other substances are less effective. The appearance of leukemic cells in the peripheral blood one week after the onset of therapy is inhibited or delayed compared to controls — corresponding to the action on median survival time. — In further experiments using busulfan, 6-mercaptopurine, and triaziquon the weekly determined nucleated cell count was taken as indication for repeated treatments. The improvement of survival time by this schedule was quite small; possible reasons are discussed.

ZUSAMMENFASSUNG

Chemotherapieversuche an der virusinduzierten Rauscher-Leukämie der Maus mit Busulfan, Cactinomycin, Cyclophosphamid, 6-Mercaptopurin, Triaziquon und Vincristin werden beschrieben. Eine einmalige Behandlungsserie vom 10. bis 12. Tag nach der Virusinfektion mit Busulfan, 6-Mercaptopurin oder Triaziquon führt fast zu einer Verdoppelung der mittleren Überlebenszeiten; die anderen Substanzen sind weniger wirksam. Die Ausschwemmung von Leukämiezellen in das periphere Blut eine Woche nach Therapiebeginn ist — analog der Wirkung der Substanz auf die mittlere Überlebenszeit — verhindert oder im Vergleich zur Kontrolle verzögert. In weiteren Versuchen mit Busulfan, 6-Mercaptopurin und Triaziquon wird die wöchentlich bestimmte Zellzahl im peripheren Blut als Kriterium für wiederholte Behandlungen genommen. Die nur geringe Verbesserung der Überlebenszeiten durch dieses Behandlungsverfahren wird diskutiert.

LITERATUR

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Transport d'Agents Cytostatiques par les Protéines Plasmatiques

II. Localisation Intratumorale du Fibrinogène

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INTRODUCTION

LA VALEUR pratique des traitements habituels des cancers inopérables, basés sur l'administration systématique d'une ou plusieurs doses de cytostatiques, est limitée du fait de la toxicité sévère survenant généralement avant qu'aucun effet tumoricide ne soit obtenu. Cette toxicité pourrait être abaissée en concentrant électivement les cytostatiques dans les tumeurs. La technique de la perfusion artérielle en est perfusion un exemple [1]. Une autre possibilité est l'utilisation de 'transporteurs' capables d'amener sélectivement les cytostatiques dans le tissu néoplasique.

Plusieurs auteurs [2–8] ont suggéré l'utilisation de certains protéines plasmatiques, telles le fibrinogène, l'albumine, les anticorps antitumoraux, qui peuvent se concentrer dans les tissus tumoraux. La fixation et la pénétration de la sérum-albumine dans les cellules tumorales ont été récemment étudiées par Cerottini *et al.* [9].

Le présent travail décrit la fixation intratumorale du fibrinogène bovin dans un sarcome transplantable du rat, fixation qui peut être favorablement influencée par différents facteurs. La concentration élevée du fibrinogène, homologue ou hétérologue, dans diverses tumeurs humaines [10, 11] et animales [12–14], en fait, avec les anticorps antifibrine [12, 13, 15], une des protéines intéressantes comme véhicule d'agent cytostatique.

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MATERIEL ET METHODES

Animaux

Les animaux utilisés sont des rats BD4 de l'élevage de l'Institut Suisse de Recherches Expérimentales sur le Cancer, à Lausanne.

Tumeur

Une souche du sarcome de Yoshida est entretenue par transplantation sous-cutanée à des rats BD4, pesant de 90 à 150 g. Le dixième jour de la greffe, la tumeur pèse environ 1 g. A ce moment, elle n'est généralement pas ulcérée.

Protéines et réactifs

Le fibrinogène bovin.—C'est un produit de la maison Kabi (Suède); sa coagulabilité est de 90 à 95%.

Les lipopolysaccharides bactériens de E. coli 0111 ont été préparés par le Dr. O. Westphal (Fribourg, Allemagne). Ils sont injectés par voie intraveineuse, à des doses de 75 μg, 6 h avant l'injection du fibrinogène radiomarqué.

L'acide ε-aminocaproïque (Fluka, Suisse) est administré dans l'eau de boisson en solution à 5%, un à trois jours avant l'injection du fibrinogène radiomarqué, et pendant la durée de l'expérience.

Iodation

Le fibrinogène est marqué selon la méthode de McFarlane [16]. L'iode radioactif (^{131}I), stérile et sans entraîneur (IBS 3), provient du Radiochemical Centre (Amersham). Moins d'un atome d'iode radioactif est couplé par molécule de protéine. L'excès d'iode libre est éliminé par dialyse contre du tampon citrate – phosphate 0,1M, pH 7,5. Le rendement de l'iodation est de 40 à 60%, avec un pourcentage d'iode soluble dans l'acide trichloracétique ne dépassant pas 2%.

Distribution du fibrinogène in vivo

Au 10e jour de la transplantation sous-cutanée du sarcome de Yoshida, des groupes de 4 rats BD4 reçoivent, par voie intraveineuse ou intrapéritonéale, 3 mg de fibrinogène radiomarqué, avec une activité spécifique de 200.000 à 2.830.000 coups/min/mg. Un à trois jours avant l'expérience, et durant celle-ci, les rats mangent *ad libitum* et reçoivent, dans leur eau de boisson, de l'iodure de potassium $6{,}25 \times 10^{-4}$N. Ils sont sacrifiés 20 et 66 h, ou 3 à 5 jours après l'injection du fibrinogène. Les tissus et les organes désirés, non perfusés, sont disséqués et pesés. Leur radioactivité est déterminée à l'aide d'un compteur à cristal (Nuclear Chicago, modile C 120-1). Les résultats sont exprimés en pour-cent de la dose injectée de ^{131}I couplé au fibrinogène, fixé dans 1 g tissu frais et sont normalisés pour des rats de 100 g. Sont indiquées les valeurs moyennes plus ou moins l'erreur moyenne de la moyenne.

L'irradiation locale

L'irradiation locale, aux rayons X, de la tumeur de Yoshida est effectuée le 7e jour de la greffe, soit un jour avant l'injection du fibrinogène radiomarqué, à l'aide d'un appareil Siemens émettant 20 rad/min (distance 10 cm, filtre de cuivre de 1 mm, 175 KV/6,6 mA). Le dispositif utilisé permet d'irradier 4 rats simultanément. Les animaux sont préalablement endormis au chloral et fermement fixés, couchés sur le flanc, autour d'une fenêtre pratiquée dans une table de bois. Leurs corps sont protégés par un couvercle de plomb, évidé en son centre, ne laissant apparaître que la tumeur. Les rayons sont encore focalisés par des plaques de plomb, de manière à n'irradier que les tissus néoplasiques.

RESULTATS

Fixation in vivo *du fibrinogène bovin*

La fixation intratumorale du fibrinogène bovin marqué au ^{131}I, 20 h après injection intraveineuse, varie selon le poids de la tumeur, c.-à-d. son âge. La Figure 1 montre que la fixation par gramme de tissu tumoral est optimale lorsque la tumeur pèse environ 1 g, ce qui correspond au dixième jour de la transplantation. Le quatorzième jour, la tumeur pèse environ 4 g, et 10 g le dix-huitième. C'est pourquoi, dans la mesure du possible, le fibrinogène radiomarqué a été injecté le 10e jour de la greffe.

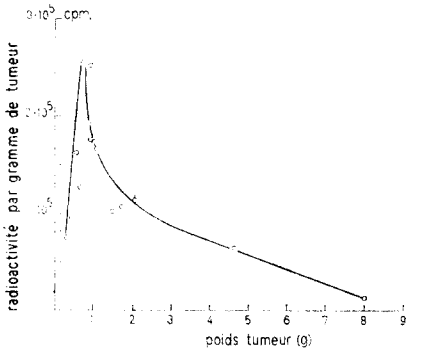

Fig. 1. Localisation intratumorale du fibrinogène bovin marqué au ^{131}I en fonction du poids de la tumeur. Localisation 20 h après injection intraveineuse de fibrinogène à des rats BD4 porteurs de sarcomes solides de Yoshida de poids différents. La tumeur pèse environ 1 g le dixième jour de la transplantation.

Effet de l'acide ε-aminocaproïque sur la fixation intratumorale du fibrinogène injecté par voie intraveineuse

Du fibrinogène bovin (3 mg), radiomarqué, est injecté, par voie intraveineuse, à trois groupes de 4 rats BD4 porteurs du sarcome de Yoshida, au dixième jour de la transplantation. Deux groupes ont reçu de l'acide ε-aminocaproïque (EACA) à 5% dans leur eau de boisson. Les rats sont sacrifiés 20 et 66 h après l'injection de la protéine. Le troisème groupe sert de contrôle. Les animaux de ce groupe sont sacrifiés 20 h après l'injection du fibrinogène. Le Tableau 1 met en évidence l'augmentation significative de la localisation intratumorale due au traitement à l'EACA ($P<0{,}05$). La sélectivité de la distribution, c.-à-d. le rapport de la fixation tumorale à la fixation tissulaire moyenne, est également améliorée par ce traitement, malgré une augmentation de la fixation hépatique et splénique.

Après 66 h, la localisation intratumorale du fibrinogène bovin est toujours aussi élevée qu'après 20 h. La sélectivité de la distribution, dans ce cas, est encore meilleure du fait que le fibrinogène, sous forme de fibrine, persiste plus longtemps dans le tissu néoplasique que dans les tissus normaux (Tableau 2).

Tableau 1. *Fixation intratumorale du fibrinogène bovin marqué au ^{131}I chez des rats BD4 porteurs du sarcome solide de Yoshida* (20 *h après l'injection intraveineuse*)

	Sans EACA 5%	Avec EACA 5%*
Nombre de rats	4	4
% ^{131}I fixé par g de tumeur	1,10±0,05	1,88±0,28†
Rapport $\frac{^{131}I \text{ par g de tumeur}}{^{131}I \text{ par g de tissu sain}}$	13	17
Poids moyen des tumeurs	0,890 g	0,720 g
Poids moyen des animaux	200 g	200 g

*EACA=acide ε-aminocaproïque. Solution à 5% dans l'eau de boisson.
†Moyenne±erreur moyenne de la moyenne.

Tableau 2. *Fixation intratumorale du fibrinogène bovin marqué au ^{131}I chez des rats BD4 porteurs du sarcome solide de Yoshida* (66 *h après injection intraveineuse*)

Nombre de rats	4
% ^{131}I fixé par g de tumeur	1,84±0,2*
Rapport $\frac{^{131}I \text{ par g de tumeur}}{^{131}I \text{ par g de tissu sain}}$	44
Poids moyen des tumeurs	3,3 g
Poids moyen des animaux	157 g

*Moyenne±erreur moyenne de la moyenne.
L'eau de boisson contient de l'acide ε-aminocaproïque à 5%.

Effet de l'acide ε-aminocaproïque sur la fixation intratumorale du fibrinogène injecté par voie intrapéritonéale

Sept rats BD4, porteurs du sarcome de Yoshida, et traités à l'acide ε-aminocaproïque (EACA) à 5%, reçoivent dans la cavité péritonéale du fibrinogène bovin (3 mg) marqué au ^{131}I. Cinq rats sont sacrifiés après 3 jours et 2 après 5 jours. Les résultats montrent que la fixation du fibrinogène marqué a lieu dans les tumeurs, mais à des taux peu importants: après 3 jours, la fixation par gramme de tissu tumoral représente 0,80± 0.06% de la dose de fibrinogène injecté. Après 5 jours, la fixation a diminué de moitié et s'élève à 0,45±0,12% (Tableau 3).

Effet de lipopolysaccharides bactériens sur la fixation intratumorale du fibrinogène injecté par voie intraveineuse

Six rats porteurs de la tumeur de Yoshida, au 9e jour de la greffe, reçoivent, par voie intraveineuse, 0,5 ml d'une solution de NaCl 0,15 M, contenant 75 μg de lipopoly-saccharides (LPS) de *E. Coli* 0111, 6 h avant l'injection intraveineuse de fibrinogène bovin radiomarqué. Six rats témoins ne reçoivent que 0,5 ml de NaCl 0,15 M stérile. Les animaux sont sacrifiés 20 h après l'injection du fibrinogène (Fig. 2). La fixation intratumorale s'est élevée à 2,4% de la dose injectée par gramme de tumeur, alors qu'elle n'atteint que 1,5% chez les rats témoins ($P<0,01$). En revanche, la sélectivité de la distribution est nettement diminuée chez les rats traités: en effet, le rapport de la fixation tumorale à la fixation tissulaire moyenne n'est que de 10, alors qu'il est de 17 chez les rats de contrôle. L'effet général des endotoxines peut être encore mis en évidence par la diminution du poids corporel des animaux traités (125,5±4,8 g) par rapport à celui des rats témoins (163,4±7,4 g). L'action des LPS sur la croissance tumorale n'est pas encore décelable 20 h après l'injection, le poids moyen des tumeurs n'ayant subi aucune modification.

Tableau 3. *Evolution de la fixation intratumorale du fibrinogène bovin marqué au ^{131}I chez des rats BD4 porteurs du sarcome solide de Yoshida* (*après injection intrapéritonéale*)

Temps écoule après injection intrapéritonéale du fibrinogène radiomarqué	3 jours	5 jours
Nombre de rats	5	3
% ^{131}I fixé par g de tumeur	0,80±0,07	0,45±0,12*
Rapport $\frac{^{131}I \text{ par g de tumeur}}{^{131}I \text{ par g de tissu sain}}$	36	55
Poids moyen des tumeurs	3,56 g	5,97 g
Poids moyen des rats	188 g	199 g

*Moyenne±erreur moyenne de la moyenne.
L'eau de boisson contient de l'acide ε-aminocaproïque à 5%.

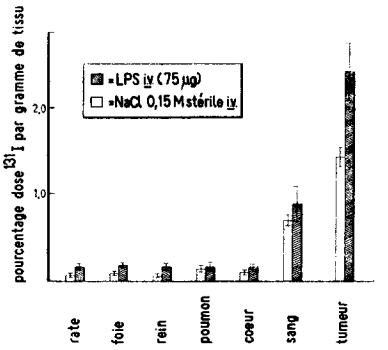

Fig. 2. Effets des lipopolysaccharides bactériens (LPS) sur la fixation intratumorale du fibrinogène bovin.

Injection intraveineuse de 75 µg *de LPS de* E. Coli 0111, *le 9e jour de la greffe, 6 h avant l'injection intraveineuse du fibrinogène radiomarqué* (270.000 *coups/min/mg*) *à un groupe de* 6 *rats BD4 porteurs d'un sarcome de Yoshida. Six rats témoins reçoivent par voie intraveineuse NaCl* 0,15 M *stérile. Les rats sont sacrifiés* 20 *h après l'injection de la protéine* ($P<0{,}01$).

Effet de l'irradiation locale aux rayons X de la tumeur sur la fixation intratumorale du fibrinogène bovin injecté par voie intraveineuse

Dans ces expériences, les tumeurs de Yoshida, forme solide, sont irradiées le septième jour de la transplantation, et le fibrinogène radiomarqué est injecté le jour suivant. Les rats BD4 sont traités à l'EACA 5% et sont sacrifiés 66 h après l'injection de la protéine. Des doses de 800, 1200 et 1500 rad ont été utilisées. La fixation intratumorale du fibrinogène a été la plus élevée avec les doses les plus faibles. Une irradiation de 800 rad permet d'obtenir une fixation tumorale de 6,6±0,1%, alors qu'avec 1500 rad, la fixation est moins importante et s'élève à 3,9±0,3%. D'une manière générale, la sélectivité de la distribution est très favorable, puisque le rapport de la fixation tumorale à

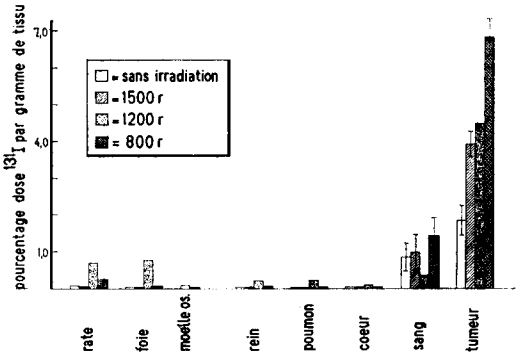

Fig. 3. Effets de l'irradiation locale aux rayons X du sarcome de Yoshida sur la fixation intratumorale du fibrinogène bovin. L'irradiation de la tumeur à des doses de 800, 1200 *et* 1500 *rad a été pratiquée le 7e jour de la greffe. L'injection du fibrinogène radiomarqué a eu lieu le jour suivant* (190.000 *coups/min/mg*). *Les rats sont sacrifiés* 66 *h après l'injection de la protéine. L'eau de boisson contient du KI* 6,25.10^{-4} N *et de l'EACA* 5%.

la fixation tissulaire moyenne varie entre 87 et 108 (Fig. 3).

DISCUSSION

Une des réactions importantes de l'organisme des mammifères à un dommage tissulaire consiste en l'activation locale du système de la coagulation qui conduit à un dépôt de fibrine dans le tissu endommagé. Le dépôt de fibrine à partir du fibrinogène plasmatique fait également partie de la réaction inflammatoire à une irritation tissulaire étendue [17, 18].

Dans le cas des tumeurs transplantables, ce phénomène se décompose en deux phases: le dépôt initial dû à la réaction inflammatoire, entravant la croissance tumorale, et le dépôt secondaire lié à la vascularisation du tissu néoplasique [19]. Ainsi, dans le cas d'un hépatome de Miller, 15% du fibrinogène circulant de l'hôte se dépose, pendant les trois premiers jours, dans le tissu tumoral en formation. Du 8ème au 22ème jour, 25% du fibrinogène contribue à la croissance tumorale. Lors de la greffe d'un lymphosarcome de Murphy-Sturm, la tumeur croît, dès le 7e jour, à un taux de 0,3 g/h, et localise, pendant le même laps de temps, 7% du fibrinogène de l'hôte. Le fibrinogène injecté par voie intraveineuse pourra donc être fixé rapidement dans les tumeurs sous forme de fibrine. Day *et al.* [12, 15] ont montré que les anticorps antifibrine et le fibrinogène humain ou de rat se déposaient dans la tumeur de Murphy-Sturm à des taux comparables, représentant 22% de la dose injectée. Lorsque la même tumeur est transplantée à des rats de race différente, la fixation des anticorps antifibrine et du fibrinogène de rat s'élève, selon Spar *et al.* [13], à 13% et 7,9%, respectivement, par gramme de tumeur. Cette fixation a lieu également dans les tumeurs spontanées du chien mais elle varie suivant la nature du néoplasme: pratiquement nulle dans les tumeurs épithéliomateuses, la fixation des anticorps antifibrine homologue peut s'élever jusqu'à 66,3% par gramme de tissu sarcomateux [14]. Le même phénomène se produit chez l'homme [10], ce qui permet, en particulier, de mettre en évidence les tumeurs sarcomateuses du cerveau [20].

Malheureusement, dans plusieurs tumeurs transplantables du rat, et dans la plupart des tumeurs spontanées du chien et de l'homme, la localisation préférentielle du fibrinogène ne se fait pas, soit que la vascularisation des tumeurs à croissance lente est moins riche, soit que le catabolisme intratumoral de la protéine est accéléré. C'est pourquoi, Bale *et al.* [21] ont étudié plusieurs substances

inhibitrices de la fibrinolysine ou de l'activation de la profibrinolysine. Leurs travaux démontrent que l'administration orale de l'acide ε-aminocaproïque (EACA) à des rats porteurs du carcinome de Walker entraîne une augmentation de la fixation intratumorale du fibrinogène cinq fois supérieure à celle mesurée chez les animaux témoins. Le même traitement, appliqué à des rats porteurs du sarcome de Murphy–Sturm, fait doubler la concentration du fibrinogène par gramme de tissu tumoral.

Dans notre système, la fixation du fibrinogéne bovin, dans le sarcome de Yoshida, est également doublée par l'administration orale d'une solution d'EACA 5%.

Chez l'homme, l'emploi de l'acide ε-aminocaproïque produit une nette élévation de la fixation intratumorale du fibrinogène et des anticorps antifibrine. Celle-ci est particulièrement élevée dans les mélanomes et les tumeurs sarcomateuses [10]. Par l'administration à des patients cancéreux de doses élevées de ^{131}I couplé à des anticorps antifibrine, une rémission clinique non négligeable a pu être obtenue, qui ne s'est cependant pas maintenue [10]. Mais il est à remarquer que l'utilisation de l'EACA est sujet à caution, du fait que ce produit favorise l'établissement des métastases pulmonaires [22].

Des essais préliminaires de *radiothérapie* ont été également tentés [8]. Lorsque le fibrinogène radiomarqué est injecté suffisamment tôt (le 7e jour de la greffe, par exemple), le volume moyen des tumeurs est nettement inférieur aux contrôles et la survie des animaux, prolongée. Aucun effet curatif n'est obtenu lorsque des doses plus importantes sont administrées à partier du 10ème jour de la transplantation.

L'irradiation locale. L'irradiation locale du néoplasme aux rayons X augmente significativement la localisation tumorale du fibrinogène [23]. Dans le cas des tumeurs de Yoshida, cette augmentation n'a pas été identique selon les doses de rayons X utilisées. Une dose de 800 rad s'est révélée plus efficace que des doses de 1200 ou de 1500 rad. Ce fait suggère qu'une dose de 800 rad agit en tant que dose élective faible suscitant principalement des réactions de type inflammatoire et favorisant le dépôt de fibrine dans les espaces interstitiels [24]. Avec des doses plus fortes, l'action cytotoxique prédomine, causant des lésions de radionécrose diffuses, qui diminuent la vascularisation du tissu tumoral, et, par conséquent, la fixation intratumorale du fibrinogène.

Les lipopolysaccharides bactériens (LPS). Ils ont la propriété de provoquer, dans les tumeurs, des hémorragies et des zones de nécrose telles qu'elles peuvent amener la disparition complète du néoplasme [25]. Ces manifestations sont dues à un effet direct sur le métabolisme de la cellule tumorale. La glycolyse aérobique qui s'ensuit peut entraîner la mort cellulaire du fait de la quantité excessive d'acides formés. Les endotoxines ont aussi un effet indirect de vasoconstriction [26], et accélèrent les processus de coagulation en augmentant le taux sanguin des facteurs V, VII, X, de la prothrombine, du fibrinogène [27], et des précurseurs plaquettaires de la thromboplastine plasmatique [28]. De plus, elles libèrent, à partir des lysosomes des granulocytes, les hydrolases acides qui semblent jouer un rôle important dans la génèse des lésions tissulaires accompagnant les phénomènes d'Arthus et de Shwartzman [29]. De par leur action sur la vascularisation tumorale et leurs effets sur le système de la coagulation, les endotoxines devaient aussi influencer la fixation intratumorale du fibrinogène. Nous avons pu montrer que l'injection intraveineuse préalable d'endotoxines permet d'augmenter la fixation tumorale du fibrinogène bovin d'un facteur de 1,5 environ. Si l'action locale des lipopolysaccharides n'est pas évidente 20 h après leur injection, en revanche, leur action générale se traduit par une diminution du poids des animaux traités et par un rapport fixation tumorale/fixation tissulaire peu favorable. Ainsi, la fixation intratumorale du fibrinogène homologue ou hétérologue est suffisamment élevée et élective pour que cette protéine puisse étre considérée comme véhicule amenant, dans les tissus néoplasiques, les quantités de cytostatiques requises pour y exercer une action thérapeutique. L'instabilité du fibrinogène en solution nuit à sa conjugaison à des agents cytotoxiques [8]. Néanmoins, des expériences préliminaires *in vivo* chez la souris indiquent que la fixation intratumorale de conjugués Méthotrexate-azofibrinogène a lieu, mais qu'elle diminue proportionellement avec le degré de conjugaison, c.-à-d. suivant le nombre de molécules d'antimétabolite couplé à la protéine [8]. Ces données sont en accord avec le fait que les azoprotéines sont phagocytées par le système réticuloendothélial d'autant plus vite que le degré de conjugaison est plus élevé [30–32].

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RESUME

L'étude de la fixation du fibrinogène bovin a été réalisée chez le rat BD4 porteur du sarcome solide de Yoshida. L'acide ε-aminocaproïque, les endotoxines, et l'irradiation locale du néoplasme, permettent une nette élévation du taux de la fixation intratumorale. Les résultats obtenus sont discutés dans l'optique d'une chimiothérapie 'dirigée' utilisant le fibrinogène comme vecteur d'agents cytostatiques, et permettant leur localisation préférentielle dans les tumeurs.

SUMMARY

The fixation of bovine fibrinogen has been studied in BD4 rats bearing a solid Yoshida tumour. ε-Aminocaproic acid, endotoxin and local irradiation of the neoplasma produced a marked increase of the concentration of fibrinogen within the tumour. The results are discussed in terms of a guided chemotherapy using fibrinogen as a vehicle of cytostatic agents in order to obtain a more selective intratumoral localisation of the latter.

ZUSAMMENFASSUNG

Die intratumorale Fixierung von Fibrinogen wurde in BD4-Ratten mit Yoshida-Sarkomen untersucht. ε-Aminocapronsäure, Endotoxine und lokale Bestrahlung des Neoplasmas bewirken eine signifikane Steigerung der intratumoralen Einlagerung von Fibrinogen. Die Ergebnisse zeigen, dass Fibrinogen als Träger von Cytostatica eine gute Lokalisierung der letzteren in Tumorgeweben erlaubt.

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Transport d'Agents Cytostatiques par les Protéines Plasmatiques

III. Activité Antitumorale *in vitro* de Conjugués Cytostatique-Azoprotéines

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INTRODUCTION

PLUSIEURS travaux ont montré que certaines protéines plasmatiques se déposaient préférentiellement dans le tissu tumoral [1–5] et qu'elles pouvaient ainsi servir de véhicules à des médicaments anticancéreux, améliorant leur index thérapeutique [5–9]. Parmi ces protéines, l'albumine a particulièrement attiré l'attention, et récemment *Cerottini et al.* [6] ont passé en revue les difficultés que pose toute chimiothérapie 'dirigée' employant des protéines comme vecteur d'agents cytostatiques.

Le fibrinogène homologue ou hétérologue [10–14], les anticorps antifibrine [12, 13, 15] se fixent dans les néoplasmes à un taux plus élevé que l'albumine et semblent, dans cette optique, d'un meilleur rendement, d'autant plus que certains facteurs, tel que l'acide ε-aminocaproïque [16] améliorent leur fixation intratumorale. Des essais préliminaires [7] ont montré que le fibrinogène couplé par diazotation à des groupes cytotoxiques garde la propriété de se fixer dans les tumeurs. Mais cette fixation diminue selon le nombre de molécules du médicament couplé, tandis qu'augmente la captation du conjugué par le système réticulo-endothélial [6, 7, 17, 18]. Le présent travail décrit le pouvoir cytostatique de quelques conjugués azoprotéiques *in vitro*, utilisant le fibrinogène et la sérumalbumine comme support d'agents antitumoraux.

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MATERIEL ET METHODES

Protéines. L'albumine humaine pure, sous forme lyophilisée, provient du Laboratoire Central de la Croix Rouge Suisse à Berne; le fibrinogène humain ou bovin, de la Maison Kabi (Suède). Sa coaguabilité est de 90 à 95%.

Cytostatiques. L'améthoptérine (Méthotrexate), reçue par l'intermédiaire du Dr. A. Lanini, est un don de l'American Cyanamid Co., Lederle Laboratories Division (Pearl River, New York). Le Méthotrexate marqué au tritium a été offert par le Dr. A. Welch, du Département de Pharmacologie de l'Université de Yale (New Haven, Connecticut). La maison Hoffmann-La Roche & Cie (Bâle) a aimablement fourni la méthylhydrazine Ro4-9374/1 (1-méthyl-2-*p*-(hydroxyethyl carbamoyl)- benzylhydrazine hydrochlorure estérifié par l'acide *p*-aminobenzoïque).

Préparation des azoprotéines. Le composé Ro4-9374/1 est couplé à l'albumine et au fibrinogène par liaison azoïque, selon les méthodes classiques décrites par Landsteiner [19] et Kabat et Mayer [20]. L'excès de diazonium est éliminé par filtration sur gel de Sephadex G-25 (Pharmacia, Uppsala, Suède). Le Méthotrexate est conjugué au fibrinogène

par l'intermédiaire de la benzidine bis-diazotée selon les procédés de DeCarvalho [21] et Kabat et Mayer [20]. Dans ce cas également, l'excès de diazonium est éliminé par filtration sur gel de Sephadex G-25.

Calcul du rendement de couplage. Le rendement du couplage est défini par le rapport de la quantité de cytostatique liée à la protéine à la quantité de cytostatique introduite dans le mélange réactionnel. La teneur en protéines est dosée par la microméthode de Kjeldahl décrite par Kabat et Mayer [20]; celle du médicament est obtenue par la méthode de Bratton et Marshall [22] après réduction du pont diazoïque par le chlorure stanneux 0,1 N [18]. Lorsque l'antimitotique est marqué au tritium, sa teneur est déterminée par le comptage direct de la radioactivité dans un compteur à scintillation liquide. Le rendement de la conjugaison du Ro4-9374/1 à l'albumine est de l'ordre de 30%. Le couplage du Ro4-9374/1 au fibrinogène est rendu difficile du fait de l'insolubilité du produit terminal: à partir d'un rapport molaire initial cytostatique/protéine de 50, le rendement devient égal ou inférieur à 20% [7]. De même, le conjugué Méthotrexate-azofibrinogène n'est soluble que jusqu'à un rapport de 15 moles d'antimitotique par mole de protéine. Le rendement est, dans ce cas, de 50% environ [7].

Souche cellulaire et méthodes de culture des cellules tumorales. Des cellules d'un mastocytome transplantable de souris de la lignée cellulaire P-815-X2 [23–25] sont cultivées, dans le milieu, selon la méthode décrite par Schindler [26, 27]. Dans ce milieu, les cellules tumorales se développent facilement, sans coller aux parois des récipients. Le temps de génération est de 10 à 15 h.

Détermination de l'activité cytostatique

(*a*) *En milieu liquide.* A 3,8 ml, 1,8 ml ou 1,6 ml de milieu contenant environ 10^5 cellules/ml, on ajoute 0,2 ou 0,4 ml de la solution du conjugué, aux dilutions requises, en utilisant 3 tubes par dilution. La protéine normale correspondante, dissoute dans le méme tampon et diluée dans les mêmes proportions, ainsi que le tampon seul, servent de controle. Après 24 heures d'incubation à 37°C, on compte le nombre de cellules par ml de milieu. Le rapport du nombre de cellules avant et après l'incubation permet de calculer le facteur de multiplication cellulaire. Si le conjugué à la concentration utilisée exerce une activité cytostatique marquée, ce facteur est inférieur à 1.

(*b*) *En milieu gélifié.* Les cellules du mastocytome sont diluées avec un milieu contenant de la thrombine [27]. A 4 ml de suspension cellulaire diluée, on ajoute 1 ml des différentes dilutions de l'azofibrinogène. Ces dilutions ont été préparées à partir de la solution initiale du conjugué avec une solution de fibrinogène à 0,5%. Le fibrinogène natif aux mêmes dilutions sert de contrôle. On utilise 2 tubes par dilution. L'incubation à 37°C dure 5 à 7 jours. Pendant ce laps de temps, les cellules se sont multipliées et ont formé des colonies visibles à l'oeil nu. La diminution du nombre des colonies, par rapport au contrôle, traduit l'activité cytostatique du produit testé.

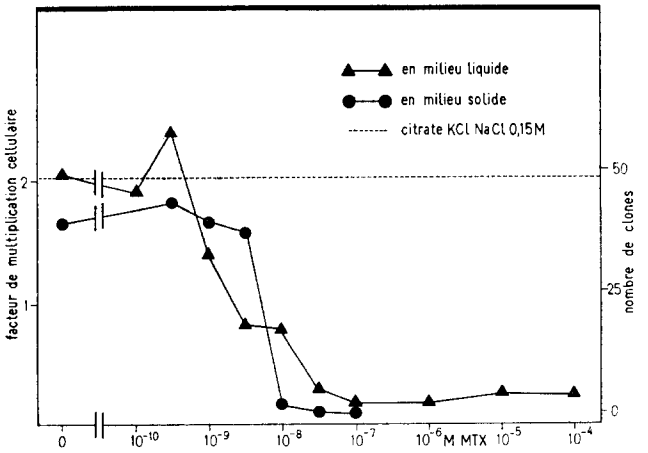

Fig. 1. *Effet cytostatique du Méthotrexate (MTX) sur les cellules du mastocytome P-815-X2. Le facteur de multiplication cellulaire (rapport du nombre de cellules par ml avant et après incubation) a été mesuré après une incubation de 24 h à 37°C (deux tubes par dilution contenant 93.000 cellules/ml). Le tampon citrate-KCl–NaCl 0,15 M sert de contrôle. Le nombre de clones a été compté après une incubation de 5 à 7 jours dans des tubes contenant 50 cellules dans un milieu gélifié par l'apport de fibrinogène (deux tubes par dilution). L'eau physiologique stérile sert de contrôle. En abscisse sont indiquées les concentrations molaires de MTX.*

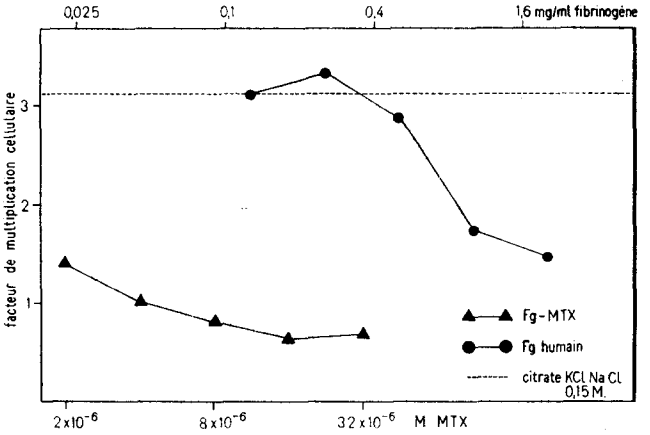

Fig. 2. Effet cytostatique d'un conjugué MTX-azofibrinogène humain (Fg.MTX) sur les cellules du mastocytome P-815-X2 après une incubation de 24 h à 37°C (trois tubes par dilution, contenant 115.000 cellules par ml). Le fibrinogène humain et le tampon citrate-KCl–NaCl 0,15 M servent de contrôles. En ordonnée est indiqué le facteur de multiplication cellulaire. L'abcisse supérieure indique les concentrations de la protéine en mg par ml, l'abcisse inférieure les concentrations molaires du MTX introduit dans le milieu cellulaire par le conjugué MTX-azofibrinogène (15 moles de MTX par mole de protéine).

RESULTATS

(a) *Effets cytostatiques du Méthotrexate et du Ro4-9374/1*

Le Méthotrexate (MTX) dissous dans le tampon citrate-KCl–NaCl 0,15M, pH 7,4, stérile, exerce, après une incubation de 24 h à 37°C, un fort effet cytostatique sur les cellules du mastocytome P-815-X2. Il est exprimé par un facteur de multiplication cellulaire inférieur ou égal à 1 pour des concentrations égales ou supérieures à $3{,}3 \times 10^{-9}$M MTX (Fig. 1). Le tampon ajouté dans les mêmes proportions (0,1 ml de tampon pour 2 ml de culture) n'a aucune action inhibitrice sur la croissance cellulaire. En milieu gélifié, l'action du MTX est comparable puisque, après une incubation de 5–7 jours, pratiquement aucun clone ne s'est développé à des concentrations de 10^{-8}M (Fig. 1).

Le Ro4-9374/1, sous forme de bromhydrate, est actif en milieu liquide à partir d'une concentration de 10^{-3}M (Fig. 4b), le bromhydrate de sodium n'ayant aucun effet toxique [7].

(b) *Effets cytostatiques d'un conjugué Méthotrexate-azofibrinogène*

En milieu liquide. Sur la Fig. 2 est résumée l'action cytostatique d'un conjugué Méthotrexate-azofibrinogène (15 moles de MTX par mole de protéine) sur les cellules du mastocytome P-815-X2 après incubation de 24 h à

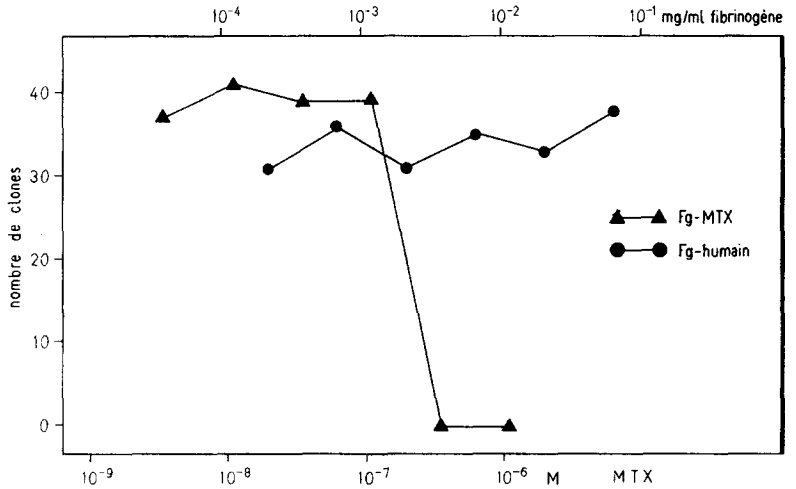

Fig. 3. Effet cytostatique d'un conjugué MTX-azofibrinogène humain (Fg-MTX) sur le développement de colonies de cellules du mastocytome P-815-X2 après 7 jours d'incubation à 37°C (deux tubes par dilution, contenant environ 40 cellules dans un milieu gélifié par l'apport de fibrinogène). Le fibrinogène humain sert de contrôle. En ordonnée est exprimé le nombre de clones; en abscisse supérieure, la concentration de la protéine en mg par ml; en abscisse inférieure, les concentrations molaires du MTX introduit dans le milieu cellulaire par le conjugué MTX-azofibrinogène (15 moles de MTX par mole de protéine).

37°C. L'azofibrinogène a un pouvoir inhibiteur aux dilutions correspondant à une concentration de Méthotrexate de 2×10^{-6}M. Le tampon seul n'exerce aucune action toxique sur les cellules tumorales. En revanche, le fibrinogène seul, à ces concentrations de 1 mg/ml, exerce une activité inhibitrice évidente sur la croissance cellulaire.

En milieu solide. Le même conjugué (15 moles de MTX par mole de fibrinogène) a un pouvoir cytostatique puissant sur les cellules du mastocytome P-815-X2 en milieu gélifiél. Après 7 jours d'incubation à 37°C aucune colonie ne s'est développée pour des concentrations de Méthotrexate de $3{,}3 \times 10^{-7}$M. Dans les tubes contrôles contenant le fibrinogène non conjugué, les colonies se développent et on peut dénombrer 31–38 clones de cellules tumorales (Fig. 3).

(c) *Effets cytostatiques de conjugués Ro*4-9374/1-*azoalbumine*

L'activité cytostatique de 3 conjugués à 6, 11 et 17 moles de Ro4-9374/1 par mole de sérum-albumine a été testée sur les cellules du mastocytome murin P-815-X2 en milieu liquide. Les résultats sont exprimés en mg par ml de protéine sur la Fig. 4a et en molarité du cytostatique libre et couplé sur la Fig. 4b. Le conjugué le plus lourdement chargé exerce l'activité cytostatique la plus intense (Fig. 4a). Il y a donc une relation entre le nombre de molécules de méthylhydrazine couplées à l'albumine et l'effet inhibiteur du conjugué azoalbumine. Lorsque ces résultats sont exprimés en molarité du cytostatique couplé à la protéine, et lorsque l'activité des azo-conjugués est comparée à celle du cytostatique libre, il apparaît que les conjugués exercent une activité inhibitrice environ deux fois plus intense que l'agent anticancéreux seul (Fig. 4b)

DISCUSSION

L'effet antinéoplasique de certaines azo-protéines est bien connu. Mathé *et al.* [8] ont décrit l'action curative de conjugués Méthotrexate-azo-immunoglobulines de hamsters sur la leucémie 1210 de la souris. Kidd [28] et Broome *et al.* [29] ont mis en évidence le pouvoir cytotoxique de conjugués arsenic-azoprotéines sur le lymphome 6C3HED de la souris.

Sur les cellules du mastocytome murin P-815-X2, plusieurs azo-protéines ont exercé une activité cytostatique. Un conjugué Méthotrexate-azofibrinogène a révélé une activité inhibitrice inférieure à celle du Méthotrexate seul. Lorsque la concentration du médicament introduit par le conjugué dans le milieu cellulaire n'est pas adéquate, celui-ci n'a pas une action inhibitrice différente de celle du fibrinogène seul: un conjugué Ro4-9374/1—azofibrinogène à 9 moles de méthylhydrazine par mole de protéine, reste inactif, parce que la concentration de la substance antitumorale, liée à la protéine vectrice, n'est que de 10^{-5}M, c.-à-d. bien inférieure à la concentration limite efficace de 10^{-9}M [7]. Il faut noter aussi que le fibrinogène seul, à de hautes concentrations, est capable d'inhiber *in vitro* la multiplication des cellules tumorales. Ce phénomène correspond probablement à l'inhibition exercée par le fibrinogène péritumoral dans le cas des tumeurs animales *in vivo* [4].

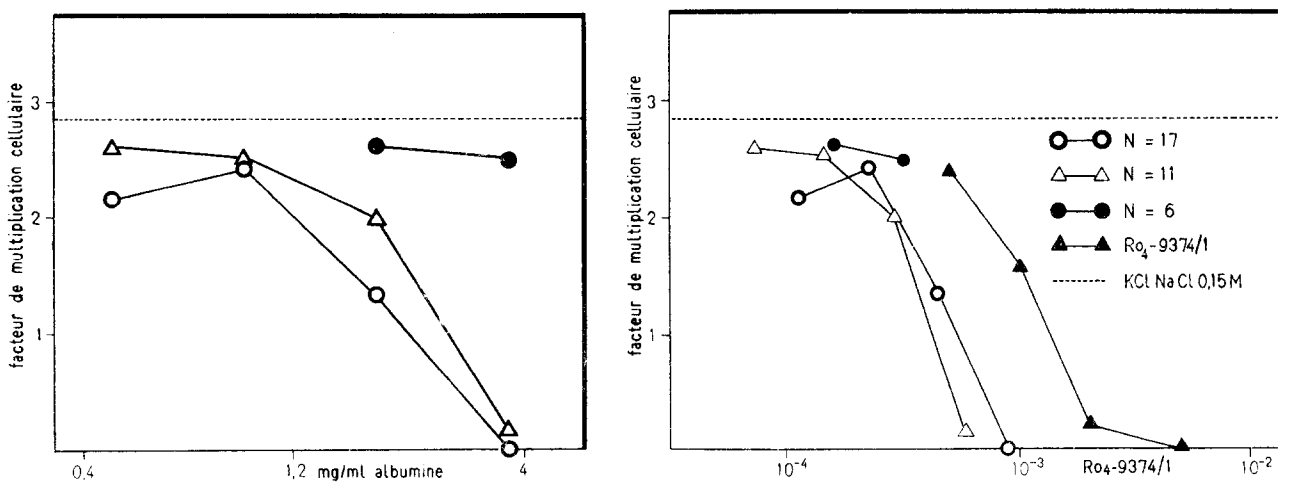

*Fig. 4. Effet cytostatique de conjugués Ro*4-9374/1-*azoalbumine humaine sur les cellules du mastocytome P-815-X2 après une incubation de* 24 *à* 37°C. *Trois conjugués à* 6, 11 *et* 17 *moles de cytostatique par mole d'albumine sont testés. Deux tubes par dilution sont mesurés, contenant* 113.000 *cellules/ml. Le facteur de multiplication cellulaire est exprimé en ordonnée. L'effet cytostatique des conjugués, en fonction de la concentration de la protéine (mg/ml), est indiqué dans la Fig.* 4*a, et en fonction de la concentration molaire du cytostatique libre, ou introduit dans le milieu cellulaire par les différents conjugués, dans la Fig.* 4*b.*

En ce qui concerne les conjugués azoalbumine, l'activité cytostatique est d'autant plus puissante que l'albumine est plus lourdement chargée en molécules de méthylhydrazine. En analysant les résultats sur une base molaire, on constate que ces conjugués se sont montrés environ deux fois plus actifs que le cytostatique libre.

Ainsi, lorsque les conjugués sont doués de propriétés antitumorales, celles-ci sont en rapport direct avec le cytostatique couplé, leur puissance dépendant du nombre de molécules fixées à la protéine vectrice. Des conclusions identiques ont été formulées par Broome *et al.* [29]: étudiant l'action antitumorale de la 4-arsono-phénylalbumine sur le lymphome 6C3HED, ils ont pu démontrer que l'activité antilymphome du produit est essentiellement due à l'arsenic, puisque le BAL (2,3-dimercapto-l-propanol) annihile complètement l'effet curatif du conjugué. La liaison axoïque en elle-même n'est pas délétère, un conjugué *p*-aminobenzoate-azoalbumine étant resté absolument sans effet sur la croissance cellulaire [7]. L'augmentation de l'activité de la méthylhydrazine Ro4-9374/1 par couplage à l'albumine serait compatible avec une accumulation intracellulaire accure du conjugué, et une perméabilité cellulaire plus élevée vis-à-vis de cette macromolécule.

La pénétration intracellulaire de protéines natives est actuellement très controversée. Différentes tumeurs animales [30–32], certains réticulosarcomes humains [33], la majorité des tumeurs de Burkitt [34], semblent doués d'un pouvoir de phagocytose. Busch *et al.* [3] pensent que l'albumine pénétrerait par pinocytose à l'intérieur des cellules tumorales sans hydrolyse extracellulaire préalable: une tumeur solide de rat pourrait capter, en 24 h, 1030 mg de protéines plasmatiques par gramme de protéines tumorales. Mais il se peut, comme le remarquent Cerottini *et al.* [6] que la très forte fixation des protéines radiomarquées par le tissu tumoral soit due surtout à une adsorption sur les cellules nécrosées. En effet, *in vitro*, l'estimation de Ryser [35, 36] est nettement inférieure: moins de 2,5 mg par gramme de protéines tumorales, par 24 h. En revanche, Cerottini *et al.* [6] n'ont pu mettre en évidence la pénétration intracellulaire de l'albumine fluorescente ou de l'albumine marquée à l'iode 125, ni dans le cas du mastocytome P-815-X2, l'intégrité de la cellule étant rigoureusement contrôlée. Les auteurs [6] soulignent néanmoins que l'introduction d'un groupe étranger sur une molécule de protéine peut entraîner une modification de la structure tertiaire suffisante pour provoquer la pinocytose, ce phénomène pouvant être en relation avec l'abaissement du point isoélectrique de la protéine conjuguée. Le fait d'ailleurs de ne pouvoir mettre en évidence, par les techniques habituelles, la présence de l'albumine dans une cellule n'est pas un critère de sa non-pénétration. On peut envisager que dans une cellule tumorale, le catabolisme de l'albumine est très accéléré, la molécule échappant ainsi à l'analyse. La découverte dans l'hépatome de Novikoff d'un système enzymatique mitochondrial capable de dégrader l'albumine [37] permet d'étayer cette éventualité.

Si la pénétration intracellulaire des azoprotéines n'est pas encore definie, le mode d'action des conjugués n'est pas élucidé non plus. En effet, on n'a pas pu déterminer si le conjugué pénètre tel quel dans la cellule ou s'il subit un certain degré de protéolyse dans le phago-lysosome, voire dans l'ambiance péricellulaire, comme le suggère la présence d'enzymes protéolytiques libérées par les cellules tumorales *in vitro* [38]. De plus, il ne peut être exclu que la liaison azoïque puisse être scindée par des réductases intracellulaires libérant l'agent cytotoxique. Bien que celle-ci semble échapper à une scission enzymatique [39], les travaux de Domagk [40] et de Tréfouel [31] sur les sulfamidés dérivés des colorants azoïques montrent que cette réduction dans l'organisme est possible. Les azodérivés des moutardes azotées aromatiques se rélèvent plus actifs lorsque la liaison azoïque est réduite [42].

Quoi qu'il en soit, il apparaît qu'*in vitro* des conjugués cytostatique-azoprotéines peuvent exercer un pouvoir antitumoral, en rapport avec le nombre de molécules d'agents anticancéreux couplés à la protéine vectrice. Dans certains cas, le conjugué se révèle plus actif que le cytostatique seul, suggérant une pénétration intracellulaire accrue, facilitée par le couplage du produit à une macromolécule. L'activité antitumorale *in vivo* de ces conjugués ne peut être extrapolée des résultats ci-dessus. Elle dépendra en particulier de la concentration intratumorale des conjugués et de l'activité épuratrice du système réticulo-endothélial [17, 18, 43].

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RESUME

Des conjugués azoprotéiques, préparés par couplage d'agents cytostatiques (améthoptérine et un dérivé méthylhydrazine) au fibrinogène et à l'albumine exercent, in vitro, *une activité antitumorale, déterminée sur les cellules du mastocytome murin P-815-X2. Certains ont montré une activité égale ou inférieure à celle de l'agent cytotoxique seul. D'autres se sont révélés plus actifs que le médicament libre. Ces résultats sont discutés selon les différentes acquisitions concernant la perméabilité des cellules tumorales aux protéines plasmatiques.*

SUMMARY

Conjugates of cytostatic agents (amethopterin and a methylhydrazine derivative) with azofibrinogen and azoalbumin have been prepared, and their inhibitory activity on cell multiplication has been determined in cultures of the murine mastocytoma P-815-X2. Certain conjugates had an activity equal or inferior to that of the free cytostatic agent, while others were more active. These results are discussed with respect to problems of tumour cell permeability to plasma proteins.

ZUSAMMENFASSUNG

Konjugate zwischen zytostatischen Substanzen (Amethopterin und ein Methylhydrazinderivat) und Azofibrinogen bzw. Azoalbumin wurden hergestellt, und ihre Hemmwirkung auf die Zellvermehrung in Kulturen eines murinen Mastozytoms wurde untersucht. Die Wirksamkeit einiger Konjugate was ähnlich oder aber geringer als diejenige der entsprechenden freien zytostatischen Verbindung. Andere Konjugate zeigten dagegen eine höhere Wirksamkeit als der freie Hemmstoff. Diese Ergebnisse werden hinsichtlich der Permeabilität von Tumorzellen für Plasmaproteine diskutiert.

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Factors Influencing the Induction of Tumours of the Urinary Bladder and Liver by 2-Acetylaminofluorene in the Mouse

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ARMSTRONG and BONSER [1, 2] demonstrated that the administration of 2-acetylaminofluorene (2-AAF) to mice of five different strains gave a wide variety of tumours, the incidence being dependent on the strain and sex of the animals.

Foulds [3] found that this carcinogen induced bladder carcinomas in male but not in female R III mice. Although the effects of strain, diet and hormonal balance on the number and distribution of tumours have been extensively studied in the rat after treatment with 2-AAF [4], little comparable work has been done in the mouse.

The purpose of the experiments described here is two fold: (1) to study factors influencing the carcinogenic action of 2-AAF in the mouse, (2) to investigate whether the early changes in the transitional epithelium can be used as a reliable indication that a particular group of animals will have a high probability of developing bladder cancer.

MATERIAL AND METHODS

Chemicals

2-AAF was obtained from Koch-Light Ltd., Colnbrook, Bucks. It was incorporated in powdered Oxo 41B diet at a level of 0·05% and compressed into pellets. In other experiments 2-AAF was suspended in corn oil at a concentration of 1% and 0·2 ml of this suspension administered to each mouse twice weekly by stomach tube in the long term experiment and 0·1 ml/day, 5 days/week in the short term investigations. Colchicine (1·0 mg/kg) was given in saline suspension by subcutaneous injection to certain mice 5 hours before death.

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Animals

Mice of the IF and R III strains were bred in the laboratory by brother–sister mating and were maintained on Oxo 41B diet and water *ad libitum*. They were weaned at 4–5 weeks and subsequently kept approximately 5 males or 5 females to a cage. They were used for experiment at about 12 weeks of age. The mice were examined daily and any found to be sick were killed. The bladder, after being distended with fixative, a piece of liver and any macroscopically abnormal tissue were fixed in Bouin's fluid and the bladder bisected sagitally.

Sections were stained with haematoxylin and eosin and one section from each piece of tissue examined histologically. Serial sections were cut from any lesions of doubtful significance.

The average degree of bladder epithelial hyperplasia for a group of mice was expressed as the hyperplastic index, calculated according to Clayson, Lawson, Santana and Bonser [5]. Five points were scored for a markedly hyperplastic epithelium consisting of 5 or more cell layers over all or an appreciable area of the surface; 3 points for a moderately hyperplastic epithelium consisting of 3 to 5 cell layers, and 1 point for an epithelium which was not normal, but which had not achieved moderate hyper-

Table 1. Results of administration of 2-AAF to RIII and IF mice

Treatment	Strain	Sex	No. of mice	Hyperplastic Index of bladder epithelium	Tumours* No. of mice dying in period 24 weeks	39–48 weeks	49 or more weeks	Tumours* Bladder Papilloma	Carcinoma I	Carcinoma II	Carcinoma III	Bladder Total	Liver Adenoma	Liver Hepatoma	Liver Total
2-AAF in diet (0·05%)	RIII	F	30	61	—	1B/10	3B/20	3	1	0	0	4	0	0	0
		M	34	58	—	0/8	3L+8B/26	3	2	1	2	8	1	2	3
2-AAF in diet (0·05%)	IF	F	32	63	—	9L+3B/22	8L+6B/10	5	4	0	0	9	5	12	17
		M	31	30	—	4L/20	5L/11	0	0	0	0	0	4	5	9
2-AAF by stomach tube (2×2 mg/ mouse/week)	IF	F	18	11	—	1L/9	0/9	0	0	0	0	0	1	0	1
		M	21	53	—	1B/10	2L+5B/11	4	1	0	1	6	0	2	2
2-AAF in diet (0·05%)+ castration	IF	F	22	63	—	2L/6	6L+4B/6	1	3	0	0	4	1	7	8
		M	24	42	—	3L+1B/6	7L+1B/7	1	0	0	1	2	2	8	10
Bladder pellet No 2-AAF	IF	F	24	46	—	1B/24	—	0	1	0	0	1	0	0	0
		M	17	56	—	0/16	0/1	0	0	0	0	0	0	0	0
Bladder pellet 2-AAF in diet (0·05%)	IF	F	13	92	0/3	3L+5B/10	—	0	3	2	0	5	2	1	3
		M	15	85	1B/3	6B/10	2L+1B/2	1	5	2	0	8	0	2	2
Controls 1 year or more of age	IF	F	31†	0	No bladder tumours								0	0	0
		M	42‡	0	No bladder tumours								1	0	1
Controls 1 year or more of age	RIII	F	15	0	No bladder tumours								0	0	0
		M	10§	0	No bladder tumours								0	1	1

L=Liver tumour.
B=Bladder tumour.
*Only most advanced lesion is listed for any tissue.
†One mouse with slight hyperplasia in the bladder.
‡One mouse with slight hyperplasia in the bladder and a high mitotic count.
§One mouse with extensive hyperplasia in the bladder and a high mitotic count.

plasia. Two points or 4 points were scored where there was felt to be some doubt into which category the hyperplasia should be classified. The total number of points for each group of mice was divided by the number of mice and multiplied by 20 to give a value on a scale of 0–100.

Operative techniques

Implantation of pellets into the bladder was carried out by the method of Jull [6] as modified by Allen *et al.* [7]. Bilateral oophorectomy was carried out using a lateral incision over each ovary. Bilateral orchidectomy was made through a lower abdominal mid-line incision.

Counts of mitotic figures

Mitotic figures were counted in the non-tumorous epithelium of two sections of each untreated or experimental bladder. There was a range of 900–3750 epithelial cells per section depending on the degree of hyperplasia. The differences in total numbers of epithelial cells were taken into account when comparing mitotic activity in various groups of mice.

RESULTS

Long term administration of 2-AAF

In Table 1, bladder carcinomas are graded according to their degree of invasion of the bladder wall i.e. grade I has invaded sub-epithelial connective tissue only, grade II has spread into muscle and grade III has grown out through the muscle into adventitia or adjacent organs. Liver tumours are listed as adenoma (benign) or hepatoma (malignant).

There were no bladder tumours in the untreated mice aged 1 year or more (Table 1). One of the IF mice and 1 of 25 RIII mice developed liver tumours.

Female IF mice to which 2-AAF had been administered in the diet developed both bladder and liver tumours whereas the male mice developed only liver tumours (Table 1). The most advanced lesion seen in the bladder of the male mice was a moderate degree of hyperplasia. The difference in the incidence of bladder tumours between male and female mice was statistically significant ($P=0\cdot001$). The female mice were also more susceptible to the induction of liver tumours. The RIII strain under the same treatment gave bladder tumours in both male and female mice and liver tumours in the males only. The RIII mice were less susceptible to the induction of liver tumours than the IF mice. In the IF strain the female was more susceptible than the male whereas in the RIII strain the male was slightly more susceptible to the induction of tumours of both the liver and bladder.

When 2-AAF was administered to IF mice by stomach tube, bladder tumours were found only in the males and the bladder epithelium of the female mice was, with a few exceptions, in an apparently normal state. The liver tumour incidence in these mice was much less than in those given 2-AAF in the diet because the amount of the chemical administered by stomach tube (4 mg/mouse/week) was much reduced from the amount given in the diet (14 mg/mouse/week). The difference in the incidence of bladder tumours between male and female mice is significant ($P=0\cdot01$).

Feeding 2-AAF in the diet to castrated male and female IF mice reduced the difference in susceptibility to bladder tumours. Four tumours were found in 12 female and two in 13 male castrates surviving for 40 weeks or more. The difference in susceptibility to liver tumours was likewise reduced by castration, the incidence in male mice being increased ($P=0\cdot005$).

The implantation of a plain paraffin wax pellet into the lumen of the IF mouse bladder gave only one carcinoma in 24 female mice after 40 weeks and none in an equivalent group of 17 male mice although moderate hyperplasia was found in most mice of each sex. Combination of the pellet in the bladder with the feeding of 2-AAF in the diet led to an enhanced incidence of carcinomas in both sexes, the final incidences being 38% in the females and 53% in the males. In one other male there was a transitional cell carcinoma of the renal pelvis. Liver tumours were also observed in this experiment but their incidence was lower than when treated by chemical alone becase few of the animals survived beyond 48 weeks.

Histology

The bladder epithelium of the majority of the experimental mice showed moderately well developed areas of hyperplasia, with patches of apparently normal bladder epithelium. The hyperplastic reaction was seen both in bladders with tumours and in tumour free bladders. There were no instances of tumour formation without accompanying hyperplasia. The presence of a paraffin wax pellet in the bladder induced a generalised confluent hyperplastic reaction, which was further accentuated when 2-AAF had been given. Epithelial mitotic figures were more frequent (6 to 20 fold the normal count) when a pellet had been present in the bladder. In mice without a pellet there

Table 2. Comparison of degree of hyperplasia and number of mitoses/section in the bladder epithelium of IF mice treated with 2-AAF (0·05%) in the diet for limited periods with and without survival to 40 weeks

Weeks on diet	Sex	Killed immediately No. of mice	Hyperplastic index	Mitoses/ sect		Killed at 40 weeks No. of mice	Hyperplastic index	Mitoses/ sect
2	F	5	36	1·9		1	20	0
	M	5	52	0·9		3	7	0
4	F	3	27	1·66		3	0	0
	M	6	37	1·83		3	0	0
8	F	10	30	1·1		7	6	0
	M	10	26	0·45		9	16	0·06
12	F	10	18	0·35		6	10	0
	M	10	18	0·45		5	0	0·1
16	F	9	40	0·76		6	10	0
	M	9	20	0·16		8	8	0·06
20	F	10	32	0·5		3	13	0
	M	8	30	0		10	14	0·05
Untreated 12–23 weeks old	F	9	0	0·06	Untreated 1 year or more of age	29	0	0·08
	M	17	0	0·03		25	0	0·08

was no correlation between the number of mitoses and degree of hyperplasia. Mitotic figures were found in all layers of the epithelium in both untreated and treated mice (Fig. 1), unlike other epithelia e.g. epidermis and intestine where only the basal layer of cells divide.

There was a tendency for 2-AAF to induce papillomatous tumours in bladders without a pellet but simple papillomas were relatively uncommon, most papillomas having invaded their own connective tissue core or the sub-epithelial connective tissue (Fig. 2). There were also sessile atypical areas in the epithelium, 4 of which were classified as carcinoma *in situ* (Fig. 3). It is of interest that all 4 of these lesions were not associated with any other area of more advanced cancer formation and resembled the similar condition described in man [8]. Carcinomas were all of transitional cell type (Fig. 4), many having originated in papillomas becoming invasive at their base.

A mild degree of chronic inflammation was a feature of most treated bladders, being more marked in those with an implanted pellet where infiltration of polymorphonuclear leucocytes was also found.

Effect of 2-AAF for limited periods, with and without a subsequent period of recovery

Groups of approximately 20 male and 20 female mice were maintained on 2-AAF in the diet for periods of 2, 4, 8, 12, 16 and 20 weeks. At the end of these times half of the mice in each group was killed and the remainder allowed to survive until 40 weeks. No tumours were found in any of these mice although a small area of carcinoma *in situ* was seen in a male bladder after 20 weeks treatment. This was unusual considering the mild response to long term treatment (Table 1). The degree of hyperplasia and the incidence of mitoses in animals killed immediately after the chemical had been given were compared with those in mice allowed to survive to 40 weeks (Table 2). Colchicine was not administered in these experiments. Mild hyperplasia was found in both male and female mice to an equal extent in the mice killed at the end of the dietary treatment, but had for the most part regressed after the recovery period. Similarly the mitotic acitivity after 2, 4 and 8 weeks treatment was raised above that in the normal bladder and after 40 weeks had regressed to within normal limits. Between 12 and 20 weeks, although there was hyperplasia the number of mitoses was near the untreated level. There was little difference in the hyperplastic or mitotic response of male and female mice.

Short term response of the IF mouse bladder to 2-AAF in the diet or by stomach tube

Groups of approximately 10 male and 10

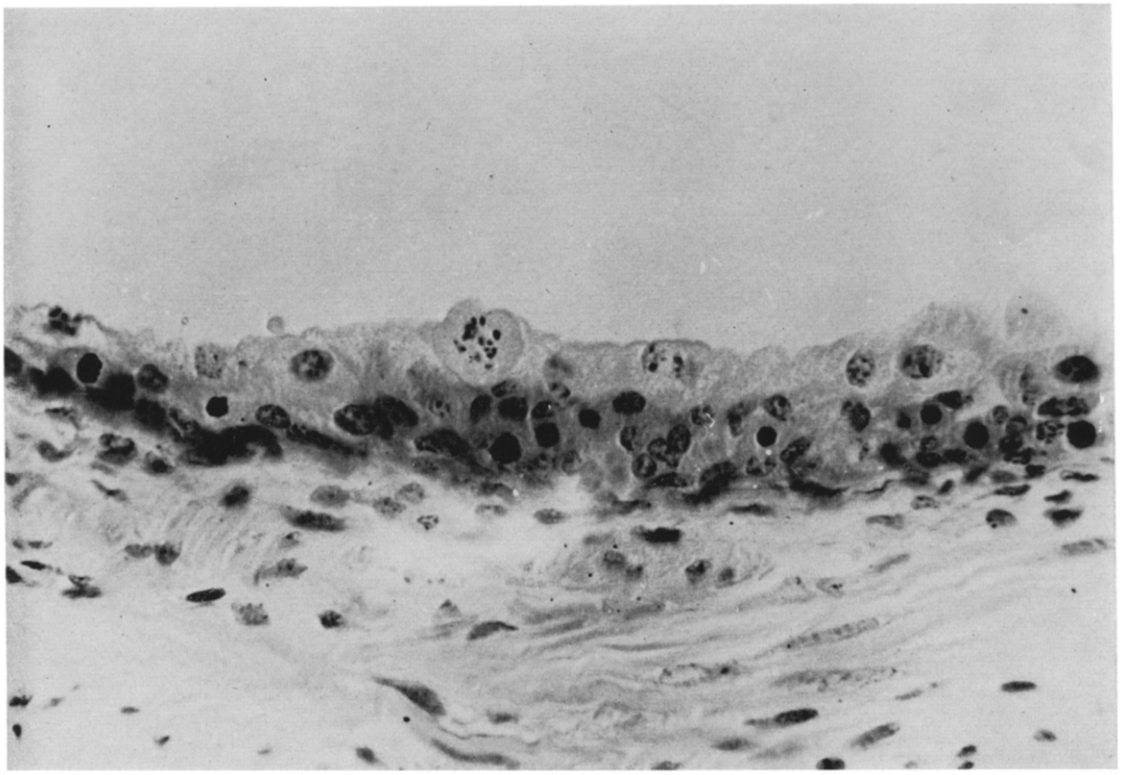

Fig. 1. *Unusually high mitotic activity in the bladder epithelium of a mouse treated only with colchicine. Mitotic figures are seen in all layers of the epithelium, including a large surface cell.* (×400)

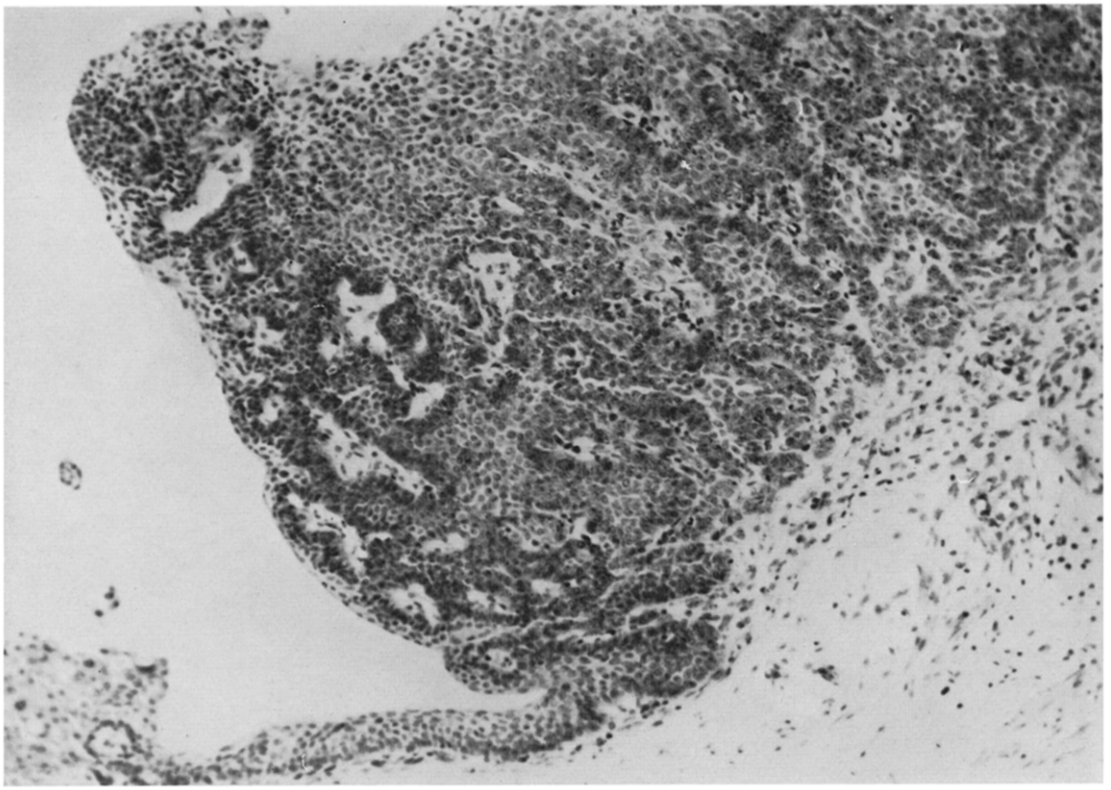

Fig. 2. *Large, solid, transitional cell papilloma of the bladder with incipient invasion of the connective tissue at the base.* (×100)

(to face p. 44)

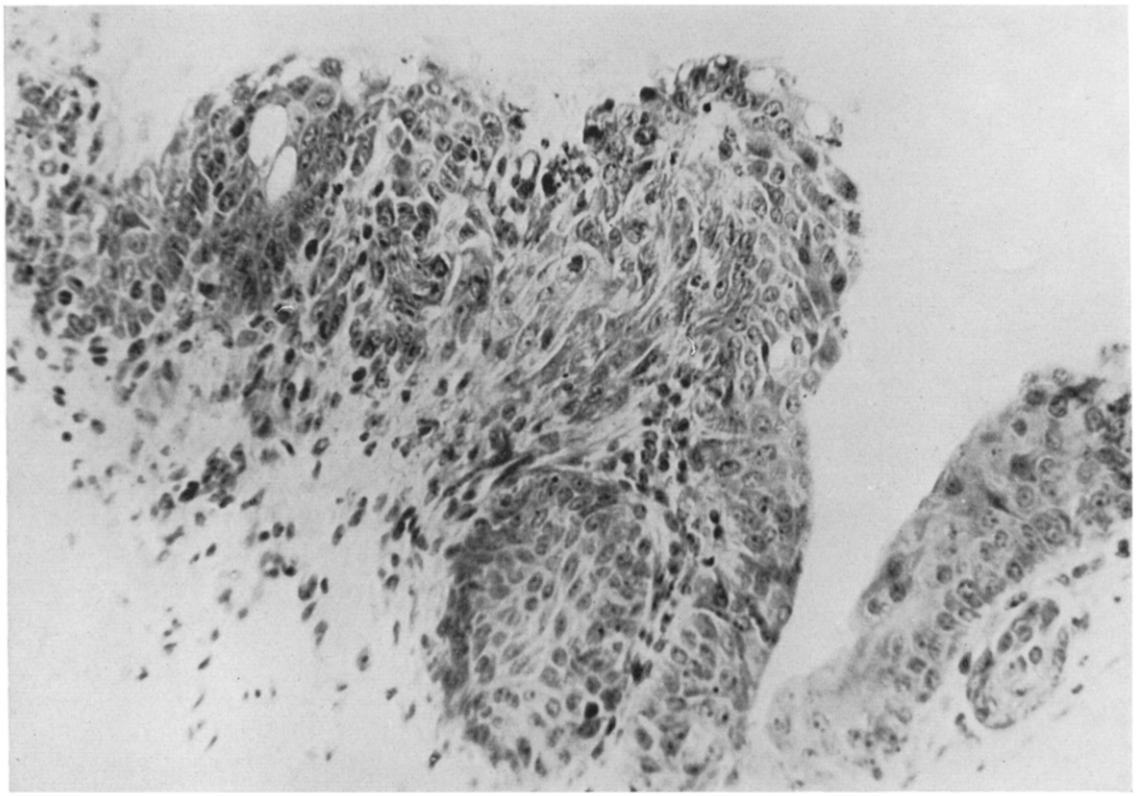

Fig. 3. *Carcinoma* in situ—*a disorganised area of epithelium with mitotic activity and early extension into the connective tissue.* (×250)

Fig. 4. *Grade II, transitional cell carcinoma invading into muscle.* (×100)

Table 3. The effects of short term administration of 2-AAF to IF mice by two different methods (male and female results are combined)

	2-AAF given by stomach tube (4 mg/week)				2-AAF given in the diet (14 mg/week approx)			
	Weeks of treatment	No. of mice	Bladder Hyperplastic index	No. of mitoses per section	No. of mice	Bladder Hyperplastic index	No. of Mitoses per section	
Without colchicine	1	6	0	0	9	0	1·5	
	2	11	0	1	10	16	0·45	
	3	13	0	1	10	10	0·68	
	4	5	0	0·5	10	18	0·1	
With colchicine	1	9	0	0·44	10	0	1·4	
	2	10	0	4·5	10	14	0·78	
	3	8	0	5·6	10	18	0·6	
	4	8	5	0·5	10	16	0·26	
Controls								
Untreated mice		64	0	0·05 Without colchicine				
+those given corn oil only		80	0	0·13 With colchicine				

female IF mice were given 2-AAF in the diet or by stomach tube for periods of 1, 2, 3 or 4 weeks. The last dose by stomach tube was given in the morning of the day of killing. Untreated mice and a group given corn oil alone by stomach tube were used as controls. Half of the mice were given colchicine 5 hours before death. Bladder sections were examined for epithelial hyperplasia and mitotic figures. There was little hyperplasia in the bladders of any of these mice, rather more occurring when the chemical was given in the diet than when it was administered by stomach tube (Table 3). In all groups there was an increased incidence of mitotic figures over the normal, this being most marked in those mice given the chemical by stomach tube. In the mice given the chemical in the diet, the highest level of mitotic activity occurred after the first week whereas, in those given the chemical by stomach tube, the peak in activity was found to be in the second and third week. Within the limits of the number of mice used, both the hyperplasia and the number of mitoses per section in colchicinised mice was higher in female than in male mice.

DISCUSSION

It has been shown that the induction of tumours by 2-AAF in the mouse is influenced by the strain of mouse; the method of administration of the chemical; the hormonal balance of the mice and the presence or absence of a pellet in the bladder. Foulds [4] gave this chemical, in the diet, to RIII mice for 25 weeks and allowed the animals to survive for a further 14 weeks or more. He failed to obtain any carcinomas of the bladder epithelium in his female mice. In the present experiments the feeding of 2-AAF to the RIII strain was prolonged up to 60 weeks, by which time it was found that although bladder tumours occurred mainly in males, some cases were seen in females. Armstrong and Bonser [3] gave 2-AAF by stomach tube to IF mice and found a preponderance of bladder tumours in males. In my experiments in which mice were killed before 60 weeks, rather than being allowed to live until 80 weeks or more, no tumours were found in females, although there was a 29% incidence in males. However, when a greater amount of the chemical was given in the diet the sex ratio of the incidence of bladder tumours was reversed, and no tumours were found in any of the 31 surviving males. The difference in the response of the bladder epithelium of the male and female mice between the two methods of administration of the chemical is statistically highly significant, but is difficult to explain. Castration of the animals followed by feeding the chemical in the diet led to the induction of bladder tumours in male as well as in female mice.

The incidence of hepatomas was highest when the chemical was administered in the diet, presumably because of the greater quantity of chemical given by this route. The liver in the female was more susceptible than that of the male when the chemical was given in the diet and this difference was reduced by castration.

Clayson, Pringle and Bonser [9] showed that 2-aminodiphenylene oxide in the diet induced bladder tumours in male but not in female C57×IF mice. The female succumbed at an earlier age to hepatomas. Nevertheless, when

a limited amount of this chemical was given with the adjuvant effect of a pellet in the bladder a similar incidence of tumours was found in both sexes. Implantation of a pellet into the bladder followed by the administration of 2-AAF in the diet gave equivalent yields of tumours in both male and female IF mice in the present experiments. The sex difference when the chemical was administered in the diet may be due to variation in the production of the proximate carcinogen, or it may be due to a direct influence of the sex hormones on the bladder epithelium. The traumatic effect of the pellet on the bladder epithelium [10], however, over-rides this difference.

The bladder of IF mice treated with 2-AAF for up to 20 weeks appeared to recover completely if the mice were allowed to survive without further treatment. The majority of the bladders appeared histologically normal in these surviving mice. This observation is of particular interest as in the RIII strain, Foulds gave 2-AAF in the diet for only 25 weeks, and then obtained carcinomas of the bladder after a further 14 weeks without treatment.

Despite the fact that only the female IF mice developed bladder tumours after 40 weeks or more on 2-AAF diet there was little consistent divergence between the sexes (Table 2) in either the hyperplastic index or the number of mitoses per section over the first 20 weeks. It therefore follows that the response of the bladder epithelium of male and female mice to the chemical in the diet must diverge at some period between 20 and 40 weeks.

Clayson *et al.* [5] suggested that there was a positive correlation between the degree of hyperplasia in the mouse bladder epithelium between 1 and 12 weeks and the ultimate development of malignancy in response to chemical carcinogens. The present results do not confirm this. The recovery experiments (Table 2) show little difference in the hyperplastic response of the bladder epithelium after the administration in the diet despite the fact that only the female mice developed tumours. Furthermore in both the stomach tubing and the dietary experiments terminated at 4 weeks, there is again little difference in hyperplastic response between the sexes and where the chemical was given by stomach tube there was little, if any, hyperplasia in the bladder epithelium in either sex before the fourth week. On the other hand, there was a considerable increase in the number of mitoses per section from the very low level normally found in the mouse bladder (0·05 mitoses per section without colchicine and 0·13 mitoses per section with colchicine); from which it can be inferred that the turnover rate of the epithelium must have been faster than normal, but new cell production was counter balanced by cell loss so that no hyperplasia developed. It would therefore appear that the explanation of the results of Clayson *et al.* [5] lies in the fact that the bladder carcinogens induce epithelial cell division which may or may not lead directly to hyperplasia of the epithelium. The distinction between reparative cell proliferation and that related to the carcinogenic process has yet to be resolved. From these experiments it appears that considerable caution must be employed in attempting to correlate hyperplasia or an increase in the number of mitoses in the bladder epithelium during the early stages of chemical treatment with the ultimate development of bladder tumours.

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RESUME

L'administration de 2-acétylaminofluorène (2-AAF) aux souris des souches IF et RIII induisit des tumeurs de l'épithélium de la vessie et du foie. On a constaté que la fréquence des tumeurs dépendait de la souche et du sexe des souris, du moyen d'administration de l'AAF et de la présence, ou non, d'un corps étranger dans la vessie. On a aussi essayé de déterminer la durée minimale d'administration de l'AAF dans la nourriture qui amènerait l'induction des tumeurs de la vessie, mais des durées de l'administration qui montaient jusqu'à 20 semaines n'induisaisent, dans la souche IF, aucune tumeur avant 40 semaines. On a montré que, dans le cas de la souche précitée, il n'y avait pas de corrélation manifeste entre le degré d'hyperplasie dans les premières phases du traitement et le développement final de la malignité.

SUMMARY

The administration of 2-acetylaminofluorene to mice of the IF and RIII strains induced

tumours of the bladder epithelium and liver. The incidence of tumours was shown to depend on the strain of mouse, the method of administration of the chemical, the sex of the mice and whether or not there was a foreign body in the bladder. An attempt was made ta determine the mimimum period of administration of 2-acetylaminofluorene in the diet required for the induction of bladder tumours, but periods of administration of up to 20 weeks did not induce tumours before 40 weeks in the IF strain. It was shown that in these mice there was no apparent correlation between the degree of hyperplasia in the early stages of treatment and the ultimate development of malignancy.

ZUSAMMENFASSUNG

Die Verabreichung von 2-Acetylaminofluoren (2-AAF) an Mäuse der Stammlinien IF und RIII induzierte Geschwülste des Blasenepithels und der Leber. Die Abhängigkeit der Geschwulsthäfigkeit von der Stammlinie und vom Geschlecht der Mäuse, von der Verabreichungsweise der Versuchssubstanz und von der An- oder Abwesenheit von Fremdkörpern in den Blasen wurde festgestellt. Es wurde auch versucht die Minimalzeit der Verabreichung des AAFs zu bestimmen, die für die Geschwulstinduzierung erforderlich war, aber die Verabreichungsdauer von bis zu 20 Wochen (bei der Stammlinie IF) führten zu einem Latenzstadium von mindestens 40 Wochen. Es wurde auch festgestellt, dass es (bei der Stammlinie IF) keine eindeutige Wechselbeziehung zwischen dem Grad der in früheren Behandlungsperioden auftretenden Hyperplasie und der Entwicklung der Bösartigkeit gab.

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Recurrence of a Transplanted Leukaemia in Mouse Radiation Chimaeras Receiving Anti-Donor Isoimmune Serum*

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INTRODUCTION

THE GROWTH of transplanted and spontaneous leukaemias may be suppressed in mice receiving lethal whole-body irradiation and allogeneic haematopoietic cells, but the majority of these radiation chimaeras die from secondary disease [1–3]. The regression of leukaemia as well as the secondary disease is attributed to an anti-host reaction of allogeneic immuno-competent cells [4]. In non-leukaemic animals, the graft-vs-host reaction could be prevented or suppressed by injecting anti-donor isoimmune serum [5–9]. In view of these data, it was decided to investigate the possibility that in radiation chimaeras with a transplanted leukaemia the graft-vs-host reaction could be controlled by anti-donor serum in such a way as to eradicate the leukaemia without a lethal secondary disease. Results of the experiments to be presented in this paper show that such a treatment is likely to result in recurrence of the leukaemia.

MATERIAL AND METHODS

Female RF mice bearing a transplanted myeloid leukaemia (and controls not inoculated with leukaemia), 12–14 weeks old, were irradiated with 530 rad X-rays (midline dose) and received, 4–6 hr after irradiation, spleen and bone marrow cells obtained from C57BL mice. Irradiation constants: 250 kVP, 30mA, HVL 2·1 mm^3, field dia. 23 cm, target distance 55 cm. Cell suspensions were prepared and counted as described by van Bekkum and Vos [10].

The transplantable myeloid leukaemia used in this study is 100% fatal and involves spleen, bone marrow, and liver [11]. It originated in an irradiated RF mouse, and has been maintained in this strain for more than 70 transplant generations by intravenous injections of 10^4–10^6 cells prepared from enlarged spleens of leukaemic mice. In the work to be described, 10^6 leukaemic cells were intravenously injected into the mice on day −4 of the experiment (day 0 being the day of irradiation).

For 30 days post-irradiation the animals were supplied with Streptomycin or Kanamycin in drinking water (4 mg/ml) in order to prevent generalised infections with Gram-negative intestinal bacteria from interfering with the experimental procedures [12].

The experiment was terminated on day 100.

Isoimmune serum was prepared as follows. Adult RF mice received 4 injections of C57BL spleen, thymus and lymph node cells at weekly intervals, and were exsanguinated 10 days after the last injection. Approximately 100×10^6 cells per mouse per injection were administered both intraperitoneally and subcuta-

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Table 1. The effect of injecting RF-anti-C57BL antiserum on the mortality of RF mice receiving 10×10^6 C57BL spleen cells after the irradiation with 530 rad of X-rays

Antiserum injected on days*	Total dose of antiserum (ml)	Mice dead within 100 days/ total No. of mice per group	Mean survival time (days)
4,5,6	0·3	0/8	>100
6,7,8†	0·3	5/6	22
—	—	10/10	16
Controls, irradiated only		0/6	>100

*Day 0 being the day of irradiation.
†RF haematopoietic cells and blood were injected as well (on days 8, 12 and 15), in the following average daily doses per mouse: bone marrow 15×10^6 i.p., spleen cells 50×10^6 i.p., blood 0·5 ml i.v.

neously. The serum was sealed in glass vials, stored at −20°C, and administered by subcutaneous injection without further purification. The highest cytotoxic titer of this serum for C57BL peritoneal cells *in vitro* was 1:64, as determined by a technique described by Balner [13].

The diagnosis of leukaemia or secondary disease was based on autopsy and histology findings [11]. In short, leukaemia was considered to be present if post-mortem examination showed splenomegaly (150 mg and more) and massive infiltration of immature myeloid cells in the spleen and liver.

Chimaerism was assessed by the electrophoretic pattern of haemoglobin [14], whereby RF haemoglobin gives 2 bands and C57BL haemoglobin a single band. Blood for testing was collected from the orbital venous sinus 2 or more months after irradiation and injection of allogeneic haematopoietic cells. The average life span of mouse erythrocytes is approximately 60 days, thus if chimaerism was present at the time of testing a majority of the animal erythrocytes should have been of donor type [15].

RESULTS

The effect of anti-serum in non-leukaemic animals

RF mice receiving 10×10^6 C57BL spleen cells immediately after irradiation with 530 rad all died within 3 weeks. Without spleen cells, all mice irradiated with the same dose of X-rays survived more than 100 days (Table 1). Histological changes attributable to an acute graft-vs-host reaction [15] as well as a bone marrow depletion [16] were found in dead animals.

Eight mice were treated with 0·3 ml RF-anti-C57BL serum, administered in 3 equal doses of 0·1 ml each on days 4, 5 and 6 after irradiation and spleen cell injection. All 8 mice survived 100 days in good health. In another group, the same amount of antiserum was administered on days 6, 7 and 8, followed by injections of RF spleen and bone marrow cells and blood on days 8, 12 and 15 (Table 1). Isogeneic cells and blood were administered in order to possibly prevent a haematopoietic failure, which might occur due to suppression by antiserum of the transplanted haematopoietic tissue at the time when the radiation syndrome develops. Five of 6 recipients died, post-mortem examination showing a variable degree of anaemia, bone marrow depletion, and signs of graft-vs-host reaction. Death of these 5 animals was postponed, however, for approximately 1 week as compared with control mice not receiving antiserum, cells and blood. The surviving mouse, as well as the 8 mice from the former group, appeared to be reversals in that they were free from donor-type haemoglobin when tested by haemoglobin electrophoresis on day 60 post-irradiation.

The effect of antiserum in leukaemia-bearing mice

The treatment schedule described in the preceding section was applied in RF mice bearing myeloid leukaemia (Table 2).

Except for 4 leukaemic mice receiving 530 rads of X-rays only, and that died with leukaemia, all the other animals were injected with 10×10^6 C57BL spleen cells and 30×10^6 C57BL bone marrow cells following the irradiation. As reported previously [16] sublethally irradiated mice receiving allogeneic spleen *and* bone marrow cells do not succumb within 3 weeks post-irradiation as do mice receiving spleen cells only. These mice usually become chimaeras and develop chronic secondary disease terminating in death 1–2 months post-irradiation.

In animals which received anti-donor serum on days 4, 5 and 6 post-irradiation (8 mice), or on days 6, 7 and 8 followed by isogeneic haematopoietic cells and blood (6 mice), leukaemia almost invariably recurred. That is, 13 of 14 treated mice died with massive leukae-

Table 2. The effect of injecting RF-anti-C57BL serum on the fate of leukaemia-bearing RF mice receiving 10×10^6 *C57BL spleen cells and* 35×10^6 *C57BL bone marrow cells after the irradiation with* 530 *rad of X-rays*

Antiserum injected on days*	Total dose of anti-serum (ml)	Mice dead within 100 days/ total No. of mice per group	
		Leukaemia	Secondary disease
4,5,6	0·3	8/8	0/8
6,7,8†	0·3	5/6	1/6
26–38	1·0	2/7	5/7
—	—	5/16	9/16‡
Controls, irradiated only		4/4	0/4

*Day 0 being the day of irradiation.
†RF haematopoietic cells and blood were injected as well (on days 10, 13, 16 and 20) in the following average daily doses per mouse: bone marrow 15×10^6, spleen cells 75×10^6, lymph node cells 50×10^6 (all i.p.), blood 0·5 ml i.v.
‡Two mice surviving 100 days post-irradiation were in good health.

mic infiltration of the spleen and liver (Table 2). On the other hand, of 16 leukaemia-bearing mice receiving no anti-serum after irradiation and injection of allogeneic cells, 9 developed secondary disease and died without morphological evidence of leukaemia, 5 died with evident leukaemia and 2 mice survived 100 days in good health. The latter 2 animals were chimaeras as judged by haemoglobin electrophoresis on day 90. Thus leukaemia recurred in one-third of the controls, i.e. less frequently than in animals treated with anti-serum.

Of 7 mice where antiserum was injected every other day between days 26 and 38 post-irradiation (a total amount of 1·0 ml of anti-serum was divided in 6 doses of 0·15–0·20 ml each), 5 died with secondary disease, and 2 animals died with gross signs of leukaemia. The incidence of leukaemia and secondary disease were thus not different from that in the control group.

In the following experiment both the spleen cell dose administered after irradiation, and the amount of anti-serum, injected after spleen cell administration, were increased (Table 3). There was no difference in the mortality pattern between the mice receiving anti-donor serum and the controls, i.e. all animals died with secondary disease and none died with leukaemia. The survival time of mice injected with the smallest amount of anti-serum (0·75 ml) was comparable to that of the controls receiving no treatment after irradiation and spleen cells (Fig. 1), but larger amounts of antiserum (1·2 and 4·0 ml), paradoxically, precipitated mortality from secondary disease. In comparison to the controls not receiving anti-serum, the mice in the latter two groups died earlier and within a more limited time-interval. However, when compared by the *t*-test, the mean survival times of these groups did not significantly differ from the value of the group receiving no antiserum.

DISCUSSION

Experiments reported by Cole and Davis [6] indicate that anti-donor serum may produce an early tolerance between grafted cells and the host. Any other available evidence [5, 7–9, 17, 18] indicates, however, that the effect of antiserum must be explained by destruction of the graft. Thereby the chimaeric state is terminated, as evidenced by rejection of donor-type skin.

Data presented here also provide evidence

Table 3. The effect of injecting RF-anti-C57BL serum on the fate of leukaemic RF mice receiving 40×10^6 *C57BL spleen cells and* 35×10^6 *C57BL bone marrow cells after the irradiation with* 530 *rad of X-rays*

Anti-serum injected on days*	Total dose of anti-serum (ml)	Mice dead within 100 days/ total No. of mice per group	
		Leukaemia	Secondary disease
7,8,9	0·75	0/24	24/24
6–11	1·2		
6–17†	4·0		
—	—	0/8	8/8

*Day 0 being the day of irradiation.
†RF blood injected as well (on days 11 and 17), 0·5 ml i.v. per mouse per injection.

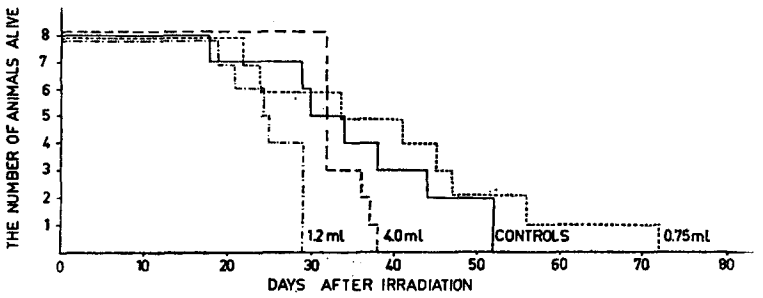

Fig. 1. Mortality of leukaemia-bearing RF mice irradiated with 530 rad, injected with 40×10^6 C57BL spleen cells and 35×10^6 C57BL bone marrow cells, and treated with RF-anti-C57BL isoimmune serum. The numbers indicate the amounts of antiserum injected. See Table 3 for details of timing of the treatment.

that anti-donor isoimmune serum is capable to terminate the chimaeric state. It is probable that the acute graft-vs-host reaction is initiated at about day 4 post-irradiation [19, 20]. Thus, when the treatment with antiserum was started early, i.e. 4 days after the recipients had been irradiated and injected with donor cells, death attributable to graft-vs-host reaction did not occur (but also the leukaemia was not sufficiently suppressed). In contrast, when the treatment with antiserum was started on day 6 post-irradiation, it failed to protect non-leukaemic recipients from death, only prolongation of the survival was attained (Table 1). Between days 4 and 6, therefore, damage produced by the graft-vs-host reaction has apparently become too advanced to be amenable to treatment. The same conclusion was reached by Davis and Cole [19], who showed that irradiated mice may survive an acute graft-vs-host reaction elicited with allogeneic spleen cells, provided that a second dose of irradiation followed by injection of isogeneic bone marrow was applied not later than on days 2, 3 or 4. A similar treatment did not prevent death when instituted on days 5 or 9 after the first irradiation.

In chimaeras bearing leukaemia and treated with antiserum leukaemia recurred (Table 2). This was probably due to the early suppression of the graft-vs-host reaction. However, when the number of allogeneic spleen cells injected after irradiation was increased, i.e. when the graft-vs-host reaction was possibly rendered more vigorous, all recipient mice died from secondary disease whether injected with antiserum or not. It was not possibly to adjust treatment with antiserum in such a way that the graft-vs-host reaction could eradicate leukaemia without killing the host. A tolerance resulting in stable chimaerism as achieved by Cole and Davis [6] could not be produced. However, the dose-schedule described by these authors was not followed precisely.

As described in a related paper [11] RF mice affected by myeloid leukaemia and treated with irradiation and allogeneic spleen cells may survive in good health provided that (a) cyclophosphamide was administered on days 4 through 15 post-irradiation, i.e. in the period of the acute graft-vs-host reaction, and that (b) chimaerism was artificially terminated by injecting isogeneic haemopoietic cells and blood after the cyclophosphamide course. Thus, a transient graft-vs-host reaction is evidently capable of eradicating this leukaemia without killing the host.

This result could not be achieved in the present experiments, where anti-donor serum was injected instead of cyclophosphamide in order to abrogate the secondary disease. The failure might be explained in terms of the following differences in action between cyclophosphamide and anti-donor serum. Cyclophosphamide is likely to have exerted an anti-leukaemic effect in addition to its effect on the haematopoietic graft. This could have compensated for the reduced anti-leukaemic effect of the graft-vs-host reaction [4, 11]. The dosage of cyclophosphamide which was used produced a depletion of the recipient's bone marrow and lymphatic tissues [11] and therefore probably facilitated the take of isogeneic haematopoietic cells, thus permitting termination of chimaerism.

An unexpected observation was that multiple injections of antiserum apparently precipitated mortality from secondary disease (Fig. 1). The following alternative explanations might be afforded for this seemingly paradoxical effect. (a) The graft-vs-host reaction, suppressed during treatment with antiserum, recurred when this treatment was discontinued, because the treatment failed to eliminate donor cells completely. (b) Antiserum treatment actually enhanced the graft's activity against the host. This speculation may find support in data published by Batchelor and

Howard [21] and Voisin *et al.* [22]. (c) Antiserum could have produced a temporary leukopaenia developing before intestinal damage caused by secondary disease had healed. Denudation of the intestinal mucosa combined with a generalised leukopaenia, might have resulted in infections with antibiotic-resistant intestinal bacteria [23].

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RESUME

Les souris RF, porteuses de la leucémie myéloïde greffée, ont été soumises à une irradiation par rayons X, à dose sublétale (530 *rads*), *suivie d'injection de* 10×10^6 *cellules de la rate C57BL, ou* $10\text{-}40\times10^6$ *cellules de la rate plus* 30×10^6 *cellules de moelle osseuse C57BL. Les animaux ont reçu de* 0,3 *ml à* 4,0 *ml de sérum RF-anti-C57BL, après irradiation et inoculation de cellules allogéniques aux jours* 4-6, 6-8, 6-11, 6-17, *ou* 26-38. *On leur a injecté occasionnellement, et à intervalles variables, des cellules RF de la rate el la moelle osseuse, ainsi que du sang. Dans un groupe de témoins non léucémiques, ce procédé peut prévenir ou retarder la mort provoquée par la réaction aiguë du greffon contre l'hôte. L'administration d'antisérum et de cellules isogéniques provoque une reprise de la leucémie chez les animaux leucémiques ayant reçu des injections de* 10×10^6 *cellules spléniques C57BL. La mort, due à la maladie homologue, a toujours été provoquée par des doses plus élevées de cellules de la rate, malgré l'administration d'antisérum. Il n'était pas possible d'accorder les temps d'administration et les doses de cellules spléniques et d'antiserum de façon telle que la réaction du greffon contre l'hôte, provoquée par les cellules de la rate, pût éliminer le leucémie en sauvegardant l'hôte.*

SUMMARY

RF mice bearing a transplanted myeloid leukaemia were subjected to sublethal X-irradiation (530 *rad*) *and were injected with* 10×10^6 *C57BL spleen cells, or* $10\text{–}40\times10^6$ *C57BL spleen cells and* 30×10^6 *C57BL bone marrow cells. At various intervals after irradiation and allogeneic cell injection* (*days* 4–6, 6–8, 6–11, 6–17 *and* 26–38) *the animals were treated with RF-anti-C57BL serum in amounts ranging from* 0·3 *ml to* 4·0 *ml. RF spleen and bone marrow cells and blood were occasionally injected as well. In non-leukaemic controls this treatment prevented, or postponed, death attributable to acute graft-vs-host reaction. In leukaemic animals receiving* 10×10^6 *C57BL spleen cells, treatment with antiserum and isogeneic cells resulted in recurrence of leukaemia. Higher doses of spleen cells invariably caused death from secondary disease in spite of the treatment with antiserum. It was not possible to adjust the timing of the treatment and the dose of spleen cells and antiserum relative to each other, in such a way that the graft-vs-host reaction elicited by spleen cells could eradicate the leukaemia without killing the host.*

ZUSAMMENFASSUNG

RF-Mäuse mit transplantierter myeloischer Leukämie wurden mit einer subletalen Röntgen-Dosis (530 *rad*) *bestrahlt. Nachträglich wurden ihnen* 10×10^6 *Milzzellen, oder* $10\text{–}40\times10^6$ *Milz- und* 30×10^6 *Knochenmarkzellen von C57BL-Mäusen injiziert. Je eine Gruppe von Versuchstieren wurde in verschiedenen Abständen nach der Bestrahlung* (4–6, 6–8, 6–11, 6–17, *oder* 26–38 *Tagen*) *mit RF-anti-C57BL-Serum* (0·3–3·0 *ml*) *behandelt. Gelegentlich injizierte man ihnen auch RF-Milz- oder Knochenmarkzellen.*

Die Seruminjektion verhinderte oder verzögerte den Tod durch 'Transplantat-Wirt'-Reaktion in nicht-leukämischen Mäusen. Die Behandlung von leukämischen Tieren mit 10×10^6 *C57BL-Milzzellen und später mit Antiserum und RF-Zellen führte zum Widerauftreten der Leukämie. Die Injektion grösserer Milzzellendosen verursachte den Tod wegen einer 'Transplantat-Wirt'-Reaktion, trotz der Behandlung mit Antiserum. Es war nicht möglich die Milzzelldosis und die Antiserumbehandlung so anzupassen, dass die mit Milzzellen induzierte 'Transplantat-Wirt'-Reaktion die Leukämie ausrotten könnte ohne gleichzeitig die Tiere zu töten.*

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Established Cell Line (ICI 101) Obtained from Frozen Human Lymphoblastic Leukaemia Cells: Morphological and Immunological Comparison with the Patient's Fresh Cells*

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INTRODUCTION

SEVERAL groups have attempted to establish continuous cell lines from human leukaemic cells, for immunological, cytological and virological studies. A certain number of cell lines have been obtained so far from fresh blood of patients with various forms of leukaemia [1–5].

We have set up in culture cells taken from a patient with acute lymphoblastic leukaemia, that had been stored at −70°C. A continuous tissue culture line has been established and maintained for six months. A comparative study of the morphology and immunology of these cultures cells and the original cells before freezing, has been made.

MATERIAL AND METHODS

A. *The cells*

The lymphoblasts were obtained from a child aged 10, coming from Reunion Island, who was admitted to the hospital in 1967, suffering from acute leukaemia. On admission he had generalized lymphadenopathy, enlarged tonsils and liver. After a period of remission he relapsed in September 1967, having a blood count of 336,000 leucocytes per mm^3, 82% being lymphoblasts. These cells were removed by repeated leukophoresis, mixed with DMSO and frozen to −70°C.

B. Setting up the cultures and maintenance of the line

Two months after freezing the cells were rapidly thawed by heating to 37°C in a water-bath and an estimate of their viability was made using the trypan blue staining technic. Three 250 ml cylindrical neutral glass flasks, with narrow necks, containing 40 ml RPMI 1640† tissue culture medium supplemented with 20% foetal calf serum‡ and containing 100 units of penicillin and 50 μg of streptomycin sulphate/ml, were inoculated with 150×10^6 viable cells.

A stationary culture system was used—the flasks were incubated at 38°C, and were only agitated for a few seconds twice a day.

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†Tissue culture medium RPMI 1640 with glutamine and without antibiotics was purchased at Grand Island Biological Co. (Grand Island, New-York 14072).

‡Foetal calf serum collected asceptically and sterilized by filtration was purchased at the SORGA establishment.

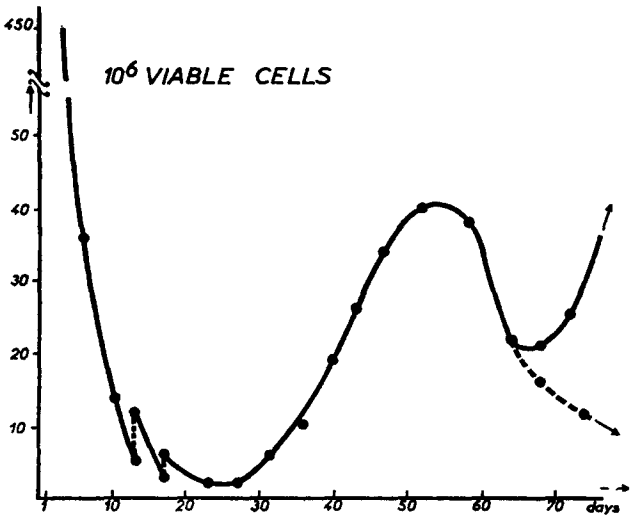


Fig. 8. *The growth of cells in culture as a function of time. After* 25 *days a marked decrease in the number of cells was seen. The cells line was definitively established from the* 6th *day.*

After a week in culture, without replacement of the medium cell counting and partial replacement of the medium was carried out every fourth day, and each time the concentration of the cells was adjusted to 5×10^5 per ml. During the first 25 days we observed a marked fall in the total number of viable cells and were obliged to pool the contents of the different flasks (Fig. 8).

The appearance of macroscopic cellular aggregates and of a marked acidity of the medium on about the 30th day indicated that the line had now become established in tissue culture. The multiplication of the cells has been followed for a month, and exponential growth was definitively established on about the 65th day (Fig. 9). Since then the line has been maintained by transplantation every 3rd day into cylindrical flasks, or fresh medium is added to the flasks to obtain a cell concentration of 3×10^5 cells/ml.

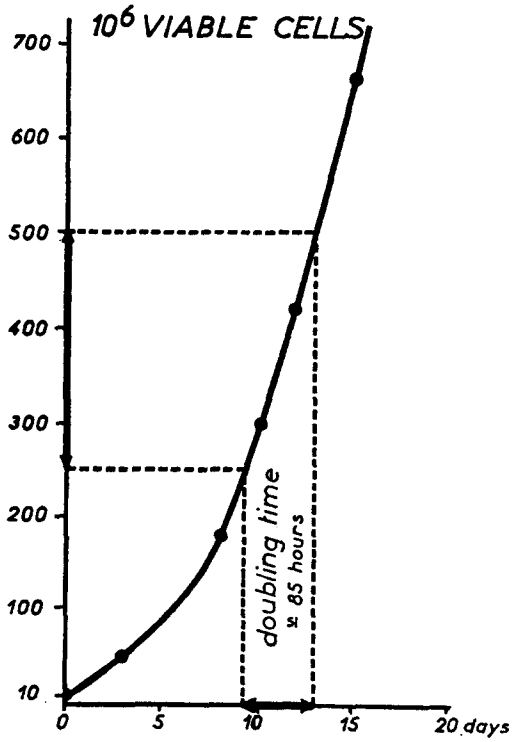


Fig. 9. *The growth of ICI* 101 *line cells as a function of time. The curve is exponential. The doubling time* in vitro *is about* 85 *hr.*

C. *Examination of cell smears*

Cell smears were made every month, and stained by the May-Grünwald-Giemsa method.

D. *Preparation for electron microscopy*

Buffy coat was obtained from the peripheral blood of the patient at the time of admission, by allowing heparinized blood to sediment for 2 hr at room temperature and then centrifuging. The buffy coat cells were aspirated into a fine polythene tube and fixed *in situ* [6]. The cells were fixed in 5% glutaraldehyde in cacodylate buffer for 2 hr and then in 2% osmic acid in Millonig's buffer; this method was also used to fix the cells after they had been in tissue culture for 3 months (they were centrifuged at 1500 rev/min. for 20 min prior to fixation). Dehydration and inclusion were effected using the Luft method, starting from 50% alcohol. The ultrathin sections were stained with saturated uranyl acetate for 2 hr and then with lead stain, using Reynold's method, for 5 min; the sections were examined in a Siemens Elmiskop I electron microscope.

E. Cytochemical tests

The following cytochemical examinations were made on the fresh cells from the patients' blood and the cells from the tissue culture: peroxydase reaction using the Graham and Knoll method, as modified by Quaglino and Flamans [7]; esterase reaction using the Wachstein and Wolf method [8]; PAS staining, using Hayhoe's technic [9]: and Sudan blue staining, using the Sheehan and Storey method [10].

F. *Study of the chromosomes*

A study was made of the chromosomes of the fresh cells of the patient and of the tissue culture line every month, using the de Grouchy and Lejeune technique [11–13].

G. *Immunological tests*

The antigenicity of the fresh cells and of the cultivated cells in the presence of the patient's

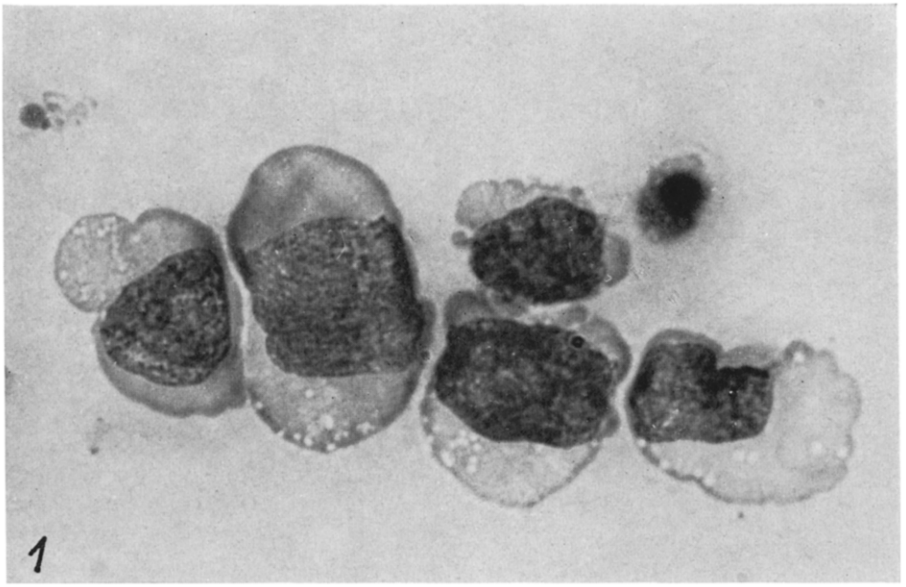
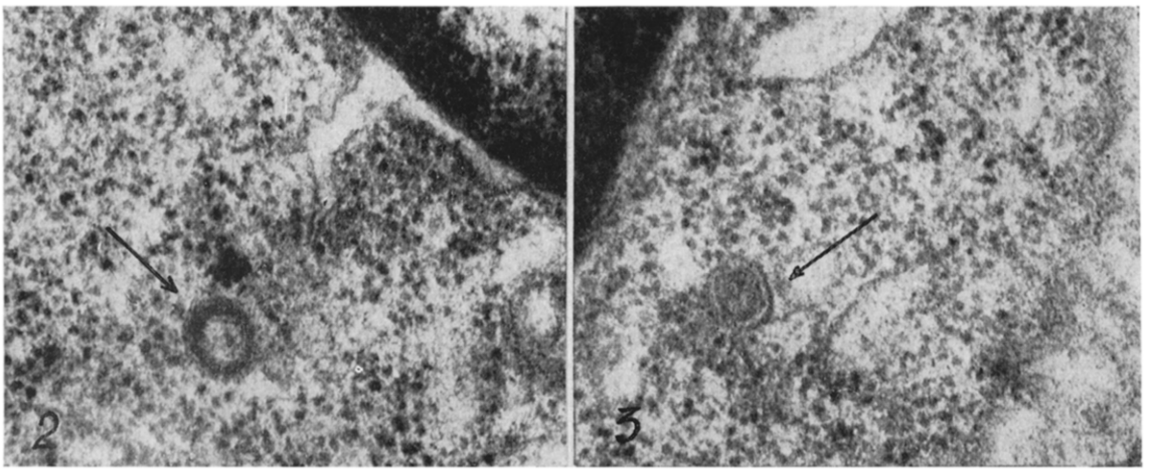


Fig. 1. *Morphology of the cells at the end of the third month. Stained by the May-Grunwald-Giemsa method.* (×1100)



Figs. 2 *and* 3. *Intracytoplasmic virus-like particles* (⟶) *in cells freshly isolated from the patient's blood. In* 2, *a double-walled particle with a clear centre. In* 3 *a particle with a double membrane and dense nucleus, resembling the C type murine particle.* (×65,000)

(to face p. 56)

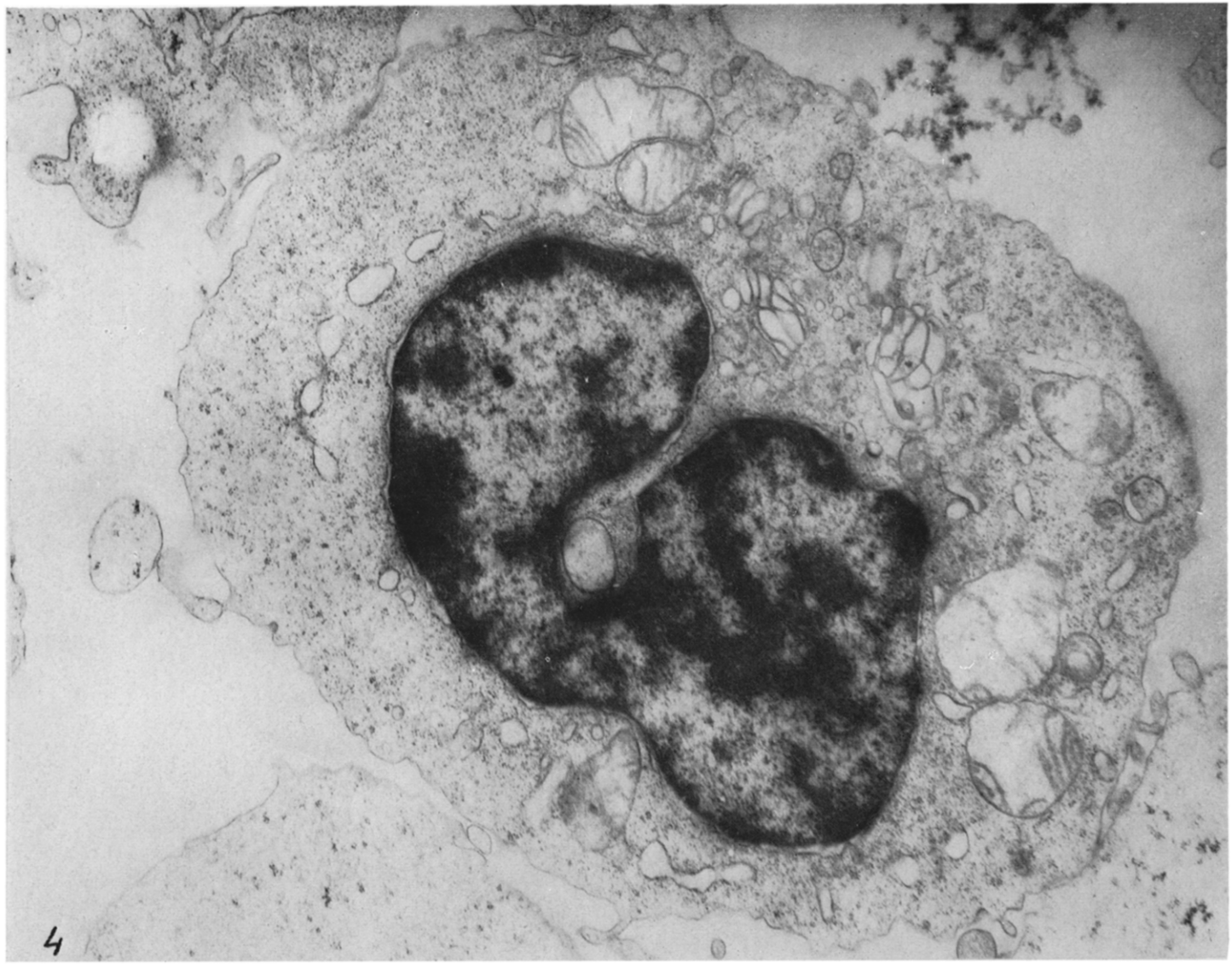
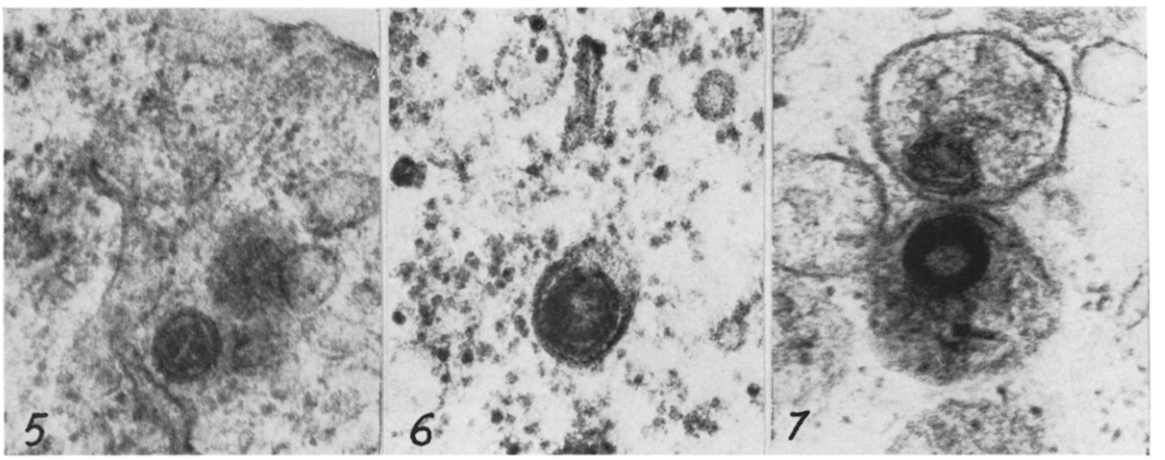


Fig. 4. Electron micrograph of the cultured cells. The cell is undifferentiated with an irregular nucleus, enlarged mitochondria of variable form, free ribosomes are uniformly dispersed.



Figs. 5, 6, 7. *Intracytoplasmic virus-like particles in the cells in culture. In* 5, *a particle with a dense 'mature' type of nucleus. In* 6 *and* 7, *immature particles with a clear centre looking like murine leukaemia virus particles.* (×65,000)

serum was studied comparatively. The serum sample that we used for this comparative study was taken and preserved at −70°C during a spontaneous relapse of the leukaemia. The following 3 tests were used Möller's immunofluorescence test, cytotoxicity test, and the test of immune adherence [14].

RESULTS

A. *Culture*

The kinetics of cell growth were of a classical exponential type, and had a doubling time in the order of 85 hr (Fig. 9).

B. Examination of the cells on smears

Smears prepared from the buffy coat of the patient showed a population of lymphoblasts without any special characteristics (Fig. 1). After three months of culture the cells were about twice the size of the original cells. The nuclei had irregular outlines and a fine, reticulated chromatin and contained one or two large nucleoli. Sometimes the nuclei were spread out and "like a flag", having a histioblastic appearance. The cytoplasm usually stained a sky-blue colour and nearly all the cells contained colourless inclusions. Only rarely a few azurophilic granules were seen; these cells resembled large, undifferentiated blast cells and have been observed in prolonged cultures of leukaemic cells [5, 15–17].

The characteristics were maintained, without change, during the course of several months.

C. *Cytochemistry*

This study did not produce any evidence of the difference between the original fresh cells and the lymphoblasts after they have been in culture (Table 1).

Table 1. *Cytochemical comparison between fresh lymphoblasts and the cells cultured for three months*

	Fresh blood	Culture
P.A.S.	+	+
Esterase	+	+
Peroxidase	−	−
Sudan Black	−	−

D. *Chromosome study*

A comparative study of the freshly isolated cells and those that had been maintained in culture showed that the karyotype of the fresh cells was normal whilst the cells in culture had differentiated from the fresh cells by a variation in their chromosomes number. There were no morphological differences (Table 2). The dominant mode was diploid, with an increased proportion of mitoses containing 45 chromosomes as well as the appearance of some hypotetraploid cells.

The examinations of the karyotypes of the mitoses with 46 chromosomes did not show any morphological abnormality. Many of the mitoses containing 45 chromosomes showed that the chromosome that was lost came from a variety of different chromosome groups. We have not observed, in any of the lines, the appearance of a marker chromosome [18].

E. *Examination of the cells by electron microscopy*

The freshly isolated buffy coat cells did not show any special morphological features; they looked like rounded lymphoblasts with small nucleoli and without any special inclusions. Nevertheless some intracytoplasmic particles, resembling murine leukaemogenic virus particles, were present in the cytoplasm (Figs. 2 and 3). The appearances of ultrathin sections of the cells after they have been 3 months in culture, are shown in Fig. 4. They are large cells without any characteristic cytoplasmic features which are indicative of differentiation; the nuclei are generally very irregular, containing one or two dense nucleoli. The cytoplasm contained relatively few and dispersed ribosomes and very few polyribosomes. Endoplasmic cisternae were numerous but short, widely spaced, and often smooth. The mitochondria were irregular and enlarged and sometimes contained filaments; their cristae were often short, leaving large clear spaces inside. The Golgi apparatus did not have any characteristics. Virus-like particles were also found in these culture cells (Figs. 5–7). No mycoplasma were seen, neither were any of the particles of the Herpes type which have been described by other workers in leukaemic cells that have been maintained for a long time in culture [2, 5, 15, 17, 19].

Table 2. *The number of chromosomes in mitotic figures present in the third month in culture**

No. of chromosomes	38	39	40	41	42	43	44	45	46	47	86	88	90	Total No.
No. of mitoses counted	2	2	1	1	2	5	6	7	13	3	1	1	1	45

*Each mitotic figure was photographed.

F. *Immunological study*

This comparative study of the fresh cells and the cultivated cells showed that the antigen, as demonstrated by the immune adherence test, on the fresh lymphoblasts, was also found in the culture originating from this line. It is noteworthy that the antigenicity of these cells in culture, with regard to the patients' serum, is greater than that of the fresh cells (Table 3)

Table 3. Immunological comparison between the fresh lymphoblasts and the cells after 3 months in culture

	Fresh blood	Culture
Immunofluorescence	−	++
Cytotoxicity	−	−
Immune-adherence	+ (1/64)	+++ (1/1280)

DISCUSSION

This work raises the following remarks.

(A) It was possible to establish a cell line (ICI 101) from deep-frozen cells; thus it appears that deep freezing to −70°C does not inhibit the viability of at least some of the stored cells. The morphology of the cells from this line is not different from the morphology of other cell lines obtained from fresh blood cells [1, 2, 5, 15, 17].

(B) The absence of any modification in the cytochemical reactions by comparison with the original lymphoblasts, suggests that the cells that have survived in the culture are the progeny of the circulating lymphoblasts from the patient's blood, and are not of granulocytic or other origin.

(C) We have found by the immune adherence test that the antigenicity of the cultured cells is similar to that of the fresh cells [14]. This antigenicity is much more marked in the cultured cells (immune adherence and immunofluorescence). We have as yet not fully elucidated this phenomenon, which may consist in unmasking the antigen as a result of culturing or freezing the cells.

(D) Finally, the detection of virus like particles in the cultured cells similar to those observed in the fresh lymphoblasts, and the demonstration of the antigenic activity of the cultured cells towards the patients' serum, indicate that these cells retain both viral material and antigenic 'marker'. This combination provides a valuable experimental cell line for *in vitro* studies on human leukaemia and for bio-assays.

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RESUME

Nous avons réussi à établir une lignée cellulaire permanente (ICI 101) à partir de sang congelé à −70°C, provenant d'un malade atteint de leucémie lymphoblastique. Nous avons fait une étude morphologique, virologique et immunologique comparative entre les lymphoblastes frais de malade et les cellules cultivées. Des particules virales évoquant celles des leucémies murines ont été détectées dans des lymphoblastes frais, et retrouvées dans les cellules à culture, à plusieurs reprises. Par ailleurs, l'activité antigénique des lymphoblastes frais vis-à-vis du propre sérum du malade, en adhérence immune, a été retrouvée sur les cellules de la lignée. Nous avons noté, de plus, que l'antigénicité des cellules en culture est plus importante que celle des fraîches (en adhérence et en immunofluorescence). Ce phénomène est à l'étude.

Cette lignée lymphoblastique ICI 101, ayant conservé son matériel viral et son marqueur antigénique, constitue donc un matériel de choix pour l'étude de la leucémie humaine. Enfin, l'obtention de cette lignée, à partir de sang congelé, démontre que la congélation des cellules originelles à −70°C n'est pas un obstacle à l'établissement de lignées continues.

SUMMARY

We have established a cell line (ICI 101) from blood taken from a patient with lymphoblastic leukaemia and stored at −70°C. A comparative morphological, virological and immunological study of this line and the fresh cells have been made. Virus particles, resembling murine leukaemia viruses, were detected in the fresh cells and found in samples from the cultures. The antigenic activity of the lymphoblasts, as demonstrated by immune adherence with the patient's serum, was present in the cultured cells. The antigenicity of the cultured cells was greater with the fresh cells (in immune adherence and fluorescence). This phenomenon is under study.

This lymphoblastic line (ICI 101) which has retained its virus content and antigenic marker, is an excellent material for the study of human leukaemia. Finally, the isolation

of this line from frozen cells shows that freezing of the original cells to −70°C is not an obstacle to the establishment of continuous cell lines.

ZUSAMMENFASSUNG

Ein permanenter Zellstamm (ICI 101) wurde aus dem Blut eines Patienten mit einer Lymphoblastenleukämie gezüchtet und bei −70°C aufbewahrt. Es wurde eine vergleichende morphologische, virologische und immunologische Untersuchung zwischen diesem Stamm und den Ausgangszellen durchgeführt. In beiden wurden Viruspartikel, welche den Viren von Geflügelleukämien ähneln, gefunden. Die Antigenaktivität zu dem Serum des Patienten war auch in dem Zellkulturen vorhanden. Sie was bei den frischen Zellen grösser als bei den kultivierten Zellen, ein Phänomen, das noch in Untersuchung ist.

Dieser Lymphoblastenstamm (ICI 101) der seinen Virusgehalt und Antigencharakter behalten hat, ist ein ausgezeichnetes Material für das Studium der menschlichen Leukämie. Die Gewinnung des Stammes aus gefrorenen Zellen zeigt, dass die Abkühlung auf −70°C die Ausbildung permanenter Zellstämme nicht verhindert.

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Some Biochemical Aspects of Leukaemias: Leucocyte Glutathione Metabolism in Chronic Granulocytic Leukaemia

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INTRODUCTION

IN A PREVIOUS study it was found that oxidised glutathione (GSSG*) occurred in the leucocytes of patient suffering from chronic granulocytic leukaemia, while abnormally low levels of GSH were detected in the leucocytes of patients who had received treatment with Myleran [1]. Several enzymic mechanisms could be responsible for these observations: the appearance of GSSG might be the outcome of deficiencies in one or more of the linked chain of enzymes responsible for reduction of the oxidised tripeptide, or alternatively to an elevation in the level of GSH peroxidase. While Roberts and Warwick have shown that Myleran can react with glutathione in the rat, it is unlikely that a similar mechanism accounts for the reduction in leucocyte GSH content following Myleran treatment in humans [2]: the dose administered would lead to an insufficient accumulation of drug in the leucocytes to permit the subsequent fall in GSH to be attributed to a stoichrometric deletion by myleran. It seems more reasonable to speculate that Myleran may: inhibit the activity of GSH-synthetic enzymes; repress their synthesis; activate γ-glutamyltranspeptidase or derepress its synthesis.

The relationships existing between the various enzymes involved are outlined in Fig. 1. GSH synthesis is catalysed by (i), γ-glutamylcysteine synthetase (EC 6.3.2.2.) and (ii) GSH synthetase (EC 6.3.2.3.), while degradation of the tripeptide is catalysed by (iii), γ-glutamyl transpeptidase (EC 2.3.2.a.). GSH oxidation is catalysed by (iv), GSH peroxidase (EC 1.11.1.a), while reduction of GSSG is catalysed by (v), GSSG reductase (EC 1.6.4.2.). Of the enzymes catalysing the reduction of NADP (vi), G6P dehydrogenase (EC 1.1.1.49) (vii) 6PG dehydrogenase (EC 1.1.1.43), and viii), isocit. dehydrogenase (EC 1.1.1.42) have been selected for study here.

In this paper we describe the results of experiments designed to investigate the possibility that the GSH-oxidoreduction may be impaired in leukaemic cells, and that myleran treatment may limit the function of the GSH-synthetic pathway in these cells.

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*The following abbreviations will be used throughout this paper: GSSG, oxidised glutathione; GSH, reduced glutathione; CySH, cysteine; CGL, chronic granulocytic leukaemia; iso cit., iso citrate; NADP, nicotinamide adenine dinucleotide phosphate; $NADPH_2$, nicotinamide adenine dinucleotide phosphate (reduced form); ATP, adenosine triphosphate; DTNB, 5-5′ dithiobis (2 nitrobenzoic acid).

MATERIAL AND METHODS

Chemicals

Reagent chemicals were obtained from Hopkin & Williams or British Drug Houses, AnalaR grades being used where available. GSH and GSSG were purchased from Sigma Chemical Co., and G6P, 6PG, isocit., $NADPH_2$

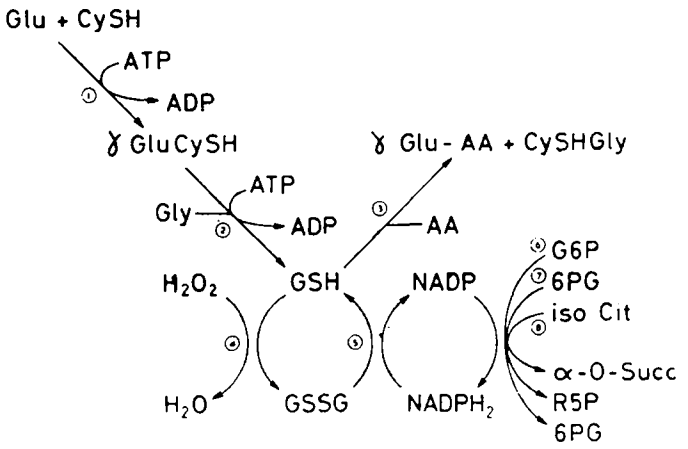

Fig. 1. *Metabolic pathways involved in the synthesis, degradation, and oxido-reduction of GSH.*

ATP from Boehringer Corporation (London). Glyoxylic acid was supplied by Koch-Light, and DTNB by Aldrich Chemical Co. γ-Glutamyl cysteine was prepared by the method of Gutcho and Laufer [3], or according to Sheehan and Yang [4]; in the former case the product contained 9% unchanged GSH, but no CySH.

Preparations of leucocyte enzyme extracts

Venous blood (generally 20 ml) was taken by venepuncture into heparin (10 units/ml whole blood) and the temperature adjusted to 0°C immediately. The bulk of the erythrocytes were removed by dextran sedimentation according to the procedure of Skoog and Beck [5], and the last contaminating erythrocytes in the leucocyte-rich preparation destroyed by 'osmotic shock' [6]. The cell concentration in the leucocyte preparation was measured in a Neubauer haemocytometer. Subsequent preparative manipulations varied according to the enzyme(s) studied. For the preparation of extracts of GSSG reductase, GSH peroxidase, 6 PG dehydrogenase, isocit. dehydrogenase, the leucocyte preparation was subjected to a 20 kc/s sonic oscillation for 2 min, and the suspensions centrifuged at 20,000 **g** for 20 min. The pellet was discarded. Extracts containing γ-glutamyl transpeptidase and γ-glutamyl cysteine synthetase (located in the nucleus) were provided by whole lysates of leucocyte suspension which had been subjected to an identical sonication procedure. For the assay of GSH synthetase it was necessary to eliminate interference from γ-glutamyl transpeptidase: sonication could not be adopted as a means of cell-rupture, since this technique damage the nuclei and released γ-glutamyl transpeptidase into the lysate. Instead, leucocytes, isolated by dextran sedimentation [5] and osmotic shock [6] were ruptured by the method of Cohn and Hirsch [7] being admitted to and expelled from a fine-bore needle (Gillette $25g \times \frac{5}{8}$) until phase contrast microscopy indicated the absence of intact cells (approximately six passages). The resultant suspension was centrifuged at 400 **g** for 10 min; the pellet, containing the nuclei (and virtually all the γ-glutamyl transpeptidase activity) was discarded, and the supernatant dialysed (to remove endogenous GSH and sucrose) against 2×200 ml of 0·01M phosphate buffer pH 7·0. The dialysed supernatant contained GSH synthetase and was devoid of γ-glutamyl transpeptidase activity. All preparative manipulations were conducted at 0–4°.

Enzyme assays

γ-glutamyl cysteine synthetase (i), was assayed according to the method described by Jackson [8], with the following modifications: (1) the final volume of assay mixture was 2·0 ml constituted as follows: 1·0 ml 0·1M triethanolamine HCl buffer (containing 0·25% v/v Triton X-100, 0·3M KCl and 0·02M $MgSO_4$), 0·42 ml distilled water, 0·50 ml leucocyte lysate from approximately $3{\cdot}0 \times 10^7$ cells, 0·04 ml 1·0M sodium glutamate, 0·02 ml 1·0M cysteine hydrochloride, and 0·02 ml 0·3M ATP. The mixture was incubated at 37°C for 120 min. Before and after incubation, 0·75 ml samples were transferred to 1·25 ml of ice-cold 3·2% sulphosalicyclic acid, the protein precipitate removed by centrifugation at 3000 **g** for 10 min, and the supernatant reduced electrolytically. Reduced supernatant in 0·5 ml quantities was added to 2 ml phosphate/ascorbate/glyoxalate assay buffer. After 5 min equilibrium at 60°C followed by re-equilibration to 25°C, 0·25 ml DTNB was added and E^{1}_{410} mμ measured, exactly 10 min

Table 1. Summary of the leucocyte composition of the peripheral blood of CGL patients

	Total WBC		% Immature granulocytes		% Blast cells	
	Mean	Range	Mean	Range	Mean	Range
I. Untreated	320,000	86,000–540,000	29	16–50	4	0–7
II. Treated* (in remission	15,000	5800–108,000	4	0–18	0	—
III. Treated* (in acute blast cell crisis)	79,000	6000–169,000	28	25–33	13	7–18

*Treatment of Group II patients was with Myleran (1–4 mg/day): Group III patients were in general treated with dibromomannitol at the time of sampling. (Prior to the development of blast-cell crisis they had been treated with Myleran.)

later, in Unicam micro cells on a SP500 spectrophotometer.

GSH synthetase (ii), was assayed according to the method of Jackson [8], modified as follows: the assay mixture included an aliquot of supernatant preparation containing about 8×10^7 cells and was incubated for 4 hr at 37°C. Initial and final samples were deproteinised by adding 1 ml assay mixture to 4 ml ice-cold 6·25% aq. metaphosphoric acid. Other stages in the procedure were identical to the erythrocyte method.

γ-glutamyl transpeptidase (iii), was assayed according to the method of Jackson [8] without modification: 5×10^7 cells were required for each determination.

GSH peroxidase (iv), was assayed by the method of Mills as modified by Harrap [9, 10], and *GSSG reductase* (v) according to Scott *et al.* [11]. *G6P dehydrogenase* (vi) and *6PG dehydrogenase* (vii) were assayed by the method of Shonk and Boxer [12]. *isocit. dehydrogenase* (viii) activity was measured by the method of Siebert *et al.* [13].

Unit of enzyme activity was defined as: μmoles substrate converted per 10^9 cells min at: 0°C for GSH peroxidase, 37°C for the GSH synthetic and degradative enzymes, 25°C for the remaining enzymes. Protein was measured in leucocyte lysates by the method of Lowry *et al.* [14].

RESULTS

Patients were classified into the following groups: CGL untreated, CGL treated (in remission), and CGL treated (in acute blast cell crisis). A broad summary of the haematologic data pertinent to these groups is provided in Table 1.

The cell fractionation procedure provided leucocytes showing negligible variation in protein content, either within or between the three groups of patients (see Table 2).

The activities of enzymes of the GSH oxido-reduction pathway in leucocytes from the four groups of donors are summarised in Table 3. Table 4 compares the levels of the GSH-synthetic and degradative enzymes in leucocytes from the four groups.

The possibility that Myleran may inhibit the enzymes of the GSH synthetic and degradative pathways has been investigated, and the results are summarised in Table 5. Human erythrocytes provided a convenient source for the synthetic enzymes, and Yoshida ascites sarcoma cells for γ-glutamyl transpeptidase. (The activity of the latter enzyme is very much higher in ascites cells than in leucocytes.) Only GSH synthetase was inhibited by Myleran (at drug concentrations of 2·5 μg/ml and above): the remaining enzymes were unaffected.

Table 2. Soluble protein content of leucocytes from CGL patients and healthy donors (controls)

Group	Sample size	Mean protein content mg/10^9 cells (S.E.)	
Controls	11	59·2	(3·4)
I. Untreated	9	54·2	(3·9)
II. Treated	11	57·9	(4·5)
III. 'Blast-crisis'	11	58·2	(4·2)

Table 3. Enzymes of the GSH oxido-reduction pathway in leucocytes

	Enzyme activity (units/10^9 cells)									
	GSSG Reductase		G6P dehydrogenase		6PG dehydrogenase		iso cit dehydrogenase		GSH peroxidase	
	No. samples	Mean (S.E.)	No. samples	Mean (S.E.)	No. samples	Mean (S.E.)	No. samples	Mean (S.E.)	No. samples	Mean (S.E.)
Controls	11	3·0 (0·2)	11	13·1 (1·4)	11	4·4 (0·4)	10	1·9 (0·2)	8	2·0 (0·1)
I. Untreated	9	2·9 (0·2)	9	18·8 (1·8)*	9	7·2 (0·5)†	9	2·4 (0·2)	8	1·4 (0·2)‡
II. Treated (in remission)	10	2·8 (0·3)	11	10·2 (1·0)	11	5·5 (0·4)	6	2·7 (0·3)	6	1·6 (0·3)
III. Treated (blast cell crisis)	8	3·0 (0·5)	8	12·4 (2·3)	8	5·8 (0·4)	8	2·3 (0·5)	8	2·5 (0·4)

*Significant: $0·02<P<0·05$.
†Highly significant: $P<0·001$.
‡Highly significant: $0·001<P<0·01$.

Table 4. Enzymes of GSH synthesis and degradation in leucocytes

	Enzyme Activity (units/10^9 cells)					
	γ-gluCySH synthetase		GSH synthetase		γ-glutamyl transpeptidase	
Group	No. samples	Mean (S.E.)	No. samples	Mean (S.E.)	No. samples	Mean (S.E.)
Controls	7	0·049 (0·007)	6	0·079 (0·005)	8	0·36 (0·02)
I. Untreated	5	0·048 (0·012)	4	0·087 (0·016)	5	0·36 (0·07)
II. Treated (in remission)	5	0·046 (0·003)	5	0·084 (0·013)	5	0·37 (0·04)
III. Treated (blast cell crisis)	4	0·045 (0·003)	3	0·086 (0·012)	3	0·32 (0·12)

Table 5. *Effects of Myleran* in vitro *on the activities of GSH synthetic and degradative enzymes*

	Relative activity (% 'uninhibited' enzyme)		
Myleran conc. (μg/ml incubate)	0	2·5	5·0
Enzyme			
γ-glutamyl cysteine synthetase	100	103	98
GSH synthetase	100	71	29
γ-glutamyl transpeptidase	100	99	96

DISCUSSION

The levels of enzymes of the GSH oxido-reduction pathway in the leucocytes of treated patients (groups I and II) were comparable with those found in healthy leucocytes. In the case of leucocytes from untreated patients, however, significant differences were noted for G6P dehydrogenase (44% raised), 6PG dehydrogenase (64% raised) and glutathione peroxidase (30% reduced) compared with the levels in healthy leucocytes. No change in the levels of GSSG reductase or isocit. dehydrogenase was detected in the leucocytes of untreated patients. Hence there is no deficiency of the apoenzymes involved in the reduction of GSSG, neither is there an excess of GSH peroxidase which might account for the accumulation of GSSG in these cells. However, sufficiency of apoenzyme does not guarantee the integrity or activity of the holoenzyme, which requires the specific binding of an exact quantity of pyridine nucleotide cofactor. It is known that abnormalities in the proportions of pyridine nucleotide cofactors occur in leukaemic cells, though what effect, if any, this altered distribution has on the binding of NADP(H), or the function of the holoenzymes of the GSH oxido-reduction pathway is unknown [15]. The present study has also established that Myleran treatment is without effect on the levels of the apoenzymes of the GSH oxido-reduction pathway in the leucocytes.

This paper presents the first comparative report on activities of GSH-synthetic and degradative enzymes in neoplastic and normal tissues. The levels of these enzymes were indentical in the leucocytes of healthy donors and patients suffering from CGL. Further, enzyme-activity was not modified by treatment. Hence the reduction in the GSH content of leukaemic leucocytes following treatment with Myleran cannot be attributed to a reduction in the cells' content of the synthetic enzymes, or to an increase in that of the transpeptidase.

It was necessary to eliminate the possibility that any effects exerted by Myleran on these enzymes *in vivo* would remain undetected due to dilution of the drug concentration during preparation of the enzyme extracts and the subsequent assembly of the assay system. Since γ-glutamyl cysteine synthetase and γ-glutamyl transpeptidase activities were not influenced *in vitro* by Myleran (cf. Table 5), it seems improbable that the drug can have any effect on these enzymes *in vivo*. Although Myleran did inhibit GSH synthetase *in vitro*, this effect is unlikely to possess any metabolic significance to the leucocyte *in vivo*: a typical drug-dose administered to a 70 kg adult would, assuming uniform tissue distribution, result in a concentration of 0·03 μg drug/ml body volume: there is no evidence from the literature to suggest a selective uptake or accumulation of alkylating agents. This is almost one hundredth of the drug concentration required to give a measurable inhibition *in vitro*, and could not influence the enzyme activity *in vivo*.

Harington has speculated on "a new hypothesis of a cause for cancer" [16, 17]. In essence this hypothesis ascribes to GSH a role in the regulation of mitosis, which is disrupted in tumour cells due to a carcinogen-induced lesion in the enzyme systems responsible for the mobilisation of GSH: during the carcinogenic process, it is postulated that elevated levels of GSH accumulate either due to a de-repression of synthesis of the GSH-synthetic enzymes, or to an inhibition of γ-glutamyl transpeptidase. Our present work, together with previous studies [1, 10], provides no support for this hypothesis: we have observed neither accumulation of GSH, γ-glutamyl cysteine synthetase, or glutathione synthetase, nor a deficiency of γ-glutamyl transpeptidase in leukaemic cells.

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RESUME

On a établi qu'il n'y avait pas de déficits dans les différentes parties du système enzymatique responsable de la réduction du glutathion oxydé dans les leucocytes de malades atteints de la leucémie myéloïde chronique. Dans les leucocytes des malades qui n'avaient reçu aucun traitement par les agents alcoylants, les niveaux de la glucose-6-phosphate déshydrogénase et de la 6-phosphogluconate déshydrogénase étaient élevés d'une manière significative, alors que les niveaux de glutathion peroxidase se trouvaient fortement abaissés. De même, on a montré que les niveaux des enzymes responsables de la dégradation du glutathion dans ces cellules étaient identiques à ceux trouvés dans les leucocytes du sang normal, et que, après la chimiothérapie, les niveaux restaient toujours les mêmes.

SUMMARY

No deficiencies exist in the components of the enzyme chain responsible for the reduction of oxidised glutathione in the leucocytes of patients suffering from chronic granulocytic leukaemia. In the leucocytes of untreated patients, the levels of glucose 6 phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were significantly elevated, while those of glutathione peroxidase were significantly reduced. Similarly, the levels of glutathione synthesising and degrading enzymes in these cells are identical to those in leucocytes taken from healthy donors, and remain unchanged by chemotherapy.

ZUSAMMENFASSUNG

In den Bestandteilen des enzymatischen Systemes, das die Reduktion des Glutathions in den Leukozyten von Patienten mit chronischer myeloischer Leukämie bewirkt, wurden keine Mängel gefunden. In den Leukozyten von nicht behandelten Patienten waren die Konzentrationen an Glucos-6-Phosphat-Dehydrogenase und 6-Phosphogluconat-Dehydrogenase signifikant erhöht, während die Konzentrationen der Glutathion-Peroxydase signifikant gesenkt waren. Die Konzentrationen an Enzymen, die Glutathion herstellen und abbauen, in diesen Zellen und in Leukozyten aus gesunden Spendern stimmten überein und veränderten sich unter der Chemotherapie nicht.

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Host Defence Mechanisms in the Spontaneous Regression of S-180*

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INTRODUCTION

S-180 is a non-specific type of tumor growing in non-inbred mice. It takes in 100% when inoculated into Swiss mice and is rejected in 5–20% of these animals [1, 2].

Old and Clarke [3] have shown that certain strains of mice infected with Bacillus Calmette–Guérin will reject the S-180. They suggested that the increased host resistance to the tumor is the result of proliferation and increased phagocytic activity of reticulo-endothelial cells.

Many publications have described experimental procedures which significantly increase the complete regression rate of S-180 [4, 1, 2].

Some evidence that immunologic mechanisms are responsible for the regression of S-180 was given by Ferrer and Mihich, who showed that S-180 does not regress in Vitamin B_6 deficient mice thymectomized shortly after birth [5, 6]. The possibility that antibodies are formed against the tumor has also been suggested [7].

The present investigation was undertaken in order to clarify the immunologic mechanism operating in the spontaneous regression of S-180 in mice.

MATERIAL AND METHODS

Tumors

Sarcoma 180 bearing Swiss mice were obtained in 1960 and again in 1965 from the Sloan Kettering Institute of Cancer Research, New York. The tumors were maintained since then by serial subcutaneous transplantation in Swiss mice. The implantation was performed by introducing through a trocar a single piece of the tumor measuring 1 mm^3. The Ehrlich's carcinoma was carried in its ascitic form in Swiss mice by intraperitoneal transfers at weekly intervals of 2×10^6 cells/mouse.

Mice

Mature inbred Swiss, as well as mature random bred Swiss mice weighing 18–22 g were used for the irradiation studies. Mice weighing 18–22 g as well as new-born mice (24 hr after birth) were used in the transplantation studies. DBA/2 Cr. I. Inbred strain was received from Texas inbred Mice Company, U.S.A. and used in some of the experiments.

X-ray studies

Total body X-radiation of the animals was administered by an X-ray machine. The dose was calculated in roentgens (R) per mouse, in air, and was given at 200 kVP and 200 mA with HVL of 1·45 mm Cu. A filter of 0·5 mm Cu, 1·0 mm Al was used. The animals were irradiated at a 40 cm skin target distance.

Histology

Growing and regressing tumors, as well as lymph nodes from all groups, were fixed in Bouin solution and sections were stained by hematoxylin–eosin and methyl green pyronin.

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Immunological studies

Antigen was prepared as follows: S-180 was removed under aseptic conditions from tumor bearing mice. The tumor was minced with scalpels and homogenized in saline (0·5 g wet tumor tissue/ml saline) using a Potter–Elvehjem homogenizer. The homogenate was centrifuged for 10 min at 1500 rev/min. The cell-free supernatant was removed and used as antigen in the complement fixation, the haemagglutination, the gel diffusion and the passive cutaneous anaphylaxis tests.

Serum was obtained from S-180 bearing mice during the logarithmic phase of growth as well as within 14 days after complete regression of the tumor had occurred. The latter serum will be referred to as immune serum. Serum from non-tumor bearing mice was used as control.

Complement fixation test

One tenth ml of serial dilutions of the tumor antigen were each mixed with 0·1 ml of the inactivated and serially diluted mice sera, and 0·1 ml guinea pig complement (Texas Biological Laboratories) diluted 1 : 30 with a 0·5 mM solution of $MgSO_4$ in saline. The tubes were incubated for 1 hr at 37°C. Then 0·4 ml of a mixture of equal volumes of 2% washed sheep cells and rabbit hemolysin 1 : 200 were added. The sheep cells were previously sensitized by contact with the rabbit hemolysin for 10 min. The tubes were incubated at 37°C and read at various intervals until hemolysis appeared in the control tube. Both the serum and the antigen were tested for anticomplementary action. Control experiments with normal serum from non tumor bearing mice as described above were run simultaneously.

Haemagglutination

The sera obtained from the S-180 immune mice were tested by the haemagglutination technique of Boyden [8] and Stavitsky [9], using red blood cells type O coated with S-180 antigen.

Agar gel diffusion

The gel diffusion technique of Ouchterlony [10] was applied using a 1% solution of Noble Agar (Difco) in veronal buffer pH 8·2 ionic strength 0·05M. Mouse antiserum (0·1 ml) and S-180 tumor antigen (0·1 ml) were each pipetted into one of the reservoirs of the agar gel plate. The plates were kept at room temperature for 24–48 hr.

Passive cutaneous anaphylaxis was performed as described by Ovary [11], using the S-180 saline cell-free extracts as antigens and sera from immune S-180 mice as antibody.

Passive transfer of immunity was attempted by the following methods. (a) 1 ml serum obtained within 14 days after complete regression of S-180 was injected i.p. into normal Swiss mice 1 hr prior to S-180 inoculation. Multiple i.p. injections of immune serum (0·5 ml), daily during 4 days, were also administered and S-180 inoculated 1 hr after the last injection.

(b) S-180 cells obtained by trypsinization of the solid tumor were incubated for 30 min at 37°C with spleen cells from mice who had rejected the S-180, in a proportion of 1 tumour cell to 200 spleen cells. Aliquots containing 2×10^6 tumor cells and 4×10^8 spleen cells were then inoculated into normal Swiss mice (Alexander's method) [12].

Cytoxic studies

Suspensions containing 20×10^6/ml Ehrlich's ascites or S-180 cells obtained by trypsinization

Table 1. *Growth and regression of S-180 in newborn and adult mice*

Strain of mice	No. of mice inoculated with S-180	Mean survival time* Day± S.D.	Mean tumor vol.† 10 days after implantation	No. of mice showing complete regression/ total	Percentage animals showing complete regression
1. Inbred Swiss mature	55	28 ± 5	0·67 ±0·03	18/55	32
2. Inbred Swiss newborn	20	12 ±0·9	1·024±0·06	0/20	0
3. Random bred Swiss mature	60	24·5±10	0·68 ±0·05	16/60	26
4. Random bred Swiss newborn	20	11 ±1·2	1·15 ±0·4	0/20	0
5. DBA/2 mature	20	26 ±1·7	0·7 ±0·1	0/20	0

*Based only on the mice who died with tumors.

†Calculated tumor volume $V=0·4\ (a.b)^2$; a, b=tumor diameters.

of the solid tumor were used; 0·2 ml suspension was incubated with 0·2 ml immune serum and 0·1 ml guinea pig complement diluted 1 : 3 at 37°C for 1 hr. The viability of the cells was ascertained by vital staining with 0·5% eosin.

RESULTS

Spontaneous regression of S-180 occurred in 32% of the inbred and 26% of the random bred Swiss mice (Table 1). Both strains of S-180 obtained from the Sloan Kettering Institute in 1960 and in 1965 showed a closely similar rate of regresssion when implanted in these animals.

Implantation of S-180 to new born mice gave 100% takes with 100% mortality. S-180 developed more rapidly in these hosts (see Fig. 1 and Table 1). No regression of S-180 was observed in DBA/2 strain of mice (Table 1).

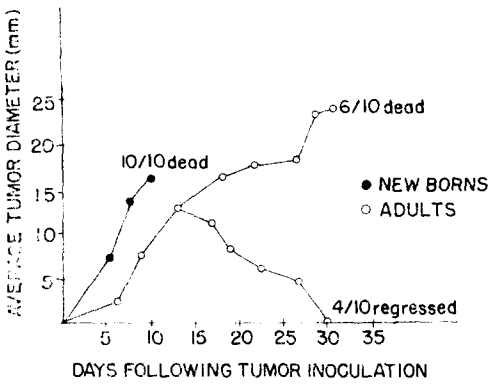

Fig. 1. *Growth curves of S-180 in mature and new-born Swiss mice.*

All of the mice whose original tumors had regressed were resistant to subsequent challenge with S-180 (Table 2).

Intraperitoneal inoculation with 2×10^6 Ehrlich ascites tumor cells into S-180 immune mice resulted however in only 50% of immediate rejection, the remaining 50% of the animals accepted the graft, but rejected it after a period of 4 weeks (Table 2).

Irradiation of the mice with 300 R one day prior to tumor implantation reduced the incidence of complete regression from 30% to 10%. Immune females did not transfer the immunity to the offsprings. S-180 implanted on new borns from previously immunized females showed no regresssion of the tumor implants. In another experiment, male and female mice who rejected the S-180 implant were mated and the offsprings inoculated with S-180 tumor at the age of 5 weeks. The percentage of rejection did not differ from the random group.

The lymph nodes of the mice who had rejected the S-180 showed follicular hyperplasia with proliferation of large pyrinophilic lymphocytes.

Histological examination of regressing tumors showed a strong perivascular or intratumoral infiltration of pyrinophilic mononuclears, and large areas of necrosis (see Fig. 2 (1–4). The non-regressing tumor showed intact S-180 cells, many of them in mitotic division [Fig. 2 (1–6)].

In order to find out if the rejection of S-180 could be correlated to humoral antibodies, the following experiment was undertaken. Serum was obtained 30 days after implantation of S-180, both from mice showing complete regression as well as from mice bearing large tumors. These sera were tested by the complement fixation, haemagglutination, gel diffusion and passive cutaneous anaphylaxis methods. Positive complement fixation was found with a 1:10 dilution of the antigen and 1:4 dilution of serum from the mice showing complete tumor regression. Complement fixation was negative when using serum from mice with unrestricted growth of S-180 (Table 3). Similar results were obtained by the tanned haemagglutination technique (Table 4).

Demonstration of humoral antibody was obtained also by the passive cutaneous anaphylaxis technique of Ovary in two different experiments. In the first, 0·1 ml tumor extract

Table 2. *Growth of S-180 and Ehrlich ascites carcinoma in mice on which the S-180 tumor had previously regressed (immune mice)*

Group of mice	No. of mice	Inoculated with	Takes (%)	Mortality (%)
Immune Mice (Swiss)	20	S-180	0	0
Normal Control (Swiss)	20	S-180	97	60
Immune Mice (Swiss)	20	Ehrlich Ascites	50	0
Normal Control (Swiss)	20	Ehrlich Ascites	100	100
Normal DBA/2	20	S-180	100	100

Table 3. Fixation of complement by S-180 antigen with serum from mice with spontaneously regressing tumors and serum from mice with non-regressing tumors

Dilutions of S-180 antigen (Saline extract)	Dilutions of serum from mice			
	With regressing tumors			With non-regressing tumors
	1 : 2	1 : 4	1 : 8	1 : 2
1 : 2	+	+	−	−
1 : 4	+	+	−	−
1 : 8	+	+	−	−
1 : 10	+	+	−	−
1 : 20	−	−	−	−

+Positive complement fixation.
−Negative complement fixation.

was inoculated intradermally in each of two mice who had shown previously tumor regression and no take on subsequent S-180 rechallenge. After 4 hr, 0·1 ml of 1% Evans Blue was injected i.v. 20 min later, a distinct blue stain appeared on the skin [Fig. 2 (7)], at the site of inoculation.

In the second experiment, 0·2 ml immune serum was injected intradermally into each of two normal mice. Four hr later, 0·1 ml of antigen plus 0·1 ml Evans Blue 1% were injected i.v. 20 min later, distinct blue stains were observed on the skin [Fig. 2 (7)].

These experiments demonstrated the presence of circulating antibodies to S-180 in the serum of mice showing spontaneous regression of the tumors.

No precipitation lines were observed in the agar gel diffusion between the S-180 saline extracts and the sera of S-180 resistant mice.

Passive transfer of immunity was not achieved by single or multiple peritoneal injection of immune serum prior to S-180 inoculation. The growth of S-180 was neither inhibited nor enhanced by the immune serum. On the other hand, passive transfer of immunity was achieved by inoculation of immune spleen cells according to Alexander's method, while spleen cells from normal Swiss mice did not prevent the growth of the S-180 tumor.

The immune serum was not found to be cytotoxic to the Ehrlich ascites cells nor to the S-180 cells. No changes in viability of treated cells were found. Furthermore, inoculation of mice with serum treated tumor cells did not prevent or delay the development of the Ehrlich ascites tumor and S-180 respectively.

Parabiosis experiments using the Bunster *et al.* technique between immune mice and normal mice were also performed [13]. Two weeks after parabiosis, the immune as well as the normal parabiont mouse were challenged with S-180 tumor. No take was found in any of the parabionts.

S-180, when transplanted to the DBA/2 mice, takes in 100% of the animals and causes a 100% mortality. Since this strain of mice is known to have an antigen configuration similar to that of S-180 [14], it was attempted to render the Swiss mice immune against S-180 by previous immunization with normal tissues from DBA/2 mice, using the methods described by Snell [15]. Thymus cells as well as spleen cells from DBA/2 inbred mice were used as immunization material. No immunity to S-180 was conferred by injection of these tissues into the Swiss mice. Furthermore, no H2d antibodies could be detected in the serum

Table 4. Tanned haemagglutination reactions of S-180 antigen with serum from mice showing spontaneous regression of S-180

Dilutions of S-180 antigen (Saline extract)	Dilutions of serum from mice showing spontaneous regression			Non-tumor bearing mice
	1 : 2	1 : 4	1 : 8	1 : 2
1 : 2	+	+	−	−
1 : 4	+	+	−	−
1 : 8	+	+	−	−
1 : 10	+	+	−	−
1 : 20	−	−	−	−

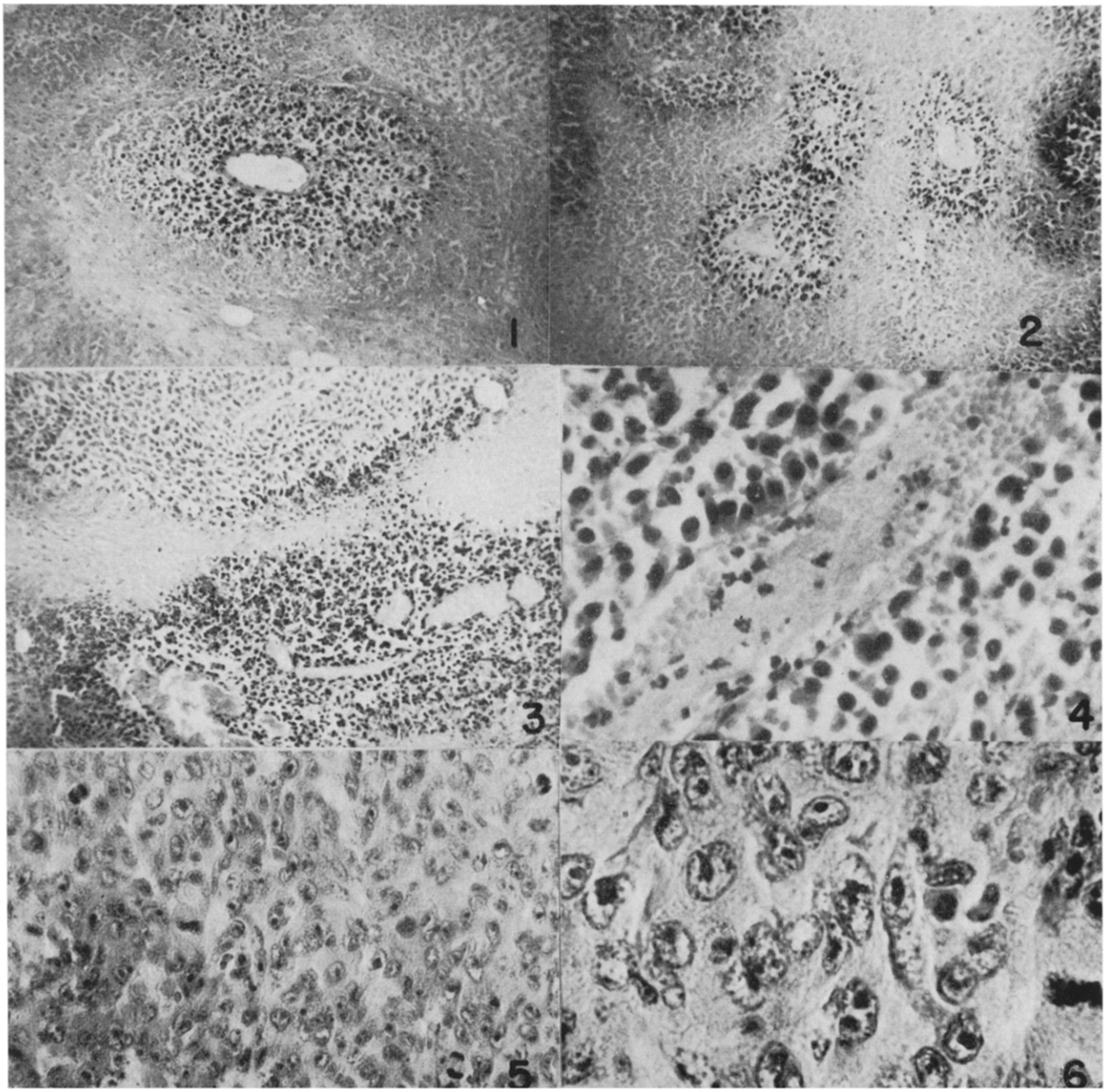

Fig. 2. (1–6). *Sections stained by hematoxylin eosin.*
(1,2) *S*-180 *regressing tumor showing perivascular infiltration of mononuclear cells.* (×80)
(3) *S*-180 *regressing tumor showing massive infiltration of mononuclear cells into necrotising tumor tissue.* (×80)
(4) *Infiltration of regressing S*-180 *tumor by mononuclear cells around a small blood vessel.* (×400)
(5) *Non-regressing S*-180 *tumor showing numerous mitotic divisions.* (×400)
(6) *Higher magnification of the S*-180 *tumor shown on Fig.* 2.(5). (×1000)

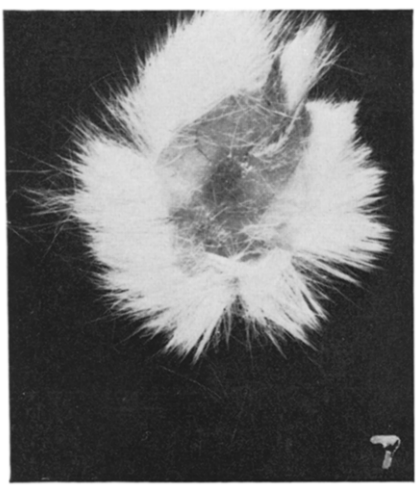

Fig. 2 (7). *Positive passive cutaneous anaphylaxis test. Blue stain at the site of antigen inoculation.*

(to face p. 72)

of immune Swiss mice by the agglutination technique of Gorrer [16].

DISCUSSION

Spontaneous regression of S-180 grown in C57BL/6 mice and in Rockland strain of mice has been reported by Pasqualini *et al.* [7]. The author suggested that S-180 is induced by a cell free agent. On this assumption, the immunity to the sarcoma could be explained by antibody formation against the supposed subcellular sarcoma inducing agent. However, this assumption was not supported by any experimental evidence.

It has been suggested that the rejection of S-180 may be the expression of an enhanced homograft rejection [17]. A demonstrable immune response to S-180 was described by Andervont as early as 1932 [18]. Sarcoma 180 was found to regress completely after initial growth in a large percentage of Swiss mice fed a pyridoxine deficient diet [1, 2]. Zymosan has been also shown to increase significantly the regression rate of S-180 [4].

Mice inoculated with S-180 after BCG infection showed definite protection with 70–75% tumor regression [3]. The increased host resistance to the tumor has been correlated to the proliferation and increased phagocytic activity of the reticulo–endothelial cells. On the other hand, Schoenberg and Moore [19] showed that stimulation of the reticuloendothelial system with complete Freund's adjuvant failed to prolong the survival of CFW Swiss mice, given injections of ascites S-180 or to alter the course of growth of S-180 implanted subcutaneously. Therefore they suggested that the host defence against S-180 is not improved simply as a result of an increase in the reticuloendothelial population.

In the present study, the occurence of humoral antibody in mice showing complete regression of S-180 tumor was demonstrated, using the passive cutaneous anaphylaxis, the complement fixation and the tanned haemagglutination techniques. No antibodies were found in sera of S-180 bearing mice which did not reject the tumor. The fact that antibodies could not be demonstrated in mice bearing large tumors does not necessarily mean that less antibodies had been produced in these mice, since large tumor masses are likely to bind considerable amounts of humoral antibodies. It remains questionable however, whether the circulating antibodies were the main reason for the observed rejection of S-180 in as much as the titers found were relatively low. Furthermore, no cytotoxic effect of immune serum on S-180 cells could be demonstrated, neither could the growth of S-180 be prevented by preincubation of the implant in immune serum, nor by inoculation of the immune serum prior to implantation of S-180. This observation as well as the presence of mononuclear pyrinophilic cells in the regressing tumors point to the possibility of cell bound antibodies as responsible for the spontaneous rejection of S-180.

A synergistic action of both types of antibodies could not be excluded. Supporting evidence to these findings may be found in Lemperle's study [20]. Synergistic action between humoral and cell bound antibodies against the various compounds of the same antigen were described by Batchelor *et al.* [21]. The absence of tumor rejection in the normal newborn mice might be due to immunological immaturity, since no transfer of immunity from parents to offspring was found. Humoral antibodies have been shown in small doses to enhance, and in larger amounts, to reject tumors [22]. The humoral antibodies found in the present study had neither inhibitory nor enhancing effect.

It might be argued whether the antibodies found are directed against a normal transplantation antigen (histocompatibility antigen) or a tumor specific antigen. The absence of anti H2d antibodies in mice which had rejected the S-180 implants, as well as the failure to render the Swiss mice refractory to S-180 implants by previous immunization with DBA/2 tissues, suggest that incompatibility at the H2d locus is not responsible for the rejection of S-180.

The presence of a common antigen between the Ehrlich ascites carcinoma and the S-180 is also inferred from the results of this study.

Evans *et al.* have already reported the presence of certain common antigens between S-180 and Ehrlich ascites carcinoma [23]. Furthermore, Koun, using the fluorescent antibody staining, suggested also the presence of an antigen common to Ehrlich ascites and the S-180 cell [24].

It is noteworthy that Easty and Ambrose [25], using Ehrlich tumor cell suspension and rabbit antisera in gel diffusion and cytotoxic tests, found that the Ehrlich ascites tumor cells contained soluble antigens absent from the normal organs of the host strain of mice. The authors did not, however, claim that these antigens were tumor specific.

The transfer of immunity achieved in this study be spleen cells from mice who had rejected the S-180, as well as by parabiosis

experiments between immune and normal mice, together with the demonstration of the humoral antibodies directed against S-180 antigen, leave no doubt that host defence mechanisms are responsible for S-180 regression.

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RESUME

Une régression spontanée de la tumeur S-180 a été observée dans 32% des cas, après inoculation à des souris de la race "Swiss", et dans 26% des cas, après inoculation à des souris de l'élevage courant. De plus, les souris survivantes devenaient complètement résistantes à l'implantation ultérieure de la tumeur S-180, et partiellement résistantes à l'administration de cellules de la tumeur ascitique d'Ehrlich. L'irradiation totale des souris, avant la transplantation, réduisait de 10 à 30% la fréquence des régressions de la tumeur S-180. L'implantation de cette tumeur chez des souris nouveau-nées de la race "Swiss" provoquait 100% de prise et 100% de mortalité.

Des études sérologiques par la technique de l'anaphylaxie cutanée passive, selon Ovary, par la fixation du complément, et par l'agglutination des hématies tannées, démontrèrent la présence d'anticorps contre les cellules tumorales S-180 dans le sérum des souris porteuses de tumeurs en régression spontanée.

L'injection intrapéritonéale d'immun-sérum ne permit pas le transfert de l'immunité passive à des souris normales. Toutefois, la croissance de la tumeur S-180, chez des souris normales, était complètement inhibée par l'injection intrapéritonéale de cellules spléniques provenant de souris immunes, ou par la parabiose avec des souris immunes.

SUMMARY

Spontaneous regression of S-180 was found in 32% of inoculated inbred Swiss mice and 26% random bred mice. Furthermore, the surviving mice became completely resistant to subsequent implantation of S-180 and partially resistant to challenge with Ehrlich's ascites carcinoma tumors. Whole body X-radiation of the mice prior to tumor inoculation reduced the incidence of regression of S-180 10–30%. Implantation of S-180 into new born Swiss mice yielded 100% takes with 100% mortality.

Serological studies using the passive cutaneous anaphylaxis technique of Ovary, complement fixation and tanned haemagglutination techniques demonstrated the presence of antibody against S-180 in the serum of mice with spontaneously regressing tumors.

Passive transfer of immunity to normal mice could not be achieved by intraperitoneal injection of immune serum. However, S-180 growth in normal mice was completely inhibited by intraperitoneal injection of spleen cells from immune mice or by parabiosis with immune mice.

ZUSAMMENFASSUNG

Spontane Rückbilding des Sarkoms 180 (S-180) wurde bei 32% geimpfter Inzucht-Swiss-Mäuse und bei 26% wahllos gezüchteter Mäuse gefunden. Weiterhin erwiesen sich die überlebenden Mäuse als völlig resistent gegen eine folgende Impfung mit S-180 und teilweise resistent bei der Prüfung mit Ehrlich'schen Ascites-Karzinom-Zellen. Ganzkörper-Röntgenbestrahlung der Mäuse vor der Tumorverimpfung senkte die Rückbildungshäfigkeit des S-180 von 10 auf 30%. Die Impfung von S-180 in neugeborene Swiss Mäuse ergab 100% Angänge mit 100%iger Mortalität.

Serologische Studien, welche die passive Haut-Anaphylaxie Technik von Ovary, Komplementbindung und Haemagglutinationtechniken verwendeten, zeigten die Gegenwart eines Antikörpers gegen S-180 im Serum von Mäusen mit den spontan zurückgebildeten Tumoren.

Die passive Übertragung der Immunität auf normale Mäuse konnte nicht durch intraperitoneale Injektion von Immunserum erreicht werden. Jedoch wurde das Wachstum von S-180 in normalen Mäusen durch die intraperitoneale Injektion von Milzzellen aus Immunmäusen oder durch Parabiose mit Immunmäusen völlig gehemmt.

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Letter to the Editor

Effect of Triton WR 1339 on Cancer Dissemination and Metastases*

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TRITON WR 1339 (polyoxyethylene ether of formaldehyde polymers of octylphenol), a non-ionic detergent, is known mainly because of its capacity to induce, after parenteral administration, a sustained hyperlipemia (1) and an antitubercular activity (2). This note will report a novel effect of this agent, namely an inhibition of cancer dissemination and metastases formation. Female Swiss mice (20 ± 2 g) transplanted with Ehrlich carcinoma or Sarcoma 180 were used in these experiments. These 2 tumors had been maintained in the ascites form in this strain of mice for more than 250 passages. As previously reported [3, 4], mice transplanted intracerebrally with Sarcoma 180 or Ehrlich Carcinoma cell showed a marked dissemination of cancer cells in blood and lung. This dissemination can be evaluated in a quantitative way by using a bioassay procedure, based upon the subcutaneous transplantation of blood or lung obtained from intracerebrally tumor bearing animals, into normal recipient mice.

Table 1 indicates that Triton WR 1339 500 mg/kg (Roland Am. Corporation) given for 6 or 11 days does not significantly affect the number of Sarcoma 180 cells present in the blood, but it markedly decreases (up to 90%) the number of cancer cells present in lung. Similar results were obtained with Ehrlich carcinoma transplanted intracerebrally, where the administration of Triton WR 1339 was limited to a period of 6 days.

It has been previously reported also that mice transplanted intratibially with Ehrlich carcinoma cells develop large metastases in the lymph nodes [5]. Table 2 shows that Triton WR 1339, given at the dose of 500 mg/kg/i.p. daily or at the dose of 1250 mg/kg/i.p. every four days till the animals death, significantly decreases the percentage of mice showing metastases. However, in mice showing metastases the weight of the metastatic tumors calculated per mouse was similar in Triton treated and control animals.

Finally, Triton WR 1339 given at the dose of 500 mg/kg/i.p. daily for 6 days beginning the day after tumor transplantation (intracerebral tumor 100,000 cells per mouse) 3 days after (intraperitoneal tumor 1,000,000 cells per mouse and intratibial tumor 100,000 cells per mouse) and 5 days after (subcutaneous tumor 5,000,000 cells per mouse) was devoid of any significant effect as judged by measuring the weight of the tumor (subcutaneous and intratibial tumor) or the survival time of the animals (intraperitoneal, intratibial and intracerebral tumor). The data reported show that a non-ionic detergent compound as Triton WR 1339 may affect cancer dissemination and metastases formation without impairing the growth

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Table 1. Dissemination from the intracerebrally transplanted tumors

Tumor	Tissue	Time*	Saline	Triton WR 1339†	*P*
Ehrlich carcinoma	Blood	7	9/24 (4)	8/24 (2)	—
	Lung	7	16/24 (155)	8/24 (7)	<0·05
Sarcoma 180	Blood	7	7/24 (4)	6/24 (3)	—
	Lung	7	11/24 (105)	3/24 (8)	<0·05
	Blood	12	14/24 (47)	15/24 (66)	—
	Lung	12	19/24 (925)	11/24 (105)	<0·05

*Between intracerebral transplantation of the tumor and bioassay.
†500 mg/kg/i.p. × 6 days or 11 days beginning the day after the intracerebral transplantation.
The numbers reported indicate the ratio between animals showing subcutaneous tumors and animals transplanted with about 40 mg of blood or lung obtained from intracerebral tumor bearing mice. From each donor mouse two blood clots and two lung fragments were obtained and each fragment of each tissue was transplanted into a recipient mouse.
In brackets is reported the number of cancer cells/cmm of blood or mg of lung mice bearing an intracerebral tumor.
For the technical details of this procedure see reference 4.

of transplanted tumor. Furthermore, it should be noticed that reduction of dissemination in the lung can take place without any change in the number of circulating cancer cells suggesting that Triton may interfere with the processes responsible for cancer cell lodgment in the normal tissue.

At the doses used Triton WR 1339 was well tolerated by the animals during the period of treatment. In this respect it is interesting to recall that Miller and Janicki [6, 7] have recently showed that Triton WR 1339 possesses *in vitro* a selective cytotoxic activity on cells of malignant origin in respect to cells derived from normal tissues.

Table 2. Effect of Triton WR 1339 on metastases in mice transplanted intratibially with Ehrlich acrcinoma cells (100,000 *cells/mouse*)

Schedule of treatment	Treatment	% mice with metastases	Total number of metastases	Average tumor* weight (g±S.E.)	Survival time (days ±S.E.)
	Saline	91 (34)	58	8·1±0·8	47±3
A	Triton WR 1339	62 (29)†	39	7·0±1·0	44±3
	Saline	60 (38)	33	9·2±0·5	47±2
B	Triton WR 1339	37 (35)†	24	10·5±0·6	46±2

A: 500 mg/kg/i.p./daily; B: 1250 mg/kg/i.p./every 4 days. With both treatments the plasma level of Triton was during two successive treatments always higher than 1 mg/ml.
In brackets is reported the number of mice.
*Tumor growing in the leg.
†$P<0·05$ vs. saline.

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Letter to the Editor

Duration of Cytotoxic Activity of Vincristine in the Blood of Leukemic Children*

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PREVIOUS work carried out in rats established that the cytotoxic activity of vincristine in the blood of the rat lasts for a period of about 2 hr [1].

We now report the duration of vincristine action in children affected by acute lymphoblastic leukemia. Fourteen hospital patients of both sexes ranging in age from 18 months to 11 years were studied. Twelve were bearing an acute lymphoblastic leukemia, 1 a myeloid leukemia and 1 a lymphosarcoma. Seven patients were at the first course of therapy, 6 at the second course and 1 at the third course after remission obtained by means of 6-mercaptopurine and prednisone or amethopterine and prednisone treatment.

Patients received 0·1 mg/kg of vincristine in 100 ml saline plus 10% glucose during 1 hr of perfusion in the cubital vein. Blood samples of 2 ml each were withdrawn from the controlateral vein before and at various times after the end of the perfusion. After clotting at room temperature, serum was stored at −20°C for the bioassay of vincristine by using tissue culture technique [2]. KB cells were grown in Eagle's basal medium with 10% calf serum. To avoid toxic effects, the serum of the patient was inactivated at 56°C for 30 min. In other experiments the effect of the same serum was tested on cells cultivated under cellophane. Vincristine added to normal human blood was not inactivated at room temperature or at 56°C or by diffusion through cellophane [3]. In the bioassay the serum of the patient replaced the calf serum. The KB cells were cultivated in 1·8 ml medium in Rose chambers. Under the experimental conditions used all the KB cells were blocked in metaphase at a concentration of 5×10^{-2} μg vincristine/ml of patient serum, while a partial block was seen (presence of ball and star metaphase with atypical anaphases at telophases) at concentration 5×10^{-3} μg/ml. No detectable effect was present at concentration 5×10^{-4} μg/ml.

All the data obtained are summarized in Table 1. It is evident that all the cases under study had a measurable level of vincristine activity in the serum for a period of about 2 hrs after the end of the infusion.

Beginning with the 3rd hr, 2 of 7 patients showed no activity in serum and after the 4th hr all the sera were negative.

No evident difference in the disappearance of vincristine activity in serum was observed in relation to the previous treatment, the status of the bone marrow or the therapeutic response.

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Table 1

Time (hr)	Activity corresponding to a concentration of vincristine (μg/ml) $<5\times10^{-3}$	$>5\times10^{-3}<5\times10^{-2}$	$>5\times10^{-2}$
Before perfusion	20/20		
End of perfusion			20/20
1*		1/11	10/11
2		1/12	11/12
3	2/7	3/7	2/7
4	5/10	5/10	
8	1/1		
10	5/5		
24	3/3		
48	1/1		

The figure represents the number of cases in a given category compared to the total cases assayed.
*Time after the end of infusion.

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Announcements

THE SECOND meeting of the Working Group on Human Carinogenesis under the Co-ordinating Committee for Human Tumour Investigations was held in the Department of Experimental Pathology and Cancer Research, University of Leeds on 7th November 1968, with participation of the following research workers: Dr. Honor M. Anthony, Leeds; Dr. Georgiana M. Bonser, Leeds; Professor E. Boyland, London; Dr. R. D. Bulbrook, London; Professor D. B. Clayson, Leeds; Dr. J. Clemmesen, Copenhagen (Convenor); Professor L. A. Elson, London; Professor P. C. Koller, London; Professor D. Schmähl, Heidelberg; Dr. G. P. Warwick, London.

Dr. Eva Klein, Professor C. Maltoni and Professor R. D. Passey were unable to be present.

The meeting was preceded by a colloquim at which members of the staff of the Department of Cancer Research, under the direction of Professor E. H. Cooper, presented experimental aspects of their research on Cancer of the Bladder. Their present work is concerned with the immediate effects of chemical carcinogens on the induction of cell division in the bladder epithelium. This epithelium is normally quiescent but can be stimulated to commence rapid proliferation by a variety of carcinogens. Both biochemical and cell kinetic studies are in progress to analyse the behaviour of this epithelium. The other aspects of the work include studies of the evolution of cell populations in transitional cell carcinoma of the bladder in man.

The working group discussed various aspects of human carcinogenesis particularly developments which had arisen since the first meeting in Copenhagen in October 1967 (*Europ. J. Cancer*, **4**, 339, 1968).

Cigarette smoking

It had been suggested that the higher incidence and higher rate of increase in bronchial carcinoma in some countries might perhaps be attributed to the use of and introduction into these countries of cigarettes containing flue-cured (high sugar content) tobacco in contrast to the slow air-dried (low sugar content) tobacco. Investigation of the relation between the acidity or alkalinity of the smoke and sugar content of the tobaccos from more than 20 countries shows that in general the flue-cured tobaccos give a smoke of acid pH whilst the air-cured tobaccos give rise to an alkaline pH smoke. The effect of the pH of the smoke on the smoking habits, including amount smoked and inhaled, and the availability of nicotine for absorption may have a considerable bearing on the incidence of lung cancer. The flue-cured cigarettes (giving an acid smoke), usually the so-called 'mild' variety, appear to be the most dangerous.

The possible relation between cigarette smoking and *bladder cancer* was discussed. An association between the two has been suggested following surveys in Denmark, the U.S.A. and France. A current survey in Leeds has so far failed to confirm this association and this may suggest a possibility that the relatively high incidence of lung cancer in cigarette smokers in England may obscure the time relation. It would be desirable to obtain reliable data on

bladder cancer incidence in smokers in other countries, both with a high and a lower incidence of lung cancer and to relate this to the type of cigarettes smoked.

Tumours of the testis

It appears that an increase in the numbers of testicular tumours is taking place in certain locations, particularly in Denmark and in the State of Connecticut among men in the age group 20–55 years. Above the age of 55 the incidence is comparable in practically all countries throughout the world. It is striking, however, that practically no cases of testicular cancer occur in the 20–55 year age group in Finland or among the Negro Americans. No indications as to the cause of these divergences are at present available, but attention should be drawn to this aspect since these observations indicate that cancer of the testis might be partly a preventable disease.

Lymphosarcomas

In continuance of the work reported at the first meeting of the group in 1967 it was emphasised that Dr. Eva Klein, Institute of Tumour Biology, Stockholm 60, Sweden, is continuing studies on the immunology of reticulosarcomas and lymphosarcomas as well as on Burkitt tumours and highly appreciates receiving biopsy tissues and serum samples from patients with such tumours.

Next Meeting

The Working Group will meet again in Heidelberg in 1969.

Année 1969-1970

Fondation 'Lady Tata'

Bourses, Allocations, Aides Financières Internationales

Les Administrateurs de la Fondation 'Lady Tata' invitent les candidats à présenter leur demande de bourses, allocations ou aides finfincières en vue de

recherches sur la leucémie

pendant l'année académique commençant le 1er octobre 1969.

En raison des relations qui existent entre la leucémie et d'autres formes de néoplasmes, les candidatures des chercheurs dont le programme, orienté vers tout genre d'affection maligne, est susceptible de faciliter la solution du problème que pose la leucémie, seront prises en considération. Les administrateurs désirent particulièrement encourager les études sur les cancers du sein, sur la formation et la prolifération de la leucémie, sur la production de caractères d'immunisation.

Ce concours est ouvert aux chercheurs qualifiés de toutes nationalités travaillant soit dans les laboratoires de leur pays, soit à l'étranger.

Les Administrateurs de la 'Fondation Lady Tata' accordent deux aides financières d'un montant de 1.500 livres Sterling par an, valables pour 3 ans, avec provisions pour réajustement et augmentation annuelles de 100 livres sterling, aux chercheurs qualifiés et ayant déjà une expérience certaine.

A titre de rémunération personnelle, il est alloué des bourses d'un montant de 800 livres Sterling par an ou moins selon que l'on y consacre tout son temps ou une partie de cellui-ci. Ces bourses seront attribuées aux chercheurs travaillant sous le controle d'un Directeur qualifié.

De plus amples renseignements, ainsi que les formules de demandes à remplir, seront obtenues en écrivant au Secretariat de la Fondation 'Lady Tata' (zone latine) à l'attention de Madame S. Bazile, laboratoire Pasteur, 26 rue d'Ulm, Paris 5°, France. Les demandes devront être retournées au Secrétariat avant le 31 Mars 1969. Les décisions seront communiquées directement aux intéressés par les Administrateurs en juin 1969.

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Modifications Métaboliques *ex vivo* des Cellules Tumorales au cours de la Croissance d'une Ascite Expérimentale de la Souris C_3H

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INTRODUCTION

La durée du cycle cellulaire et de ses composantes s'allonge dans l'ascite expérimentale de la souris C_3H au cours de sa croissance. En effet, quatre jours après inoculation, la durée du cycle est de 17 heures, et elle s'allonge pour atteindre 40 heures au 12e jour [1]. Si l'on cultive ces mêmes cellules *in vitro*, en couche monocellulaire, en renouvelant fréquemment le milieu, la durée du cycle paraît constante à toutes les phases de l'évolution [2]. La différence ainsi constatée est probablement liée aux modifications des conditions métaboliques régnant au cours de l'évolution de l'ascite de la souris. L'étude *in vivo* de la pO_2, au cours de la croissance de l'hépatome ascitique de Yoshida, a amené U. Del Monte à conclure à l'inadaptation de l'apport en oxygène aux stades tardifs de l'évolution tumorale. Dans le présent travail, nous avons étudié les modifications des pressions partielles gazeuses et de l'équilibre acido-basique, ainsi que du glucose et des acides lactique et pyruvique au 5e et au 12e jour de l'évolution de l'ascite tumorale.

MATERIEL ET METHODES

La tumeur ascitique a été obtenue en injectant à des souris C_3H, par voie intrapéritonéale, des cellules NCTC 2472, suivant un protocole déjà décrit [1].

Les prélèvements ont été effectués sur 19 souris et on a examiné 7 ascites à 5 jours et 12 ascites à 12 jours. Les liquides d'ascite ont été recueillis dans des seringues étanches de manière strictement anaérobie et toutes les déterminations ont été effectuées *le plus rapidement possible:* dans les secondes suivant le prélèvement, le liquide servant aux déterminations de glucose (technique de Folin et Malmros), de l'acide lactique (technique de Barker-Summerson) et de l'acide pyruvique (technique de Friedemann et Haugen) est déféqué.

Les mesures de pression partielle d'O_2 et de CO_2 sont déterminées à l'aide du dispositif d'Eschweiler utilisant des macro-électrodes avec agitation et un bloc de mesure comportant un dispositif de compensation [3].

Le pH est évalué au moyen d'une micro-électrode de verre thermostatée (radiometer) dont la pente d'étalonnage est établie avec des tampons NBS.

RESULTATS

(1) Les numérations cellulaires ont montré des chiffres légèrement plus élevés dans des liquides d'ascite à 12 jours (100×10^6 par mm^3) que dans des liquides d'ascite à 5 jours (80×10^3 par mm^3).

(2) Les résultats concernant le glucose, l'acide lactique, l'acide pyruvique et le pH

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Tableau 1. *Résultats des mesures directes pour les liquides d'ascite à* 5 *jours et pour les liquides d'ascite à* 12 *jours*

	pH		Glucose mg (%)		Acide lactique (mEq./l)		Acide pyruvique (mEq./l)		Rapport L/P	
	5j	12j	5j	12j	5j	12j	5j	12j	5j	12j
	7,09	6,55	45	20	10,67	16,67	0,79	0,58	13,5	28,7
	7,11	6,71	50	25	12,00	18,00	0,47	0,74	25,5	24,3
	7,12	6,50	15	22	8,67	13,33	0,43	0,38	20,2	35,1
	7,13	6,86	20	40	8,80	11,55	0,60	0,42	14,7	27,5
			20	5	12,00	14,66	0,52	0,56	23,1	26,2
			15	30	13,06	14,66	0,54	0,44	24,2	33,3
			30	7		15,33		0,42		36,5
				25		14,00		0,31		45,2
				7		16,00		0,42		38,1
				15		17,33		0,42		41,3
m=	7,11	6,66	27,9	19,6	10,87	15,15	0,56	0,47	20,2	33,6
$S/\sqrt{n}$=	0,008	0,08	5,4	3,6	0,74	0,61	0,05	0,04	2,1	2,2

sont indiqués dans le Tableau 1.

La concentration en glucose est légèrement inférieure dans le liquide d'ascite à 12 jours que dans le liquide d'ascite à 5 jours, mais sur cet échantillon les différences ne sont pas significatives. Les différences observées sur le pH sont très significatives ($P<0,01$) et affirment une acidose beaucoup plus marquée pour l'ascite à 12 jours. A cette dernière date, le taux d'acide lactique s'élève également de manière très significative ($P<0,01$), tandis que le taux d'acide pyruvique (0,3 à 0,8 mEq/l) ne montre pas de différence appréciable entre les deux groupes d'ascites.

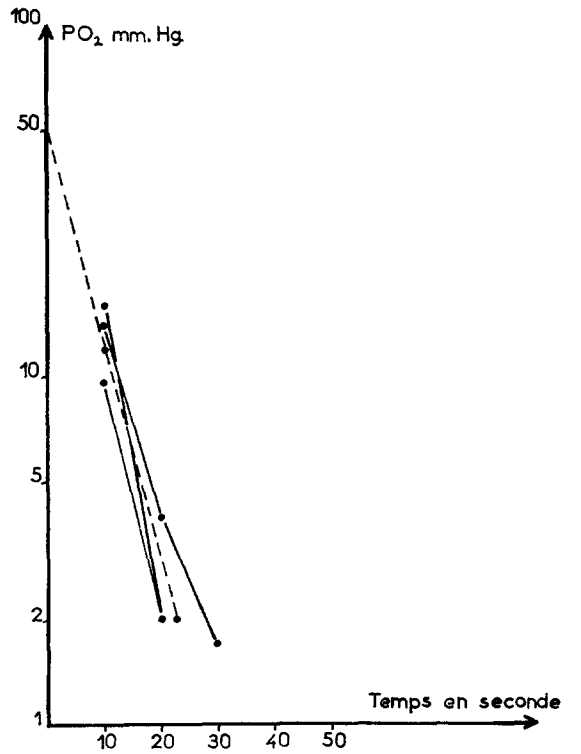

Fig. 1. *Etude de la consommation d'oxygène de liquides d'ascite à* 12 *jours.*
——— *mesures directes.*
..... *tonométrie sous* 50 *mm Hg de* pO_2

(3) Les mesures de pressions partielles de CO_2 et d'O_2 n'ont pu être pratiquées que sur les liquides à 12 jours, étant donné la quantité de liquide nécessaire pour cette détermination:

(a) la pCO_2 est constamment élevée, après deux minutes (temps nécessaire à l'équilibre dans l'électrode utilisée), on enregistre des valeurs entre 85 et 110 mm Hg; mais les valeurs continuent à monter durant les 5 minutes suivantes, sans atteindre de chiffre stable.

(b) la pO_2, évaluée 10 secondes après l'introduction de l'échantillon dans la cellule de mesure, se situe entre 9 et 16 mm Hg; 20 à 30 secondes plus tard, la valeur est nulle. Dans la Fig. 1, on a représenté l'évolution de ce phénomène dans lequel on constate que la décroissance de l'oxygène est exponentielle. La disparition de l'oxygène dissous correspond à sa consommation dans le liquide étudié [4].

DISCUSSION

La mesure de l'état métabolique régnant dans un milieu biologique à haut niveau énergétique est particulièrement difficile. Puisque les mesures directes *in vivo* ne peuvent donner qu'exceptionnellement des informations utilisables, force est de recourir à des mesures *ex vivo* dont les résultats risquent d'être entachés d'erreur, si le temps entre prélèvement et dosage est déjà susceptible de permettre une évolution métabolique de l'échantillon. L'analyse de ce dernier ne conduit plus alors à reconnaître une situation biologique instantanée *in vivo*, mais détermine un état biologique modifié par de nouvelles conditions *ex vivo*

où l'évolution des processus est largement modifiée du fait des conditions particulières dans la seringue de prélèvement, qui sont forts différentes de celles régnant dans la partie de l'organisme d'où l'échantillon a été prélevé.

Dans la cas présent, les ascites tumorales sont représentatives de cette situation particulière; leur niveau énergétique est considérablement élevé, tant du fait du grand nombre de cellules que du haut niveau énergétique de chacune d'elles.

Avant toute discussion, il y a lieu de souligner que toutes les mesures ont été effectuées avec la plus grande célérité possible. Les délais ont été suffisamment réduits pour que l'on puisse accorder plein crédit aux valeurs du glucose et des acides organiques. En revanche, celles concernant le pH et les pressions partielles gazeuses ont été obtenues après un délai obligatoirement plus important puisqu'il n'est pas possible de suspendre instantanément l'activité métabolique du milieu, comme dans le cas précédent.

Les taux de glucose des liquides d'ascite, dans ce type de tumeur, chez la souris C_3H, sont élevés, si l'on considère les taux très faibles observés au cours de la tumeur ascitique d'Ehrlich de la souris [5], ou au cours de l'hépatome ascitique de Yoshida chez le rat [6]. Depuis les travaux de Warburg, il est classique de considérer que le glucose n'existe qu'a l'état de traces dans les ascites tumorales; or, dans nos expériences, le taux de glucose trouvé est certainement supérieur à la concentration minimum pour que soient assurés les besoins énergétiques des cellules [5]. L'absence de différence significative entre les taux de glucose dans les liquides au 5e et au 12e jour permet d'éliminer ce facteur métabolique pour expliquer les modifications de durée du cycle cellulaire.

Dans la plupart des ascites, on a constaté une élévation très significative du taux d'acide lactique [6]. Au cours de nos propres expériences, ce caractère est largement confirmé. Mais aux faits précédemment rapportés, nous pouvons ajouter les notions suivantes:

— l'évaluation de la hausse de l'acide lactique par rapport à celle de l'acide pyruvique permet de conclure à une élévation de l'index lactique. Cette hausse de l'index lactique est significativement plus marquée au 12e jour qu'au 5e jour.

— cet accroissement de l'index lactique s'accompagne d'une acidification très marquée du liquide d'ascite, avec, là encore, une différence très notable suivant l'âge du liquide.

— l'acidose importante constatée résulte de phénomènes mixtes, respiratoires d'une part puisque nous avons noté, dans tous les cas étudiés (liquide au 12e jour), une forte hypercapnie, métabolique d'autre part, dont le témoin est justement la hausse importante du taux d'acide lactique.

Les pH des liquides d'ascite au 12e jour sont extrêmement bas; mais il faut moins retenir la valeur absolue des chiffres trouvés pour les raisons préalablement exposées que la différence très significative constatée suivant l'âge du liquide d'ascite. Dans ces conditions, il est fondamental de reconnaître l'état d'oxygénation du liquide d'ascite. Le but de nos expériences n'est pas de définir le chiffre exact d'oxygène disponible dans le liquide. Afin d'essayer d'obtenir des résultats parfaitement valables sur le plan de la mesure, nous avons utilisé une méthode d'analyse *in vitro*, en employant une macro-électrode avec agitation. Cette dernière nécessite une quantité de liquide à analyser notable, de telle sorte que nous n'avons pu étudier les liquides les plus jeunes. Le développement de l'ascite par rapport à l'âge de la tumeur a permis des mesures du liquide au 12e jour; à cette époque, la seule conclusion que nous puissions tirer est qu'il existe toujours de l'oxygène dans le liquide, malgré la consommation intense qui se poursuit dans la seringue ou il a été recueilli. Le niveau de l'oxygène *in vivo* est obligatoirement supérieur aux chiffres que nous avons obtenus *in vitro*, après un délai de quelques secondes.

La pente de décroissance de l'oxygène dans le liquide a été comparée à celle du même liquide tonométré sous 50 mm Hg de pO_2. On s'aperçoit, dans ces conditions, que les pentes sont peu différentes les unes des autres (Fig. 1). A partir de ce fait, nous nous croyons autorisés à penser que le niveau d'oxygénation des liquides d'ascite au 12e jour, et à fortiori des plus jeunes, est peut-être proche d'un niveau sanguin veineux. Le fait de retrouver un niveau d'O_2 dans le liquide ne doit pas faire conclure que l'oxygène disponible est suffisant pour réaliser un métabolisme aérobie satisfaisant pour les cellules. Mais ce résultat s'inscrit en faux contre les propositions classiques issues des expériences *in vitro* de Warburg selon lesquelles les ascites tumorales ne contiennent que des traces de glucose et d'oxygène [7]. De même, ces résultats sont en contradiction avec ceux de U. Del Monte, selon lesquels la pO_2 devient pratiquement nulle au stade tardif de l'évolution des liquides d'ascite de l'hépatome de Yoshida [8].

Quoique ce dernier auteur ait utilisé une mesure polarographique *in vivo*, on ne peut retenir, sans restriction, cette conclusion, quand on sait toutes les difficultés à recueillir des informations valables concernant la mesure de l'oxygène dissous dans des milieux ne contenant pas d'hémoglobine [9].

Les conclusions auxquelles nous sommes amenés seront obligatoirement limitées par le fait que l'étude *ex vivo* d'un milieu où le niveau énergétique est très élevé est particulièrement difficile, la consommation de l'oxygènation du milieu et le confinement conduisant à des informations ne reflétant plus l'état régnant *in vivo*. Cependant, on pourra retenir les éléments suivants:

Quel que soit le stade évolutif du liquide d'ascite, celui-ci contient une concentration de glucose notable puisque approchant 20 mg %. Ce résultat est très différent de ceux obtenus dans les expériences *in vitro*, ou le glucose n'est présent qu'à l'état de traces.

Aux deux stades envisagés, l'index lactique est élevé et le pH acide; mais on note une différence significative suivant l'âge, le vieillissement conduisant à un état d'acidose lactique majeure. Cette augmentation de l'index lactique est en faveur d'une perversion du métabolisme glucidique, du fait d'une diminution de l'oxygène disponible [10].

La présente étude sur la teneur en oxygène du liquide d'ascite ne permet pas de conclusion à ce sujet: on a reconnu la présence d'oxygène dans les liquides plus âgés, mais on a été incapable de préciser les valeurs absolues.

Cependant, l'existence d'un niveau de pO_2 relativement élevé, vis-à-vis du K_m pour l'oxygène de ces cellules [11], n'est absolument pas contradictoire avec le fait que l'oxygène disponible est insuffisant pour satisfaire la consommation d'oxygène de toute la population cellulaire; en effet, *le niveau de* pO_2, dans le liquide d'ascite, est fonction de l'irrigation sanguine régionale, tandis que *la quantité d'oxygène* mise à la disposition des cellules est fonction du volume de l'ascite et du coefficient de solubilité de O_2 dans ce milieu.

En conclusion, l'acidose mixte, lactique en particulier, témoin de la diminution de l'oxygène disponible (alors que le niveau de la pO_2 reste important) doit être reconnu comme l'élément métabolique primordial expliquant l'évolution de la durée du cycle cellulaire de ce type de tumeur.

Remerciements—Nour remercions Madame A. Marc et Mademoiselle F. Clavier pour leur collaboration technique.

RESUME

Dans le but d'expliquer les causes de allongement du cycle cellulaire au cours de la croissance de l'ascite expérimentale de la souris C3H, le présent travail rapporte certaines modifications métaboliques constatées au 5e et au 12e jour de l'évolution. Quel que soit le stade, la concentration en glucose reste supérieure à 20 mg %.

Le fait le plus significatif est consigné dans l'existence d'une acidose s'accroissant avec l'âge. Cette acidose est mixte, mais son caractère lactique est à souligner. L'augmentation de l'index lactique est en faveur d'une perversion du métabolisme glucidique, du fait d'une diminution de l'oxygène disponible. *Les études faites sur l'état de l'oxygène dans le liquide d'ascite ont permis de reconnaître la présence d'oxygène, mais sa mesure quantitative n'a été qu'approximative, contrariée par le fait que l'étude* ex vivo *de ce milieu à niveau énergétique très élevé conduit à des informations ne reflétant plus l'état* in vivo.

SUMMARY

In order to try to elucidate the reasons for the prolongation of the cell cycle during the growth of an experimental ascites tumour of the C3H mouse, the present work reports certain modifications of the metabolism in 5 and 12-day ascites tumours.

In both these stages of growth, the concentration of glucose remains above 20 mg/100.

The most significant fact to report is the existence of acidosis which increases with age. This acidosis is mixed but is mostly due to the accumulation of lactate. The increase in the lactic index is in favour of a perversion of glucose metabolism due to the decrease of oxygen at the disposal of the cells.

Our studies on the oxygen state in the ascitic fluid enabled us to recognize the presence of oxygen but quantitative measurements were only a rough estimate since the studies were conducted ex vivo *which do not correspond to quantities* in vivo *because of the very rapid consumption of oxygen by the cells.*

ZUSAMMENFASSUNG

Als Beitrag zur Klärung der Ursachen der Verlängerung des Zellcyclus während des Wachstums eines experimentellen Ascitestumors der C3H Maus, berichtet die vorliegende Arbeit über gewisse Stoffwechselveränderungen in 5 *und* 12 *Tagen alten Ascitestumoren.*

In beiden Wachstumsstadien bleibt die Glukosekonzentration über 20 *mg%.*

Auffallend ist das Vorhandensein einer mit dem Alter zunehmenden Azidosis. Diese Azidosis hat verschiedene Ursachen, geht aber hauptsächlich auf eine Laktatanhäufung zurück. Die Zunahme des Milchsäureanteils begünstigt eine Fehlleitung des Glukosestoffwechsels, die einer Verminderung des, den Zellen zur Verfügung stehenden, Sauerstoffs zuzuschreiben ist. Die Untersuchungen über den Nachweis des Sauerstoffs haben es möglich gemacht die Anwensenheit des Sauerstoffs in der Ascitesflüssigkeit zu erkennen, jedoch quantitative Messungen sind nur eine ungefähre Abschätzung, da die Studien ausserhalb des Organismus durchgeführt worden sind und bei dem sehr raschen Sauerstoffverbrauch der Zellen nicht mit den Verhältnissen in vivo *übereinstimmen.*

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Studies on the Circulation of Experimental Tumours

I. Effect of Induced Fibrinolysis and Antifibrinolysis on Capillary Blood Flow and the Capillary Transport Function of two Experimental Tumours in the Mouse

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INTRODUCTION

THE GROWTH rate and the spontaneous metastases to the lungs of a transplanted mouse mammary carcinoma was significantly inhibited by induced antifibrinolysis and stimulated by induced fibrinolysis. Moreover, the growth rate of a 20-methylcholanthrene induced sarcoma in animals of the same strain was stimulated by induced fibrinolysis, but was not influenced by induced antifibrinolysis. The spontaneous metastasis formation from this tumour remained unchanged [1–3].

The discrepancy between the two tumours was thought to be explained by different properties of the tumour tissue, i.e. the mammary carcinoma had a high thromboplastic activity, but no spontaneous fibrinolytic activity, while the sarcoma had a comparably lower thromboplastic activity and spontaneous fibrinolytic activity.

In preliminary studies on the mouse mammary carcinoma by vital microscopy [4] wide, tortuous vessels with slow blood flow and a tendency to thrombus formation were noted. From this it was suggested that induced fibrinolysis might decrease the tendency to thrombus formation and thus increase the tumour blood flow, the tumour growth rate and the shedding of cells from the tumour. On the other hand induced antifibrinolysis might have the reverse effect. Such effects could then explain the results obtained in the studies on tumour growth and on spontaneous metastasis formation from the mouse mammary carcinoma. It might also be suggested that such mechanisms are less important for the 20-methylcholanthrene induced sarcoma, which has a spontaneous fibrinolytic activity.

The aim of the present investigation was to study the blood flow and the capillary transport function in the two mouse tumours and the effect of induced fibrinolysis and antifibrinolysis on these parameters.

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MATERIAL AND METHODS

Animals

C_3H hybrid mice 3–4 months old and with a mean body weight of about 30 g were used. The animals were fed *ad libitum* a diet of rat

pellets* and water. They were kept in plastic cages, 10 animals in each, in an air-conditioned room at about 22°C. Animals of the same sex were used in each experiment.

Tumours

A spontaneous mammary carcinoma. This tumour developed in a female mouse of the C_3H-strain in 1963 and was later continuously transplanted intramuscularly in one hindleg in animals of the same strain. The microscopic picture of this tumour was that of a low differentiated carcinoma with cells of different shape and size. It grew infiltrating muscle tissue and spontaneous metastases to the lungs of the animals were found to occur between 7 to 9 days after transplantation of tumour tissue, when a local tumour was palpable at the site of the tumour transplantation.

A 20-methylcholanthrene induced sarcoma. This tumour developed in February 1967 in a C_3H mouse, injected subcutaneously 3 months earlier with 1 mg of 20-methylcholanthrene suspended in sesame oil. This tumour was also continuously transplanted intramuscularly in animals of the same strain. The microscopic picture was that of an undifferentiated sarcoma with rounded and slender shaped cells of different size. It infiltrated into muscle tissue and spontaneous metastases to the lungs were noted as for the carcinoma.

Tumour transplantation. Local tumours in animals were obtained by implanting a solid piece of tumour tissue, about 1 mm^3, intramuscularly in one hindleg of the animals by a trochar technique. Tumour circulation was studied 14 days after transplantation when the tumour had a mean diameter of about 10 mm.

Fibrinolysis and antifibrinolysis

Fibrinolysis was induced by injecting animals intraperitoneally twice daily with 0·3 casein units (Sgouris) of human plasminogen activated with 1000 units of streptokinase ("Kabikinas")† in a volume of 0·1 ml per 10 g body wt. A continuous high fibrinolytic activity in blood was obtained in this way. It was induced for 3 days before the tumour circulation studies.

Antifibrinolysis was induced by giving animals a 3% concentration of an antifibrinolytic agent, tranexamic acid—AMCA ("Cyclokapron")† in their drinking water for 3 days before the investigation. Each animal consumed about 150 mg of tranexamic acid per day.

Tumour circulation

Xenon is a small, lipid-soluble, highly diffusible substance that comes rapidly into diffusion equilibrium with the tissues following local injection. Blood flowing through capillaries receives xenon by diffusion through presumably the whole of the capillary membrane. This xenon is then carried away in the venous return from the tissue and expired virtually entirely in its first passage through the capillary bed of the lung. The amount of xenon removed per unit time is thus proportional to the capillary flow. Using radioactive xenon this can easily be measured by noting the rate of decline detected externally over the injection side. For a discussion of the xenon clearance method for the measurement of tumour blood flow the reader is referred to Gelin *et al.* [5].

While the clearance of xenon is limited only by capillary flow, the clearance of water-soluble, more slowly diffusible substances, e.g. $^{24}Na^+$, is limited both by blood flow and by the transport characteristics of the capillary bed. The most important features here are the size of the capillary bed open to flow and the permeability of the capillary wall to the substance in question. Since therefore, the level of clearance of sodium following local injection is dependent both on blood flow and on the transport characteristics of the capillary bed, measurement of this parameter alone can give only the resultant of these two factors.

However, by determining xenon clearance simultaneously, in the same capillary bed, the blood flow factor can be measured independently. Knowing then the capillary flow, the level of sodium clearance, at that particular flow level, indicates the combined permeability –surface area factor (transport function) of the capillary bed. For a detailed discussion of this subject the reader is referred to Appelgren and Lewis [6] and Lassen and Trap-Jensen [7].

Thus it can be said that capillary blood flow was measured by the clearance of locally injected radioactive xenon (^{133}Xe) and the capillary transport function of the vascular bed measured by comparison of the simultaneous clearance of two substances, one water-soluble and relatively slowly diffusible ($^{24}Na^+$) and the other lipid-soluble and relatively rapidly diffusible (^{133}Xe).

Technique. Radioactive xenon (^{133}Xe) dissolved in saline (4mCi/0·4 ml) and radioactive sodium (^{24}Na, 8mCi/1·0 ml) were obtained from the Swedish Atomic Energy Commission, Sweden. The two isotopes were mixed anaero-

*Manufactured by AB EWOS, Södertälje, Sweden.
†Kindly supplied by AB Kabi, Stockholm, Sweden

bically in one syringe so as to give a final count rate of xenon approximately 3–5 times that of sodium.

The animals were lightly anaesthetized intraperitoneally with pentobarbital (0·5 mg/10 g body weight in a volume of 0·1 ml). The tumour in the thigh of the animals was carefully exposed by dissecting away the overlying tissues. The isotope (0·05 ml–0·1 ml) was injected into the tumour tissue. Each animal was injected only once. An attempt was made to inject into a viable portion of the tumour. If subsequent inspection showed that in all likelihood injection had been made into a grossly necrotic region, these data were discarded. In a number of instances the reproducibility of the method was checked by making a second injection after the activity of the first had disappeared. In those situations in which the state of the animal was unchanged, neither flow nor transport was significantly different than at the first inspection. With changes in the status of the animal, mostly due to changes in the level of anesthesia, there were differences in the flow but not in the transport function.

Activity of the two isotopes was detected with one $1\frac{1}{2}$ in. NaI crystal in a cylindrical thick lead collimator positioned directly over the tumour. The activity was then led to two pulse height analyzers and ratemeters with a time constant of 10 sec. Initial count rates for ^{133}Xe were of the order of 50·000–100·000 cpm and 10·000–30·000 for ^{24}Na. ^{24}Na activity was completely independent of ^{133}Xe activity, since the sodium channel was completely insensitive to the ^{133}Xe gamma emission. At the settings used here the interference of ^{24}Na on the ^{133}Xe channel was of the order of 8–12 % and with the higher ^{133}Xe activity, correction of the ^{133}Xe curve for ^{24}Na interference was not found necessary.

The disappearance curves for both isotopes were written directly using linear potentiometric recorders. These were then replotted on semilogarithmic paper so as to obtain the half-times of disappearance ($t_{1/2}$), that is the time it takes the curve to go down to one-half of any given value. Capillary flow in ml/min/g tissue$=\lambda \cdot \ln 2/t_{1/2}$, where λ is the partition coefficient for xenon between tissue and blood and ln 2 is the natural logarithm of 2($=0·693$). Since λ is not at present known for these experimental tumours, it was not possible to calculate flow in ml/min/g. Assuming, however, that this is most likely the same for all of the animals within one tumour group, a value proportional to flow can be calculated, i.e. $\ln 2/t_{1/2}$. This value, the so-called clearance constant (k) is that reported here, and is as mentioned, proportional to the flow, for the xenon disappearance curve. For the ^{24}Na curve it is also an expression of the rate of disappearance of the isotope, directly comparable to that for xenon.

RESULTS

A. *Mammary carcinoma.* Twenty-eight animals were used, 10 controls, 9 animals given tran-

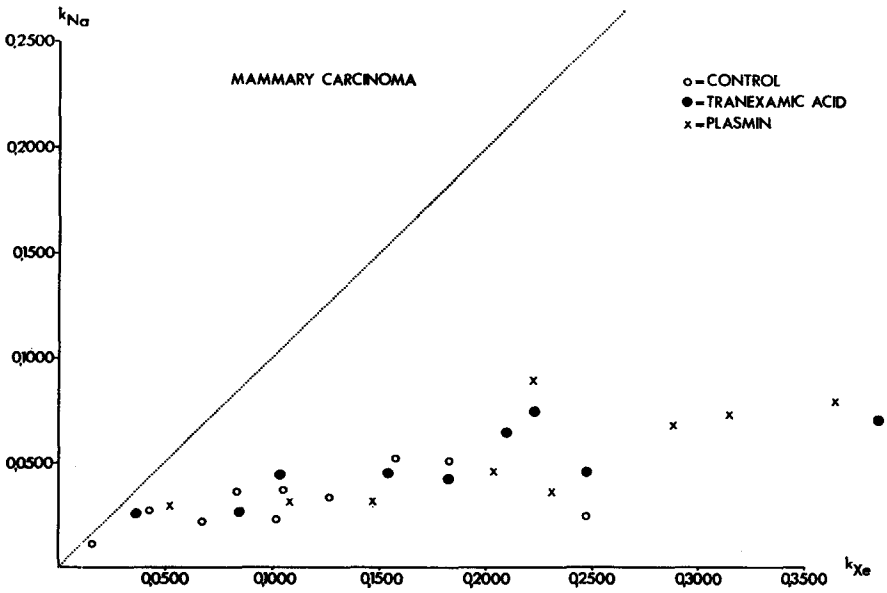

Fig. 1. *Mammary carcinoma. Clearance constant for sodium* (k_{Na}) *vs. that for xenon* (k_{Xe}) *for controls (open circles), animals treated with tranexamic acid (closed circles) and those treated with plasmin (crosses). One injection per animal. Note the wide spread of flow values* (k_{Xe}) *but relatively uniform transport characteristics with no difference between the three groups. The dotted line is the line of identity.*

examic acid and 9 animals given plasmin. The data are shown graphically in Fig. 1. Here the clearance constant for ^{24}Na (k_{Na}) has been plotted on the ordinate against the corresponding value for xenon (k_{Xe}) on the abscissa. For the control animals there is a wide spread of flow values as evidenced by k_{Xe} which ranged from approximately 0·0160–0·2470. This is very likely due not only to differences in the individual tumours, but also to variations in the state of the animals and in injection sites within the tumours. The relationship between k_{Na} and k_{Xe} described virtually one line which begins at or near the origin and deviates more and more from the line of identity with increasing k_{Xe}, reaching eventually a plateau. This is in agreement with the theory, and indicates that in these animals, despite the wide variations in flow, there were relatively uniform capillary transport characteristics.

For both of the treated groups (tranexamic acid and plasmin) the spread of flow values and the relationship between k_{Na} and k_{Xe} were almost identical and the same as the control.

B. 20-*methylcholanthrene induced sarcoma.* Twenty-seven animals were used, 9 controls, 9 animals given tranexamic acid and 9 animals given plasmin. The data are shown graphically in Fig. 2, which has been plotted in the same way as Fig. 1. As in the mammary carcinoma there is a wide spread of flow values for the animals within each group, but almost exactly the same spread for all 3 groups. Also similar to the mammary carcinoma, the relationship between k_{Na} and k_{Xe} for each group can be reasonably described by one line, indicating relative uniformity in capillary transport characteristics within each group. Note also that the 3 "lines" are virtually identical. Here too neither tranexamic acid nor plasmin affected the two flow parameters measured.

For the sarcoma animals as a whole the plateau value appears to be reached at a k_{Na} value lower than in the mammary carcinoma, k_{Na}=approximately 0·0200 (sarcoma) versus approximately 0·0400 (carcinoma). From these preliminary data it is difficult to say if this difference is statistically significant, but it does suggest that the maximal capillary transport function is greater in the mammary carcinoma than in the 20-methylcholanthrene induced sarcoma.

DISCUSSION

All flow techniques have their disadvantages as well as advantages, and most techniques are applicable only under certain circumstances. Until the advent of the various recent isotope techniques for the quantitation of capillary blood flow in peripheral tissues, virtually no method was applicable to the measurement of tumour blood flow, either in man or in the experimental animal. Gullino and Grantham [8], in an ingenious application of the venous outflow method, measured the total blood flow through experimental tumours which had completely replaced isolated organs. Wartnaby *et al.* [9] used the bromsulphonphthalein

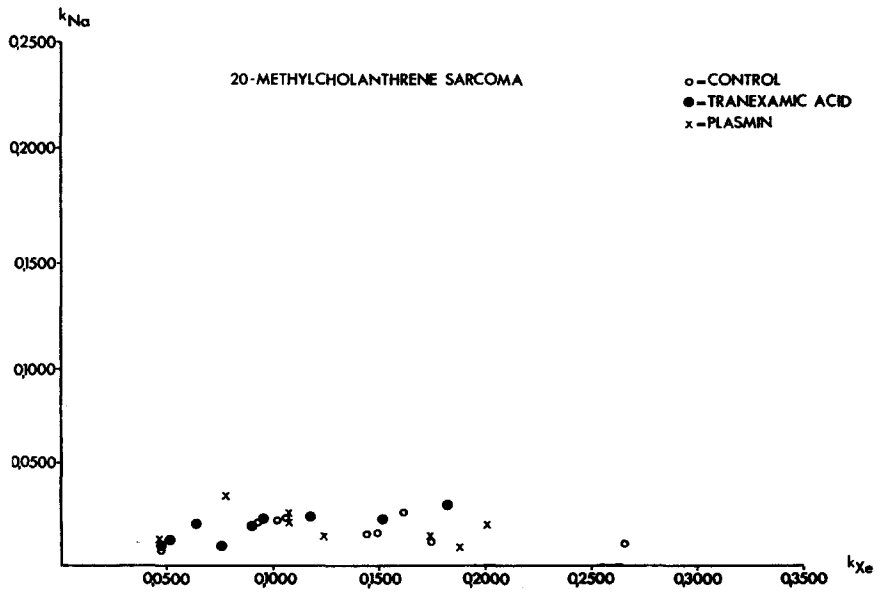

Fig. 2. Methylcholanthrene-20 induced sarcoma. Data plotted in the same way as in Fig. 1. Note, as in mammary carcinoma, the wide spread of flow values but relatively uniform transport characteristics and the lack of difference between the 3 groups. Note also that for any given k_{Xe} value, the k_{Na} values for this tumour are at a lower level than for the mammary carcinoma.

method to measure blood flow through a large hepatic metastasis in a patient by passing a catheter into the vein draining the tumour. Such methods are obviously applicable only in situations in which the venous blood sample drains exclusively tumour tissue.

Sapirstein's method [10] for the measurement of capillary blood flow by the fractional tissue uptake of a highly diffusible ion, for which the cells act as a sink, was applied by Cataland *et al.* [11] for the measurement of blood flow in two transplantable mouse tumours. The advantage of this method is that it does measure capillary flow of the tissue studied. It has the disadvantages, however, that to express flow in ml/min the cardiac output must be known and that the animals must be killed to make the measurement of tumour blood flow.

The clearance techniques using radioactive inert gases also measure capillary blood flow and avoid the necessity of measuring cardiac output and of killing the animals to quantitate tumour blood flow. Gump and White [12] measured blood flow of a transplantable carcinoma in rabbits by the tissue clearance of radioactive krypton (^{85}Kr). Gelin *et al.* [5] measured the clearance of radioactive xenon (^{133}Xe) from metastatic tumours in human livers at the time of operation.

These isotopes have been used in various ways and the variations depend upon the method of delivery of the isotope to the tissue and the method of detection. Delivery of the isotope may be made via the arterial supply of the tissue or by local injection. Local injection was used by Gelin *et al.* [5]. To get the isotope into the arterial blood supply, it may be injected close intra-arterially or it may be inhaled. This latter approach was used by Gump and White [12].

Depending upon the type of emissions from the isotope, detection may be made locally (β emissions) or at a distance (γ emissions). The advantages and disadvantages of the various combinations of delivery and detection need not to be gone into detail here, but several features, related both to previous studies and to that reported here, can be mentioned. As pointed out by Gump and White [12] and by Gelin *et al.* [5], regardless of the isotope used, the partition coefficient for the isotope in question between tumour tissue and blood must be known before the disappearance curve can be converted into flow in ml/min/unit weight of tissue. When gamma detection is used the delivery of the isotope must be exclusively to the tumour tissue. When a β detector is placed on the surface of a tumour only the flow immediately under the detector is measured. To explore other areas of the tumour, a β detector incorporated in a needle can be used [13], but this is also not free from problems [14].

In the present study capillary blood flow in two transplanted experimental tumours in the mouse was estimated by the clearance of locally injected radioactive xenon (^{133}Xe) at the same time that the capillary transport function of the tumour vascular bed was estimated by the simultaneous clearance of xenon and sodium (^{24}Na). Simultaneous determination of these two parameters has not only given additional valuable information about the tumour, but has also simplified comparison between the various treatment schedules employed. This was of importance, since there was such a wide spread of flow values.

In most experimental tumours studied, a low blood flow rate was found compared to the flow rate in the corresponding normal tissue [11, 8]. In the present investigation determination of the partition coefficient for xenon between tumour tissue and blood has not been made, so that only clearance constants have been reported. The xenon partition coefficient for tissues with a low fat content is of the order of 0·72 [15]. Using this value, the flow range for the mammary carcinoma would have been 0·01–0·17 ml/min/g tissue and the corresponding value for the sarcoma 0·04–0·19 ml/min/g tissue.

Cataland *et al.* [11] observed that the tumour blood flow in small tumours was comparably greater than that of larger, older tumours. This was explained by the regular observation of central necroses developing in the experimental tumours during growth. In the two mouse tumours studied in the present investigation, the tendency to the development of central necrosis in growing tumours was higher in the sarcoma than in the carcinoma. This may well be correlated to the observed tendency to a lesser capillary transport in the sarcoma than in the carcinoma.

On the basis of previous studies [1, 3] it was thought that the changes observed in tumour growth and metastases as a result of induced fibrinolysis and induced antifibrinolysis could be due to changes in the blood flow through the tumour. The results of the present study indicate clearly that neither induced fibrinolysis (plasmin) nor induced antifibrinolysis (tranexamic acid) alter the capillary blood flow or the capillary transport function of these tumours. It would thus appear most likely

that the observed influence of plasmin and tranexamic acid on tumour growth and spontaneous metastasis formation is not due to changes in tumour circulation.

Spontaneous metastasis formation reflects in part the shedding of cells from the transplanted tumour. Since no changes in tumour circulation were found, the influence of induced fibrinolysis and antifibrinolysis on the shedding of tumour cells is probably due to other factors, among them an increased or decreased growth rate of the transplanted primary tumour or an influence on the adhesiveness of cells in the transplanted tumours [2].

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RESUME

1. *Le débit sanguin et la fonction de transport de capillaires de deux tumeurs expérimentales de la souris (un carcinome mammaire et un sarcome induit par le 20-méthylcholanthrène) ont été déterminés par la mesure de la clearance de deux isotopes radioactifs injectés dans les tumeurs, le ^{133}Xe et le ^{24}Na.*
2. *Les résultats ont montré de grandes variations individuelles du débit sanguin dans les différentes tumeurs, mais néanmoins des valeurs assez uniformes de la fonction de transport.*
3. *Une fibrinolyse fut provoquée par la plasmine, et une antifibrinolyse par l'acide tranexamique, dans deux groupes d'animaux avec tumeurs transplantées; le débit sanguin et la fonction de transport ne furent pas modifiés par ces méthodes.*
4. *Par conséquent, les effets de la fibrinolyse et de l'antifibrinolyse sur la progression et la dissémination des tumeurs, effets qui furent précédemment observés dans ces deux tumeurs, ne sont pas dus à des modifications de la circulation.*

SUMMARY

1. *In two transplanted experimental tumours in the mouse (mammary carcinoma and 20-methylcholanthrene induced sarcoma) studies were carried out on the capillary blood flow and the capillary transport function of the tumour vasculature by measuring simultaneously the clearance of two locally injected radioactive isotopes, ^{133}Xe and ^{24}Na.*
2. *Results indicated a wide spread of flow values from animal to animal for both tumours, but despite this fact relatively uniform transport characteristics.*
3. *Fibrinolysis was induced with plasmin and antifibrinolysis with tranexamic acid in two additional groups of animals with the transplanted tumours. Neither procedure altered the blood flow or the transport characteristics of either tumour.*
4. *It is concluded that the previously observed effects of plasmin and tranexamic acid on the growth rate and spontaneous metastasis formation of these two tumours in the mouse are not due to changes in tumour circulation.*

ZUSAMMENFASSUNG

1. *In zwei bei Mäusen transplantierten Tumoren (ein Mammacarcinom und ein mit 20-Methylcholanthren erzeugtes Sarkom) wurde die Kapillardurchblutung, sowie der Vorgang des kapillaren Transportes durch gleichzeitige Bestimmung der Clearance von zwei lokal injizierten Isotopen, ^{133}Xe und ^{24}Na, bestimmt.*
2. *Trotz gleichguter Transporteigenschaften, zeigten die Ergebnisse in beiden Tumoren grosse Durchblutungsdifferenzen bei den verschiedenen Tieren.*
3. *In einer weiteren Gruppe von Tieren mit transplantierten Tumoren wurde Fibrinolyse durch Plasmin und Antifibrinolyse durch AMCA hervorgerufen. Keine dieser Behandlungen konnte die Durchblutung oder Transporteigenschaften in den beiden Geschwülsten ändern.*
4. *Aus diesen Ergebnissen kann man den Rückschluss ziehen, dass der früher festgestellte Einfluss von Plasmin und AMCA auf die Zuwachsgeschwindigkeit und die Bildung von spontanen Metastasen dieser beiden Tumoren bei der Maus nicht auf Änderungen der Tumordurchblutung zurückgeführt werden kann.*

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Tumeurs Hypophysaires Induites par les Oestrogènes chez le Rat

I. Activité Fonctionnelle, Histologie et Ultrastructure

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INTRODUCTION

L'ADMINISTRATION prolongée d'oestrogènes provoque la formation de tumeurs hypophysaires [1–3], composées de cellules à prolactine, et exerçant un effet mammo-lutéotrope [4, 5]; en même temps se produit une diminution de l'activité gonadotrope [6] avec, pour corollaire, une atrophie progressive des glandes génitales [7–10]. Au cours de transplantations répétées chez des animaux isologues, les tumeurs acquièrent la capacité de poursuivre leur croissance en l'absence de stimulation oestrogénique. Les effets des transplants, hormonodépendants et autonomes, ne se limitent pas toujours aux activités mammotrope et lutéotrope; il s'y associe généralement une activité somatotrope [11] et parfois corticotrope dans le cas de certaines souches tumorales devenues autonomes [12].

Nous avons répété ces expériences d'induction et de transplantations tumorales, et nous nous sommes notamment intéressés aux conséquences éloignées de l'arrêt définitif du traitement oestrogénique. Peu d'auteurs ont étudié cette dernière question et les résultats qu'ils ont publiés comportent des discordances. On a généralement décrit une régression du volume hypophysaire mais, d'après les auteurs, celle-ci serait complète [13, 14], incomplète [5, 15], ou même nulle [16, 17]. De même, la restauration des fonctions sexuelles est totale pour les uns [15, 18–20] et partielle pour les autres [13, 14, 21]. Emmens [19] observe, chez la souris, que l'activité sexuelle se rétablit progressivement, le degré de normalisation étant inversement proportionnel à la dose et à la longueur du traitement oestrogénique antérieur. Ce dernier résultat suggère que les divergences constatées dans la littérature tiennent en grande partie à des différences dans les modalités expérimentales mises en oeuvre.

Un autre but de nos recherches était de vérifier si les tumeurs induites ne peuvent, spontanément, devenir autonomes après l'arrêt du traitement. C'est la raison pour laquelle nous avons pris soin d'interrompre l'oestrogénisation à un moment où les tumeurs se trouvaient encore dans la phase de dépendance hormonale; malgré cela, certains de nos transplants mis en carence oestrogénique ont continué à pousser. D'autres tumeurs greffées sont devenues indépendantes au cours de transplantations répétées. L'accession à l'indé-

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Tableau 1. *Répartition des animaux dans les groupes I*

Groupe	Nombre d'animaux mâles	femelles	Traitement oestrogénique (jours)	Phase postoestrog. (jours)
I.A.	5	4	28	0
	3	5	84	0
	3	5	140	0
	5	4	196	0
I.B.	5	5	28	112
	5	4	84	28
	3	8	84	112
	7	5	140	112
	5	4	196	112

pendance s'est généralement accompagnée d'un virage dans l'activité fonctionnelle et d'une transformation correspondante de la morphologie.

La littérature consacrée aux tumeurs hypophysaires induites par les oestrogènes a été récemment passée en revue par Furth et Clifton [22]. Dans l'ensemble, les auteurs se sont intéressés aux activités endocriniennes et au comportement biologique de leurs tumeurs beaucoup plus qu'à la cytologie de celles-ci, ce qui peut expliquer l'opinion défendue par Furth et Clifton [22], selon laquelle un type cellulaire peut sécréter plusieurs hormones hypophysaires. Les études cytologiques publiées ont été basées sur des techniques histologiques qui précisent mal les différentes catégories de cellules acidophiles [23–26]. Les publications consacrées à l'ultrastructure des tumeurs hypophysaires induites par les oestrogènes sont peu nombreuses [27–30]. Aussi nous avons jugé utile de procéder à une analyse histologique soigneuse de notre matériel tumoral; nous l'avons complétée par des examens au microscope électronique.

Dans la présentation de nos résultats, nous mettrons à part les tumeurs greffées devenues indépendantes, soit au cours de transplantations répétées, soit après l'interruption du traitement inducteur. Elles feront l'objet d'un second chapitre dans lequel seront évoqués les problèmes de tumorigénèse que soulève l'ensemble de nos constatations.

MATERIEL ET METHODES

Animaux et traitements

Des rats blancs de souche pure, appartenant à la lignée R Amsterdam, et âgés de 6 à 7 mois au début de l'expérience, ont été oestrogénisés par l'implantation, sous la peau du cou, d'un comprimé de 25 mg de dipropionate de diéthylstilboestrol qui a été laissé en place pendant des temps variables. Des fragments de tumeurs hypophysaires, induites par ce traitement, ont été transplantés sous la capsule du rein gauche chez des animaux normaux de même souche. A chaque transplantation, la tumeur est distribuée à 3 animaux dont 2 sont oestrogénisés; le troisième hôte, non traité et châtré lorsqu'il s'agit d'un mâle, sert à vérifier si la tumeur a acquis son autonomie. Lorsque l'hôte a été soumis au traitement oestrogénique, l'implantation du comprimé hormonal a été effectuée dans la même séance que la transplantation du tissu tumoral.

Quatre groupes expérimentaux ont été

Tableau 2. *Evolution de* 25 *tumeurs hypophysaires induites par les oestrogènes et entretenues par des transplantations successives chez des rats oestrogénisés*

Souches tumorales	Nombre						
Restées dépendantes (Groupe II A)	17	15	12	10	8	5	?
Devenues autonomes (Groupe III A, rat N°)	1 (1)	—	—	1 (2)	—	—	1 (3)
Perdues	7	2	3	1	2	3	?
Transferts n°	1	2	3	4	5	6	7

constituées en vue d'étudier les effets du traitement oestrogénique et de l'arrêt de celui-ci sur les hypophyses respectivement *in situ* et greffées.

Groupe I. A. Les animaux de ce groupe, 16 mâles et 18 femelles, ont été soumis à une oestrogénisation continue, au terme de laquelle ils ont été sacrifiés. Quatre séries ont été constituées, qui diffèrent par la durée de l'oestrogénisation: celle-ci a été respectivement de 28, 84, 140 et 196 jours (Tableau 1).

Groupe I. B. Les animaux de ce groupe, 25 mâles et 26 femelles, ont été oestrogénisés dans les mêmes conditions que ceux du groupe précédent. Ils ont ensuite été abandonnés, sans traitement, pendant une période de 28, ou, le plus souvent, de 112 jours avant d'être sacrifiés. (Tableau 1). A ce groupe se rattache une série supplémentaire de 3 animaux dont l'hypophyse a été réservée à l'examen en microscopie électronique. Dans cette série, l'oestrogénisation a duré de 146 à 211 jours, tandis que la phase post-oestrogénique a varié entre 133 et 290 jours.

Groupe II. A. Des tumeurs hypophysaires ont été induites chez 25 rats par une oestrogénisation continue dont la durée a varié entre 139 et 246 jours. Elles ont ensuite été transplantées, de génération en génération, à des rats le plus souvent mâles, chez lesquels leur séjour a duré 6 mois en moyenne, avec des variations conditionnées par la cachectisation plus ou moins rapide de l'hôte. Au cours de ces transplantations répétées, 3 tumeurs sont devenues autonomes et ont été incluses dans le groupe III. A. (Tableau 2).

Groupe II. B. Des fragments de tumeurs hypophysaires ont été greffés à 12 animaux, 11 mâles et 1 femelle, qui ont été oestrogénisés pendant une période allant de 146 à 210 jours. Au moment de l'ablation du comprimé hormonal, une laparotomie exploratrice a été pratiquée chez 11 rats pour apprécier la taille atteinte par le greffon tumoral. Les animaux ont été sacrifiés entre 126 et 361 jours après l'arrêt de l'oestrogénisation. Les greffes sont de sources différentes. Dans 5 cas, elles proviennent d'une hypophyse rendue tumorale *in situ* par une oestrogénisation poursuivie jusqu'au moment du prélèvement. Les autres fois, le greffon consiste en un fragment d'une tumeur ayant déjà été transplantée chez d'autres rats: le greffon en est à son 2e transfert dans 4 cas, à son 3e dans 2 cas et à son 5e dans 1 cas. Trois de ces tumeurs ont manifesté, au cours de la phase post-oestrogénique, des signes de vitalité marquée, se traduisant, chez leur hôte, par un effet mammosomatotrope important. De ce fait, elles ont été considérées comme autonomes et transférées dans le groupe III. B.

Groupe III. A. Ce groupe comprend les 3 tumeurs du groupe II. A., devenues indépendantes au cours des transplantations répétées (Tableau 2).

Groupe III. B. Ce groupe comprend les 3 tumeurs qui, au cours de l'interruption définitive du traitement oestrogénique, ont accédé à l'indépendance. Le Tableau 3 indique les conditions expérimentales auxquelles elles ont été soumises.

Méthodes d'observation

(1) *Vérification de l'autonomie.* Les tumeurs qui n'ont pas régressé après l'interruption du traitement oestrogénique et celles dont la greffe s'est développée chez un animal non traité ont été présumées autonomes. Leur propriété d'autonomie a été en outre mise à l'épreuve par la transplantation à un nouvel hôte non traité.

(2) *Etudes des effets fonctionnels.* Pendant leur vie, les rats porteurs d'une greffe ont subi une pesée hebdomadaire; chez les femelles, un frottis vaginal a été examiné chaque jour de la semaine. A l'autopsie, les organes suivants ont été prélevés, pesés et fixés dans du liquide de Bouin pour l'examen histologique: testicules,

Tableau 3. *Souches tumorales du groupe III B ayant acquis leur indépendance à la suite de l'interruption du traitement oestrogénique. Traitements subis avant l'accession à l'indépendance*

Tumeur n°	Transfert n°	Traitement oestrogénique	Phase post-oestrogénique
		jours	jours
4	2	210	225
5	3	160	126
6	2	167	361

Tableau 4. *Poids moyen ± déviation standard des hypophyses des rats appartenant aux groupes I*

Groupe	Phase oestrogénique	Phase post-oestrogénique	N^bre. d'animaux males	femelles	Hypophyse
	jours	jours			mg
IA	28	0	5	—	29,9±1,8
			—	4	40,9±2,1
IB	28	112	5	—	11,1±1,4
			—	5	17,7±1,9
IA	84	0	3	—	48,1±5,5
			—	5	48,1±8,2
IB	84	28	5	—	21,0±1,1
			—	4	24,1±4,8
IB	84	112	3	—	24,9±3,5
			—	8	36,7+11,3
IA	140	0	3	—	63,9±5,5
			—	4	62,4±15,9
IB	140	112	7	—	52,2±19,3
			—	5	63,0±26,4
IA	196	0	5	—	173,6±46,1
			—	4	87,2±15,5
IB	196	112	5	—	79,9±14,5
			—	4	130,3±27,6

vésicules séminales et prostate, ovaires et tractus génital, surrénales, thymus, foie, reins et rate. Les glandes mammaires ont été prélevées *in toto* chez la plupart des animaux.

(3) *Etudes morphologiques des hypophyses* in situ *et transplantées.* Après pesée, ces organes ont été fixés pour l'analyse histologique dans du Bouin–Hollande-sublimé. Les coupes à la paraffine ont été colorées au Dominici, à l'azocarmin de Heidenhain, au PAS-orange G, à l'aldéhyde-fuchsine et au tétrachrome de Herlant [31]. Pour la microscopie électronique, les fragments ont été fixés pendant 1 à 2 h. à la température de la chambre dans une solution à 1% de tétroxyde d'osmium dans du tampon Sörensen à pH 7,2; ils ont été enrobés dans du Durcupan "Fluka" ou dans des résines époxy "Dow" [32]. Les coupes ont été contrastées par l'application successive, et pendant 2

Tableau 5. *Poids moyen±déviation standard du corps, du foie, et des surrénales, des rats appartenant aux groupes I et II*

Groupe	Sexe	Phase oestrogén.	Phase post-oestrogén.	N^bre. de rats	Corps	Foie	Surrénales
		jours	jours		g	g	mg
IA	M	28 à 84	0	8	190±12	9,22±1,22	68±5
	M	140 à 196	0	8	185±24	8,11±1,38	72±4
	F	28 à 84	0	9	147±6	6,75±0,47	67±6
	F	140 à 196	0	9	140±11	7,31±1,06	61±4
IB	M	28 à 84	112	8	283±24	10,14±0,49	34±3
	M	140 à 196	112	12	272±31	10,16±,037	55±4
	F	28 à 84	112	13	206±14	7,49±0,74	48±6
	F	140 à 196	112	9	198±10	8,15±0,58	49±7
IIA	M	130 à 247	0	74	163±18	8,19±1,20	74,3±12
IIB	M	146 à 210	126 à 361	9	275±37	9,88±1,10	85±23

minutes chaque fois, d'une solution à 2% de permanganate de potassium et d'une solution de citrate de plomb [33]. Les grilles ont été examinées dans un microscope Zeiss EM-9.

I. TUMEURS HYPOPHYSAIRES IN SITU ET GREFFEES EN PHASE DE DEPENDANCE HORMONALE

RESULTATS

Groupe I. A.: Evolution des tumeurs hypophysaires in situ *au cours de la phase d'induction tumorale*

Observations générales: Pendant toute la durée de l'oestrogénisation se produit un amaigrissement continu aboutissant, en 6 mois, à la cachexie de l'animal. A l'autopsie, les organes sont de taille réduite, à l'exception de l'hypophyse (Tableau 4) et des surrénales (Tableau 5): celles-ci ont doublé de volume et montrent une hypertrophie du cortex. Du point de vue gonadique, chez la femelle, les résultats de l'oestrogénisation varient avec la durée de celle-ci. Lorsqu'elle ne dépasse pas 104 jours, on observe un dioestrus permanent, des ovaires garnis de volumineux corps jaunes actifs, un vagin de type gravidique et un important développement lobulo-alvéolaire des glandes mammaires qui montrent des phénomènes de sécrétion active. Avec des temps d'oestrogénisation plus longs, allant de 140 à 196 jours, le dioestrus vaginal est entrecoupé de brèves phases oestrales ou fait place à des oestrus prolongés; les ovaires sont devenus atrophiques, les vagins sont kératinisés et les glandes mammaires sont en involution, ne montrant plus que quelques kystes lactés. Chez le mâle se produit une atrophie gonadique et annexielle progressive avec arrêt de la spermatogénèse.

Hypophyse: Le Tableau 4 indique le poids moyen des hypophyses au terme des différentes périodes d'oestrogénisation. On voit que, pendant les 28 premiers jours, le rythme de croissance de l'hypophyse est plus lent chez les mâles ($P<0{,}05$); ensuite, cette distinction s'efface et d'importantes variations individuelles apparaissent. A l'examen histologique, le lobe antérieur de l'hypophyse se montre composé d'un nombre important de cellules à prolactine, identifiées grâce à la coloration rouge de leurs grains de sécrétion au tétrachrome de Herlant; l'hypertrophie et la basophilie intense de leur cytoplasme, ainsi que la présence d'un volumineux idiosome paranucléaire, sont autant de signes d'une activité élaboratrice considérable. Au sein des nappes diffuses de cellules à prolactine, on parvient encore à reconnaître des cellules somatotropes; les éléments basophiles, par contre, disparaissent à peu près totalement. Après 28 jours de traitement, l'hypophyse présente déjà une homogénéisation cellulaire complète mais sa structure générale est encore normale. Après 84 jours de traitement on voit apparaître des lacs séreux, une dilatation diffuse des sinusoïdes et une accumulation de colloïde dans la fente hypophysaire. Après 140 jours s'individualisent des nodules cellulaires d'allure adénomateuse; l'hypophyse est, à ce moment, parsemée de foyers hémorragiques et nécrotiques. Chez aucun de nos animaux, nous n'avons observé d'atypies cellulaires.

Ultrastructure hypophysaire: L'image est dominée par la présence d'innombrables cellules à prolactine, facilement identifiables par leur granulation densément osmiophile et polymorphe; le trait caractéristique, pendant cette phase d'induction, est la présence d'un ergastoplasme très développé, de structure feuilletée et présentant de multiples enroulements en 'Nebenkerne' (Fig. 1). Les éléments appartenant aux autres catégories cellulaires sont rares, à tel point qu'il devient difficile d'apprécier leur niveau d'activité.

Groupe I. B.: Evolution des tumeurs hypophysaires in situ *après l'interruption du traitement inducteur*

Observations générales: L'ablation du comprimé hormonal est immédiatement suivie d'une ascension du poids du corps qui, en 4 à 8 semaines, rejoint le niveau atteint dans notre élevage par les rats de même âge et de même sexe. A partir de ce niveau, la croissance se poursuit suivant le rythme physiologique et, à l'autopsie, les organes sont de taille normale (Tableau 5). Font exception: l'hypophyse (Tableau 4) et la surrénale (Tableau 5); celles-ci gardent un poids supérieur à la normale. Du point de vue gonadique, chez les femelles, l'interruption du traitement même prolongée pendant 112 jours, n'apporte aucun changement aux modifications produites par l'oestrogénisation, et mises en évidence chez les rat femelles du groupe précédent. Les femelles, qui avaient été soumises à une oestrogénisation de 28 jours, montrent maintenant encore des signes manifestes de stimulation mammo-lutéotropique active; celles qui avaient subi une oestrogénisation de 196 jours conservent des ovaires atrophiques. Les séries ayant subi une oestrogénisation de durées intermédiaires sont composées de rats femelles montrant indifféremment l'un ou l'autre des états que nous venons de signaler. Chez les

mâles, en revanche, l'interruption du traitement oestrogénique est suivie d'une régénération testiculaire et annexielle plus ou moins complète, et qui s'accompagne d'une reprise de la spermatogénèse.

Hypophyse: Son volume diminue au cours de la phase post-oestrogénique, mais sans jamais revenir à des valeurs normales (Tableau 4). A l'examen histologique, l'architecture de la glande reste fortement remaniée: des travées fibreuses dissèquent le parenchyme, dans lequel s'isolent des nodules adénomateux, et que parsèment des lacs séreux, ainsi que des foyers hémorragiques anciens et récents. La composition cellulaire est hétérogène. Des nappes de petites cellules agranulaires, d'aspect peu actif, forment l'essentiel du tissu tumoral. Ill apparaît une redifférenciation des cellules acidophiles et basophiles, qui reste souvent confinée à quelques territoires bien vascularisés. Les cellules basophiles sont grandes; elles montrent un idiosome bien développé, et parfois de la vacuolisation cytoplasmique. Il persiste un nombre important de cellules à prolactine qui ne présentent plus d'hypertrophie de l'appareil de Golgi.

Ultrastructure hypophysaire: Trois hypophyses ont été étudiées au microscope électronique. On y trouve une quantité appréciable d'éléments appartenant aux diverses catégories cellulaires de l'hypophyse normale, mais aussi de nombreuses cellules à prolactine et des éléments non indentifiables. Parmi les éléments des catégories normales, on identifie sans peine de nombreuses cellules somatotropes et gonadotropes (Fig. 8), ainsi que des cellules corticotropes (Fig. 7) telles que les ont définies Herlant et Klastersky [34], ainsi que Kurosomi et Kobayashi [35]; les cellules thyréotropes sont les moins abondantes. A en juger par les critères cytologiques habituels, tous ces éléments se trouvent dans des états variables d'activité. C'est ainsi que certaines cellules gonadotropes sont grandes, riches en grains bien formés, et contiennent un appareil de Golgi bien développé; d'autres, au contraire, sont chargées de multiples lysosomes et montrent de nombreuses images de 'grains éclatés' (Fig. 8). Parmi les cellules thyréotropes, certaines ont un aspect cytologique typique de cellules de thyroïdectomie (Fig. 9). Les cellules corticotropes présentent parfois des images d'extrusion corpusculaire de la sécrétion (Fig. 7). Les cellules somatotropes enfin paraissent avoir une charge granulaire souvent réduite, et un appareil de Golgi bien développé. Les cellules à prolactine sont abondantes; elles aussi paraissent se trouver dans des états variables d'activité; jamais pourtant elles ne montrent les volumineux Nebenkerne ergastoplasmiques qui étaient caractéristiques dans le groupe expérimental précédent (Fig. 3). Dans les préparations figurent encore un nombre variable d'éléments relativement petits, dépourvus de granulations et qu'il est dès lors difficile d'identifier; leur ergastoplasme modérément développé revêt une structure tantôt vésiculaire et tantôt feuilletée; l'appareil de Golgi, quand il est visible, est peu étendu; ces cellules contiennent fréquemment des lysosomes (Fig. 5).

Groupe II. A.: Evolution de tumeurs greffées au cours de transplantations successives

Observations générales: L'activité fonctionnelle propre aux greffons est impossible à dissocier de celle des tumeurs hypophysaires *in situ* qui se développent parallèlement sous l'influence du traitement oestrogénique. Tout comme dans le groupe I.A., on observe une cachectisation progressive et des signes de stimulation mammo-lutéotrope décelable par les frottis vaginaux. Nous n'avons pas observé d'effets somatotrope ou corticotrope évidents, tout au moins tant que les greffons ont gardé leur caractère d'hormono-dépendance.

Greffons tumoraux: Ils poursuivent leur croissance à des rythmes variables, suivant l'hôte qui les héberge, en restant localisés à l'endroit de leur implantation; dans quelques cas, on trouve, à l'autopsie, un chapelet de petits nodules péritonéaux qui représentent des greffes accessoires. A l'examen histologique, les greffons ont une structure multi-nodulaire et sont constitués par des cellules à prolactine typiques présentant des caractères cytologiques de grande activité. Au cours des générations néanmoins, ces éléments, tout en conservant leurs caractères cytologiques propres, ont tendance à devenir plus petits et moins granuleux. Des cellules somatotropes typiques ne peuvent plus être reconnues.

Ultrastructure des greffons tumoraux: Deux greffons ont été étudiés au microscope électronique. Ils sont composés de cellules à prolactine caractéristiques, reconnaissables à leurs grains densément osmiophiles et polymorphes. Leur appareil de Golgi est bien développé. L'ergastoplasme est abondant et de structure généralement feuilletée, les enroulements en Nebenkerne sont nombreux (Fig. 2). Dans un des deux cas étudiés, les cellules à

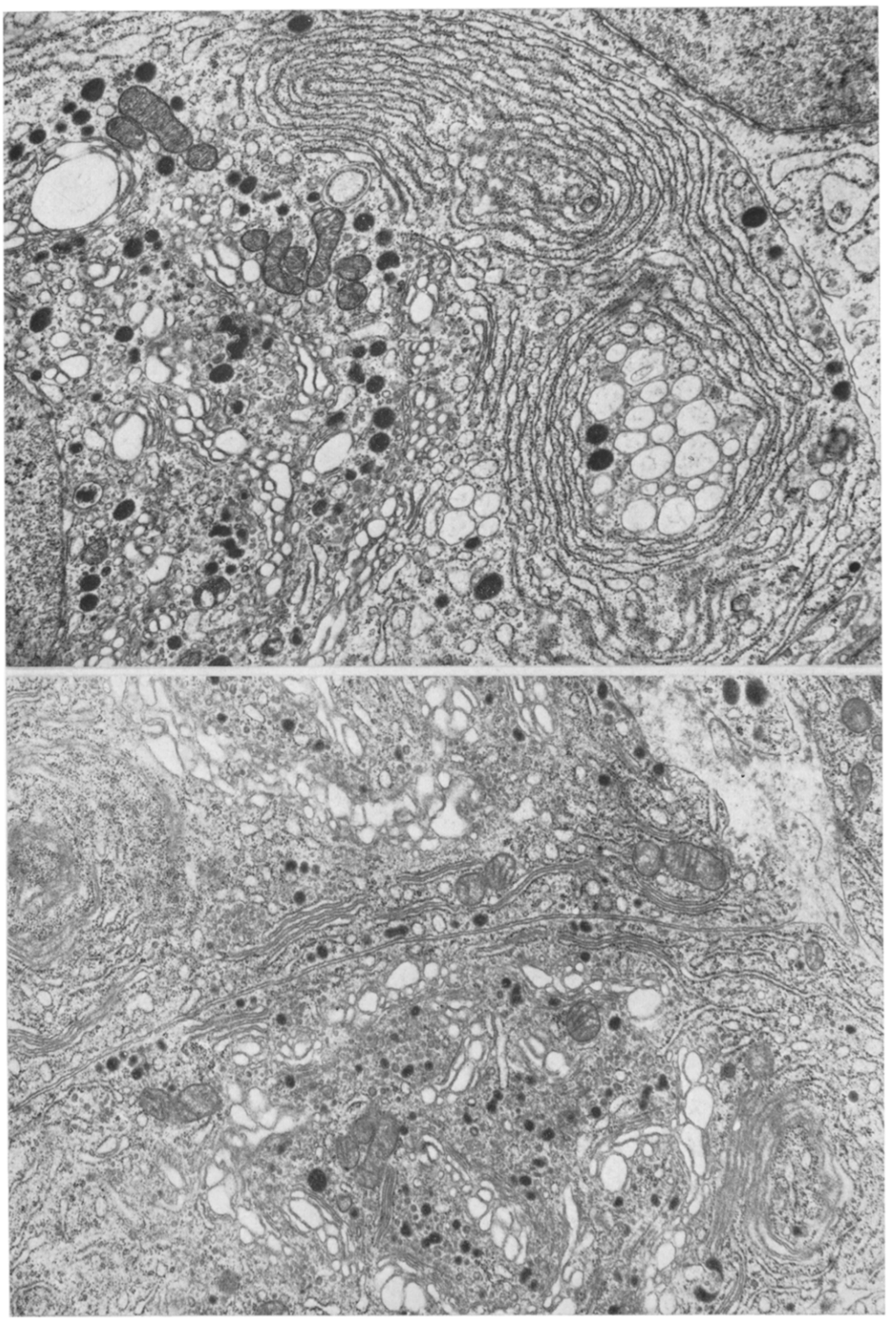


Fig. 1. *Hypophyse* in situ. *Rat femelle sous traitement oestrogénique depuis* 112 *jours. Cellule à prolactine à grains de sécrétion caractéristiques: densément osmiophiles et polymorphes, les plus petits figurant dans la zone golgienne où certains sont de forme vermiculaire. L'ergastoplasme est très développé et s'enroule en Nebenkerne* (× 14.400).

Fig. 2. *Greffon tumoral provenant de l'hypophyse d'un rat oestrogénisé pendant* 112 *jours et entretenu pendant* 196 *jours chez un hôte oestrogénisé. Les deux cellules à prolactine ont un aspect comparable à celui illustré dans la figure précédente* (× 16.000).

(to face p. 104)

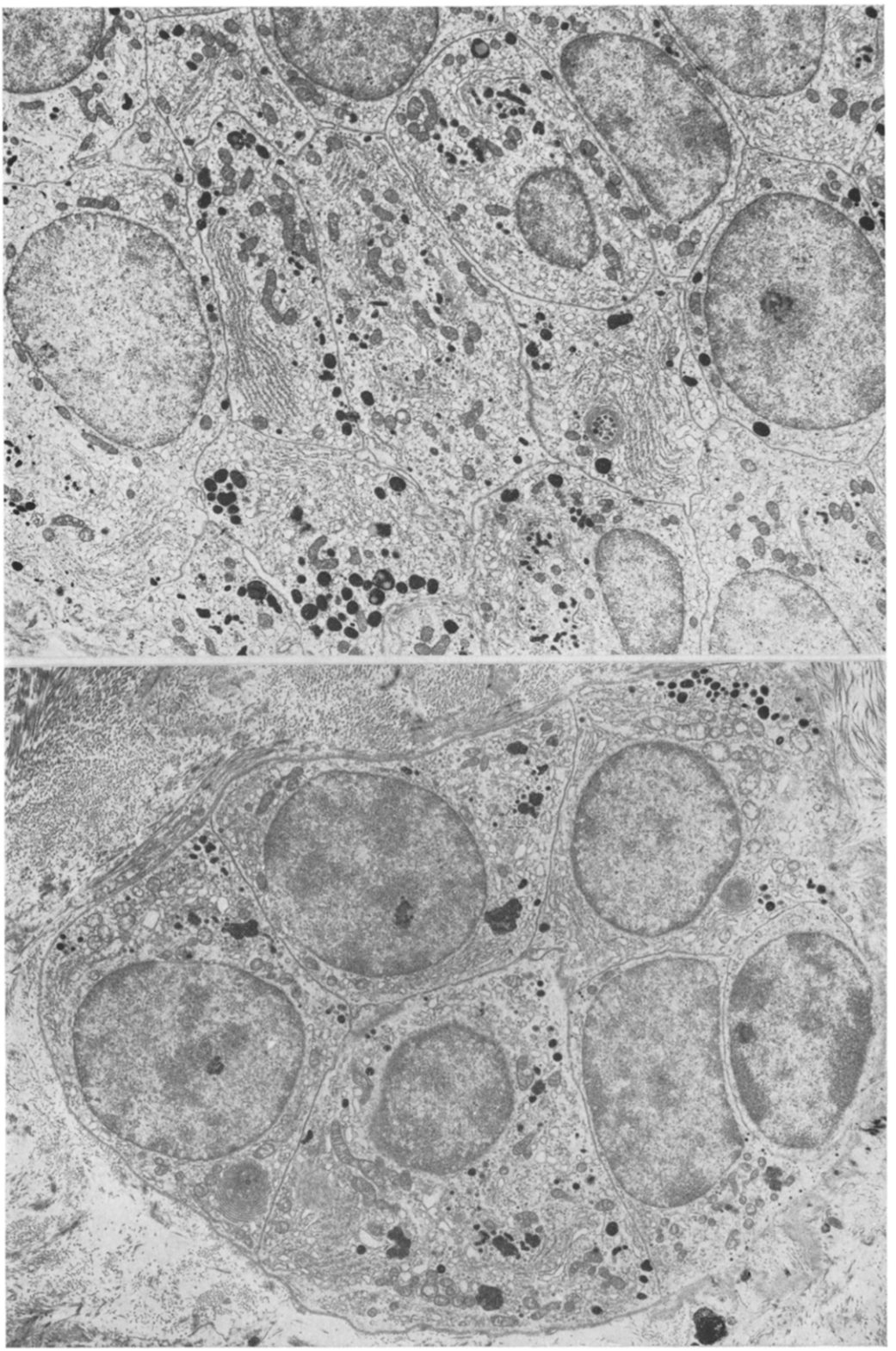


Fig. 3. *Hypophyse* in situ. *Rat oestrogénisé pendant* 165 *jours, puis abandonné sans traitement pendant* 290 *jours. Champ composé exclusivement de cellules à prolactine d'aspect actif, mais moins volumineuses que pendant le traitement hormonal et pourvues d'un ergastoplasme moins développé. Noter la présence de quelques lysosomes* (× 6.8000).

Fig. 4. *Greffon tumoral. Transplant soumis à l'oestrogénisation pendant* 146 *jours, puis abandonné sur place sans traitement pendant* 230 *jours. Groupe de* 6 *cellules à prolactine d'aspect comparable à celui illustré dans la figure précédente* (×6.800).

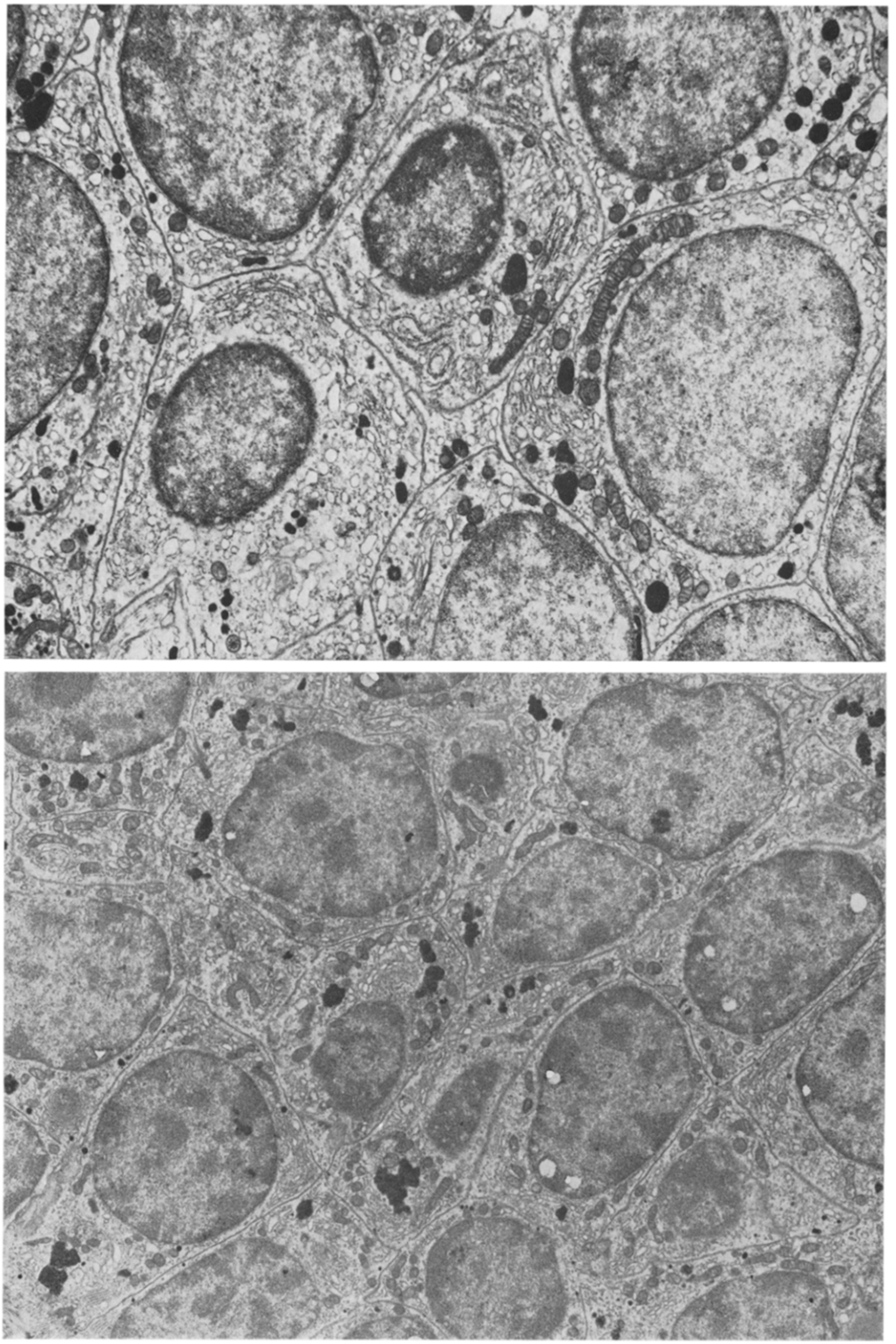
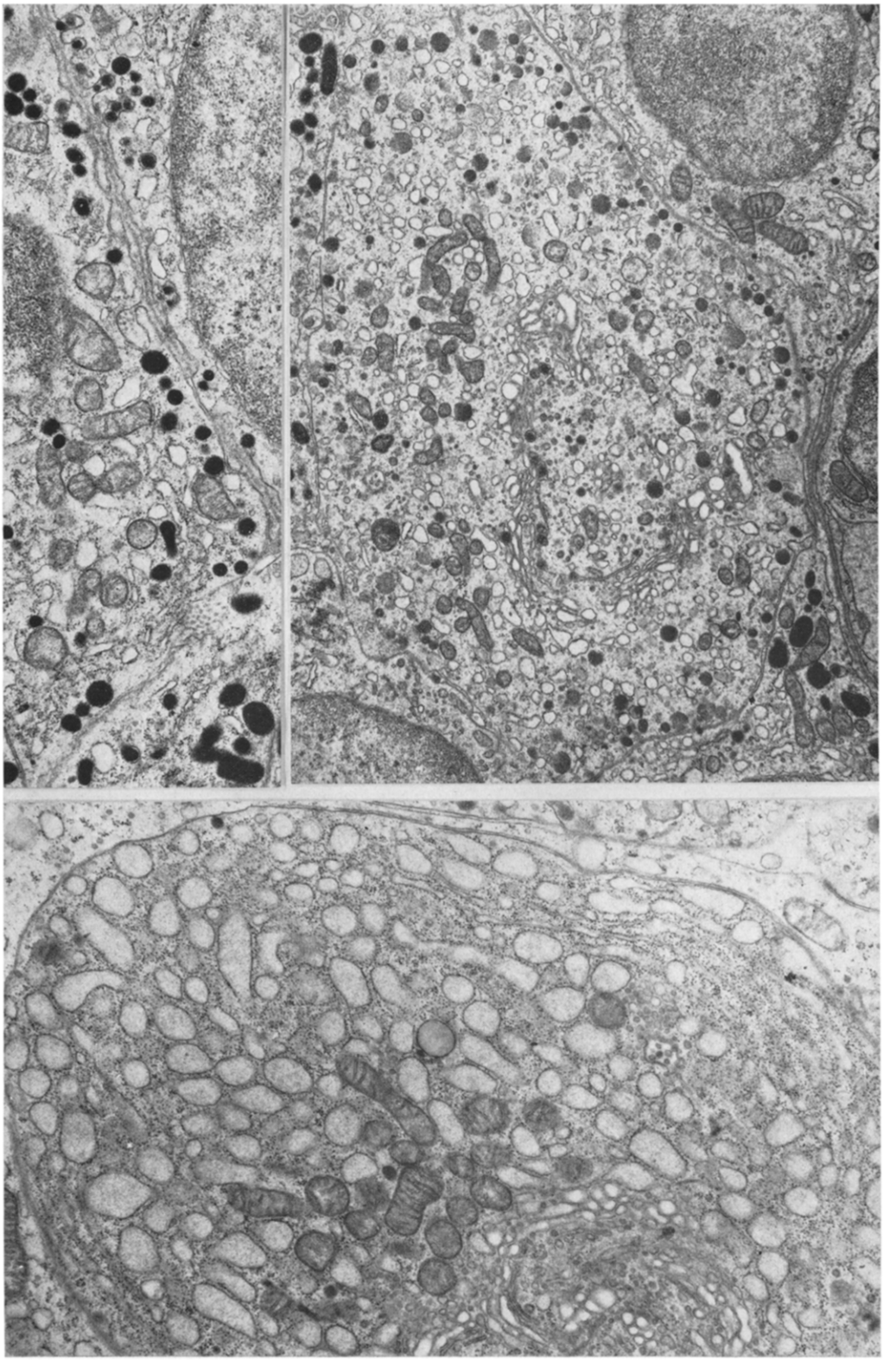


Fig. 5. *Hypophyse* in situ. *Même animal que dans la Figure* 3. *Groupe de cellules à prolactine reconnaissable à la présence de quelques grains typiques, mais pauvres en cytoplasme et à ergastoplasme peu développé* (× 10.000).

Fig. 6. *Greffon tumoral. Même animal que dans la figure* 4. *Champ composé de petites cellules à prolactine, quiescentes, très pauvrement granuleuses* (× 6.800).

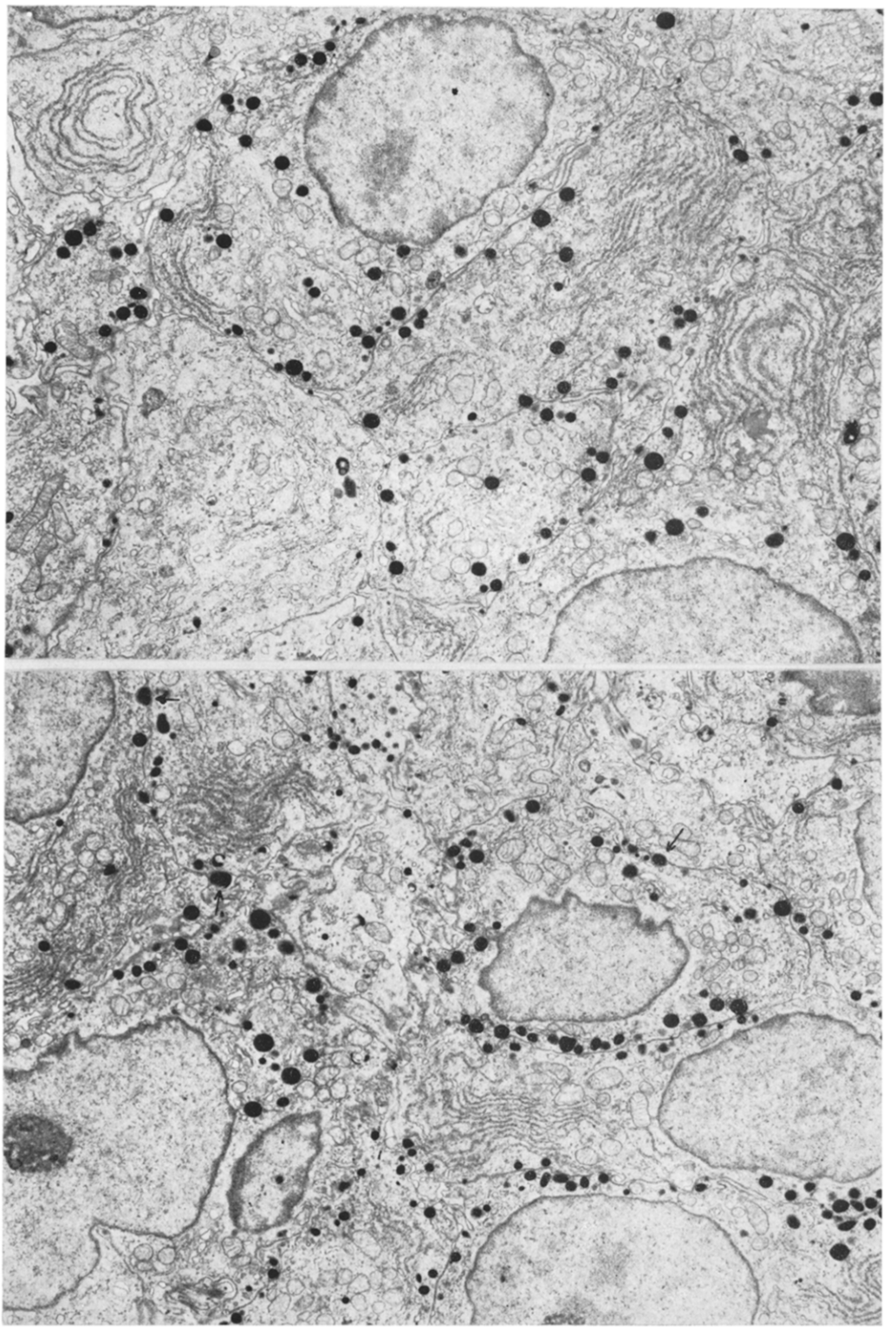


Hypophyse in situ *après interruption du traitement oestrogénique. Même matériel que dans les Fig.* 3 *et* 5.

Fig. 7. *Le coin supérieur droit de la préparation est occupé par un fragment de cellule 'corticotrope'; sous la membrane cellulaire sont alignés de multiples grains arrondis, denses et petits* (150 – 200 mμ), *dont plusieurs sont en voie de libération dans l'espace intercellulaire. Le reste de la préparation montre une portion de deux cellules à prolactine dont les grains, beaucoup plus gros, sont de forme parfois ovalaire* (× 16.000).

Fig. 8. *Cellule gonadotrope (à FSH), reconnaissable à sa granulation de densité variable, souvent entourée d'une membrane 'éclatée'. L'aire golgienne est bien développée et renferme des grains de petite taille* (× 12.000).

Fig. 9. *Cellule très pauvrement granulée, caractérisée par un appareil de Golgi bien développé et surtout par une dilatation vésiculaire de son ergastoplasme. L'aspect est celui d'une cellule de thyroïdectomie* (× 17.500).



Souche tumorale indépendante maligne, à effet mammosomatotrope, obtenue chez le rat n° 2 à la suite de transplantations répétées.

Fig. 10. *Groupe de cellules chargées d'un petit nombre de grains densément osmiophiles, régulièrement arrondis et ne dépassant pas* 450 mμ *de diamètre: ces caractères permettent d'identifier des cellules somatotropes. Les citernes ergastoplasmiques ont une disposition feuilletée et suivent des trajets capricieux* (× 6.800)

Fig. 11. *Les cellules sont ici plus riches en grains; certaines de ceux-ci sont ovalaires et se trouvent parfois dans les espaces intercellulaires (flèches): ce type de sécrétion rappelle celui de la cellule à prolactine* (× 6.800).

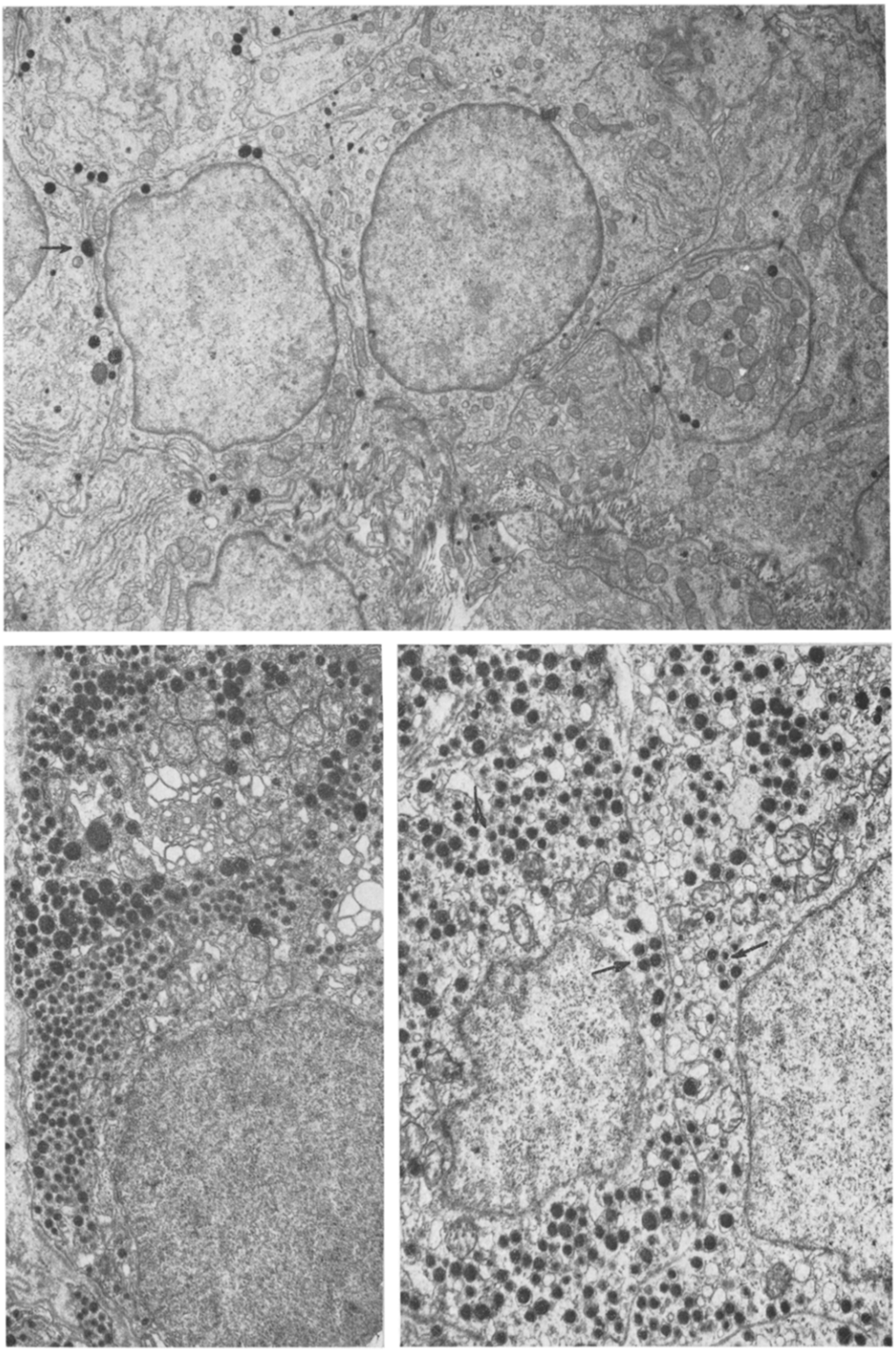
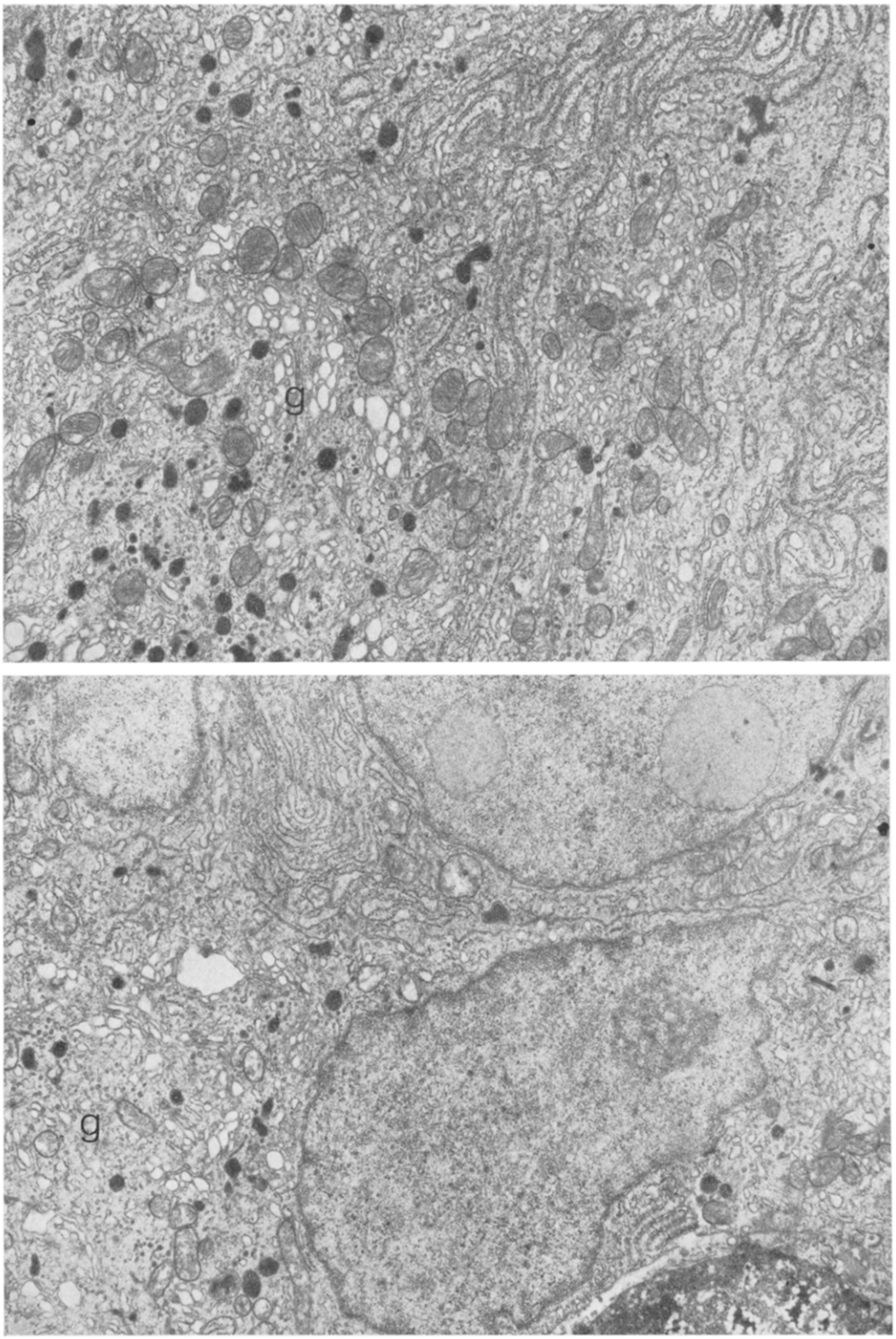


Fig. 12. *Même matériel que dans les fig.* 10 *et* 11. *Group de cellules pauvres en grains; l'un d'eux est ovalaire et situé dans l'espace intercellulaire (flèche). Absence d'appareil golgien individualisé* (× 6.000).

Fig. 13. *Tumeur autonome à effet mammosomatotrope dévelopée chez le rat n° 5 à la suite de l'arrêt du traitement oestrogénique. La préparation montre un fragment de deux cellules superposées différant par la taille de leurs grains. Les petits grains de la cellule inférieure* (125 mμ *en moyenne*) *caractérisent la cellules 'corticotrope'; les plus gros grains arrondis* (375 mμ) *de la cellule supérieure caractérisent une cellule somatotrope* (× 12.000).

Fig. 14. *Même tumeur que dans la Fig.* 13. *Les deux cellules visibles sur ce cliché montrent un halo clair autour des grains de sécrétion; ce caractère morphologique est typique de la cellule 'corticotrope'* (× 12.000).



Souche tumorale indépendante à action uniquement mammotrope, obtenue chez le rat n° 6 à la suite de transplantations répétées.

Fig. 15 *et* 16. *Cellules tumorales dont les caractères cytologiques généraux, et notamment la granulation, sont ceux des cellules à prolactine. L'appareil de Golgi est bien individualisé. Disposition capricieuse des citernes ergastoplasmiques et présence de ribosomes libres en grande quantité* (× 12.000).

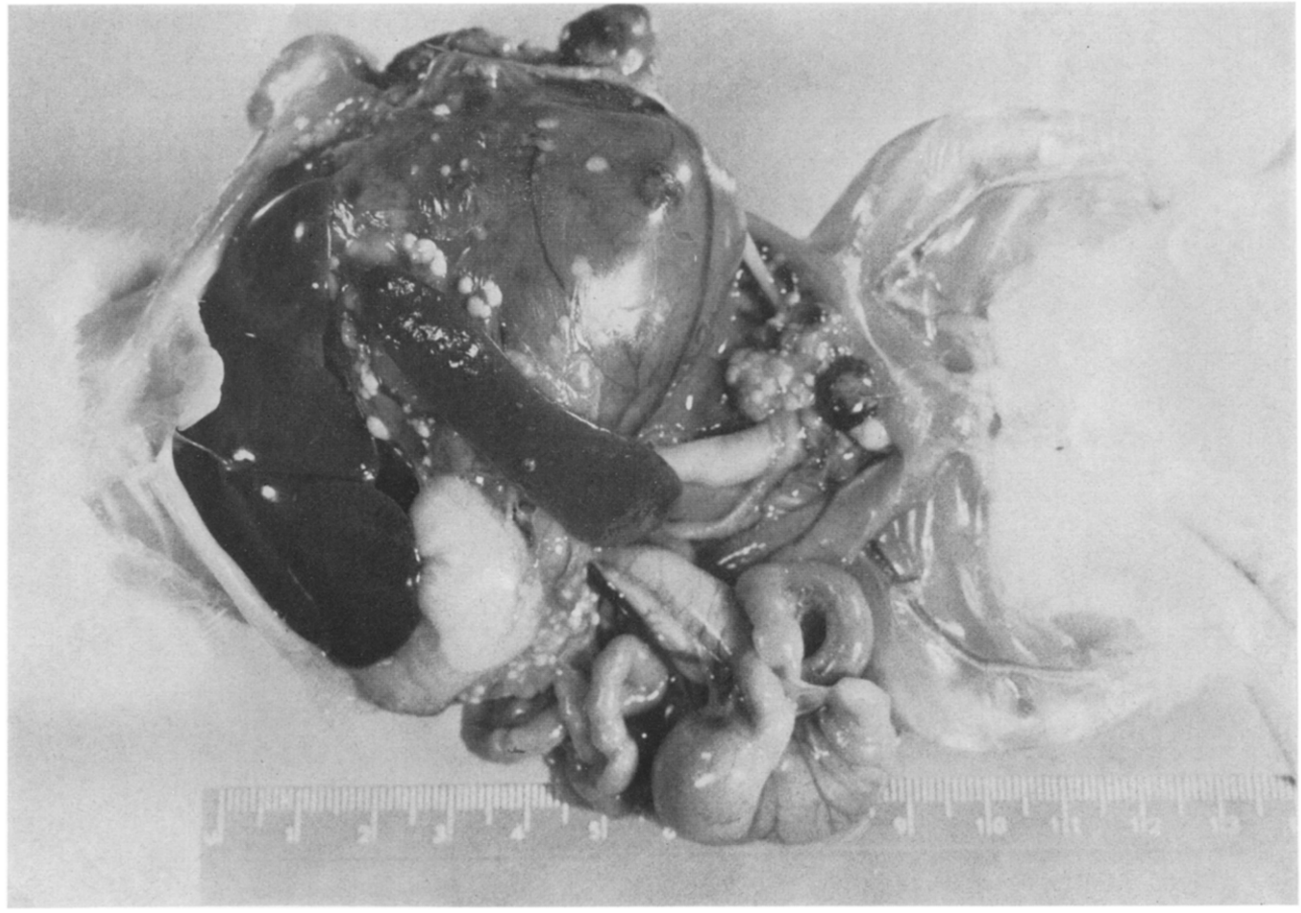


Fig. 17. *Rat porteur d'une tumeur indépendante dérivant de la souche tumorale n° 2. Présence d'une volumineuse masse tumorale englobant le rein gauche, et d'infiltrats métastatiques sur le péritoine.*

prolactine montrent des aspects dégénératifs; elles se présentent sous la forme d'éléments rétractés, fort opaques aux électrons, dont les citernes ergastoplasmiques sont collabées. Nous n'avons pu identifier aucune cellule glandulaire appartenant à d'autres catégories que celle des cellules à prolactine.

Groupe II. B.: Evolution des tumeurs greffées après l'interruption du traitement inducteur

Observations générales: Comme dans le groupe I. B, il se produit une réascension rapide de la courbe pondérale après l'ablation du comprimé d'oestrogène (Tableau 5). A l'autopsie, les organes sont de taille normale, exception faite des surrénales dont le poids ne dépasse pas le double de la normale (Tableau 5). Du point de vue gonadique, les 9 mâles de ce groupe montrent une récupération testiculaire et annexielle plus ou moins prononcée suivant les cas; leurs glandes mammaires contiennent de petits kystes lactés.

Greffon tumoral: Chez les 9 mâles, grâce à la laparotomie pratiquée au départ de la phase postoestrogénique, nous pouvons affirmer qu'au cours de celle-ci une diminution de volume du transplant s'est produite. A l'examen histologique, les greffons se montrent disséqués par des travées fibreuses; ils sont parsemés d'infiltrats plasmocytaires, ainsi que de dépôts d'hémosidérine. Le tissu tumoral est principalement formé par des cellules agranulaires de petite taille, d'allure plasmocytoïde et qui paraissent se trouver au repos. En périphérie des foyers tumoraux, et en bordure des sinusoïdes, figurent un petit nombre d'éléments plus volumineux dont le cytoplasme contient des grains acidophiles. Parfois, on rencontre un nodule formé de grandes cellules, pauvrement granulées, à noyau nettement atypique.

Ultrastructure: Trois greffons ont été étudiés au microscope électronique. Les grandes cellules à noyau atypique, observées en microscopie optique, n'ont pas été retrouvées. En revanche, on rencontre régulièrement des cellules à prolactine typiques et des éléments de petite taille d'aspect quiescent. Les cellules à prolactine s'identifient sans peine grâce à leur granulation. Leurs indices cytologiques d'activité sont inégalement marqués: dans certains cas, l'appareil de Golgi est bien développé et contient quelques grains de sécrétion de petite taille; l'on observe aussi des images d'extrusion corpusculaire de la sécrétion. Toutefois, l'ergastoplasme ne montre jamais de Nebenkerne et, de plus, ces éléments contiennent souvent des formations lysosomiales (Fig. 4). Les éléments d'aspect quiescent sont pauvres en cytoplasme et contiennent de volumineux phagosomes. On pourrait douter de la nature hypophysaire de ces cellules, n'était qu'occasionnellement, elles contiennent quelques rares grains de sécrétion et portent un cil; certaines, en outre, possèdent encore quelques citernes ergastoplasmiques aplaties (Fig. 6).

DISCUSSION

En oestrogénisant des rats de la souche R Amsterdam par l'implantation sous-cutanée d'un comprimé de 25 mg de dipropionate de diéthylstilboestrol, laissé en place pendant au moins 28 jours, nous avons obtenu des tumeurs hypophysaires qui ont été transplantées en séries et qui ont exercé une activité mammolutéotrope. Contrairement à Furth [11], nous n'avons pas observé de stimulation somatotrope chez nos rats porteurs d'une tumeur dépendante, mais il se peut que les doses particulièrement élevées d'oestrogène que nous avons administrées aient inhibé l'action de l'hormone de croissance. Nos tumeurs sont formées de cellules à prolactine qui, au bout de 140 jours de traitement, édifient dans l'hypophyse *in situ* des nodules tumoraux; l'ultrastructure de ces éléments est caractérisée, pendant toute la durée de la phase oestrogénique, et aussi bien dans l'hypophyse *in situ* que dans les transplants tumoraux, par un développement énorme de l'ergastoplasme qui s'enroule en Nebenkerne, traduisant ainsi une activité élaboratrice intense. Dans les hypophyses *in situ*, l'acroissement de la population des cellules à prolactine aboutit à submerger les autres catégories cellulaires; au microscope optique, on parvient encore à déceler la présence de cellules somatotropes, mais il faut la microscopie électronique pour repérer les quelques cellules gonadotropes, thyréotropes et corticotropes qui subsistent encore. Selon Meyer et Clifton [6], le dosage biologique du contenu hormonal des hypophyses traitées par les oestrogènes a montré que leur teneur totale en prolactine est augmentée, celle des hormones gonadotropes étant abaissée; d'autre part, si leur concentration en hormones thyréotrope et corticotrope est normale, celle de l'hormone somatotrope est abaissée. Nos observations morphologiques sont en accord satisfaisant avec ces résultats. Dans les transplants de tumeurs dépendantes, la prédominance des cellules à prolactine est encore beaucoup plus accusée, et, même au microscope électronique, on ne parvient pas à identifier

d'éléments appartenant aux autres catégories cellulaires de l'hypophyse.

Dans un second temps, nous avons étudié les conséquences d'un arrêt définitif du traitement oestrogénique, les délais d'observation variant de 28 à 361 jours. Notre but était de vérifier si, dans ces circonstances, les tumeurs dépendantes régressent complètement, et si l'hypophyse peut reprendre une morphologie et une activité normales. Dans la discussion de nos résultats, nous aurons à distinguer les effets obtenus dans les hypophyses *in situ* de ceux observés dans les transplants tumoraux. Par suite de leur déconnexion avec l'hypothalamus, ceux-ci se trouvent en effet dans la situation particulière d'être soustraits à l'influence modératrice que les centres nerveux exercent sur les cellules à prolactine [36]. Nos principales constatations ont été les suivantes.

Chez les rats portant uniquement une tumeur hypophysaire in situ:

(1) On assiste à la réapparition, dans l'hypophyse, d'un nombre important d'éléments appartenant aux diverses catégories cellulaires normalement représentées dans le lobe antérieur. Ceci témoigne, selon toute apparence, de la reprise de l'effet régulateur normalement exercé par l'hypothalamus. Toutefois, les fonctions ovariennes restent profondément perturbées et ne redeviennent jamais cycliques. Chez les mâles, la restauration gonadique est souvent incomplète, mais, à la différence de ce qui se passe chez les femelles, elle reste possible. Des constatations similaires ont été faites chez le rat hypophysectomisé, porteur d'une greffe hypophysaire; elles ont conduit à envisager que l'hypophyse déconnectée de l'hypothalamus sécrète plus d'hormone gonadotrope chez les mâles que chez les femelles, ou que le testicule est plus sensible que l'ovaire à la présence d'une faible quantité d'hormone gonadotrope [37]. Ce déséquilibre endocrinien persistant peut être imputé à l'oestrogénisation prolongée et au vieillissement de nos animaux soumis à des expériences de longue durée. Ces deux facteurs peuvent avoir altéré la réponse des organes effecteurs aux hormones trophiques, ou déréglé le fonctionnement de l'axe hypothalamo-hypophysaire. Des travaux antérieurs [38] nous ont montré que les ovaires prélevés chez des rats femelles soumis à un traitement oestrogénique prolongé, puis à un long arrêt de celui-ci, reprennent une activité cyclique lorsqu'ils sont greffés à des animaux simplement castrés. Dans une expérience comparable, portant sur des ovaires de rats femelles séniles ayant d'ailleurs atteint un âge nettement plus avancé que celui de nos animaux, Ascheim [39] a obtenu des résultats similaires. Il apparaît ainsi que ni l'oestrogénisation ni le vieillissement ne sont capables de modifier les qualités intrinsèques des récepteurs périphériques, et que le lieu de leur action doit se situer, dès lors, au niveau des organes de commande. La présence, dans l'hypophyse, de cellules basophiles anormalement actives, pouvant même revêtir l'aspect de cellules de thyroïdectomie, indique à nouveau que l'action régulatrice de l'hypothalamus reste perturbée. Dans nos expériences, les rats n'ont pas atteint l'âge sénile défini par Ascheim [39], et c'est par conséquent à l'oestrogénisation prolongée que l'on songe, en premier lieu, pour expliquer le défaut de régulation. Celui-ci implique l'altération durable, soit des fonctions hypothalamiques, soit de la réceptivité des cellules hypophysaires.

(2) Quelle qu'ait été la durée de la phase post-oestrogénique, et malgré la diminution du volume de l'hypophyse *in situ*, il persiste, dans cet organe, un grand nombre de cellules à prolactine. Elles revêtent deux aspects différents. Les unes sont morphologiquement typiques et montrent des indices cytologiques de grand activité, moins intense néanmoins qu'elle ne l'est pendant la stimulation oestrogénique: c'est ainsi que leur cytoplasme est diminué de volume et que leur ergastoplasme ne s'enroule plus en Nebenkerne. Les autres apparaissent en microscopie optique sous la forme de petites cellules chromophobes difficilement classables; leur ultrastructure permet de reconnaître en elles des cellules à prolactine profondément involuées. Dans une mesure très différente, ces deux catégories de cellules tumorales se montrent donc sensibles à la privation d'oestrogène. Suivant le mécanisme que l'on invoque pour expliquer l'effet des oestrogènes sur les cellules à prolactine, on peut penser que l'arrêt du traitement hormonal intervient, soit pour supprimer la stimulation directe des cellules hypophysaires [40], soit pour restaurer l'action inhibitrice de l'hypothalamus. Même si l'on adopte le second point de vue, la survie des cellules à prolactine, sous deux formes distinctes, indique que, parmi elles, se sont sélectionnées deux populations inégalement réceptives aux influences hypothalamiques. Cette altération de la réceptivité doit être liée à une modification cellulaire irréversible résultant d'une action directe des oestrogènes sur les cellules à prolactine.

Chez les rats porteurs d'un transplant tumoral:

(1) En opposition avec ce que l'on voit dans

les hypophyses *in situ*, on n'observe pas de redifférenciation de cellules basophiles, corticotropes ou somatotropes dans les transplants. Ceux-ci semblent réfractaires à toute influence hypothalamique, ce que l'on peut expliquer le plus simplement par l'interruption des relations anatomiques normales entre les deux organes. Si l'on adopte le point de vue suivant lequel les oestrogènes stimulent les cellules à prolactine uniquement par l'intermédiaire d'une action inhibitrice sur l'hypothalamus [36], on peut se demander si une cause accessoire ne réside pas dans la dépression durable des centres nerveux. Enfin on peut encore envisager que, au cours de la phase d'induction tumorale, les cellules à prolactine, spécifiquement stimulées à la fois par le traitement oestrogénique et par la déconnexion hypothalamo-hypophysaire, se sont développées au point d'éliminer complètement les autres catégories cellulaires interdisant ainsi toute redifférenciation ultérieure. Pour admettre cette explication, il faut postuler, en corollaire, que les cellules de l'hypophyse sont incapables de se transformer d'un type dans un autre.

(2) Il subsiste, dans les transplants, des cellules à prolactine dont l'aspect morphologique est très comparable à celui des éléments de la même catégorie présents dans l'hypophyse de l'hôte. Il semble donc que le degré d'activité des cellules à prolactine restées vivaces ne soit guère différent dans les deux localisations, et, par conséquent, que l'effet modérateur qu'exerce l'hypothalamus ne soit pas plus efficace au niveau de l'hypophyse *in situ* qu'il ne l'est au niveau des transplants. Ceci pourrait encore une fois s'expliquer de deux manières différentes, soit par une dépression de l'hypothalamus, soit par une altération dans la réceptivité des cellules à prolactine. L'hypothèse que celles-ci appartiennent à une lignée de cellules tumorales, irréversiblement modifiées dans leur possibilité de répondre aux influences régulatrices, cadrerait bien avec la seconde explication.

(3) A côté des cellules à prolactine morphologiquement typiques se développent des foyers de petites cellules chromophobes d'allure plasmocytoïde. La microscopie électronique démontre qu'elles sont des éléments glandulaires, mais à peu près totalement dépourvues de grains de sécrétion et d'appareil golgien; la présence de multiples lysosomes fournit un argument supplémentaire pour penser qu'il s'agit de cellules hypophysaires en involution profonde [41]. Si l'on considère les circonstances de leur apparition, on voit mal de quels éléments elles pourraient dériver, si ce n'est des cellules à prolactine. On est tenté de faire d'elles les homologues des cellules quiescentes visibles dans les hypophyses *in situ* et de conclure qu'elles font partie d'une lignée en régression de cellules tumorales restées hormono-dépendantes.

II. TUMEURS HYPOPHYSAIRES INDEPENDANTES

RESULTATS

Groupe III. A.: Tumeurs transplantées devenues indépendantes chez l'hôte non-oestrogénisé

Effets fonctionnels:

A l'exception du rat n° 1, les animaux de ce groupe ont montré des signes d'une stimulation somatotrope qui s'est exprimée par l'accélération anormale de la croissance (Fig. 18) et par le développement d'une splanchnomégalie manifeste (voir Tableau 6). En même temps, une très importante augmentation du poids des surrénales s'est produite chez l'hôte non oestrogénisé, avec une délipidation et une hypertrophie marquées des cellules de la fasciculée et de la réticulaire. Le thymus de l'un de ces deux animaux était atrophié, l'autre n'a pas été examiné.

Les trois animaux chez lesquels les tumeurs sont passées à l'indépendance étant de sexe mâle, l'effet lutéotrope n'a pas pu être observé. Un effet mammotrope net s'est manifesté chez le rat n° 2; il doit être attribué exclusivement à l'action de la tumeur puisque, dans ce groupe-ci, les hôtes n'ont subi aucune oestrogénisation.

Observations morphologiques:

La plus grande partie des tumeurs prélevées chez les rats n° 1 et n° 3 a été utilisée pour des retransplantations; de ce fait, leur étude morphologique n'a été approfondie que dans les stades ultérieurs de leur développement chez de nouveax hôtes non traités.

La tumeur obtenue chez le rat n° 2 est de structure hétérogène. Elle est disséquée par des travées fibreuses et parsemée de foyers de nécrose et d'hémorragie. Elle est composée par un mélange de cellules chromophobes, somatotropes et lutéotropes à noyau fréquemment atypique. La distinction entre cellules à prolactine et cellules somatotropes se fait aisément au tétrachrome de Herlant. Les premières ont des grains érythrosinophiles; leur cytoplasme contient un volumineux idiosome paranucléaire et présente un large ourlet

Tableau 6. Poids de la greffe tumorale, du corps, et de divers organes, chez les rats porteurs d'une tumeur hypophysaire autonome, et, dans deux séries de rats normaux différant par leur âge

Groupe n°	Tumeur n°	Sexe	Age au sacrifice (jours)	Corps (g)	Greffe (g)	Hypoph. (mg)	Foie (g)	Reins (g)	Surrén. (mg)	Thymus (mg)
III A	1	M	325	272	0,359	13	8,477	1,932	50	?
	2	M	322	510	22,432	15	31,380	5,170	180	atrophié
	3	M	364	368	23,274	28	21,219	4,520	223	?
III B	4	M	600	460	14,842	?	19,975	6,094	139	?
	5	F	361	345	0,234	61	19,325	2,900	106	36
	6	M	738	470	10,600	29	25,080	5,723	116	26
Contrôle	Série 1+	M	300	322 ±12	—	10 ±0,4	10,963 ±0,278	2,590 ±0,042	36 ±1	177 ±22
	Série 2+	M	540	338 ±34	—	11 ±0,3	11,692 ±0,549	2,135 ±0,128	32 ±3	178 ±24

+ moyenne de 5 rats±déviation standard.

basophile bien mis en évidence par la coloration de Dominici. Les secondes ont des grains orangeophiles; leur cytoplasme est de taille relativement réduite et ne contient pas d'idiosome bien individualisé.

Groupe III. B.: Tumeurs transplantées devenues indépendantes après l'arrêt du traitement oestrogénique de l'hôte

Effets fonctionnels:

Les 3 tumeurs retenues dans ce groupe ont exercé, pendant la phase post-oestrogénique, une activité somatotrope nette. Celle-ci s'est manifestée, déjà du vivant des animaux, par l'allure particulière de leur courbe pondérale. Après le retour au poids normal, qui se produit régulièrement dès qu'on enlève le comprimé d'oestrogène, une poussée de croissance qui a porté le poids des animaux à un niveau supérieur à la normale est survenue dans des délais plus ou moins brefs (voir Fig. 19). L'autopsie a démontré, chez eux, l'existence d'une forte splanchnomégalie, ainsi qu'une importante

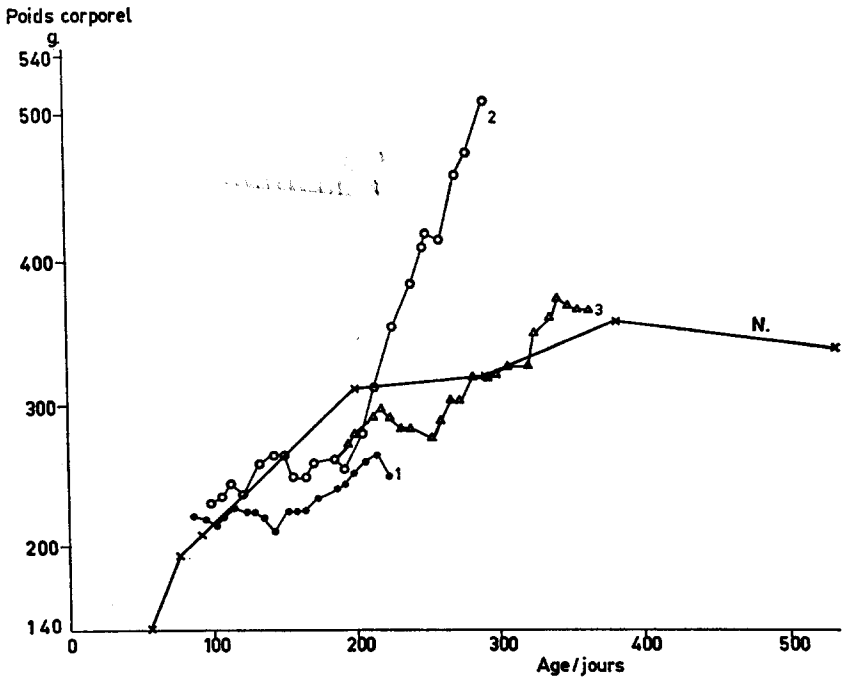


Fig. 18. Evolution du poids des rats n° 1, 2 et 3 (groupe III. A) depuis le moment où une tumeur hypophysaire autonome leur a été greffée jusqu'à celui du sacrifice. N est la courbe pondérale moyenne de rats mâles normaux appartenant au même élevage.

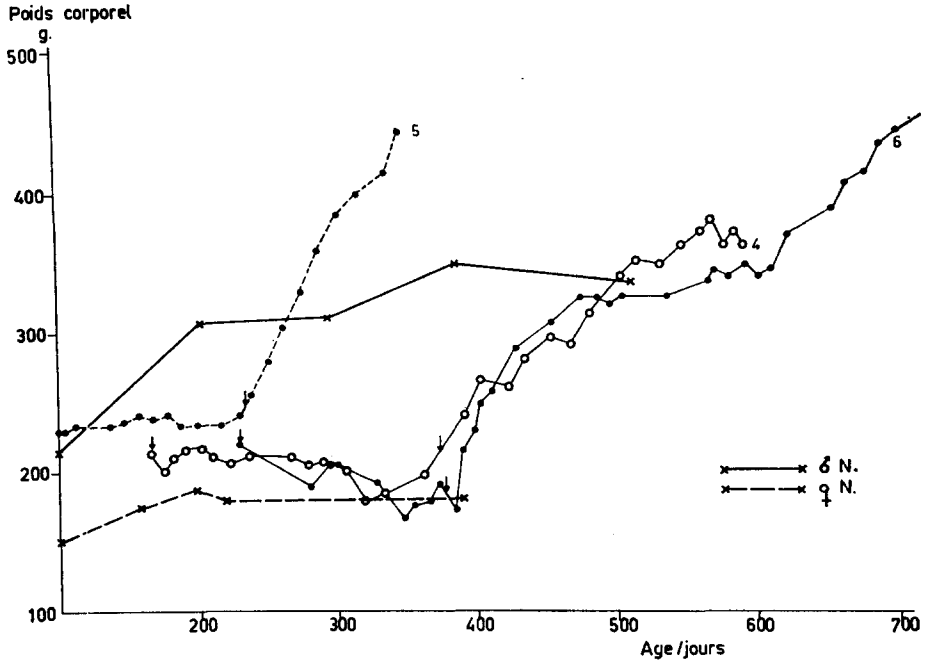


Fig. 19. *Evolution du poids des rats n° 4, 5 et 6 (groupe III. B.) chez lesquels la tumeur hypophysaire transplantée est devenue indépendante après l'interruption du traitement oestrogénique. L'intervalle compris entre deux flèches indique la durée du traitement oestrogénique de l'hôte. La courbe pondérale de deux groupes de rats normaux mâles et femelles est présentée à titre de comparaison.*

augmentation du volume des surrénales (Tableau 6); les cellules de la fasciculaire et de la réticulaire sont hypertrophiées et délipidées. Le thymus est atrophié dans les deux cas où il a été examiné. Du point de vue des glandes génitales et mammaires, ainsi que de l'activité sexuelle, ces animaux ont montré des altérations comparables à celles que montrent les rats porteurs d'une greffe dépendante; elles témoignent d'une stimulation mammolutéotrope soutenue, et peuvent être attribuées en partie à l'activité de l'hypophyse *in situ* qui, au cours de l'expérience, a subi les effets de l'oestrogénisation.

Observations morphologiques:

Chez le rat n° 4, la laparotomie, lors de l'ablation du comprimé hormonal, a fourni un repère permettant d'affirmer que le transplant a poursuivi sa croissance malgré la privation d'oestrogènes. A l'autopsie du rat n° 5, il a été découvert un transplant de taille importante, mais qui ne semblait pas avoir grandi pendant la phase post-oestrogénique. Chez le rat femelle n° 6, il a été trouvé, à l'endroit de la greffe, une tumeur maligne qui englobe la surrénale gauche, infiltre le rein gauche et parsème le grand épiploon de nodules métastatiques.

A l'examen histologique, nous avons fait les constatations suivantes. Le greffon provenant du rat n° 6 est formé de grandes cellules d'aspect oncocytaire qui sont groupées en plages à l'intérieur de nappes d'éléments plus petits contenant des grains acidophiles; le tétrachrome de Herlant permet de distinguer, parmi eux, des cellules à prolactine et quelques très rares cellules somatotropes. Les deux autres greffons contiennent un mélange de cellules chromophobes et de cellules acidophiles bien différenciées appartenant aux deux types somatotrope et lutéotrope; les cellules à prolactine ont un idiosome bien développé qui témoigne d'une activité sécrétoire importante. Dans le greffon du rat n° 4 figure en outre un nodule de cellules atypiques à noyaux irréguliers, inégaux et souvent en mitose.

Un fragment du greffon, prélevé chez le rat n° 5, a été examiné au microscope électronique. Les préparations confirment la présence d'un grand nombre de cellules somatotropes abondamment chargées de grains arrondis densément osmiophiles, et mesurant en moyenne 350 mμ de diamètre (Fig. 13); d'autres cellules contiennent des granulations assez semblables, mais beaucoup plus petites(150 mμ) et logées au large à l'intérieur de petites vésicules: il pourrait s'agir de cellules corticotropes (Figs. 13 et 14).

Tableau 7. Comportement des tumeurs indépendantes transplantées à des rats non oestrogénisés

Tumeur n°	Groupe	Reprise chez les rats non oestrog.	Activité des transplants somatotr.	mammotr.	lutéotr.
1	III A	+	+	+	+
2		+	+	+	+
3		0	—	—	—
4	III B	+	+	+	+
5		0	—	—	—
6		+	0	+	+

Comportement des tumeurs au cours de nouvelles transplantations chez des rats non traités (Tableau 7):

Les souches tumorales obtenues chez les rats n° 3 (groupe III. A.) et n° 5 (groupe III. B) se sont éteintes dès la première retransplantation.

Les quatre autres tumeurs ont été indéfiniment entretenues sur des rats non oestrogénisés. Suivies pendant une période d'observation d'un an et demi, trois d'entre elles ont continué à exercer une activité à la fois somatotrope et mammolutéotrope, cette dernière étant particulièrement évidente lors du passage chez un hôte femelle. La tumeur issue du rat n° 6 a fait exception; chez elle, l'activité somatotrope a disparu à la première transplantation, et seule l'action mammolutéotrope s'est conservée. Chez les animaux porteurs d'une tumeur à effet somatotrope, une forte augmentation de volume des surrénales s'est régulièrement produite, accompagnée d'une délipidation et d'une hypertrophie des cellules corticales, mais non d'une atrophie thymique.

Le rythme de croissance adopté par chaque souche tumorale, ainsi que le degré de son activité fonctionnelle, sont restés assez constants tout au long de sa carrière (Fig. 20). Les tumeurs à effet somatotrope se sont toutes développées rapidement: en 2 mois, le poids de leur greffe atteint souvent le gramme, et, après 3 mois, il peut dépasser 20 g. En revanche, la tumeur mammotrope a eu une croissance lente: après 4–5 mois d'évolution, le poids de la greffe reste inférieur au gramme.

Les deux tumeurs du groupe III. A. se sont révélées hautement malignes: leurs transplants débordent du site de la greffe pour infiltrer les organes voisins, se disséminer dans la cavité

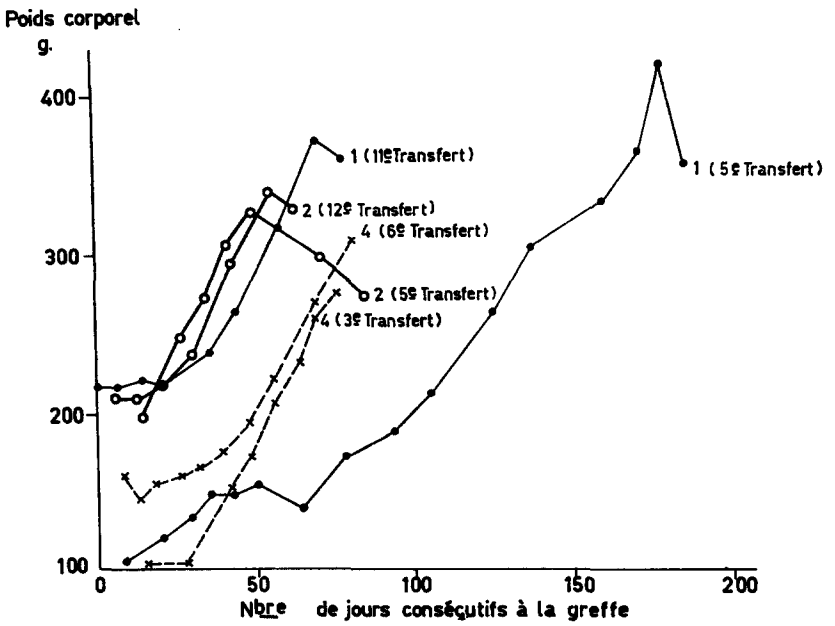


Fig. 20. Courbes pondérales de certains rats chez lesquels les souches tumorales provenant des rats n° 1, 2 et 4 ont été entretenues par transplantations successives. Pour chaque souche tumorale, deux courbes choisies à des étapes très écartées de leur carrière sont représentées. Elles illustrent la constance relative de l'effet somatotrope au cours des générations.

péritonéale et donner fréquemment des métastases ganglionnaires et thymiques (Fig. 17). Par contre, les greffons obtenus à partir des deux tumeurs du groupe III. B. restent localisés à l'aire pararénale gauche.

L'examen histologique des tumeurs mammo-somatotropes a montré la présence, dans celles-ci, de cellules à prolactine et de cellules somatotropes en proportions variables avec, dans l'ensemble, une nette prédominance de cette dernière espèce cellulaire. Dans la tumeur mammotrope, on n'a pu reconnaître que des cellules à prolactine. Les cellules tumorales montrent, dans tous les cas, des atypies nucléaires importantes.

Deux tumeurs ont été étudiées au microscope électronique. L'une est un transplant dérivé de la tumeur obtenue chez le rat n° 2 à ce stade, il s'agit d'un néoplasme infiltrant à croissance rapide, exerçant un effet mammo-somatotrope. L'identification du type sécrétoire auquel appartiennent les cellules est souvent rendue impossible par l'absence presque totale de granulation. Lorsqu'ils existent, les grains de sécrétion sont densément osmiophiles et ne dépassent jamais 500 mμ de diamètre. Certains éléments ont des grains uniformément arrondis, ce qui permet de les ranger dans la catégorie des cellules somatotropes (Fig. 10). D'autres contiennent quelques grains ovalaires et, de ce fait, peuvent être classées parmi les cellules à prolactine (Fig. 11); cette identification est encore renforcée par la présence de grains de sécrétion dans l'espace intercellulaire qui entoure ces éléments. Quels que soient l'abondance et le type de leur granulation, les cellules tumorales ont en commun de nombreux caractères atypiques. Le cytoplasme n'est abondant que chez les éléments chromophobes. Le noyau est de forme irrégulière et contient un volumineux nucléole. L'appareil golgien est mal individualisé et se présente souvent sous la forme d'amas dispersés de saccules à parois lisses (Fig. 12). Les citernes ergastoplasmiques sont toujours aplaties; à la périphérie du cytoplasme, elles s'empilent en feuillets qui décrivent des courbes d'une capricieuse sinuosité (Figs. 10–12). Des ribosomes et des polysomes libres sont extrêmement abondants. La seconde tumeur étudiée au microscope électronique est dérivée du transplant devenu autonome chez le rat n° 6. Au moment de l'examen, elle exerce, depuis plusieurs générations, une action purement mammolutéotrope et pousse de manière lente et non infiltrante. Le tissu tumoral ne comprend ici qu'un seul type cellulaire dont les caractères cytologiques sont, de toute évidence, ceux de la cellule à prolactine active. Les grains de sécrétion sont polymorphes et peuvent même prendre un aspect vermiculaire; ils ont tendance à se grouper au centre d'une aire golgienne bien individualisée, comportant souvent, à sa périphérie, une couronne de vésicules dilatées à parois lisses (Fig. 15 et 16). Dans certains éléments, l'ergastoplasme, de structure feuilletée, s'enroule en typiques 'Nebenkerne'. Il existe en outre des atypies variées de l'ultrastructure. Les noyaux sont très irréguliers. Le cytoplasme est abondant et ses limites sont difficiles à définir. Les citernes ergastoplasmiques, toujours aplaties, prennent des dispositions bizarres (Fig. 15). Les ribosomes et les polysomes libres sont abondants.

DISCUSSION

Les six tumeurs hypophysaires autonomes étudiées ont été induites par les oestrogènes et se sont développées à partir de transplants dépendants. Trois ont été obtenues par la méthode classique des transplantations répétées. Les trois autres ont acquis leur autonomie à la faveur d'un arrêt définitif du traitement inducteur. De ces 6 tumeurs, 4 ont confirmé leur autonomie en poursuivant indéfiniment leur croissance chez des hôtes non traités. Une cinquième tumeur n'est restée autonome que pendant une génération et, retransplantée, elle n'a continué à croître que chez des hôtes oestrogénisés. La sixième n'a pas fait la preuve de son autonomie lors des transplantations ultérieures; toutefois, son aspect morphologique, ses caractères fonctionnels, et le fait qu'en l'absence de traitement inducteur elle n'ait pas régressé, nous l'ont fait ranger dans la classe des tumeurs indépendantes.

Comme nous l'avons exposé dans le premier chapitre, nos tumeurs dépendantes n'ont exercé qu'une action purement mammolutéotrope. Par contre, 5 de nos 6 tumeurs autonomes ont manifesté en outre des propriétés somatotropes qui se sont traduites, chez leur hôte, par du gigantisme et de la splanchnomégalie. Des 4 tumeurs qui ont fait la preuve de leur autonomie, 3 ont exercé une activité somatotrope tout au long de leurs transferts successifs; chez la quatrième, celle-ci a disparu lors de la première transplantation pour ne laisser subsister par la suite que la seule action mammolutéotrope. Les surrénales des rats géants montrent régulièrement une forte activation de leur cortex, plus prononcée que chez les animaux porteurs d'une greffe dépendante; elle résulte peut être d'une stimulation corticotrope; toutefois, l'inconstance de l'atrophie thymique ne permet pas de conclusion formelle.

Sur le plan morphologique, le passage à l'autonomie se manifeste par deux ordres de transformations cytologiques. Le premier, commun à toutes nos tumeurs indépendantes, consiste dans le développement de caractères anaplasiques: les cellules tumorales montrent un important polymorphisme nucléaire, leur cytoplasme renferme de nombreux ribosomes libres et leur réticulum endoplasmique prend une disposition désordonnée. Le second, particulier aux tumeurs qui exercent une activité mammosomatotrope, consiste dans l'apparition de cellules somatotropes qui viennent se substituer aux cellules à prolactine de façon plus ou moins complète, suivant les cas. Leur identification, quoique rendue plus difficile par les atypies cytologiques, est clairement établie par les propriétés tinctorielles et l'ultrastructure de leurs grains de sécrétion. Il existe donc, dans notre matériel, une corrélation étroite entre le type cellulaire et l'activité fonctionelle de la tumeur.

Un dernier caractère propre à nos tumeurs mammosomatotropes est leur mode de croissance infiltrant et particulièrement rapide lorsqu'on le compare à celui de la tumeur autonome devenue mammotrope pure. Il semble donc ressortir de nos expériences que, dans les tumeurs hypophysaires induites par les oestrogènes, ce sont les cellules somatotropes qui sont dotées du potentiel prolifératif le plus élevé. Le point de départ de cette lignée est difficile à établir; elle reste totalement inaperçue dans les tumeurs dépendantes, et ne se développe, de manière apparente, qu'au moment du passage à l'autonomie. Plusieurs hypothèses sont possibles.

(1) Il se pourrait que des cellules somatotropes non tumorales restent présentes pendant la stimulation oestrogénique et qu'elles passent inaperçues simplement à cause de l'énorme développement de la population des cellules à prolactine. N'étant pas assujetties aux oestrogènes, ces cellules pourraient reprendre le dessus dès la suppression du traitement inducteur. Mais on voit mal, dans ces conditions, quel pourrait être le facteur responsable de leur transformation maligne.

(2) Il se pourrait, comme le suggère Schelin [29], que les différentes catégories de cellules hypophysaires se développent à partir de cellules souches pluripotentes dont la différenciation est conditionnée par les influences inductrices auxquelles elles se trouvent exposées. On peut s'attendre à ce que de telles cellules n'existent qu'en petit nombre et soient dépourvues d'attributs morphologiques bien particuliers, deux circonstances qui explique raient la difficulté de leur détection. La stimulation oestrogénique les fait se différencier en cellules à prolactine; l'arrêt du traitement hormonal leur imposerait une nouvelle orientation, cette fois dans le sens somatotrope. Outre que cette théorie fait appel à une cellule hypothétique, elle ne précise ni quel facteur est responsable du virage somatotrope, ni à quel niveau de la différenciation des cellules souches s'effectue la transformation maligne.

(3) Il se pourrait que les cellules somatotropes et les cellules à prolactine représentent deux formes interchangeables d'une même lignée de cellules hypophysaires acidophiles à laquelle, si l'on accepte les vues de Herlant [34], on pourrait encore adjoindre les cellules corticotropes. Suivant le degré de différenciation qui se produit au moment du passage à l'autonomie, la cellule acidophile tumorale prendrait l'un ou l'autre de ses trois aspects possibles, la forme somatotrope correspondant à l'état le moins évolué et doté du potentiel proliférative le plus élevé.

Il semble que la troisième explication rende le mieux compte de l'ensemble de nos observations. Elle peut se concilier avec la conception suivant laquelle la diversité des effets fonctionnels qu'exercent les tumeurs hypophysaires induites par les oestrogènes implique la sécrétion par la cellule tumorale de plusieurs hormones différentes [22, 43]. Elle s'accorde avec les données expérimentales obtenues en culture de tissu hypophysaire par Pasteels [42].

Au contraire de Furth [11], nous n'avons jamais observé d'effet somatotrope chez nos rats porteurs d'une tumeur hypophysaire dépendante. Cette discordance tient sans doute à des différences dans les modalités expérimentales mises en oeuvre. Mais on peut aussi se demander si les tumeurs dépendantes de Furth ne contiennent pas déjà des cellules somatotropes. De même, les observations de Clifton [44] qui, au cours des transplantations successives de ses tumeurs dépendantes, constate un abaissement progressif du seuil de la sensibilité hormonale et une accélération correspondante du rythme de croissance de celles-ci pourraient s'expliquer par le développement d'une proportion croissante de cellules somatotropes. Ces auteurs ne nous fournissent malheureusement pas de données morphologiques précises à ce sujet.

La méthode classique pour déceler l'accès à l'autonomie d'une tumeur induite consiste à transplanter celle-ci chez 2 animaux dont un seul est soumis au traitement inducteur, et à vérifier si le transplant pousse également chez l'hôte non traité. Etant donné les aléas que

comporte toute greffe, on peut se demander si ce procédé est bien le meilleur qui convienne pour détecter les stades initiaux du passage à l'autonomie. On peut imaginer en effet que celui-ci ne se produit pas d'emblée dans l'entièreté du tissu tumoral mais débute dans une population restreinte de cellules. C'est une des raisons qui nous ont incités à étudier l'évolution des tumeurs dépendantes abandonnées sur place, à elles mêmes, après l'arrêt du traitement oestrogénique. Nous avons constaté que même les tumeurs qui, dans ces conditions, ont diminué de volume et sont restées dépendantes lors de transplantations ultérieures, contiennent encore un grand nombre de cellules à prolactine, les unes involuées, les autres fonctionnellement actives et morphologiquement typiques. Il est difficile de voir, dans ces dernières, autre chose que des cellules tumorales qui ont accédé à un certain degré d'autonomie leur permettant de survivre en état de carence oestrogénique. La capacité, non pas simplement de survivre mais encore de proliférer en l'absence de stimulation hormonale, caractérise mieux l'état d'indépendance tel qu'il est habituellement compris. D'après nos expériences, ces deux propriétés sont sans doute liées, mais restent distinctes; la seconde, en effet, ne se déclare que sporadiquement dans un certain nombre de transplants, elle s'accompagne de phénomènes anaplasiques et s'associe généralement à une transformation du type cellulaire de la tumeur. Cette dernière métamorphose n'affecte pas d'emblée la totalité du tissu tumoral puisque certaines de nos tumeurs indépendantes mammosomatotropes ont une composition cellulaire mixte, comprenant des cellules à prolactine et des cellules somatotropes.

De l'ensemble de nos résultats, nous pensons pouvoir conclure que l'oestrogénisation prolongée laisse, à sa suite, des effets irréversibles qui se répartissent inégalement dans la population des cellules hypophysaires tumorales, provoquant ainsi la sélection, parmi elles, de lignées distinctes. Celles-ci diffèrent, au début, par leur réceptivité aux influences régulatrices, ensuite par leur tendance à devenir autonomes et par leur capacité de changer de type. L'étude cytogénétique de notre matériel tumoral vient à l'appui de cette conclusion. L'un de nous a constaté en effet [45] que, dans les tumeurs *in situ*, et dans les greffes dépendantes, il existe déjà des anomalies numériques du caryogramme. Le passage à l'indépendance s'accompagne d'une accentuation très nette des anomalies chromosomiales qui, à ce stade, sont d'ordre à la fois numérique et structurel. Certaines d'entre elles se reproduisent avec une telle régularité que leur transmission héréditaire apparaît fort probable; elles apportent ainsi un argument supplémentaire en faveur de l'existence de lignées cellulaires distinctes.

Une étude comparable à la nôtre, et portant également sur l'évolution d'une tumeur induite par les oestrogènes après l'arrêt du traitement a été publiée par Andervont *et al.* [46, 47]. Ceux-ci ont étudiés les tumeurs testiculaires à cellules de Leydig chez la souris. Leurs résultats concordent avec les nôtres. Comme nous, ils constatent que l'interruption du stimulus inducteur ne produit pas nécessairement l'extinction du tissu tumoral. Dans leurs expériences, qui ont été conduites systématiquement, la moitié seulement des tumeurs régressent, les autres persistent ou continuent à se développer surtout chez les rats jeunes [47]. Pour ces auteurs, l'interruption du traitement hormonal favorise le passage à l'indépendance, en produisant, selon eux, une sélection plus précoce de cellules tumorales autonomes. En effet, à l'épreuve de la transplantation, la proportion des tumeurs testiculaires qui, dès le premier transfert, se comportent en néoplasmes indépendants, atteint 42% lorsque le greffon provient d'une tumeur qui a continué à croître après l'arrêt du traitement; elle est nulle lorsque l'oestrogénisation est poursuivie jusqu'au prélèvement de la greffe [46]. D'autre part, le délai de latence nécessaire à l'apparition d'une tumeur testiculaire est raccourci lorsqu'une première oestrogénisation a été appliquée, serait-ce 8 mois auparavant [47], ce qui prouve, encore une fois, le caractère irréversible des modifications induites par le traitement hormonal.

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RESUME

Un traitement oestrogénique prolongé détermine, chez le rat, la formation de tumeurs hypophysaires mammolutéotropes qui peuvent être transplantées en séries, et qui sont composées de cellules à prolactine. L'arrêt définitif du traitement inducteur, à un moment

où les tumeurs, in situ *et transplantées, se trouvent encore dans la phase de dépendance hormonale, n'entraîne pas la disparition des cellules à prolactine. Celles-ci subsistent sous une double forme, quiescente et active; la première semble correspondre à une population de cellules tumorales restées strictement hormono-dépendantes; la seconde, à des cellules tumorales dont les possibilités de réponse aux influences régulatrices ont été irréversiblement modifiées par l'oestrogénisation. En même temps, les autres catégories cellulaires, qui réapparaissent dans l'hypophyse* in situ, *ne récupèrent ni une fonction, ni une morphologie parfaitement normales; ce phénomène doit résulter d'une altération durable de la réceptivité des cellules hypophysaires ou d'une dépression durable de l'hypothalamus.*

Le passage à l'indépendance des tumeurs hypophysaires peut se produire au cours de transplantations répétées, ou à la suite de l'interruption du traitement oestrogénique. L'application des deux méthodes nous a fourni trois tumeurs mammosomatotropes, ainsi qu'une tumeur à fonction exclusivement mammotrope, qui ont été entretenues indéfiniment chez des rats oestrogénisés et dont les cellules ont une cytologie atypique. L'acquisition de l'activité somatotrope s'accompagne d'une transformation correspondante de la morphologie, consistant dans l'apparition de cellules somatotropes, associées, dans un cas, à des cellules du type corticotrope. Un autre caractère, propre à nos tumeurs somatotropes, et qui les différencie également de notre tumeur autonome à fonction exclusivement mammotrope, est leur rythme de croissance particulièrement rapide et leur comportement agressif; celui-ci les classe parmi les authentiques néoplasmes malins.

Il semble que l'oestrogénisation prolongée laisse à sa suite des effets irréversibles qui se répartissent inégalement dans la population des cellules hypophysaires tumorales, provoquant ainsi la sélection, parmi elles, de lignées distinctes. Celles-ci diffèrent, au début, par leur réceptivité aux influences régulatrices, ensuite par leur tendance à devenir autonomes. Le point de départ des cellules somatotropes tumorales ne peut être établi avec certitude. Il est possible que, au même titre que les cellules corticotropes, elles dérivent de lignées cellulaires acidophiles dont la pluripotentialité se manifeste à la faveur du passage à l'indépendance.

SUMMARY

A long term oestrogenic treatment induces in the pituitary of rats the development of transplantable mammoluteotrophic tumors composed of prolactin cells. Complete interruption of the hormonal treatment, at a time when the pituitary tumors are still in a dependant state, does not lead to the disappearance of all prolactin cells; some survive under the form of either active or quiescent cells. It would appear that the latter represent a strictly hormone dependent group of cells and that, in the former, the capacity of response to regulatory mechanisms has been permanently altered by the oestrogenic treatment. At the same time, cells belonging to the different normal categories of pituitary cells make their reappearance in the in situ *tumors. Their morphology and functional activity however remain abnormal; this must result from either a permanent disturbance in the receptivity of pituitary cells or an enduring depression of the hypothalamic control.*

Pituitary induced tumors may become autonomous in the course of repeated transplantations or after a complete interruption of the oestrogenic treatment. Both methods provided us with a total of three mammosomatotrophic and one purely mammotrophic tumors which could be indefinitely maintained in non-oestrogenized rats and were composed of cytologically atypical cells. The development of a somatotrophic activity was accompanied by a corresponding morphological change consisting of the appearance of somatotroph cells associated, in one case, with corticotroph cells. The three mammosomatotrophic tumors showed a faster and more agressive growth than the purely mammotrophic tumor; they represented true malignant neoplasms.

It would appear that long term oestrogenization produces irreversible effects which are unevenly distributed among the pituitary cells and lead to the selection of different cell lineages. These differ, in the beginning, by their receptivities to regulatory influences and, later on, by their tendencies to autonomous growth. The origin of the tumoral somatotroph cells could not be determined with any certainty; one possibility is that, like the corticotroph cells, they derive from a common acidophil stemcell, the pluripotentiality of which becomes manifest when the tumor attains its autonomy.

ZUSAMMENFASSUNG

Eine Langzeitbehandlung von Ratten mit Ostrogen erzeugt in der Hypophyse transplantierbare mammoluteotrope Tumoren, die aus Prolactin-Zellen bestehen. Vollständige Unterbrechung der Hormonbehandlung zu einer Zeit, da die Hypophysen-Tumoren noch in einem abhängigen Stadium sind, führt nicht zum Verschwinden aller Prolactin-Zellen. Einige davon überleben in der Form aktiver oder ruhender Zellen. Es scheint, als würden die letzteren eine Gruppe streng hormonabhängiger Zellen repräsentieren und dass in den ersteren die Fähigkeit des Ansprechens auf einen Regulations-Mechanismus durch die Ostrogen-Behandlung permanent verändert wird. Gleichzeitig erscheinen die Zellen, die zu den normalen Kathegorien der Hypophysenzellen gehören, in dem in situ *Tumor wieder. Ihre Morphologie und Funktionsfähigkeit bleibt jedoch abnormal; die Ursache hierfür liegt entweder in der Störung der Aufnahmefähigkeit der Hypophysen-Zellen oder in der andauernden Unterdrückung der Kontrolle durch den Hypothalamus. Hypophysen-Tumoren können im Verlauf der wiederholten Transplantation oder nach einer vollständigen Unterbrechung der Ostrogenbehandlung autonom werden. Beide Methoden lieferten uns insgesamt drei mammosomatotrope une einen reinen mammotropen Tumor, die unbegrenzt auf nicht-Ostrogen-behandelten Ratten erhalten werden konnten und bestanden aus cytogenetisch atypischen Zellen. Die Entwicklung der somatotropen Aktivität war begleitet von einer entsprechenden morphologischen Veränderung, die im Erscheinen von somatotropen Zellen und in einem Fall von corticotropen Zellen bestand. Die drei mammosomatotropen Tumoren zeigten ein rascheres und aggressiveres Wachstum als der reine mammotrope Tumor; es waren echte maligne Neoplasmen.*

Es scheinte so, als würde eine Langzeitbehandlung mit Ostrogen irreversible Effekte hervorbringen, die ungleichmässig auf die Hypophysenzellen verteilt sind und die zu einer Auswahl verschiedener Zellinien beitragen. Diese unterscheiden sich anfänglich durch ihre Empfänglichkeit gegenüber Regulationseinflüssen und später durch ihre Neigung zum autonomen Wachstum. Der Ursprung der somatotropen Zellen konnte nicht bestimmt werden; es besteht die Möglichkeit, dass sie, wie die corticotropen Zellen, aus einer gemeinsamen acidophilen Stammzelle stammen, deren Pluripotenz manifest wird, wenn der Tumor seine Autonomie erreicht hat.

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Tumeurs Hypophysaires Induites par les Oestrogènes chez le Rat

II. Etude cytogénétique

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INTRODUCTION

Notre matériel d'étude comprend une série de tumeurs hypophysaires qui ont été induites par les oestrogènes et ont été entretenues par des transplantations isologues répétées chez des rats de la souche R Amsterdam. Certaines d'entre elles ont été suivies jusqu'à leur accession à l'indépendance, c'est-à-dire jusqu'au moment où leur croissance s'est poursuivie en l'absence de traitement hormonal. Leur évolution, leur morphologie et leurs effets fonctionnels ont été décrits dans un précédent travail [1]. Nous avons observé que l'arrêt définitif du traitement hormonal n'entraîne pas la disparition totale du tissu tumoral, même lorsque la croissance de celui-ci nécessite encore l'administration d'oestrogènes. Bien plus, certaines de ces tumeurs mises en carence oestrogénique sont devenues indépendantes et ont acquis tous les caractères d'un néoplasme malin à croissance rapide et infiltrante. Nous avons conclu que le traitement oestrogénique prolongé laisse à sa suite des modifications irréversibles qui se répartissent inégalement dans la population des cellules hypophysaires tumorales, et nous avons envisagé que l'accession à l'indépendance se produit dans des lignées cellulaires distinctes dont la sélection débute pendant la phase d'induction tumorale.

Dans le présent travail, nous avons voulu vérifier si, en parallèle avec les transformations cytologiques et fonctionnelles, il n'apparaissait pas, dans nos tumeurs, des anomalies chromosomiales indiquant le développement, éventuellement précoce, de modifications génétiques telles que Furth [2] en a postulé l'existence dans les tumeurs hypophysaires indépendantes. Les tumeurs malignes expérimentales et humaines ont été amplement explorées du point de vue cytogénétique. Généralement, on a observé une association entre le caractère de malignité et la présence d'anomalies chromosomiales [3]; toutefois, cette liaison ne paraît pas être absolument constante et l'on a rencontré des tumeurs malignes euploïdes [4–6], tout comme on a décrit des lésions prénéoplasiques aneuploïdes [7–11]. Il a été reconnu, d'autre part, que les infections virales peuvent provoquer, pour leur propre compte, des altérations chromosomiales qui ne sont pas l'indice d'une transformation maligne [3, 12].

En ce qui concerne plus spécialement les tumeurs induites expérimentalement par un traitement hormonal, des études cytogénétiques ont été publiées portant sur des tumeurs hypophysqires mammotropes [13], corticotropes [13] et thyréotropes [14], ainsi que sur des tumeurs testiculaires induites par les oestrogènes [15], et des tumeurs mammaires devenues autonomes [13]. En revanche, les lésions prénéoplasiques [7–11] et les tumeurs bénignes d'étiologie non virale [3, 16] ont été peu étudiées.

L'intérêt particulier de notre matériel est

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Tableau 1. *Origine et constitution cytogénétique des tumeurs et des prélèvements médullaires*

Groupe	Rat no.	Transfert no.	Tumour *in situ* no.	Total	Nombre de mitoses										
					36 et <36 chrom.	37 chrom.	38 chrom.	39 chrom.	40 chrom.	41 chrom.	42 chrom.	43 chrom.	44 chrom.	>44 chrom.	Tétraploïde
Tumeurs *in situ*	96			54	2	2	2		5	8	30	4	1		
	97			35				1	2	5	17	2	4	4	
	99			6						1	1	4			
	112			19	1			2	2	2	12				
	116			17						3	12	2			
	118			12						3	9				
	137			14					1	1	7	2	1		2
	144			18				2	1	1	8				
	162			23					1	3	18	1			
	194b			13		1		1		5	4	2			
	198b			11		3			1	5	2				
	206			9				1		4	4				
	209			29						3	23	2	1		
	345			25						2	21	2			
Tumeurs dépendantes greffées	116	1	83	33					1	5	20	4	1		2
	137	1	88	3							3				
	141	1	90	18	1		2	1	6	3	5				
	162	1	93	5							4	1			
	198	1	140	40		1	2	3	1	2	30	1			
	192b	2	81	12						1	9	1	1		
	194b	2	84	27	1	1		2		6	13	4			
	345	2	93	20			1	2	6	3	8				
	388	3	86	22					1	9	10	1			1
	403	2	123	29	3		2		2	5	8	6		1	2
	707	3	208	13	2		1	2			5	3			
Tumeurs autonomes	1003	6	81/642	24		2	5	9	7	1					
	1264	12	81/642	20		1	1	2	1	3	7	3	1		1
	1277	12	81/642	24				1	1	3	3	14	1		1
	1190	11	81/634	8				1	1	3	2				
	1184	11	81/634	27		1		1	5	7	12	1			
	1188	11	81/634	9		1	1		1	4	2				
	1216	10	86	36		2	5	3	5	18	3				
	1186	10	86	24				3	5	7	5	4			
	1191	5	212	49			1		4	2	17	9	12	2	2
Moelle	95			35	1			1	1	3	27	2			
	96			42			1	1	1	2	37				
	97			5							5				
	143			5					1		4				
	209			10							10				

que celui-ci permet de suivre la progression du processus tumoral depuis le stade initial d'induction jusqu'à la phase de l'autonomie.

MATERIEL ET METHODES

Induction et transplantation tumorales:

Des rats mâles de la souche pure R d'Amsterdam reçoivent, sous la peau de cou, un implant de 25 mg de diéthylstilboestrol. Ces rats sont sacrifiés 6 mois après l'implantation; ils portent, à ce moment, une volumineuse tumeur hypophysaire. La tumeur *in situ* est greffée sous la capsule du rein à 3 rats mâles, 2 d'entre eux sont oestrogénisés, le troisième est châtré. Six mois après la transplantation, la greffe, devenue tumeur, est elle-même retransplantée dans des conditions identiques. Après plusieurs passages successifs, la tumeur devient indépendante et n'est dès lors plus greffée que chez des rats non oesgrogénisés, normaux ou châtrés.

Examen cytogénétique:

Sept heures avant le sacrifice, nous faisons une injection sous-cutanée de colchicine, à raison de 14γ par 10 g de poids du corps.

La tumeur est prélevée immédiatement après le sacrifice et morcelée dans du liquide de Hanks, de façon à obtenir une suspension de cellules tumorales. Les cellules de la moelle fémorale, qui a été prélevée en même temps, sont également mises en suspension dans du liquide de Hanks. Après deux lavages sucessifs, les cellules tumorales et médullaires subissent un choc hypotonique de 10 minutes, à 37°C, dans du liquide de Hanks dilué au huitième. Nous fixons ensuite les culots de centrifugation à 4°C, en prenant soin de ne pas les remuer, dans un mélange d'acide acétique glacial–alcool méthylique (1 part–3 part). Le fixateur est renouvelé après 30 minutes. La deuxième fixation dure 30 minutes, mais peut tout aussi bien se prolonger pendant toute la nuit. Après fixation, les cellules sont resuspendues dans du fixateur frais, puis étalées sur des lames propres préalablement immergées dans de l'eau distillée froide. Nous séchons l'étalement à l'air par agitation. Lex cellules sont colorées au mélange d'Unna ou au Feulgen modifié [17]. Les mitoses sont soit photographiées, soit examinées directement au microscope. Un examen microscopique attentif permet aisément de déterminer le nombre global de chromosomes et de déceler les anomalies chromosomiales majeures.

Matériel cytogénétique:

Nous avons examiné 14 tumeurs *in situ*, 11 tumeurs dépendantes de 1ère, 2e et 3e générations, ainsi que 9 tumeurs indépendantes de 5e, 6e, 10e, 11e et 12e générations. Comme matériel de contrôle, nous avons utilisé la moelle fémorale de 5 rats porteurs de tumeurs dépendantes.

RESULTATS

1. *Moelle osseuse:*

Quatre-vingt-dix-sept cellules provenant de cinq moelles fémorales différentes (Tableau 1) vont constituer les données de contrôle nous permettant d'apprécier la marge des erreurs dues à des facteurs techniques; 85,6% des mitoses ont un caryotype normal. Il y a 12,4% de cellules hypodiploïdes principalement à 39, 40 et 41 chromosomes et 2% de cellules hyperdiploïdes à 43 et 44 chromosomes (Tableau 2).

Nous n'observons aucune anomalie structurelle. Les fragmentations sont extrêmement rares. L'hypodiploïdie est vraisemblablement due à des artéfacts techniques ayant provoqué une rupture de la membrane cellulaire.

2. *Tumeurs* in situ:

Sur 285 cellules provenant de 14 tumeurs *in situ* différentes, nous trouvons 58,9% de cellules diploïdes, 27% de cellules hypodiploïdes groupant principalement des mitoses à 40 et 41 chromosomes et 14% de cellules hyperdiploïdes surtout à 43 et 44 chromosomes (Tableaux 1 et 2).

Nous pouvons suspecter l'existence de lignées cellulaires nouvelles dans 4 tumeurs (209H

Tableau 2. Pourcentage de cellules diploïdes, hypodiploïdes et hyperdiploïdes dans les 4 groupes expérimentaux

Groupes	Nombre total	Cellules Hypodiploïdes (%)	Diploïdes (%)	Hyperdiploïdes (%)
Moelle	97	12,37	85,57	2,06
Tumeurs *in situ*	285	27,02	58,95	14,04
Tumeurs dépendantes greffées	222	35,13	51,80	13,06
Tumeurs indépendantes greffées	221	53,39	23,08	23,53

41,18—; 206H 41,20—; 144H 43,18+; 99H 43,19+).

Les anomalies structurelles, fragmentation et translocation sont rares.

3. *Tumeurs dépendantes:*

Nous avons analysé 222 cellules réparties parmi 11 tumeurs dépendantes, transplantées depuis une et deux générations. A une exception près, ces greffes proviennent toutes de tumeurs *in situ* différentes.

Parmi les mitoses étudiées, 51,8% sont euploïdes (Fig. 1), 36,1% hypodiploïdes (Fig. 2) et 13,0% hyperdiploïdes (Tableau 2). Il s'agit, dans 26% des cas, d'hétéroploïdie de la perte ou de l'addition d'un seul chromosome qui se situe, le plus souvent, dans le groupe des métacentriques (Tableau 1).

Ici également, nous suspectons, dans une tumeur, la présence de deux lignées cellulaires: 116G 43,10+; 41,19—. Au niveau de la tumeur 403G, la présence d'un chromosome marqueur, morphologiquement constant, identifie clairement une lignée de cellules variantes: 403G 43, métacentrique marqueur+(Fig. 4).

Comme pour les tumeur *in situ*, les anomalies structurelles se rencontrent rarement. Seule la tumeur 403G se distingue par l'existence, dans les trois quart des mitoses, d'un chromosome marqueur formé par la fusion centromérique de deux télocentriques.

4. *Tumeurs indépendantes:*

L'examen porte sur 221 cellules provenant de 9 tumeurs. Il s'agit de transplants de 5e, 6e, 10e, 11e et 12e générations, issus initialement de 3 tumeurs *in situ* différentes (Tableau 1).

L'hypodiploïdie intéresse 53,4% du contingent cellulaire et l'hyperdiploïdie 23,6% (Tableau 2), 23,1% des mitoses, seulement sont diploïdes. Nous rencontrons 38% de mitoses à 41 et 43 chromosomes. Les autres aberrations portent sur cinq éléments au plus. Les mitoses tétraploïdes sont rares.

D'importantes anomalies structurelles se rencontrent. Les fragmentations restent exceptionnelles; en revanche, nous observons de fréquentes translocations ou fusions centromériques des chromosomes, particulièrement évidentes au sein de certaines tumeurs, notamment la tumeur 1191G issue de la tumeur *in situ* 212 (Fig. 5); toutefois, ces anomalies sont également présentes dans d'autres greffes, mais en plus faible pourcentage. Il est important de signaler que la combinaison des deux chromosomes qui s'unissent par leur centromère, varie suivant la mitose étudiée.

Nous voyons, d'autre part, s'isoler nettement des lignées cellulaires nouvelles: 1003 40,18—, 19— et 1277 43,2+ (Fig. 3). La reduplication d'un chromosome 2 s'observe fréquemment dans les 2 tumeurs provenant de la tumeur *in situ* 86 (1186G 3 mitoses sur 24,2+, 1216G 18 mitoses sur 36,2+). On retrouve également la même anomalie dans 2 tumeurs de la 12e génération provenant de la tumeur *in situ* 81, souche 642 (1264G 6 mitoses sur 20,2+; 1277G 22 mitoses sur 23,2+). Il est à noter que ce 3e chromosome 2 n'existe pas à la 6e génération de la même souche tumorale 81/642. A cette reduplication du chromosome 2 s'ajoutent d'autres anomalies différenciant les tumeurs les unes des autres. Ainsi, les 2 tumeurs de la 12e génération d'ascendance 81/642, qui possèdent en commun une trisomie de la deuxième paire, présentent l'une une formule à prédominance diploïde (1264G), l'autre une formule hyperdiploïde à 43 chromosomes (1277G) (Fig. 5).

Tests de probabilité:

Le test de χ^2 démontre qu'il existe une différence significative entre la constitution cytogénétique de la moelle et celle des tumeurs *in situ* (P: 0,01), ainsi qu'entre les greffes dépendantes et indépendantes (P: 0,01). En revanche, il n'y a pas de différence significative entre les tumeurs dépendantes *in situ* et greffées (P: 0,1).

Ensemble des éléments qui font suspecter l'existence de lignées cellulaires:

Tumeurs in situ: Dans 4 tumeurs *in situ*, parmi les cellules aneuploïdes à formule chromosomique variable, se détache nettement un groupe cellulaire possédant la même structure cytogénétique aberrante. Leur fréquence nous permet de les considérer comme des cellules variantes par application des critères définis par Turpin et Lejeune [18]. Ces critères sont basés sur la fréquence des cellules exceptionnelles par rapport au nombre de cellules examinées. Ils sont appliqués par Turpin et Lejeune à une pathologie génétique constitutionnelle.

Tumeurs dépendantes transplantées: De la même façon que pour les tumeurs *in situ*, au niveau d'un transplant dépendant, nous voyons s'isoler deux lignées de cellules variantes. Dans une autre tumeur, un métacentrique marqueur caractérise les trois quart des cellules, qu'elles soient diploïdes ou aneuploïdes; parmi ces éléments on observe une lignée cellulaire à 43 chromosomes, pourvue du chromosome marqueur.

Tumeurs indépendantes: Deux tumeurs possèdent chacune une lignée de cellules variantes

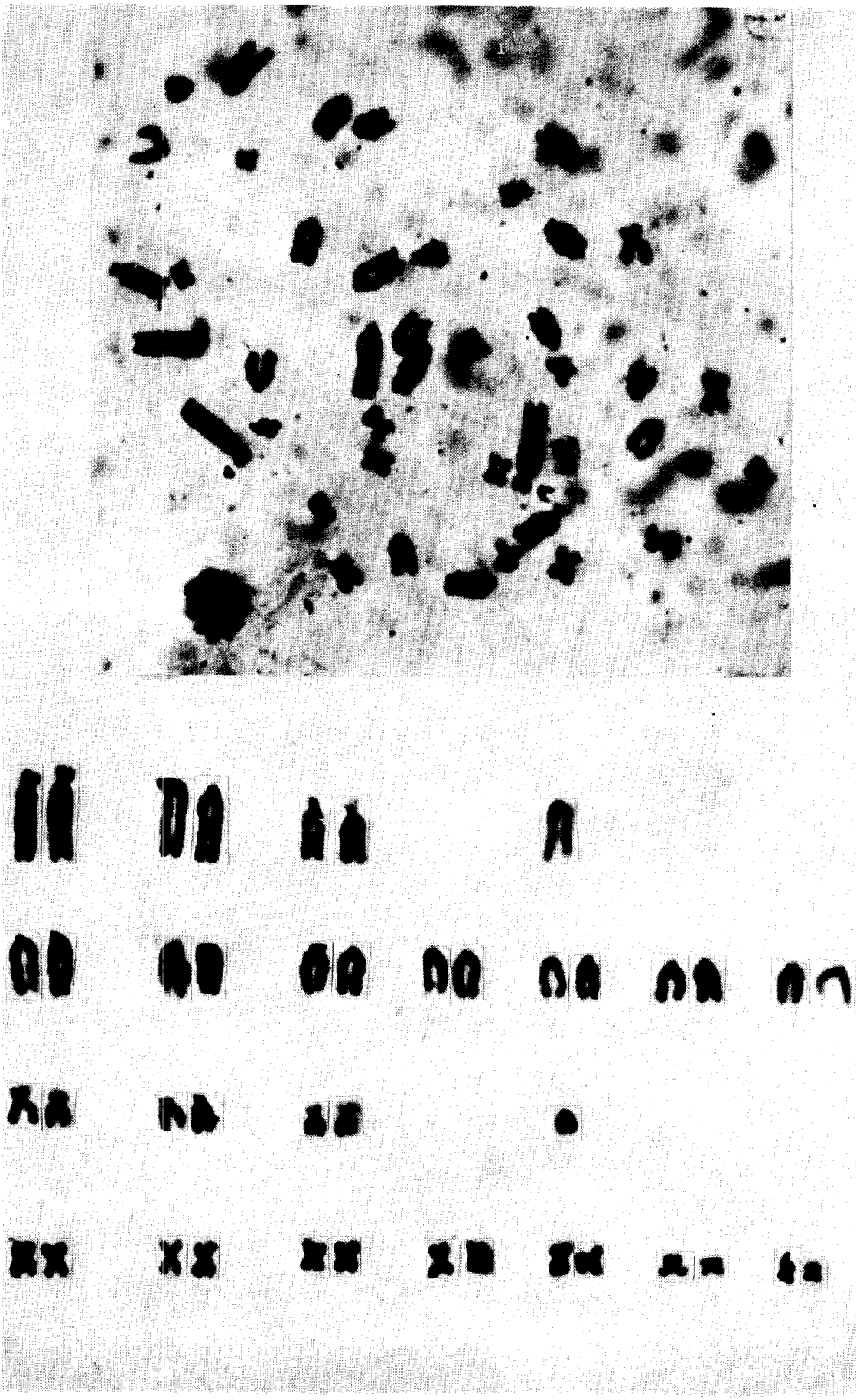

Fig. 1. *Cellule diploïde normale provenant d'une greffe tumorale dépendante portée par le rat* 198.

(to face p. 122)

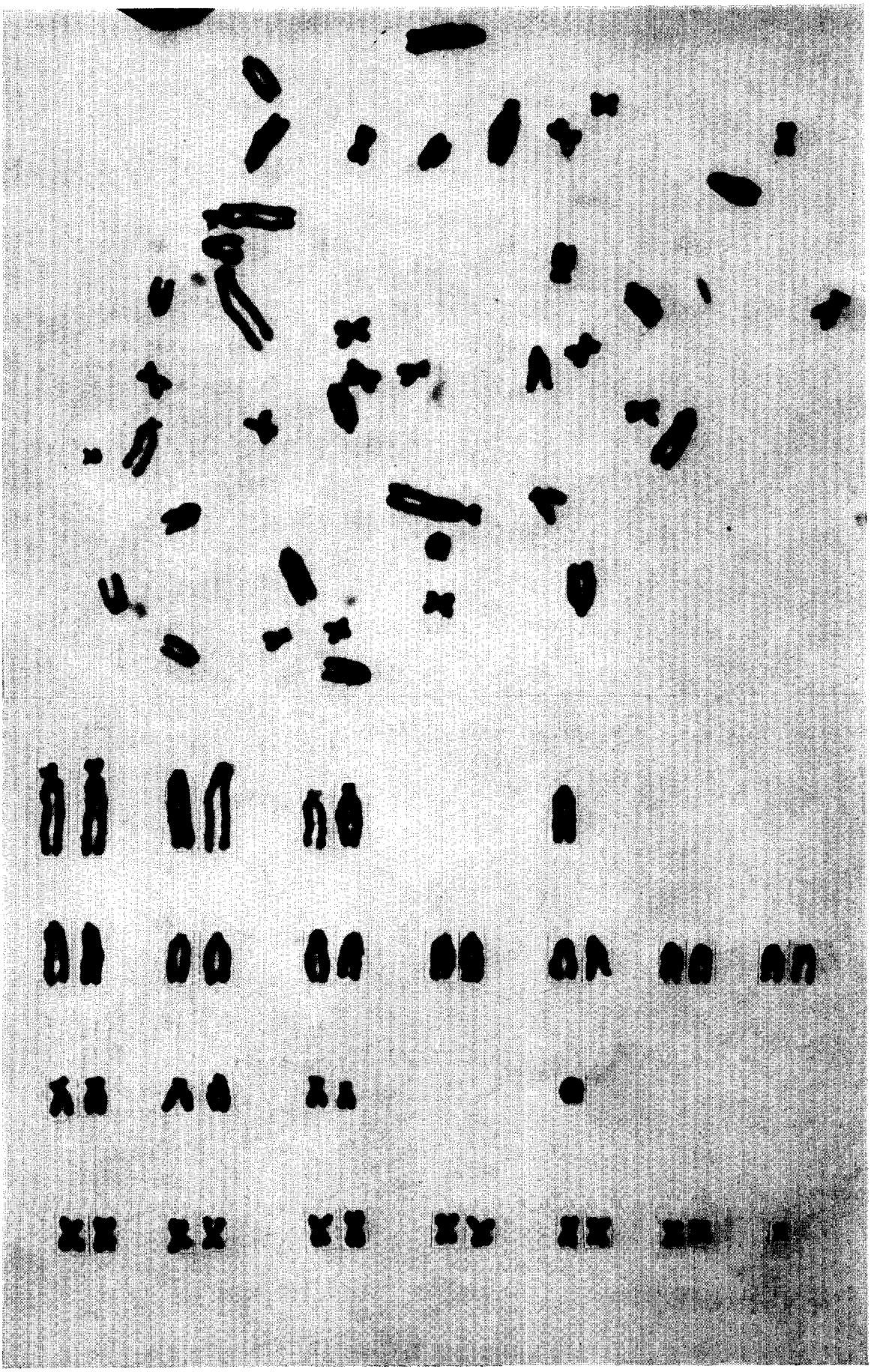

Fig. 2. *Cellule aneuploïde à* 41 *chromosomes* (20—) *provenant d'une greffe tumorale dépendante portée par le rat* 388 *G.*

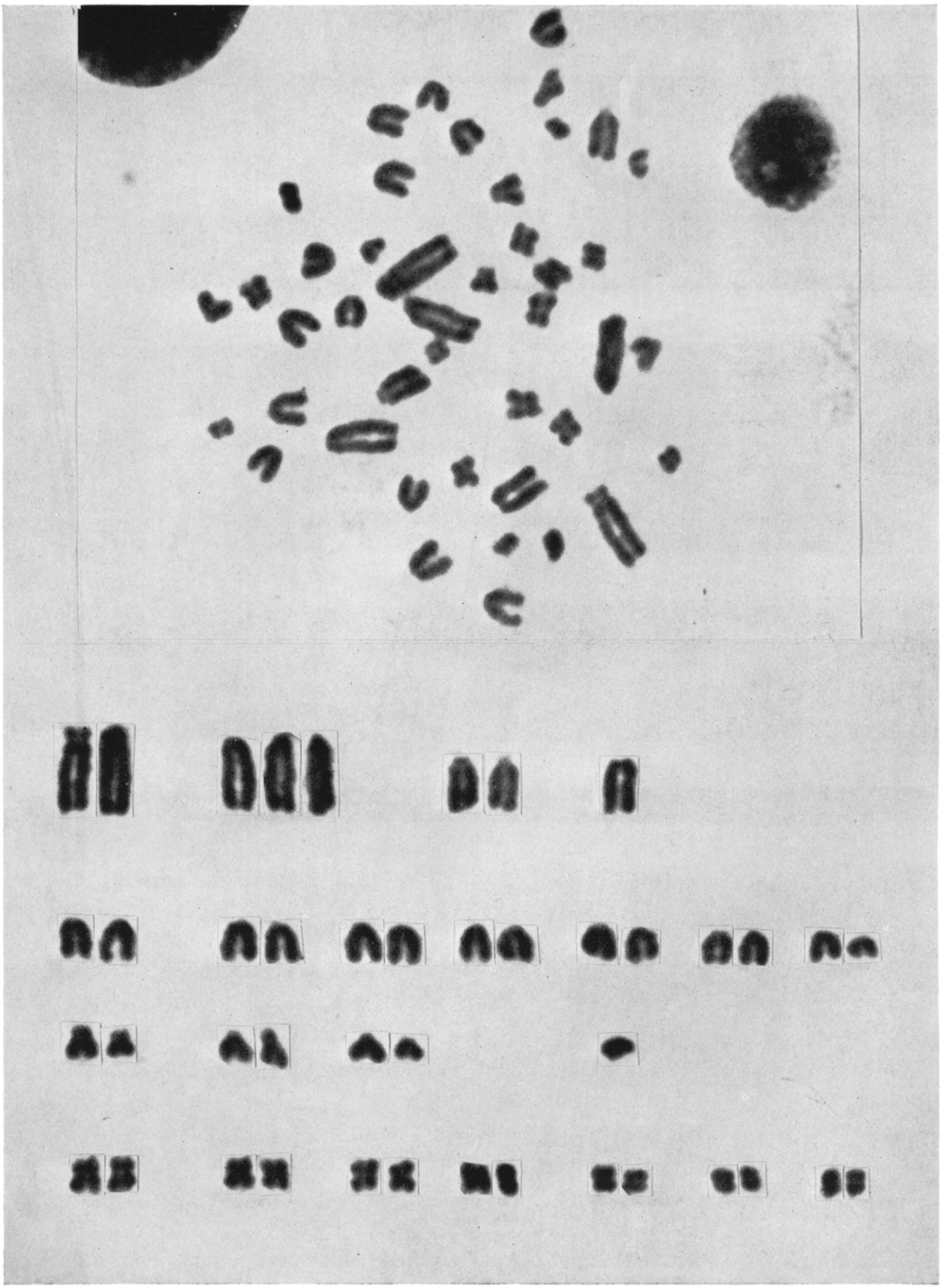

Fig. 3. *Reduplication d'un chromosome* 2 *au niveau d'une cellule tumorale provenant d'une greffe indépendante portée par le rat* 1277.

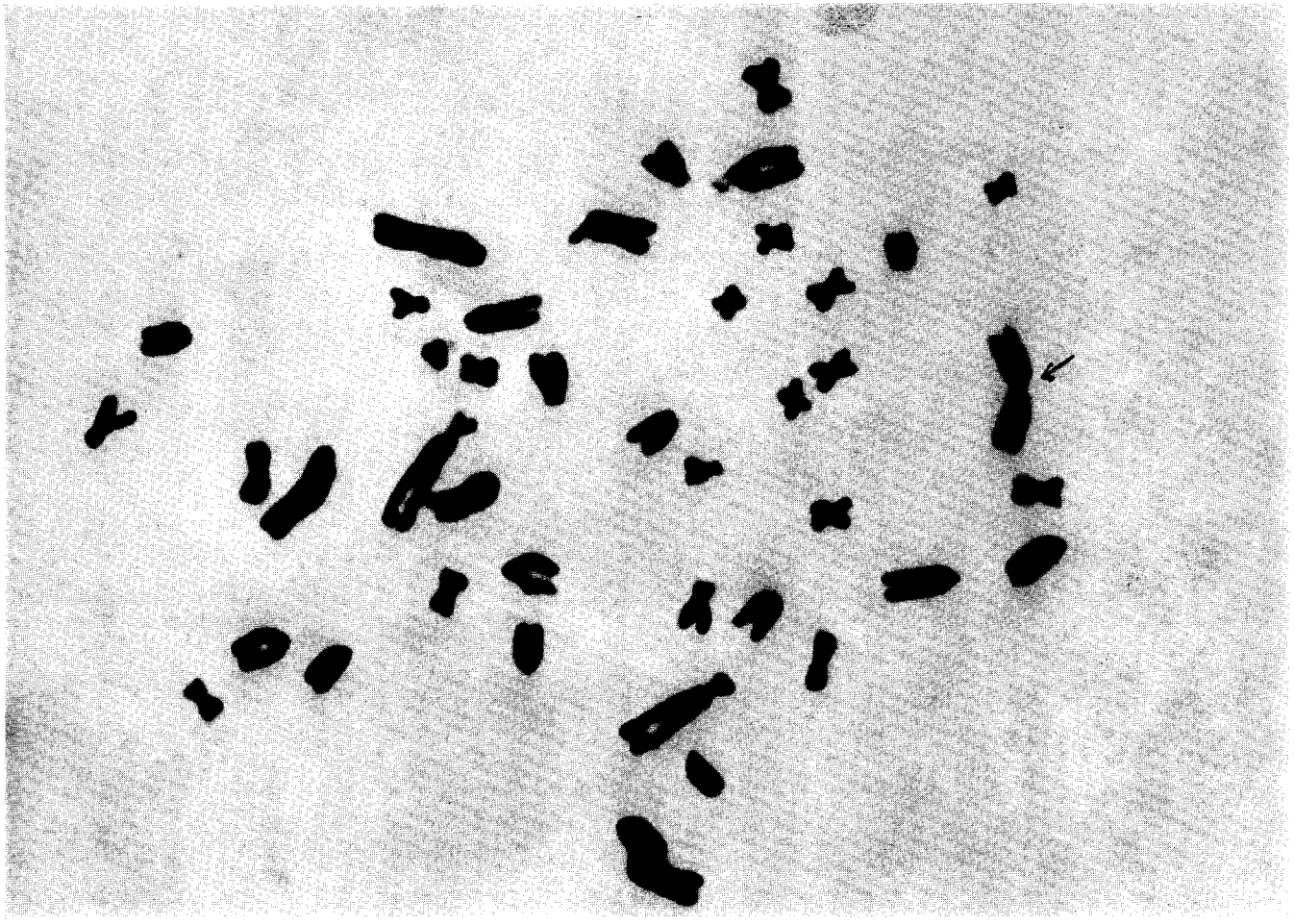

Fig. 4. *Chromosome marqueur métacentrique au niveau d'une cellule tumorale appartenant à une greffe dépendante portée par le rat* 403.

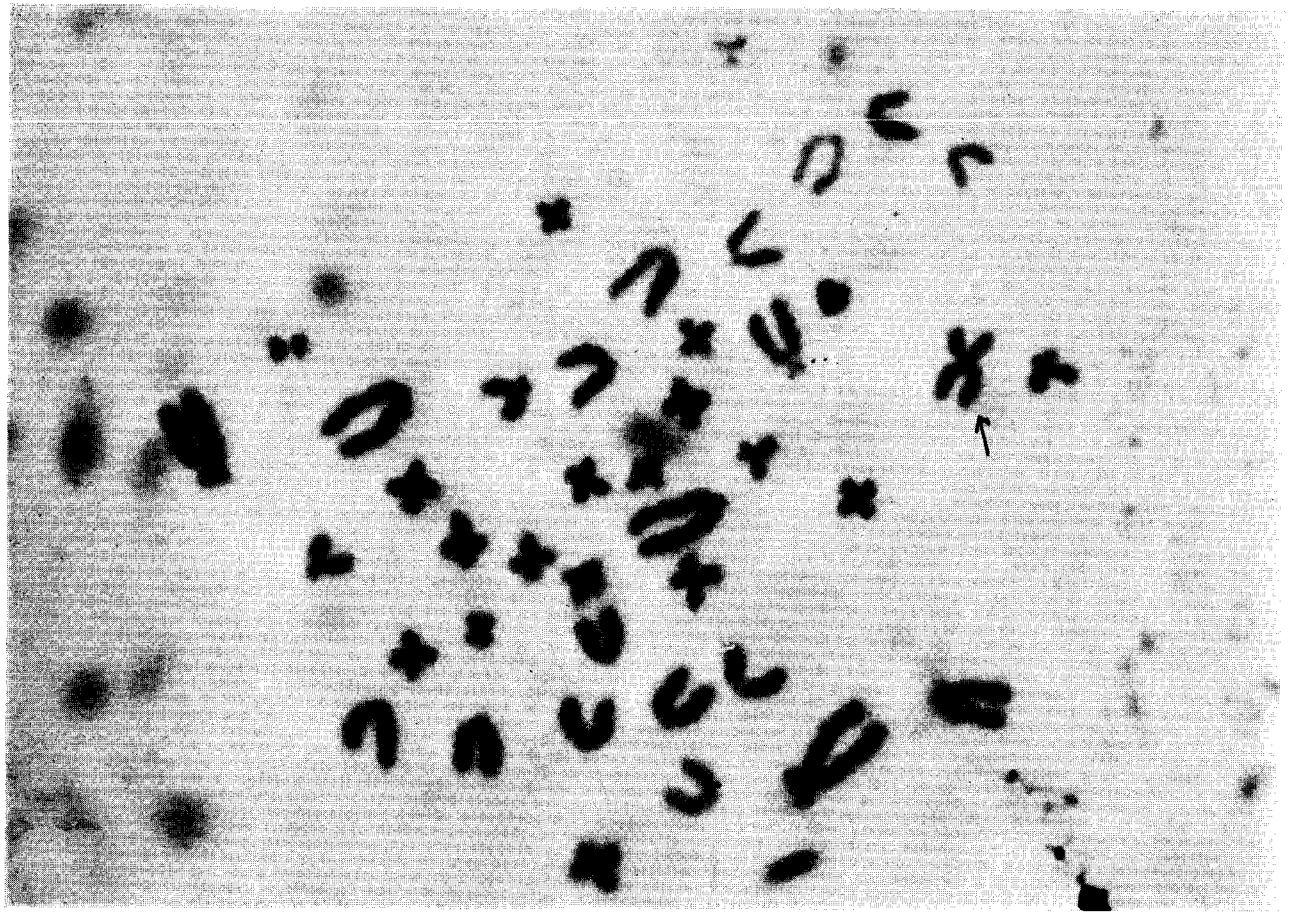

Fig. 5. *Fusion centromérique de deux chromosomes télocentriques au niveau d'une cellule tumorale appartenant à une greffe indépendante portée par le rat* 1191.

qui se distinguent des autres cellules aneuploïdes par leur formule chromosomiale identique. Une reduplication du chromosome 2 s'observe, avec une grande fréquence, dans 4 tumeurs issues de deux souches tumorales différentes et apparentées deux à deux. Toutefois, la formule cytogénétique des cellules qui présentent cette trisomie de la 2e paire n'est pas constante d'une tumeur à l'autre, ni d'une cellule à l'autre, au sein d'une même tumeur. Dans plusieurs autres tumeurs, l'apparition secondaire de fréquentes fusions centromériques rend impossible toute tentative d'identification de lignées cellulaires.

DISCUSSION

Cinq faits principaux se dégagent de nos résultats.

(1) Nos tumeurs, dépendantes et indépendantes, montrent des aberrations chromosomiales qui, comme dans d'autres modèles de néoplasie, consistent essentiellement dans des modifications du nombre des chromosomes et dans la présence de chromosomes marqueurs, ceux-ci résultant vraisemblablement de translocations ou de fusions centromériques. Par contre, nous n'avons pas observé les anomalies qui se rencontrent fréquemment après une irradiation [19–22] ou une contamination virale [23–26]: cassures chromatiniennes et chromosomiales, présence de fragments acentriques et de chromosomes dicentriques ou en anneau.

(2) Dans une même tumeur, on observe une grande diversité d'anomalies chromosomiales et de caryotypes. Ceci traduit l'existence, au sein de la population cellulaire, d'une instabilité cytogénétique que l'on voit s'installer dès le stade primaire. Déjà dans les tumeurs *in situ*, la proportion des cellules euploïdes n'atteint que 59% ce qui, par rapport aux séries témoins, représente une augmentation de 27% du nombre des aberrations numériques. L'instabilité cytogénétique s'accentue par la suite, faiblement dans les tumeurs dépendantes où la proportion des cellules euploïdes s'abaisse à 52% au cours des transplantations successives, puis de manière très marquée dans les tumeurs indépendantes chez lesquelles ne subsistent plus que 23% de mitoses euploïdes. Dans chacun de ces groupes, le nombre des chromosomes ne s'écarte que peu du chiffre normal.

(3) La présence d'un chromosome marqueur dans plusieurs cellules appartenant à la même greffe tumorale dépendante, de même que la répétition de la même anomalie numérique, au sein de plusieurs tumeurs dépendantes *in situ* et greffées, suggère l'individualisation de lignées cellulaires aberrantes dès un stade précoce de la tumorigénèse. L'apparition dans certaines tumeurs indépendantes de cellules variantes, et le fait qu'une trisomie de la 2e paire se retrouve dans plusieurs tumeurs dérivées de la même souche plaident dans le même sens. On pourrait envisager que l'action continue des oestrogènes induise, dans les cellules, des aberrations chromosomiales qui surgissent *de novo* à chaque génération successive et ne se transmettent pas par les mécanismes de l'hérédité [27]. Contre cette interprétation plaide la diversité des types d'aberrations qui sont apparues dans notre matériel. En outre, l'émergence, dans une greffe dépendante, d'une lignée cellulaire identifiable par un chromosome marqueur, indique, au minimum, que certaines des anomalies sont héréditaires.

(4) Le type et l'importance des anomalies cytogénétiques sont comparables dans nos deux groupes de tumeurs dépendantes, qu'il s'agisse des tumeurs *in situ* ou des transplants (Fig. 6).

(5) Le passage à l'indépendance s'accompagne d'une augmentation sensible du nombre et de l'importance des anomalies numériques et structurelles (Fig. 6).

Voyons comment ces faits s'intègrent avec les données de la littérature et nos résultats précédents.

Au cours de l'évolution de nos tumeurs, il se manifeste un certain parallélisme entre les modifications cytologiques et fonctionnelles d'une part et le développement d'anomalies chromosomiales d'autre part. Pendant la phase de dépendance hormonale, aussi bien les tumeurs *in situ* que les tumeurs greffées se montrent composées de cellules à prolactine, morphologiquement typiques et fonctionnellement actives [1]; de leur côté, les anomalies chromosomiales restent qualitativement et quantitativement similaires dans les deux groupes. Le passage à l'autonomie s'accompagne de bouleversements [1] à la fois dans le domaine de l'activité fonctionnelle (acquisition fréquente de propriétés somatotropes), dans celui de la cytologie (développement de caractères atypiques et transformations du type cellulaire avec apparition de cellules tumorales somatotropes et corticotropes) et dans celui du comportement (croissance agressive de rythme accéléré). En corrélation, on assiste à une accentuation marquée de l'hétérogénéité des caryotypes; toutefois, les types d'anomalies chromosomiales restent pratiquement constants.

L'apparition d'aberrations cytogénétiques

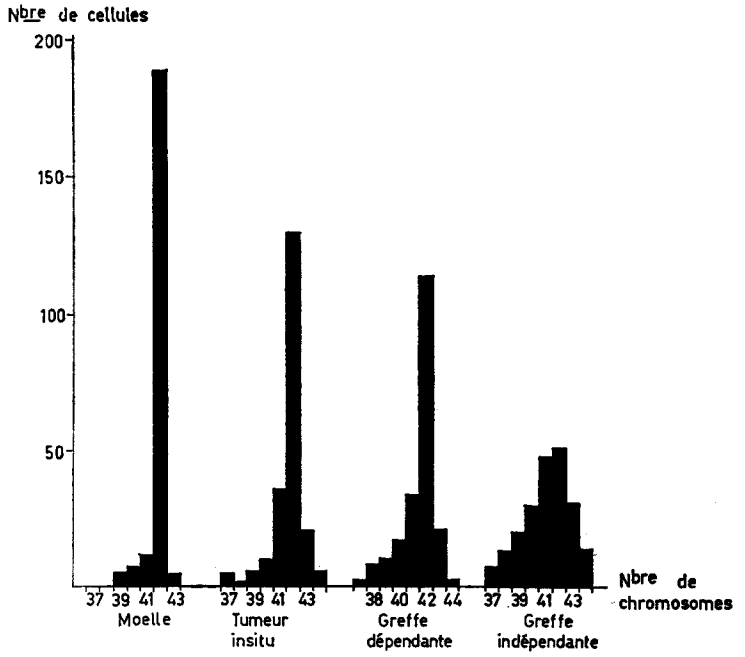

Fig. 6. *Répartition cytogénétique des cellules médullaires et des cellules tumorales dépendantes et indépendantes.*

dans les tumeurs *in situ* complète et appuie les conclusions auxquelles avait abouti le travail précédent [1]: le traitement oestrogénique chronique détermine une transformation irréversible des cellules hypophysaires avant que la tumeur induite ne soit devenue indépendante.

La présence d'aberrations chromosomiales, au premier stade de la carcinogénèse, est rapportée par plusieurs auteurs. Kirkland [8] ainsi que Wakonig-Vaartaja *et al* [9] observent une aneuploïdie au niveau de carcinomes *in situ* du col utérin chez la femme; en revanche, les lésions de dysplasies inflammatoires restent, de façon prédominante, diploïdes.

Dans une étude consacrée aux hépatomes et aux leucémies lymphoïdes, induits par agents chimiques, Stich [10] décrit le développement rapide d'une hétérogénéité cytogénétique des cellules hépatiques et lymphoïdes. Cet auteur constate cependant que la formation de tumeurs, à partir de ces populations cellulaires anormales, n'est pas un phénomène constant. Des facteurs mal définis, extra- et intracellulaires, interviennent en freinant ou en stimulant la sélection des lignées cellulaires à haute potentialité prolifératives, qui donneront naissance aux tumeurs.

Al Saadi *et al* [7] rencontrent un important pourcentage d'anomalies chromosomiques au niveau du parenchyme thyroïdien rendu hyperplasique par trois mois de carence iodée. Des altérations cellulaires irréversibles ont été induites, puisque la restitution d'un régime riche en iode ne modifie guère l'importance des aberrations chromosomiales.

Ces observations, comme les nôtres, montrent que les altérations cytogénétiques qui se développent dans divers tissus, sous l'effet de stimuli inducteurs variés, correspondent à une transformation cellulaire profonde irréversible, de nature prénéoplasique, qui progressera vers la malignité dans des circonstances favorables.

Il est intéressant de signaler toutefois qu'en ce qui concerne les tumeurs hypophysaires *in situ* ces conclusions semblent en désaccord avec celles de Clifton *et al* [28] et Boot *et al* [29]. En se basant sur des modèles mathématiques, Clifton *et al* [28] établissent que, sous l'effet d'une oestrogénisation prolongée, la réponse mitotique des cellules hypophysaires reste normale et peut aboutir à la formation d'une tumeur macroscopique, sans qu'il soit besoin d'invoquer une modification de la physiologie cellulaire ou une action anormale des oestrogènes. Pour Boot *et al* [29] également, la régularité de la croissance tumorale et l'uniformité de ses caractéristiques semblent signifier qu'aucune transformation maligne ne s'est instaurée au sein des cellules hypophysaires soumises à une oestrogénisation prolongée ou à une déconnection hypothalamique. L'orientation différente de nos investigations et celles de Clifton *et al* [28] et Boot *et al* [29] peut expliquer la discordance des conclusions. De fait, une prolifération cellulaire régulière et uniforme n'exclut pas l'installation, au sein de certains éléments prolactiniens, de bouleverse-

ments internes profonds, amorce d'une transformation néoplasique maligne.

Bien qu'il existe une association nette entre l'acquisition de l'indépendance et l'aggravation des anomalies chromosomiques, nous ne pouvons, au vu de nos résultats, apprécier le sens dans lequel s'établit la relation de cause à effet. Faut-il accorder aux modifications cytogénétiques un rôle prédominant dans la genèse de l'indépendance hormonale [2] ou, au contraire, les considérer comme un épiphénomène secondaire à la transformation autonome?

Parmi les anomalies chromosomiques que nous observons, aucune ne se rencontre de façon constante dans toutes nos tumeurs indépendantes. Ce fait plaide contre l'origine mutagénique de l'acquisition de l'indépendance. Toutefois, nos méthodes d'exploration trop grossières ne nous permettent pas d'éliminer l'intervention de mutations cryptostructurelles ou ponctuelles.

D'autre part, Bayreuther [13] constate que l'augmentation de taille de la tumeur peut conditionner l'apparition d'anomalies chromosomiques secondaires. Le même phénomène peut intervenir au sein de nos tumeurs autonomes souvent fort grandes et abondamment nécrosées.

Quelle que soit la signification que l'on veuille accorder à ce changement cytogénétique, on peut en tout cas le considérer comme un signe de progression tumorale.

Nos résultats divergent partiellement de ceux des autres auteurs. Bayreuther [13] n'observe pas d'anomalies dans 7 tumeurs hypophysaires *in situ* induites par oestrogénothérapie chez la souris. Une tumeur hypophysaire adrénotrope maintient une formule diploïde jusqu'au stade indépendance [13]. En revanche, les tumeurs hypophysaires thyréotropes, étudiées par Bayreuther [13] et Di Paolo *et al.* [14], sont aneuploïdes dès le stade primaire, suivant Di Paolo [14]. Dans un travail portant sur 3 tumeurs testiculaires de la souris, induites par les oestrogènes, Hellström [15] observe, dès le stade de dépendance, des modifications chromosomiales avec apparition de lignées cellulaires hétéroploïdes. Le passage à l'indépendance n'apporte aucune modification cytogénétique.

Selon Bayreuther [13] encore, huit tumeurs mammaires indépendantes possèdent une constitution chromosomiale normale.

Dans le cas des tumeurs mammotropes, la différence entre les résultats de Bayreuther [13] et les nôtres tient peut-être aux conditions expérimentales telles que l'intensité et la durée du traitement. Nous préférons ne pas comparer nos tumeurs aux tumeurs thyréotropes. Le traitement radiothérapique peut induire dans les cellules tumorales des modifications cytogénétiques secondaires. Il est également difficile de mettre valablement en parallèle des tumeurs hypophysaires et testiculaires; dans les deux cas cependant, les anomalies chromosomiales se sont développées dès le stade de dépendance.

En conclusion, nous sommes tentés de considérer les modifications cytogénétiques de nos tumeurs comme le témoin d'un processus de tumorigénèse qui s'installe précocement et aboutit, au cours des transplantations, à l'autonomie complète. La déconnection hypothalamique, en soustrayant le tissu hypophysaire à ses mécanismes régulateurs physiologiques, pourrait favoriser cette progression tumorale.

Remerciements—Je remercie Monsieur le Professeur Desclin, les Docteurs Govaerts et L. Koulischer de m'avoir aidée pendant l'élaboration de ce travail, ainsi que Mademoiselle Buelens pour son aide technique, et Madame Baum qui a bien voulu en assurer la dactylographie.

RESUME

Un examen cytogénétique a été effectué sur des tumeurs hypophysaires mammotropes, primaires et transplantées, induites chez le rat R Amsterdam par un traitement oestrogénique prolongé. Déjà, dans les tumeurs in situ, *les cellules tumorales manifestent une grande variabilité du nombre de chromosomes et des lignées cellulaires anormales apparaissent, individuellement différentes pour chaque tumeur. Les aberrations chromosomiques observées dans les tumeurs dépendantes transplantées sont comparables à celles des tumeurs* in situ. *En revanche, le passage à l'indépendance s'accompagne d'une augmentation du nombre des anomalies cytogénétiques; les lignées cellulaires aberrantes, qui s'isolent dans chaque souche tumorale, restent différentes les unes des autres. La signification des modifications cytogénétiques, présentes dès le stade* in situ, *est discutée et est notamment mise en parallèle avec les résultats recueillis dans le travail précédent* [1]. *L'accentuation des anomalies chromosomiales au stade d'indépendance peut être considérée comme le signe d'une progression tumorale.*

SUMMARY

We have effected a cytogenetic examination of primary and transplanted pituitary tumours, induced by a long term oestrogenic treatment in the R Amsterdam rat. In the in situ *tumours already a large variation in the number of chromosomes of the tumour cells is observed, and abnormal cellular stemlines appear, different for each specific tumour. The chromosomal aberrations noticed in the transplanted dependent tumours are comparable to those of the* in situ *tumours. On the other hand, the number of karyological anomalies increases significantly when the tumours become independent; the aberrant cellular stemlines isolated in each tumour line remain different from each other. We discuss the significance of the cytogenetic changes, already present in the* in situ *stage, and compare it, in particular, with the results presented in the preceding paper* [1]. *The increase in chromosomal anomalies at the independent stage can be considered as a sign of tumoral progression.*

ZUSAMMENFASSUNG

Es wurde eine cytogenetische Untersuchung durchgeführt und zwar an primären und transplantierten Hypophysentumoren, die durch Langzeitbehandlung von R Amsterdam Ratten mit Oestrogen erzeugt waren. An den Tumoren in situ *wurde bereits eine grosse Verschiedenheit der Chromosomenzahl beobachtet und es erschienen abnorme 'Stemlines', die in jedem einzelnen Tumor verschieden waren. Die Chromosomenveränderungen, die in den transplantierten abhängigen Tumoren beobachtet wurden, entsprechen den* in situ *Tumoren. Andererseits nimmt die Zahl der Chromosomenveränderungen signifikant zu, wenn die Tumoren unabhängig werden. Die abweichenden Zellstammlinien, die aus jeder Tumorlinie isoliert wurden, bleiben voneinander verschieden. Die Bedeutung der cytogenetischen Veränderungen, die bereits im* in situ*-Stadium vorhanden sind, werden diskutiert und besonders mit den Ergebnissen in einer vorangegangenen Arbeit verglichen* [1]. *Die Zunahme der chromosomalen Abnormalitäten in unabhängigen Stadien können als Zeichen des Fortschreitens des Tumors betrachtet werden.*

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The Biochemical Mechanism of Selective Heat Sensitivity of Cancer Cells

I. Studies on Cellular Respiration*

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INTRODUCTION

As PREVIOUSLY reported, heat causes a selective irreversible inhibition of respiration in some types of cancer cells [1, 2]. Therapeutic applications of these studies have been successfully carried out [1, 2]. Literature in this field is reported in reference 1. Since the mechanism of action of high temperature on tumors is so far unknown, our studies were directed to ascertain the first biochemical alteration responsible for the damage of cancer cells. In the present work three main lines of investigation were explored.

(a) The experimental conditions necessary to ascertain the effect of heat on minimal deviation hepatoma Morris 5123. Our previous investigations [1] had been negative or doubtful.

(b) The possible influence of variations in the incubation mixture.

(c) The possibility of observing the effect of high temperature on respiration in systems less rigidly organized than whole cells.

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MATERIALS AND METHODS

Male Sprague Dawley rats were used as the source of the Novikoff hepatoma, which was studied 6 days after transplantation, and of regenerating liver, which was taken 24 hr after partial hepatectomy. Rats bearing the Morris 5123 minimal deviation hepatoma were kindly provided by Dr. M. C. Pitot of the McArdle Laboratory, University of Wisconsin, and the tumors were used when they had reached a size of 1 cm dia. Cell suspensions were prepared as previously described [1] and incubated in anaerobic or aerobic conditions in a conventional Warburg apparatus. All values referred to 10 mg dry wt. of cells, as reported [1].

Except when indicated otherwise, the medium used for the suspension was the Krebs–Ringer phosphate solution [3], with the addition of antibiotics, as previously described [1]. Mitochondria were prepared in sucrose according to Schneider's method [4], as modified by Lardy and Wellman [5]. Oxygen consumption by isolated mitochondria was assayed at 30°C in a Gilson Electronics Oxygraph model KM, according to MacLennan and Tzagoloff [6], using either 1 mM pyruvate +1 mM malate or 1 mM succinate as substrates. Lactate was determined according to Barker and Summerson [7]. Controls were carried out with the cell suspension as such and in the presence of substrates and inhibitors, by heating at 100°C for 10 min the cell suspension before the incubation, or by adding 0·1 vol. 100% trichloroacetic acid.

The chemicals used for the preparation of the incubation mixture were obtained from C. Erba, Milano, Italy; D-glucose and sodium succinate from E. Merck, Darmstadt, Germany; lactic acid from British Drug Houses, BDH Laboratory Chemical Division, England;

filipin, 52% pure, was generously supplied by Dr. George B. Whitefield of the Upjohn Comp., Kalamazoo, Michigan. Other chemicals were commercial products of pure grade.

RESULTS

The effect of heat on minimal deviation hepatoma

When a minimal deviation hepatoma 5123 cell suspension was incubated at 38°C, 42°C and 43°C, the variations in oxygen uptake were, as previously reported [1], irrelevant. However, if hepatoma 5123 cells were incubated for 3·5 hr at 43°C and then returned to 38°C in the presence of glucose and succinate, it was found that oxygen uptake was lower in the cells pre-incubated at 43° (Fig. 1a).

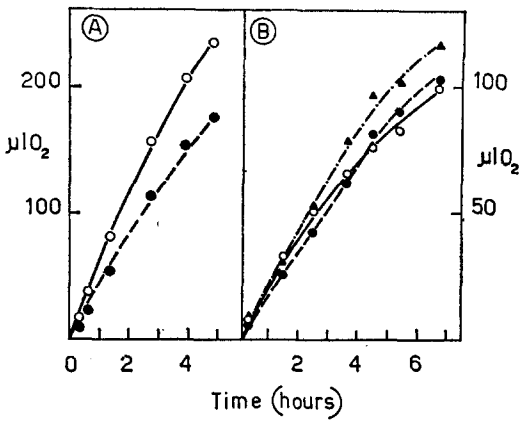

Fig. 1. O_2 *uptake by hepatoma* 5123 *and regenerating liver.* O_2 *uptake at* 38° *by* (*A*) *hepatoma* 5123 *cells;* (*B*) *regenerating liver cells, in the presence of* 0·015 M *glucose* + 0·013 M *succinate, after* 3·5 *hr aerobic preincubation at* 38°C (○—○), 43°C (●----●) *or* 44°C (▲-·-▲).

Conversely, no inhibition of oxygen uptake was instead observed in regenerating liver cells treated in identical conditions or for 3·5 hr also at 44°C (Fig. 1b).

Neither in hepatoma 5123 nor in regenerating liver was anaerobic glycolysis sensitive to elevated temperatures.

Influence of the incubation mixture on the heat effect

In order to ascertain whether the inhibition by heat resulted from a modification of the cells or from the liberation in the medium of some inhibitor, the cells were incubated 90 min at 42°C or 43°C then washed and successively assayed at 38°C. Oxygen uptake was lowered by exposure to the higher temperature (Fig. 2a).

Conversely, no inhibition was observed on fresh cells incubated at 38°C, by adding the supernatant obtained after centrifugation of a cell suspension incubated at 42°C, 43°C or 44°C. Negative results were also obtained by adding the supernatant at 23,000 **g** from an homogenate of heat treated tumor cells (Table 1).

When Novikoff hepatoma cells were incubated at 43°C under aerobic conditions, washed, and then assayed at 38°C for anaerobic glycolysis, a slight but significant (i.e. about 25%) heat inactivation was found (Fig. 2b). On the other hand it was confirmed that, if both preincubation and assay were performed in the absence of oxygen, no effect of elevated

Table 1. *Effect of extracts from pre-heated Novikoff hepatoma cells on* O_2 *uptake of Novikoff hepatoma cells*

	Temperature (°C)	O_2 uptake (µl/3 ml incubation mixture) in the first 90 min	O_2 uptake (µl/3 ml incubation mixture) in the following 90 min
1·5 ml fresh cell suspension (20 mg dry wt/ml) + 1·5 ml Krebs–Ringer phosphate solution	38	230	220
1·5 ml fresh cell suspension + 1·5 ml supernatant from A	38	270	260
1·5 ml fresh cell suspension + 1·5 ml supernatant from B	38	262	243
1·5 ml fresh cell suspension + 1·5 ml supernatant from C	38	240	220
1·5 ml fresh cell suspension + 1·5 ml Krebs-Ringer phosphate solution	43	235	75

A Novikoff hepatoma cell suspension, containing 40 mg net dry wt. of cells, was incubated aerobically at 4°C (A), 38° (B) or 43°C (C). After 6 hr, the samples were cooled to 0°C, homogenized at 1,500 rev/min in a Potter–Elvehjem apparatus, and centrifuged 30 min at 23,000**g**. The effect of the supernatant from A, B and C on the respiration at 38°C of a fresh Novikoff hepatoma cell suspension is shown in the Table. In the last line, the effect of a temperature of 43°C is shown for comparison; as indicated in the text, the inhibition is evident, in this case, only after more than 2 hr.

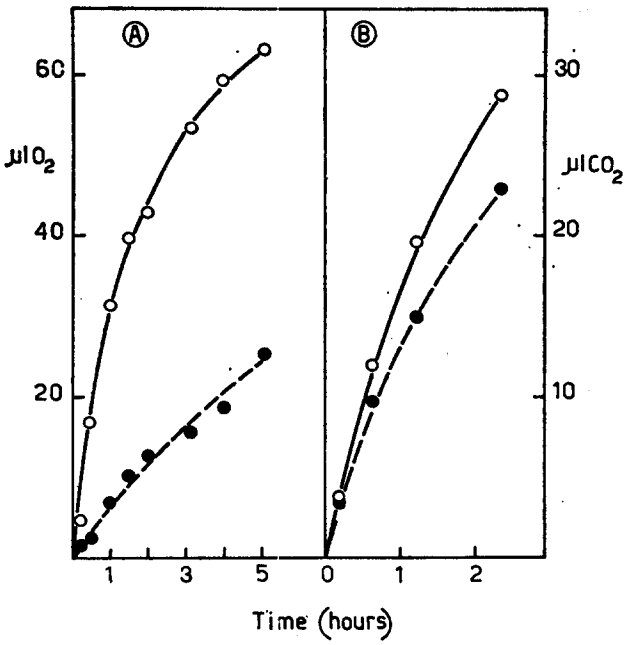

Fig. 2. O_2 *uptake (A) and anaerobic glucolysis (B) by Novikoff hepatoma cells preincubated 90 min in air at 38° (○—○) or 43°C (●----●).*

The cells, suspended in Krebs–Ringer phosphate solution, were washed three times with the same solution by centrifuging them at 30 **g**, *resuspended in Krebs–Ringer phosphate and incubated in the presence of air at 38°C or at 43°C, with gentle shaking. After 90 min, the cells were collected by centrifuging at 20* **g**, *and washed three times with either Krebs–Ringer phosphate (A) or Krebs–Ringer bicarbonate (B) solutions.*

O_2 *uptake (A) was measured at 38°C in the presence of* 0·015 M *glucose*+0·013 M *succinate. The rate of anaerobic glycolysis (B) was indicated by the amount of* CO_2 *liberated at 38°C from the Krebs–Ringer bicarbonate solution in an atmosphere of* 95% N_2+5% CO_2, *using* 0·015 M *glucose as substrate.*

temperature on anaerobic glycolysis was observed [1, 2]. In all cases chemical determinations of lacate were in good agreement with the manometric results.

In order to avoid any influence of pH on the results, the cell suspensions were checked at the pH meter before, during and after the incubation. In some experiments with Krebs–Ringer phosphate buffer, the pH fell from 7·4 to 6·5 in Novikoff hepatoma.

Therefore, experiments were performed by incubating a suspension of Novikoff cells in the following solutions:

(1) Krebs–Ringer bicarbonate–phosphate solution: in the usual Krebs–Ringer phosphate solution, 42 vol. (out of 100) of NaCl were substituted by isotonic $NaHCO_3$ (gassed with air+5% CO_2). The cells were washed before assay with Krebs–Ringer phosphate solution.

(2) 0·3 M sucrose+0·3 M imidazole+0·15 M HCl (41·5: 37·5: 21).

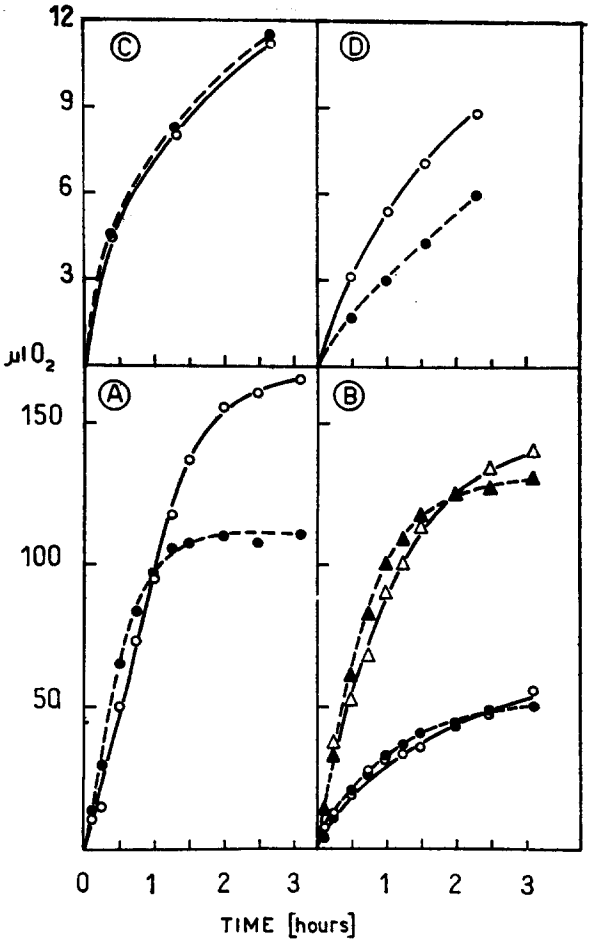

Fig. 3. Effect of cell disruption on O_2 *uptake by Novikoff hepatoma.*

(A) O_2 *uptake by intact cells, at 38°C (○—○) or 43°C (●----●).*

(B) O_2 *uptake by disrupted cells, at 38°C (——) or 43°C (---), in the absence (○—○, ●----●) or presence (△——△, ▲----▲) of* 11 µM *cytochrome c.*

(C) O_2 *uptake at 38°C by an acellular homogenate preincubated aerobically 2 hr at 38°C (○—○) or 43°C (●----●).*

(D) O_2 *uptake at 38°C by cells preincubated aerobically 2 hr at 38°C (○—○) or 43°C (●----●), and then disrupted by sonication.*

Incubations were performed as described in the text, in the presence of 0·015 M *glucose*+0·013 M *succinate.*

No differences in pH values were observed before and after incubation. In this case the difference in oxygen uptake, determined in the cells incubated at 38°C and 43°C, was also similar to the experiments described above. According to Poole [8], when the intracellular pH varies, that of the external fluid also varies.

Heat insensitivity of disrupted cells

The cells were disrupted by freezing and thawing (4 times) followed by homogenization in a Potter–Elvehjem apparatus or by sonication (20 min in a MSE ultrasonic disintegrator at 20 kc/s).

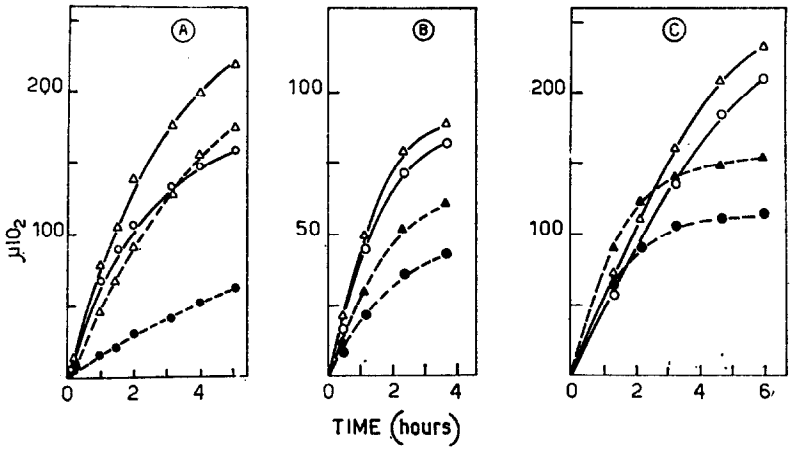

Fig. 4. Effect of cytochrome c on O_2 uptake by Novikoff hepatoma cells.

(A) Cells preincubated aerobically, 90 min at 38°C (——) or 42°C (- - -), and assayed at 38°C in the presence (△—△, △- - -△) or in the absence (○—○, ●- - -●) of 11 μM cytochrome c.

(B) Cells preincubated 2 hr at 38°C (——) or 43°C (- - -) and assayed at 38°C in the presence (△—△, ▲- - -▲) or in the absence (○—○, ●- - -●) of cytochrome c.

Cytochrome c concentrations were varied from 4·2 μM to 35·5 μM, without causing appreciable differences in the results.

(C) Cells assayed (without preincubation) at 38°C (——) or 43°C (- - -); in the presence (△—△, ▲- - -▲) or in the absence (○—○, ●- - -●) of 14 μM cytochrome c.

Incubations were performed as described in the text, in the presence of 0·015 M glucose+0·013M succinate.

In all experiments, the results were similar (Fig. 3): the temperature effect on oygen uptake is evident in intact cells (Fig. 3a), while in the acellular preparation no difference between the two temperatures can be detected (Fig. 3b, c). When the oxygen uptake was assayed in the homogenate performed with a cell suspension incubated 2 hr at 38°C or 43°C, a heat inhibition was observed (Fig. 3d).

Oxygen uptake is however very low in disrupted cells. It can be restored to higher values, similar to those obtained with intact cells, by the addition to the incubation mixture of cytochrome c. Also in this case, however, no difference between 38°C and 43°C could be detected with acellular preparations (Fig. 3b).

Addition of cytochrome c to intact cells incubated for 90 min at 42°C and then returned to 38°C enhances oxygen consumption to values similar to those given by cells incubated always at 38°C (Fig. 4a). Reduced cytochrome c is as effective as the oxidized form. However, when cells had been inhibited by more intense heat treatment, such as 2 hr at 43°C, addition of cytochrome c, even in increasing amounts (4·2 μM up to 35·5 μM) did reduce the inhibition, but was unable to reverse it completely (Fig. 4b). On the other hand, addition of cytochrome c to intact cells before incubation at 43°C did not prevent the heat inactivation effect (Fig. 4c).

Effect of temperature on mitochondrial activity

Since total disruption of the cells abolishes the heat effect, experiments were carried out by incubating for various times (5–45 min) at 38°C and 43°C isolated mitochondria from Novikoff hepatoma cells. No significant differences were found, between tumor and normal liver mitochondria, in the respiratory pattern, using either pyruvate–malate or succinate as substrates. A considerable inactivation of mitochondrial activity at both temperatures was however observed, which made the results uncertain. Addition of 10 mM ATP and 3 mM $MgCl_2$ to the incubation medium, according to MacLellan and Tzagoloff [6], did not give more encouraging results.

In the study of the heat inactivation on intact tumor cells, it was shown that neither the addition of 10 mM ATP nor of 10 mM ADP brought about any significant modification of the results. The presence of $3 \cdot 10^{-5}$ M 2,4-dinitrophenol, although this chemical by its uncoupling effect on oxidative phosphorylation had a stimulating effect on oxygen consumption, did not affect the heat sensitivity of Novikoff hepatoma cells (Fig. 5).

The effect of filipin on intact cells

Since the polyene antibiotic filipin has been reported to increase considerably the permeability of cellular membranes [9–13], its effect on cell respiration was studied. Oxygen

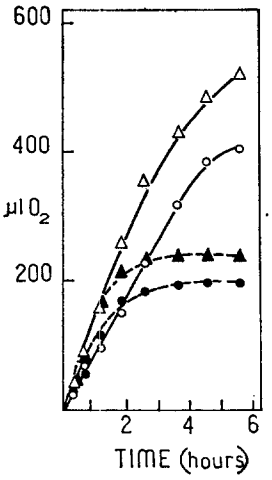

Fig. 5. Effect of 2,4-dinitrophenol on O_2 uptake by Novikoff hepatoma cells.
O_2 uptake was assayed at 38°C (——) or 43°C (- - -), using 0·015 M glucose+0·013 M succinate as substrates, with (△—△, ▲- - -▲) or without (○—○, ●- - -●) $3 \cdot 10^{-5}$ M dinitrophenol.

uptake of Novikoff hepatoma cells, if incubated in the presence of glucose and succinate at 38°C or 43°C, is considerably inhibited by the addition of filipin. Dimethylsulfoxide, which was used as the solvent for filipin, did not affect respiration at the concentrations used (0·03–0·25 M). As shown in Fig. 6, the inhibition is roughly proportional to filipin concentration, up to $2 \cdot 5 \times 10^{-5}$M; with higher concentrations, inhibition tends towards an asymptotic value around 80%.

A lag-time, the duration of which is inversely proportional to the concentration of the antibiotic and to the temperature, was observed in the onset of the inhibition (Fig. 7a).

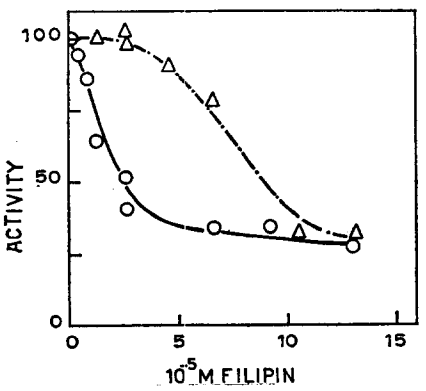

Fig. 6. Effect of filipin on O_2 uptake at 38°C of Novikoff hepatoma cells and of regenerating liver cells.
All assays were performed in the presence of 0·015 M glucose +0·013 M succinate as substrates. O_2 uptake is expressed as residual activity, taking the assay in the absence of filipin as 100. Owing to the lag-time phenomenon (see Fig. 7), the activity considered is the one reached after the lag-time.
○—○: Novikoff hepatoma cells.
△-·-△): regenerating liver cells.

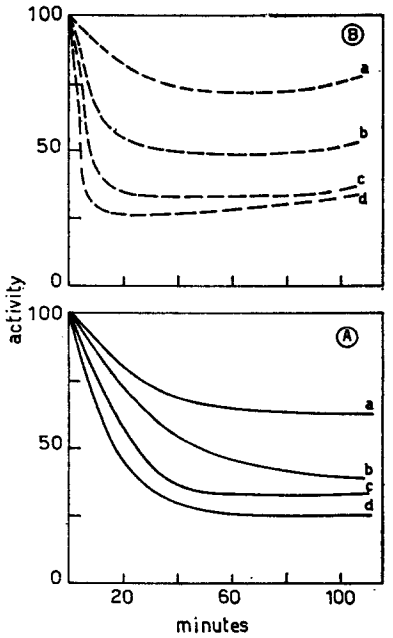

Fig. 7. Onset of inhibition by filipin of respiration by Novikoff hepatoma cells at 38°C (A) or 43°C (B).
O_2 uptake expressed as residual activity, taking the assay in the absence of filipin as a constant value of 100, is plotted vs. time. The various curves correspond to different concentrations of filipin: (a) 13·2 µM; (b) 26·4 µM; (c) 33 µM; (d) 66 µM.

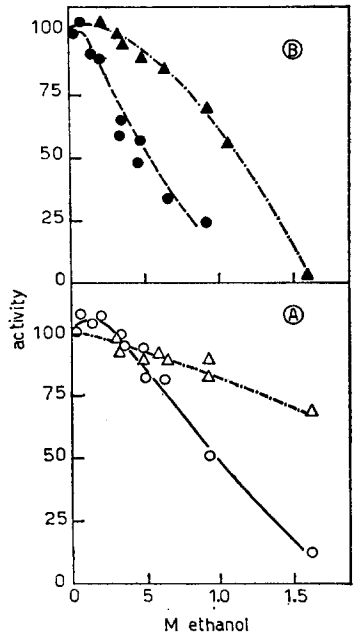

Fig. 8. Effect of ethanol on O_2 uptake at 38°C (A) or 43°C (B) of Novikoff hepatoma cells and of regenerating liver cells.
All assays were performed in the presence of 0·015 M glucose +0·013 M succinate as substrates. O_2 uptake is expressed as residual activity, taking the assay in the absence of ethanol as 100.

△-·-△: regenerating liver cells at 38°C
▲-·-▲: regenerating liver cells at 43°C
○—○: Novikoff hepatoma cells at 38°C
●- - -●: Novikoff hepatoma cells at 43°C

The inhibition by filipin in the assays at 43°C, although it reached its full values sooner, was the same as at 38°C. The effect of heat and filipin are not additive (Fig. 7b).

Regenerating liver was much less sensible to filipin than Novikoff hepatoma, concentrations up to 4×10^{-5} M being practically without effect (Fig. 6). Higher quantities of the antibiotic produced an inhibition, identical at 38°C and at 43°C.

The effect of ethanol on intact and disrupted cells

Figure 8 shows the effect of ethanol at various concentrations on O_2 uptake at 38°C and 43°C of Novikoff hepatoma and regenerating liver cells. Glucose and succinate were used as substrates. The inhibitory effect of ethanol on tumor cells was considerable, especially at 43°C: in this case 0·4 M ethanol is able to inhibit oxygen uptake by about 50% while at 38° only 0·9 M ethanol shows a similar effect. The inhibitory effect of ethanol is evident from the very beginning of incubation, and the decrease in oxygen uptake is a direct function of ethanol concentration. The curve obtained is however much steeper at 43°C than at 38°C.

Oxygen uptake of acellular preparations from Novikoff hepatoma was not significantly inhibited by 0·4 M ethanol. Also on regenerating liver cells, the effect of ethanol is enhanced by a higher temperature. At 38°C, 1·5 M ethanol gives only 30% inhibition; at 43°C, instead, about 50% inhibition is found at 1·15 M.

DISCUSSION

In the present experiments our previous results on irreversible damage to the respiration of some types of malignant tumors by incubation at 42–43°C have been confirmed. Also minimal deviation Morris 5123 hepatoma is sensitive to this treatment. Oxygen uptake of cells from regenerating liver, even when incubated 3·5 hr at 44°C, is instead completely insensitive to heat. These results indicate therefore that the damage by heat to respiration in tumor cells should really be considered as a general phenomenon. As shall be seen in the following paper, similar and much sharper results can be obtained by following the incorporation of radioactive precursors into nucleic acids and proteins. Anaerobic glycolysis, on the other hand, is also damaged by a previous aerobic exposure to heat, though inhibition was, in this case, very small.

The absence of selective heat sensitivity of respiration in tumor acellular preparations seems to indicate that the first biochemical damage can be localized in some cellular organization. The differences in oxygen uptake observed in the material obtained from cells incubated at 38°C or 43°C and disrupted after incubation seem to indicate that some damage to one or more enzymatic systems can be caused by heat, but only if the high temperature acts on the intact cells.

The experiments in which cytochrome c was added to intact Novikoff hepatoma cells indicate that some oxidoreductive system may be affected by heat. It may be recalled that Von Ardenne [14] has supposed, although no conclusive evidence has been presented, that heat treatment caused some damage on mitochondria of Ehrlich ascites cells. In our experience, it cannot yet be decided whether the restoring action of cytochrome c is specific or not. The addition of this substance seems to be able to repair the damage caused by heat only when the damage is not yet complete; but this effect could be either specific or substitutive for some other damaged pathway. Since, on the other hand, its presence does not prevent the onset of heat damage, the second hypothesis, of a non-specific action, seems to be more probable. It can moreover be recalled that variations in oxidative phosphorylation or in the availability of high energy compounds do not appear to modify the heat sensitivity of tumor cells.

The experiments carried out with filipin and ethanol on Novikoff hepatoma cells and regenerating liver cells indicate a selective depression of these substances on oxygen uptake of tumor cells.

Novikoff hepatoma cells appear to be much more sensible to filipin than regenerating liver cells. According to Kinsky *et al.* [9, 10] Sessa and Weissmann [11, 12] and Morton and Lardy [13] polyene antibiotics such as filipin can interact with sterols localized in the cell membrane, producing permeability alteration. On the other hand, they have been found by Weissmann *et al.* [15] to have a rather specific lytic effect on isolated lysosomes. Similarities between external and lysosomal membranes were emphasized by Thinès-Sempoux [16].

Because of the low purity of the filipin sample used, an inhibition by impurities (e.g. peroxidation or degradation products of filipin itself) cannot be excluded.

Experiments with pure samples of other polyene antibiotics are in progress.

The inhibitory effect of ethanol can also be tentatively ascribed to an action upon cellular membrane systems. According to Quastel [17] alcohols interact with the cell membrane.

Alcohols may inhibit active transport by inhibiting the membrane ATPase activity [18, 19] and/or more specially by increasing the membrane permeability as shown in mitochondria by Christophersen [20]. Von Ardenne and Reitnauer [21] have found an enhancement by ethanol of the high temperature effect on tumor cells. The effect of ethanol described by these authors seems however to be different from the one described in the present paper, being evident at concetrations as low as 0·054 M, but only at rather low pH values. Moreover, Von Ardenne and Reitnauer have performed their experiments only on Ehrlich ascites carcinoma cells, and do not report controls with normal cells.

The inhibitory effect of high temperature on tumor cells is paralleled by a higher sensitivity of these cells to filipin and ethanol.

A possible conclusion is that the first damage in cancer cells treated with high temperature resides in some organized system, i.e. lysosomal or surface membranes. Many results in the literature [22–24] lead in fact to the conclusion that tumor membranes have a different composition than those of normal cells, the levels of cholesterol, in particular, being frequently higher.

The possibility that other subcellular organelles or the whole cellular organization may be involved in the heat sensitivity effect in tumors cannot be ruled out at the present time.

RESUME

On a étudié l'effet de la température sur la respiration et sur la glycolyse anaérobique dans l'hépatome de Novikoff et dans l'hépatome 5123. *Le traitement des cellules tumorales, à* 43°C, *en aérobiose, inhibe la consommation d'oxygène, et, dans une moindre mesure, la glycolyse anaérobique. Les cellules du foie régénérant ne sont pas inhibées. Les cellules cancéreuses désintégrées ne sont pas sensibles à la température. L'éthanol inhibe fortement la respiration des cellules de l'hépatome de Novikoff, et cet effet s'accroît à température élevée. La "filipine" inhibe la consommation d'oxygène de l'hépatome de Novikoff; cet effet n'est pas accru par la température. Le foie régénérant n'est inhibé que par des concentrations beaucoup plus élevées d'éthanol ou de filipine; dans ce cas également l'inhibition par l'éthanol est augmentée par la température. Pour expliquer les données obtenues, une labilité de certains systèmes organisés (p. ex. les membranes cellulaires) pourrait être envisagée.*

SUMMARY

The effect of temperature on respiration and anaerobic glycolysis of Novikoff and minimal deviation 5123 *hepatoma was investigated. Exposure of tumor cells at* 43°C *in aerobic conditions caused an inhibition of oxygen uptake and also, to a minor extent, of anaerobic glycolysis. Regenerating liver cells were not inhibited. Disrupted tumor cells were temperature insensitive. Ethanol caused a severe inhibition of respiration in Novikoff hepatoma cells. The effect of ethanol is increased by the higher temperature. Filipin inhibited oxygen uptake by Novikoff hepatoma. The effect was not enhanced by temperature. Regenerating liver was inhibited only by much higher concentrations of ethanol and filipin. The inhibition by ethanol was also in this case enhanced by a higher temperature. The lability of some organized system (i.e. surface or lysosomal membranes) is suggested to explain the observed behaviour of tumor cells.*

ZUSAMMENFASSUNG

Der Einfluss von Temperatur auf Atmung und anaerobe Glykolyse von Novikoff und Morris 5123 *Hepatomen wurde untersucht. Eine Temperatur von* 43°C *bewirkte in Tumorzellen unter aeroben Bedingungen eine Hemmung der* O_2 *Aufnahme und, zu einem geringeren Grad, auch der anaeroben Glykolyse. Regenerierende Leberzellen wurden nicht gehemmt. Zerstörte Tumorzellen zeigten sich temperaturunempfindlich. Aethanol bewirkte eine starke Hemmung der Atmung in Novikoff-Hepatomzellen. Diese Wirkung von Aethanol nimmt mit höherer Temperatur zu. Filipin hemmte die* O_2*-Aufnahme von Novikoff-Hepatomen. Temperatur hatte keinen Einfluss auf die Wirkung. Regenerierende Leberzellen wurden nur durch viel höhere Konzentrationen von Aethanol und Filipin gehemmt. Auch in diesem Fall verstärkte eine Erhöhung der Temperatur die Hemmung durch Aethanol. Zur Erklärung dieses Verhaltens der Tumorzellen wird die Labilität eines organisierten Systems (z.B. Oberflächen oder lysosomale Membranen) in Betracht gezogen.*

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Note added in proof

The filipin sample is a filipin complex. According to M. E. Bergy and T. E. Eble: The filipin complex *Biochemistry* **7,** 653, 1968, it appears to contain eight filipin-like pentaenes. In the sample used filipin III was present as 52%. The concentration of filipin in the text indicates that of filipin III.

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The Biochemical Mechanism of Selective Heat Sensitivity of Cancer Cells

II. Studies on Nucleic Acids and Protein Synthesis*

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AN INHIBITION by heat of the respiration of Novikoff hepatoma cells, hepatoma 5123 (minimal deviation) and some human malignant tumors has been demonstrated [1–3]. Indications of a possible involvement of cell membranes has been suggested [3, 4].

In the present paper the investigation was extended to the effect of heat on the incorporation of labeled compounds into DNA, RNA and proteins.

MATERIALS AND METHODS

Sources of tissues are indicated in the previous paper [3].

The preparation of cell suspension was performed as previously described [2], without antibiotics in the incubation mixture. The incubations at various temperatures were carried out in a Krebs–Ringer phosphate solution [5] or an Eagle's minimum essential medium adjusted to pH 7 with $NaHCO_3$.

Chemicals used for the preparation of incubation mixture and formamide were obtained from C. Erba, Milano, Italy; Eagle's minimum essential medium no. 109 (without L-glutamine) from GIBCO, Grand Island, New York; D-glucose, sodium succinate and toluene (scintillation grade), from E. Merck, Darmstadt., Germany; 6-^{3}H thymidine (spec. activity 20,600 mC/mmole), ^{3}H uridine (U.L.) (spec. activity 3330 mC/mmole) from Radiochemical Centre, Amersham, England; ^{14}C amino acids (U.L.) mixture (spec. activity 1·5 mC/mg) from New England Nuclear Corp., Boston, Mass; Filipin, 52% pure†, was generously supplied by Dr. George B. Whitefield of the Upjohn Comp., Kalamazoo, Michigan; PPO (2,5-diphenyloxazole), POPOP (1,4 bis [2,5 phenyloxazolyl] benzene) and hyamine hydroxide were from Nuclear Chicago.

Incorporation studies

Cells were washed 3 times with Krebs–Ringer–phosphate solution by low speed centrifugation (50**g**), resuspended in 9 vol. Eagle's medium and incubated for a suitable period, as described below, in the presence of ^{3}H thymidine (for DNA labeling) or ^{3}H uridine (for RNA labeling) or ^{14}C amino acids mixture (for protein labeling). The number of cells was calculated by a Thoma–Zeiss chamber at various times of incubation. pH values were recorded at various times of incubation with a Radiometer pH-meter.

At the desired time, the cells were treated with 0·4 M cold $HClO_4$. The precipitate was collected by centrifugation, washed 3 times with ice cold 0·4 M $HClO_4$, defatted according to Tyner *et al.* [6], and dissolved in 1 ml formamide at 140°C for 12 hr.

Separation of labeled DNA, RNA and proteins was perfomed as follows. The defatted precipitates were suspended in 1 ml of 1N KOH and incubated 1 hr at 37°C. DNA and proteins were precipitated by adding 0·2 ml of 6N HCl and 1·2 ml of 0·4 M $HClO_4$ in an ice bath and collected by centrifugation. The supernatants containing digested RNA [7] were pooled. The precipitate was washed 3 times with 1 ml 0·2 M $HClO_4$ and DNA was then extracted with 0·5 M $HClO_4$ for 45 min at 96°C with occasional stirring [8]. The precipitate, containing proteins, was collected by centrifugation and washed 3 times with 0·5 M $HClO_4$. The supernatants containing DNA were pooled.

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*Some preliminary results of this work were presented at the 6th Meeting of the Coordinating Committee for Human Tumour Investigations (Tissue and Organ Culture Study Group), Amsterdam 6 May 1968.

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$HClO_4$ was removed from RNA and DNA extracts according to Birnie and Heidelberger [9]. The precipitates were discarded by centrifugation and the supernatants freeze-dried and redissolved in 1 M hyamine hydroxide. The protein precipitate was dissolved in hyamine hydroxide and insoluble salts centrifuged off.

Protein was determined by a biuret procedure [10]. Crystalline bovine serum albumin (Sigma) was used as the standard. DNA and RNA were determined by the diphenylamine and orcinol reactions, respectively [11]. Scintillation analysis were carried out with formamide-treated samples to which 8·6 ml of 95% ethanol and 10·4 ml toluene containing 6g of PPO and 0·1g of POPOP per liter were added. To hyamine-treated samples two ml 95% ethanol and 13 ml toluene solution were added. The samples were counted in a Nuclear Chicago Mod. 725 liquid scintillation Counter. Counting efficiency was calculated by a channels ratio method or, when necessary, by internal standardization with ^3H labeled toluene. Radioactivity was expressed as disint./min.

RESULTS

Effect of temperature on Novikoff hepatoma cells

The incorporation of radioactive precursors into Novikoff hepatoma cells as a function of temperature was studied.

Two series of experiments were carried out:

(A) the incubations were performed at different temperatures in the presence of radioactive precursors.

(B) the incubations were performed at different temperatures, the cells were washed and their ability to incorporate radioactive material was assayed at 38°C.

Isolation of DNA, RNA and proteins was made only when indicated. Counting of cells before and after incubations did not reveal any appreciable difference. ^3H thymidine incorporation rate into DNA of Novikoff hepatoma cells incubated up to 6 hr at 38°C and 43°C is shown in Fig. 1. After 1 hr the incorporation curve at 43°C plateaued rapidly and was about 50% less than at 38°C. In order to investigate at which time the inhibitory effect of higher temperature became evident, Novikoff hepatoma cells were incubated for various times at 43°C and 38°C and incorporation of ^3H thymidine was performed at 38°C for 60 min. Figures 2 and 3 show the results obtained on preincubation of Novikoff hepatoma cells at 38°C and 43°C for 30 and 120 min respectively. It is clearly demonstrated that a 30 min exposition of the cells at 43° is able to inhibit the incorporation of ^3H thymidine into DNA. When the incubations at 43°C were prolonged to 2 hr, inhibition reached 90–100%. Glucose and succinate added to the incubation mixture inhibited the incorporation of labeled thymidine into DNA also at 38°C (see Figs 2 and 3). Experiments carried out in order to study ^3H uridine incorporation into RNA of Novikoff hepatoma cells gave results similar to those obtained with ^3H thymidine. As reported in Fig. 4 when the cells were incubated 30 min at 43°C the incorporation of

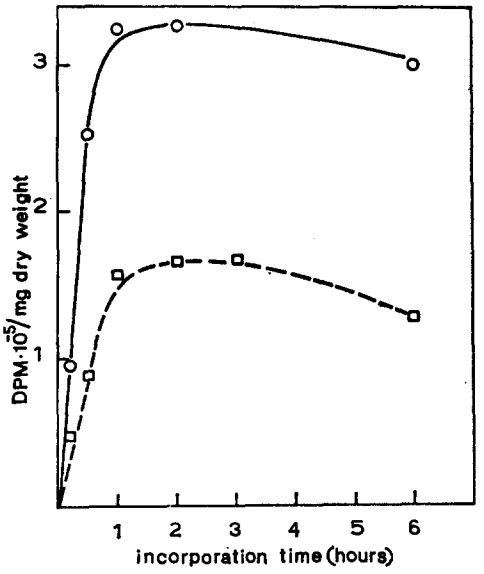

Fig. 1. ^3H thymidine incorporation into Novikoff hepatoma cells incubated at 38°C and 43°C.
DPM = disint/min.
○—○ incubation performed at 38°C.
□---□ incubation performed at 43°C.
Medium: Krebs–Ringer phosphate solution. At 0 time 0·6 μC/mg dry wt. of ^3H thymidine were added.
Incubations were performed with continuous shaking under air.

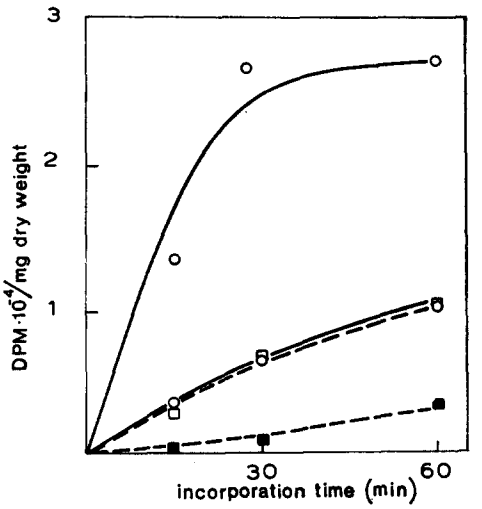

Fig. 2. ^3H thymidine incorporation at 38°C into Novikoff hepatoma cells preincubated at 38°C and 43°C for 30 min.
^3H thymidine (0·6 μC/mg dry wt.) was added at 0 incorporation time
DPM = disint./min.
○—○ preincubations at 38°C.
□—□ preincubations at 43°C.
●—● preincubations at 38°C in the presence of 0·015M D-glucose and 0·013 M succinate.
■---■ preincubations at 43°C in the presence of 0·015M D-glucose and 0·013 M succinate.
Other experimental conditions are reported in Fig. 1.

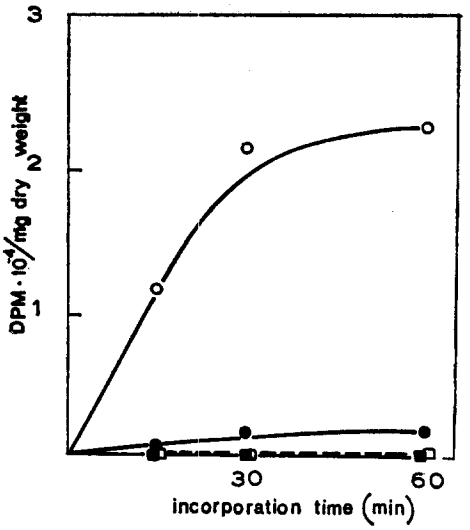

Fig. 3. ³H thymidine incorporation at 38°C into Novikoff hepatoma cells preincubated at 38°C and 43°C for 2hr—³H thymidine (0·6 μC/mg dry wt.) was added at 0 incorporation time.
Other experimental conditions are reported in Fig. 1.
Symbols are as in Fig. 2.
DPM=disint/min.

labeled compound is considerably inhibited. Fig. 5 show the results obtained by studying the incorporation of labeled uridine into RNA of the cells preincubated 2 hr at 38°C and 43°C. Glucose and succinate added to the incubation mixture lowered also the incorporation of ³H uridine into RNA (see Figs. 4 and 5).

Inhibition of ³H thymidine incorporation into Novikoff hepatoma cells as a function of temperature is reported in Table 1. The inhibitory effect of temperature is evident especially above 40°C.

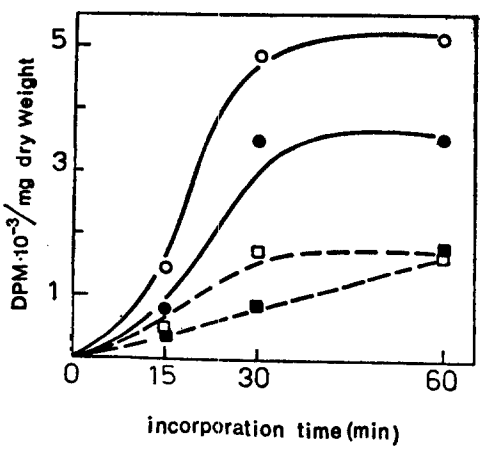

Fig. 4. ³H uridine incorporation at 38°C into Novikoff hepatoma cells preincubated at 38°C and 43°C for 30 min. ³H uridine (0·6 μC/mg dry wt.) was added at 0 incorporation time.
Other experimental conditions are reported in Fig. 1.
Symbols are as in Fig. 2.
DPM=disint/min.

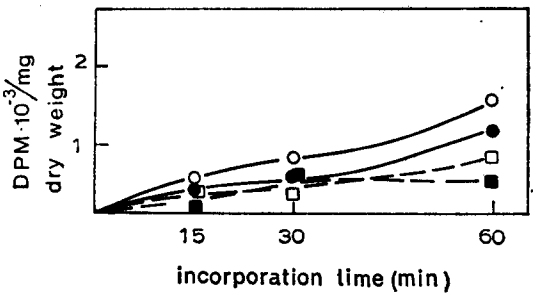

Fig. 5. ³H uridine incorporation at 38°C into Novikoff hepatoma cells preincubated at 38°C and 43°C for 2 hr. ³H uridine (0·6 μC/mg dry wt.) was added at 0 incorporation time. Other experimental conditions are reported in Fig. 1.
Symbols are as in Fig. 2.
DPM=disint/min.

In another series of experiments DNA, RNA and proteins were isolated after incubation of cells at 38°C and 43°C and incorporation of labeled precursors. As indicated in Table 2 ³H thymidine incorporation into DNA, ³H uridine incorporation into RNA and ¹⁴C amino acids incorporation into proteins of Novikoff hepatoma cells show at 43°C a 100%, 88% and 95% inhibition respectively. ATP added before or after preincubation at 43°C of Novikoff hepatoma cells did not prevent the inhibition of incorporation of ³H thymidine into the cells (see Table 3).

Effect of succinate, D-glucose and pH on ³H thymidine incorporation

Since the results reported in Fig. 2, 3, 4 and 5 indicated an inhibition of ³H thymidine incorporation into Novikoff hepatoma cells

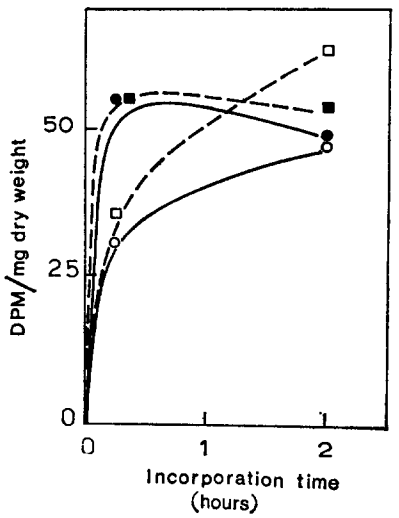

Fig. 6. ³H thymidine incorporation into rat regenerating liver cells preincubated at 38°C and 43°C for 30 min.
³H thymidine (0·6 μC/mg dry wt.) was added at 0 incorporation time.
Medium: Eagle's medium.
Symbols are as in Fig. 2.
DPM=disint/min.

Table 1. *Effect of temperature on* ^{3}H *thymidine incorporation into Novikoff hepatoma cells*

	Temperature of preincubation				
	38°C	40°C	41°C	42°C	43°C
Disint/min/μg DNA	776	326	68	40	36
Inhibition (%)	0	52	90	94	95

Cells suspensions were preincubated for 2 hr in Krebs–Ringer phosphate solution at 38°, 40°, 41°, 42° and 43°C. Incorporation of the labeled compound was carried out by adding 0·6 μC/mg dry wt. of ^{3}H thymidine to the washed cells resuspended in Egale's medium and by incubating for 1 hr at 38°C.

Table 2. *Effect of temperature on labeled precursors incorporation into DNA, RNA and proteins isolated from Novikoff hepatoma cells*

	Temperature		Incorporation at 43°C (incorporation at 38°C=100) (%)
	38°C	43°C	
Disint/min/μg DNA	400	0	0
Disint/min/μg RNA	387	44	12
Disint/min/μg Protein	84	4	5

Cell suspension was preincubated 2 hr in Eagle's medium at 38° and 43°C. Incorporation of ^{3}H thymidine (0·6 μC/mg dry wt.), ^{3}H uridine (0·6 μC/mg dry wt.) and ^{14}C amino acids (0·3 μC/mg dry wt.) was performed by incubating the washed cells in Eagle's medium at 38°C for 1 hr. DNA, RNA and proteins were there isolated for scintillation counting as described in the text.

Table 3. *Effect of* ATP *on* ^{3}H *thymidine incorporation into Novikoff hepatoma cells*

Temperature of preincubation (°C)	Added ATP (1·6 mM)	Incorporation of ^{3}H thymidine (%)
38	No	100
38	Yes (before preincubation)	70
38	Yes (after preincubation)	92
43	No	6·4
43	Yes (before preincubation)	3·9
43	Yes (after preincubation)	6·3

Cell suspension was preincubated 2 hr in Krebs–Ringer phosphate solution at 38° and 43°C. Incorporation of ^{3}H thymidine (0·6 μC/mg dry wt.) was performed by incubation at 38°C for 30 min in Eagle's medium. Incoporation is expressed in per cent with respect to the values obtained with the cells preincubated at 38°C.

imputable to the addition of glucose and succinate to the incubation mixture, controls were carried out on the action of these substrates. The pH of the medium was accurately controlled in order to discriminate between the effect of acidification due to lactic acid production and the action of added substrates.

The results reported in Table 4 indicate an increased ^{3}H thymidine incorporation into the cells which had been preincubated at 38°C in the presence of succinate. No significative differences were observed in the cells which had been preincubated at 43°C in the presence or absence of succinate. Table 5

Table 4. *Effect of succinate on* ^{3}H *thymidine incorporation into Novikoff hepatoma cells*

Temperature of preincubation (°C)	Added succinate (0·013 M)	Disint/min/μg DNA
38	No	4,000
38	Yes	12,800
43	No	42
43	Yes	15

Cell suspension was preincubated 2 hr in Eagle's medium at 38°C and 43°C in the presence or absence of 0·013 M succinate. Incorporation of ^{3}H thymidine (0·3 μC/mg dry wt.) was performed by incubating the washed cells in Eagle's medium.

Table 5. *Effect of* D-*glucose and pH on* ^{3}H *thymidine incorporation into Novikoff hepatoma cells*

Buffer used	Temperature of pre-incubation (°C)	D-glucose added to 0·015 M	Initial pH	pH after incubation	pH after incorporation	Disint/min/ µg DNA
A	38	No	7·1	6·3	6·1	380
A	38	Yes	7·1	6·1	5·7	110
A	43	No	7·1	6·3	5·9	53
A	43	Yes	7·1	6·3	5·8	15
B	38	No	7·5	8·4	7·8	11,000
B	38	Yes	7·5	7·4	7·7	2,550
B	43	No	7·5	8·4	7·6	0
B	43	Yes	7·5	7·2	7·8	0

Cell suspension was preincubated 2 hr in the following solutions at 38°C and 43°C in the presence or absence of 0·015 M D-glucose.
A—Eagle's medium.
B—Krebs–Ringer bicarbonate–phosphate solution: in the usual Krebs–Ringer-phosphate solution, 42 vol. (out of 100) NaCl were substituted by isotonic $NaHCO_3$ (gassed with air+ 5% CO_2).
Incorporation of ^{3}H thymidine (0·6 µC/mg dry wt.) was performed by incubating the washed cells at 38°C for 1 hr: A—in Eagle's medium, B—in a solution containing equal parts of Eagle's medium and Krebs–Ringer phosphate [6].

shows the results obtained when Novikoff hepatoma cells were incubated with different buffer systems at 38°C and 43°C in the presence and absence of D-glucose. The incorporation of thymidine into the cells was considerably inhibited when D-glucose was added to the mixture incubated at 38°C. By comparing the results obtained in the presence of buffers of different strength, an inhibition imputable to acidification of the medium is evident, but also a more specific and pH independent inhibition by D-glucose seems to be present.

Table 6. *Effect of filipin and ethanol on* ^{3}H *thymidine incorporation into Novikoff hepatoma cells*

Temperature of pre-incubation (°C)	Filipin µM)	Ethanol (M)	Incorporation (%)
38	0	0	100
43	0	0	4
38	13·7	0	17·5
38	27·3	0	10·5
38	0	0·36	100
38	0	0·51	30
38	0	0·85	3

Cell suspension was preincubated 2 hr in Krebs–Ringer phosphate solution at 38° and 43°C in the presence and absence of filipin or ethanol. Incorporation of ^{3}H thymidine (0·6 µC/mg dry wt.) was performed by incubating the washed cells 1 hr at 38°C in Eagle's medium. Filipin was previously dissolved in dimethyl-sulfoxide at concentration of 0·1%. Controls carried out with equal volumes of dimethyl sulfoxide did not reveal any effect of this substance.
The results are expressed in per cent of incorporation, taking as 100% the incorporation value at 38°C.

Effect of ethanol and filipin on Novikoff hepatoma cells

According to our previous paper [3] ethanol and filipin inhibit the respiration of Novikoff hepatoma cells. As reported in Table 6, when filipin was added to the cells at 38°C, about 90% inhibition of ^{3}H thymidine incorporation into the cells was demonstrated. When 0·36 M ethanol was added to the incubation mixture, no inhibitory effect on ^{3}H thymidine incorporation into Novikoff hepatoma cells was demonstrated. Seventy and 100% inhibitions were observed with 0·51 M and 0·85 M ethanol, respectively.

Heat insensitivity of disrupted cells

The incorporation of ^{3}H thymidine into a supernatant obtained after disruption of Novikoff cells and centrifugation at 105,400 **g** was studied as a function of preincubation temperature. No significative differences were observed (see Table 7).

Effect of temperature on minimal deviation hepatoma 5123 *and human osteosarcoma*

Table 8 shows the results obtained with minimal deviation hepatoma 5123 after 2hr preincubation at 38°C, 42°C and 43°C. ^{3}H thymidine, ^{3}H uridine and ^{14}C amino acids incorporation into the cells was considerably inhibited by increasing the temperature. The effect is present at 42°C as well as 43°C, but in

Table 7. Effect of temperature on ^{3}H *thymidine incorporation into a cell free homogenate from Novikoff hepatoma cells*

Temperature (°C)	Disint/min/ µg DNA
38	50
43	60

Cells were washed three times with Krebs–Ringer phosphate solution, then disrupted by adding an equal volume of distilled water and by homogenizing in a glass Potter apparatus. 9·41 g/l of Eagle's medium were added to the homogenate and pH was adjusted to 7 with bicarbonate. The suspension was then centrifuged at 105,400 **g**. The supernatant fluid was incubated for 2 hr at 38°C and 43°C. Incorporation was performed in the presence of 5 µC/ml of ^{3}H thymidine, 6 mM ATP and 50 µg DNA and RNA/ml as primers, for 4 hr.

the latter case is evident especially in the experiment with ^{3}H thymidine as precursor. The inhibitory effect of glucose was observed also in the case of hepatoma 5123 (see Table 9). No changes of pH values were observed during incubations. The results obtained with human osteosarcoma are reported in Table 10. The incorporation of ^{3}H thymidine into the cells was inhibited by the higher temperature in this case also.

Effect of temperature on rat regenerating liver cells

To determine whether an increase in the proliferative rate of normal cells would affect the response to elevated temperature, the incorporation of regenerating liver was studied. Fig. 6 shows incorporation curves of regenerating liver cells after a 30 min pre-incubation at 38°C and 43°C. No inhibition by temperature is present, whereas, in the same conditions, Novikoff hepatoma cells are strongly inhibited (see Fig. 2).

As reported in Table 11 the incorporation of ^{3}H thymidine and ^{14}C amino acids is higher after preincubation at 43°C than at 38°C while ^{3}H uridine incorporation is the same at both temperatures studied. A slight inhibition was observed in the incorporation of ^{3}H thymidine when glucose had been added to the incubation mixture. No changes of pH were observed during incubations in the various experimental conditions analysed.

Table 8. Effect of temperature on labeled precursors incorporation into minimal deviation hepatoma 5123 cells

Temperature	38°C	42°C	43°C
Disint/min/µg/DNA	530	320	195
Inhibition, referred to 38°C preincubation values (%)	0	39	63
Disint/min/µg RNA	70	—	58
Inhibition, referred to 38°C preincubation values (%)	0	—	17
Disint/min/mg protein	2700	1480	1075
Inhibition, referred to 38°C preincubation values (%)	0	45	60

Cell suspension was preincubated 2 hr in Eagle's medium at 38°, 42° and 43°C. Incorporation of ^{3}H thymidine (1·2 µC/mg dry wt.), ^{3}H uridine (1·2 µC/mg dry wt.) and ^{14}C amino acids (0·6 µC/mg dry wt.) was performed by incubating the washed cells in Eagle's medium at 38°C for 3 hr.

Table 9. Effect of glucose on ^{3}H *thymidine incorporation into minimal deviation hepatoma 5123 cells*

Temperature of preincubation (°C)	Added D-glucose (to 0·015 M)	Initial pH	pH after pre-incubation	Disint/min/µg DNA
38	No	7·1	7·22	530
38	Yes	7·1	7·22	340
43	No	7·1	7·22	195
43	Yes	7·1	7·22	280

Cell suspension was preincubated 2 hr in Eagle's medium at 38°, 42° and 43°C in the presence or absence of 0·015 M D-glucose. Incorporation of ^{3}H thymidine (0·2 µC/mg dry wt.) was performed by incubating the washed cells at 38°C for 3 hr in Eagle's medium.

Table 10. *Effect of temperature on* ^{3}H *thymidine incorporation into human osteosarcoma cells*

Temperature of preincubation (°C)	Disint/min/μgDNA
38	85
43	32

Cell suspension was preincubated 3 hr in Eagle's medium at 38°C and 43°C. Incorporation of ^{3}H thymidine (0·6 μC/mg dry wt.) was performed by incubating the washed cells at 38°C for 3 hr in Eagle's medium.

cells [12] and by Rao and Enderlburg in HeLa cells [13]. Langen [14] was unable to find differences in the inhibition of protein and nucleic acids synthesis in Ehrlich ascites mouse and normal thymus cells incubated at 44°C for 30 min.

The experiments reported in the present paper demonstrate a selective and irreversible damaging effect of heat on the incorporation of ^{3}H thymidine, ^{3}H uridine and ^{14}C amino acids into the tumor cell, DNA, RNA and proteins respectively. This effect was present

Table 11. *Effect of temperature on* ^{3}H *thymidine,* ^{3}H *uridine and* ^{14}C *amino acids incorporation into regenerating liver cells*

Temperature of preincubation (°C)	Added D-glucose (to 0·015 M)	Disint/min/ μg DNA	Disint/min/ μg RNA	Disint/min/ mg protein
38	No	85	8·8	1050
38	Yes	74	—	—
43	No	100	8·5	1260
43	Yes	78	—	—

Cell suspension was preincubated 2 hr in Eagle's medium at 38°C and 43°C in the presence or absence of 0·015M D-glucose. Incorporation of ^{3}H thymidine (0·3 μC/mg dry wt.), ^{3}H uridine (0·3 μC/mg dry wt.) and ^{14}C amino acids (0·15 μC/mg dry wt.) was performed by incubating the washed cells at 38°C for 4 hr in Eagle's medium.

Effect of ethanol and filipin on rat regenerating liver cells

Both ethanol and filipin inhibited regenerating liver-cells but at a much lower rate than tumour cells (see Table 12).

DISCUSSION

The sensitivity of cells during their mitotic cycle to external agents has prompted many authors to investigate the effect of heat on DNA synthesis and growth rate.

Inhibition by heat was shown under cell culture conditions by Sisken *et al.* in amniotic

Table 12. *Effect of filipin and ethanol on* ^{3}H *thymidine incorporation into regenerating liver cells*

Filipin (μM)	Ethanol (M)	Incorporation (%)
0	0	100
27·3	0	66
54·6	0	41
0	0·51	66
0	0·85	55

Cell suspension was preincubated for 3 h in Eagle's medium at 38°C in the presence and absence of filipin and ethanol. Incorporation of ^{3}H thymidine (0·6 μC/mg dry wt.) was performed by incubating the washed cells for 3 h at 38°C in Eagle's medium. For other details see Table 6.

in all tumor tested, including minimal deviation hepatoma 5123 and human tumors, while the incorporation of labeled precursors into regenerating rat liver cells is higher at the higher temperature.

It seems that the sensitivity of the cells to heat is related to the malignancy of the tumor. While in Novikoff hepatoma a preincubation of the cells for 2 hr at 42°C gave a 94% inhibition of the incorporation of ^{3}H thymidine into DNA (see Table 1), only 45% inhibition was observed (see Table 8) in minimal deviation hepatoma 5123 cells which has been treated in the same manner. The effect of temperature can be revealed by incubating Novikoff hepatoma cells at 43°C for 30 min or less (see Fig. 1 and 2). Therefore the heat damage of cancer cells seems to become apparent earlier in DNA, RNA and protein synthesis than at respiratory level [1–3, 15, 16]. A rather critical temperature point is present in Novikoff hepatoma cells at about 41°C (see Table 1): by increasing the temperature above this value, no additional effect is obtained.

The stimulating effect of succinate on ^{3}H thymidine incorporation into Novikoff hepatoma cells may be related to the effect of this substrate on the respiration of tumor cells [1–3, 17]. The inhibitory effect of D-glucose

is not easy to explain. Ciaccio *et al.* [18] have described a necrosis of Walker carcinoma 256 as a consequence of hyperglycemia. The influence of pH and glucose concentration on thermosensibilization of tumor cells has also been discussed by Von Ardenne [19–21]. Certainly in the presence of glucose there is a decrease in the intracellular pH (see Table 5): determination of the pH of the medium at various times in the presence of glucose revealed a shift towards lower values, which correspond presumably to an acidification of the intracellular milieu [22]. Nevertheless, when the pH of the mixture was maintained above neutrality, the inhibitory effect of D-glucose maintained. Also in minimal deviation hepatoma 5123 and in regenerating liver, (see Tables 9 and 11), an inhibitory effect of D-glucose was observed. No changes of pH during incubation were observed in these cases.

The inhibitory effect of high temperature was completely abolished when incorporation of ^{3}H thymidine into DNA was performed in cell-free preparations from Novikoff hepatoma (see Table 7). This result, according to our previous respiration data [3], may relate the sensitivity to high temperatures to the cellular organization.

The experiments performed with different concentrations of filipin and ethanol showed an extreme sensitivity of Novikoff hepatoma cells to these substances. The inhibitory effect of filipin and ethanol on regenerating liver is much lower than that on tumor cells, even at much higher concentrations of the inhibitory compounds. A discussion on the action of filipin and ethanol on cell membranes is reported in our previous paper [3].

As reported by Plagemann [23] the treatment of exponentially growing Novikoff hepatoma cells with cytostatic concentrations of phenethyl alcohol results in an immediate inhibition of the synthesis of DNA, RNA, protein and phosphorylcholine. According to this author it seems unlikely that the synthesis of DNA, RNA and protein is the primary site of action of phenethyl alcohol and it is suggested that the effect of phenethyl alcohol could occur on the cellular membrane. In this contest it should be recalled that Scornik *et al.* [24] demonstrated that the rat liver microsome fractions contain two kinds of inhibitors of amino acids incorporation *in vitro;* the first one is derived from lysosome contamination while the second inhibitor is a heat-labile factor associated with the endoplasmic reticular membrane.

Thus, tumor membranes could be altered by the action of physical or chemical agents at some critical levels which are different from those of normal cells. The inhibition by heat of DNA, RNA and protein synthesis in tumor cells is more pronounced and earlier detectable than the inhibition of the respiration. Also the action of filipin and ethanol is more pronounced at DNA synthesis than at respiratory level.

It can be concluded that inhibition of DNA, RNA and protein synthesis may be related to a primary alteration of cellular membranes of tumors. These membranes, which are probably different from that of normal cells [25, 26] may be damaged by exposing the cells to heat or to chemical agents. This alteration might result either in a leakage of enzymes or substrates or in a release of inhibitor or lytic enzymes of lysosomes. Researches in this field are in progress.

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RESUME

On a étudié l'effet de la température, entre 38 *et* 43°C, *sur l'incorporation des précurseurs marqués dans le DNA, RNA et les protéines des cellules de l'hépatome de Novikoff et de l'hépatome* 5123 *de Morris.*

La température a provoqué une inhibition marquée; celle-ci a été observée dans les deux types de cellules cancéreuses, alors que les cellules de foie régénérant ne sont pas affectées. Cet effet de la température a été observé aussi dans les cellules d'un ostéosarcome humain. Les préparations acellulaires sont insensibles à la température. Le D-*glucose, ajouté au mélange d'incubation, cause une inhibition très évidente. La filipine et l'éthanol inhibent l'incorporation dans les cellules cancéreuses à des concentrations qui n'ont pas d'effet appréciable sur les cellules du foie régénérant. Pour expliquer le mécanisme d'action de la chaleur sur les cellules cancéreuses, on peut envisager des altérations des membranes.*

SUMMARY

The effect of temperature in the range from 38° *to* 43°C *upon incorporation of labeled precursors into DNA, RNA and protein of Novikoff and Morris* 5123 *hepatoma cells was investigated. A striking inhibition by temperature was found in both kind of tumor cells whereas regenerating liver cells were not affected. The temperature effect was observed also in cells from a human osteosarcoma. Cell free preparations were temperature insensitive.* D-*glucose added to the incubation mixture caused a marked inhibition. Filipin and ethanol were found to inhibit the incorporation into tumor cells at concentrations which did not significantly affect regenerating liver cells. A possible involvement of membranes in the mechanism of heat sensitivity of tumor cells is suggested.*

ZUSAMMENFASSUNG

Untersucht wurde der Einfluss der Temperatur im Bereich von 38–43°C *auf den Einbau von markierten Bausteinen in DNA, RNA und Proteine von Novikoff und Morris* 5123 *Hepatomen. Eine auffallende Hemmung durch Temperatur wurde in beiden Arten von Tumorzellen festgestellt, während regenerierende Leberzellen nicht beeinflusst wurden; derselbe Effekt wurde auch in Zellen eines menschlichen Osteosarkoms festgestellt. Zellfreie Präparationen waren Temperaturunempfindlich. Eine starke Hemmung wurde durch Zusatz von* D-*Glukose zu der Inkubations-Mischung hervorgerufen; Filipin und Aethanol hemmten den Einbau in Tumorzellen bein Konzentrationen, die regenerierende Leberzellen nicht bedeutsam beeinflussten. Eine mögliche Beteiligung der Membranen am Mechanismus Hitzeempfindlichkeit von Tumorzellen ist anzunehmen.*

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Retrospective Search for Asbestos Bodies in Necropsies and Biopsies on Cases of Primary Malignant Disease of the Lung

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INTRODUCTION

INCREASING awareness of the hazards associated with inhalation of asbestos dust justifies a search for asbestos bodies in all biopsy specimens taken from lung and pleura, whatever the clinical diagnosis, and in lungs examined *post mortem* as a routine procedure, with the object of establishing the frequency with which they may be encountered in such material in different localities and their possible significance.

MATERIAL

The material examined is from cases referred to the Institute of Pathological Anatomy and Histology, and Division of Cancer Research, University of Perugia, for *post mortem* (23 cases) or for diagnosis (30 cases) during the years 1965–1967 (Tables 1–3).

The total number of cases was 53, all primary malignant tumours of the lung; 48 of these were confirmed histologically and the remaining 5 were diagnosed by scalene node biopsy. In these latter, of course, the search for asbestos bodies was not carried out.

The specimens examined from the 53 cases are divided as follows: *post mortem* specimen series, 23 cases (Table 1); operation specimen series, 10 cases (Table 2); scalene node biopsy series, 5 cases (Table 2); bronchial biopsy series, 15 cases (Table 3).

The size of the specimens varied from very small biopsy specimens to relatively large amounts of tissue from lesions removed during necropsy.

To provide an arbitrary standard for comparison of the material examined in each case, the surface area of all the sections studied is recorded (Tables 1–3).

In the surgical specimens and bronchial biopsy series the greater part of the section was formed by the tumour, but in the *post mortem* series the tumour area occupied about 60 to 70 per cent of the section.

METHODS

Most of the specimens were fixed with formalin in aqueous solution (10%), and embedded in paraffin; sections were cut at the conventional thickness (5–7 μ) and these were given routine staining with haematoxylin and eosin or, occasionally, with Prussian blue. When available unstained serial sections were also scanned for asbestos bodies, first under lower power objective and then, in negative or doubtful cases, under high power. Frequently macrophages loaded with pigment and inorganic dust particles were present in fibrotic

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Table 1. Pulmonary carcinomas in post mortem specimen series

Case No.	Necropsy No.	Age	Sex	Residence	Occupation	Possible causative factors: Smoking — Smokers	Smoking — Non-smokers or ex-smokers	Asbestos — Search	Asbestos — Areas exam. (mm²)	Antituberculosis drugs	Site of origin — Side	Site of origin — Lobe	Site of origin — Part of lung affected	Microscopical structure
1	89/65	61	M	Perugia	Retired	+		—	180		Right	Upper	Peripheral	Anaplastic
2	105/-	51	M	Todi	Retired	+		—	2335	+	Left	Upper	Peripheral	Anaplastic
3	110/-	55	M	Gubbio	Butcher	+		—	180		Right	Upper	—	Anaplastic
4	146/-	65	M	Terni	Steelworker	+		—	160*		Right	Upper	—	Anaplastic
5	154/-	47	M	Terni	Driver	+		—	220		Left	Lower	Lobar bronchus	Anaplastic
6	165/-	62	M	Terni	Steelworker		+	—	373		Left	U. & L.	Pleural	Anaplastic
7	176/-	59	M	Perugia	Bricklayer	+		—	883		Right	All lobes	Pleural	Anaplastic
8	9/66	58	F	Assisi	Retired		+	—	1800	+	—	—	—	Squamous
9	55/-	63	M	Corciano	Retired	+		—	335		Left		Lobar bronchus	Anaplastic
10	72/-	67	M	Foligno	Retired	+		—	1680		Right		Lobar bronchus	Squamous
11	77/-	38	M	Perugia	Labourer		+	—	5300†	+	Right		Lobar bronchus	Squamous
12	81/-	69	M	Assisi	Retired	+		—	3504††	+	—	—	—	Anaplastic
13	117/-	60	M	Perugia	Farmer	+		—	1130	+	Right		Main bronchus	Anaplastic
14	166/-	71	M	Panicale	Farmer		+	—	200		Right		Peripheral	Anaplastic
15	247/-	75	F	Perugia	Housewife		+	—	664		Right		Peripheral	Anaplastic
16	274/-	68	M	Foligno	Retired		+	—	1280	+	—	—	—	Combined: anaplastic and squamous
17	275/-	64	M	Assisi	Retired	+		—	2090		Left		Main bronchus	Anaplastic
18	24/67	66	M	Gualdo T.	Retired	+		—	3538		Right		Main bronchus	Anaplastic
19	44/-	78	M	Gubbio	Retired	+		—	420	+	Left		Main bronchus	Squamous
20	79/-	72	M	Perugia	Farmer	+		—	2258		Right	Upper		Adenocarcin.
21	118/-	70	F	Perugia	Retired		+	—	720		Right		Up. & mid bronchi	Anaplastic
22	120/-	55	M	Castig. Lago	Retired	+		—	1562		Right		Main bronchus	Anaplastic
23	240/-	83	F	Perguia	Retired		+	—	1540		Right	Lower		Squamous

*Elongated black bodies in fibrotic tissue; Prussian blue reaction negative.
†Nodular brown and black bodies; positive Prussian blue reaction, no asbestos bodies.
‡Nodular golden-brown bodies; positive Prussian blue reaction, no asbestos bodies.

Table 2. Pulmonary carcinomas in operation specimen series

Case No.	Biopsy No.	Age	Sex	Residence	Occupation	Possible causative factors: Smoking: Smokers	Smoking: Non-smokers or ex-smokers	Asbestos: Search	Asbestos: Areas exam. (mm^2)	Type of specimen	Microscopical structure
1	19/65	57	M	Magione	Ind. worker	+		—	200	Operation specimen lower left lobe	Anaplastic
2	1.743/-	61	F	Marsciano	Housewife		+	—	175	Pleural biopsy upper right lobe	Squamous
3	2.233/-	50	M	Ancona	Clerk	+		—	102	Pleural biopsy upper right lobe	Squamous
4	2.444/-	50	F	Todi	Housewife		+			Right scalene node biopsy	Anaplastic
5	2.519/-	59	M	Terni	Steelworker	+				Left scalene node biopsy	Anaplastic
6	1.885/66	45	M	Castig. Lago	Labourer	+		—	200	Operation specimen lower right lobe	Combined: squamous -adenocarc.
7	4.216/-	55	M	Fabriano	Businessman	+		—	330	Operation specimen lower left lobe	Combined: anaplastic-squamous
8	1.688/67	47	M	Spoleto	Mechanic	+				Right scalene node biopsy	Adenocarcinoma
9	1.918/-	50	F	Assisi	Farmer		+	—	120	Operation specimen lower right lobe	Anaplastic
10	2.324/-	45	F	Bastia	Tobacco worker		+	—	256	Operation specimen upper left lobe	Anaplastic
11	2.652/-	63	M	Terni	Railwayman	+				Left scalene node biopsy	Anaplastic
12	3.418/-	55	M	Perugia	Labourer	+		—	200	Operation specimen upper right and lobes	Adenocarcinoma
13	3.881/-	39	M	Perugia	Labourer	+		—	540	Operation specimen upper right lobe	Squamous
14	3.894/-	62	F	Terni	Housewife		+	—	50	Biopsy at thoracotomy upper right lobe	Anaplastic
15	4.358/-	69	M	Perugia	Retired	+				Right scalene node biopsy	Anaplastic

Table 3. Pulmonary carcinomas in bronchial biopsy series

Case No.	Biopsy No.	Age	Sex	Residence	Occupation	Possible causative factors: Smoking: Smokers	Smoking: Non-smokers or ex-smokers	Asbestos: Search	Asbestos: Areas exam. (mm^2)	Part of bronchi affected Type of specimen(+)	Microscopical structure
1	712/65	60	M	Magione	Retired	+		—	88	Main right bronchus	Squamous
2	1.675/-	56	M	Ancona	Clerk	+		—	54	Lower left bronchus	Squamous
3	2.095/-	61	M	Pesaro	Farmer	+		—	160	Middle right bronchus	Squamous
4	2.397/-	56	M	Terni	Steelworker	+		—	72	Middle right bronchus	Anaplastic
5	2.671/-	62	M	Perugia	Labourer	+		—	100	Lower right bronchus	Combined anapl.-squamous
6	162/66	54	M	Perugia	Retired	+		—	80	Upper right bronchus	Squamous
7	620/-	69	M	Perugia	Retired	+		—	72	Lower right bronchus	Squamous
8	2.069/-	40	F	Terni	Housewife	+		—	240	Main left bronchus	Squamous
9	2.311/-	68	M	Perugia	Retired	+		—	100	Main right bronchus	Squamous
10	2.363/-	63	F	Perugia	Housewife		+	—	40	Lower left bronchus	Squamous
11	2.527/-	62	M	Foggia	Bricklayer	+		—	56	Main left bronchus	Anaplastic
12	3.331/-	46	M	Norcia	Clerk	+		—	486	Middle right bronchus	Squamous
13	3.529/-	56	M	C. di Cast.	Clerk	+		—	67	Main right bronchus	Anaplastic
14	1.297/67	49	M	Perugia	Priest	+		—	67	Main left bronchus	Squamous
15	3.012/-	46	M	C. di Cast.	Labourer	+		—	84	Main right bronchus	Squamous

(+) Main bronchi or lobar bronchi.

areas, and these were micro-incinerated, washed to remove water-soluble crystalline salts and examined under polarised light for possible asbestos bodies or free asbestos fibres.

RESULTS

In many cases anthracosis and local fibrotic areas were present in addition to tumour, but although some 'curious bodies' with black irregularly-shaped shafts and heads were found, these were mostly destroyed by micro-incineration. Suspected 'bodies' were further subjected to ultrasonication and re-examined under polarised light, but in no case were birefringent fibres found.

For control purposes smears and sections from cases of asbestosis from other sources were similarly examined and these yielded many asbestos bodies, some of which were shown to contain heat-resistant birefringent fibres insoluble in water and in dilute hydrochloric acid and considered to be asbestos. The technique has been described elsewhere [1].

In the 23 necropsy tumours the average age was 63·3 years; 19 were in males and 4 in females, 8 in non- or ex-smokers and 15 in smokers; antituberculosis drugs had been used in 7 cases in which there was also active pulmonary tuberculosis associated with the malignant disease (Table 1).

The tumours originated in the right lung in 14 cases and in the left lung in 6, in the remaining 3 cases the side of neoplasia was impossible to establish because the tumour was obscured at necropsy by diffuse bilateral ulcerative tuberculosis. The lobar and bronchial distribution of the 14 tumours in the right lung was as follows: upper lobe 5, lower lobe 2, all lobes 1, main bronchus 3, upper lobar bronchus 2, upper and middle bronchi 1; for the 6 tumours in the left lung the distribution was: upper lobe 1, both lobes 1, main bronchus 2, lower lobar bronchus 2 (Table 1). The affected part of the lung is shown in Table 1.

The microscopic findings for these 23 carcinomas show the following variation of structure: anaplastic 16, squamous 5, adenocarcinoma 1, combined (anaplastic-squamous) 1.

In the 15 tumours of the operation specimen series, the average age was 53·8 years; 10 were in males and 5 in females (Table 2). Other data, such as the smoking habit, the type of specimen and the microscopical structure are shown in Table 2.

In the 15 tumours of the bronchial biopsy series, the average age was 59·5 years; 13 were in males and 2 in females (Table 3). Other data, such as the smoking habit and the part of the bronchi affected and the microscopic structure, are shown in Table 3.

DISCUSSION

Evidence for the presence of asbestos bodies in the lungs of about 20 to 30 per cent of adults of both sexes and in different parts of the world, even in the absence of clinical disease, first reported by Thomson and his associates [2], has been confirmed by independent observation [3, 4]. Most of the evidence comes from industrial centres or rapidly expanding cities where modern construction methods employ lagging of boilers and water pipes, and roof and wall insulation, with asbestos products; these might account for some degree of atmospheric pollution with asbestos dust. The almost indestructible nature of asbestos fibres and the ease with which they can penetrate the alveolar walls, as evidenced by their presence in connective tissue and macrophages when found as asbestos bodies, suggests that, once inhaled, the fibres are very difficult to eliminate, this despite the observation of asbestos bodies in the sputum in some cases.

In the present small series there is no evidence of any association between asbestos bodies and primary neoplasia of the lung, though failure to find such bodies does not exclude the possibility that they might have been present in other parts of the lung or pleura.

Kreyberg [5] has drawn attention to the prevalence of the solid types of carcinoma in association with cigarette smoking, whereas adenocarcinoma shows no such association.

In this small series the majority of tumours are of the solid type, while the two pure adenocarcinomas were in men, both of them smokers.

The incidences of the different histological types in the series of carcinomas studied are as follows: in the 23 necropsy cases, anaplastic 16, squamous 5, adenocarcinoma 1 and combined 1 (Table 1); in the 15 operation specimen series, anaplastic 8, squamous 3, adenocarcinoma 2 and combined 2 (Table 2); in the 15 bronchial biopsy series, anaplastic 3, squamous 11 and combined 1 (Table 3).

The squamous type, therefore, is prevalent in the bronchial biopsy series and the anaplastic in the other two series.

The association between asbestosis and mesothelioma is generally accepted as a causal relationship and in this connection it is not surprising that no case of mesothelioma has yet been seen in this institute.

Our failure to find a single asbestos body in the material here reported does not exclude the possibility that such bodies were present in other parts of the lung, but parallel studies on smears taken from routine necropsies in this institute [6] confirm that the incidence of demonstrable asbestos bodies is very low (one case in 109 consecutive necropsies).

It is understandable that the hazard of inhaling asbestos should be low in a city like Perugia, which is very little industralised and has a notably clear atmosphere; however, the increasing use of asbestos products here, as elsewhere, may be followed in due course by increasing evidence of inhalation of asbestos dust by the general population and, of course, particularly by those working with asbestos products.

It is to be hoped that similar studies may be made in the future and that the present record may provide a useful standard for comparison.

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RESUME

On a étudié 53 *carcinomes du poumon, confirmés par examen histologique, diagnostiqués entre* 1965 *et* 1967: 23 *après autopsie,* 15 *sur spécimens opérés, parmi lesquels figurent* 5 *biopsies d'un ganglion du scalène, et* 15 *par biopsie bronchique.*

Toutes les tumeurs, à l'exception des 5 *diagnostiquées par biopsie du ganglion du scalène, ont été réexaminées en vue d'une association possible avec des corps d'asbeste. Ces derniers n'ont été trouvés dans aucun cas. D'autres facteurs causatifs ont également été recherchés, mais il n'a pas été possible d'y trouver une association quelconque avec les tumeurs.*

Dans la série des autopsies soumises à l'examen histologique, l'âge moyen était de 63,3 *ans, la fréquence, chez la mâle, de* 19 *sur* 23, *et le type anaplastique, était le plus fréquent* (16 *sur* 23). *Dans la série diagnostiquée sur spécimens chirurgicaux, l'âge moyen était de* 53,8 *ans, la fréquence, chez le mâle, de* 10 *sur* 15, *et le type anaplastique était le plus souvent observé* (8 *sur* 15). *Dans les cas diagnostiqués par biopsie bronchique, l'âge moyen était de* 59,5, *la fréquence, chez le mâle, de* 13 *sur* 15, *et le type squameux apparaissait dans* 11 *cas sur* 15.

SUMMARY

Fifty-three histologically confirmed carcinomas of the lung, diagnosed between 1965 *and* 1967, *were studied:* 23 *in a necropsy series,* 15 *in operation specimens,* 5 *of which in scalene node biopsy, and* 15 *in bronchial biopsy.*

All the tumours, with the exception of the 5 *observed in scalene node biopsy, were re-examined for possible association with asbestos bodies. No asbestos bodies were found in any case. Other possible causative factors were investigated, but it was not possible to establish a definite association with the tumours.*

In the necropsy series, the average age was 63·3 *years, there were* 19 *tumours in males and* 4 *in females and, histologically, the anaplastic type was the most frequent,* 16 *out of* 23.

In the operation specimen series the average age was 53·8 *years, there were* 10 *tumours in males and* 5 *in females and* 8 *out of* 15 *were of anaplastic type.*

In the bronchial biopsy series the average age was 59·5 *years, there were* 13 *tumours in males and* 2 *in females, the squamous type was the most frequent,* 11 *out of* 15.

ZUSAMMENFASSUNG

Dreiundfünfzig histologisch bestätigte Lungencarcinome, die zwischen 1965 *und* 1967 *diagnostiziert worden waren, wurden untersucht:* 23 *in Nekropsieproben,* 15 *in Operationsproben* (*hiervon* 5 *Biopsien aus Scalenusknoten*) *und* 15 *Bronchialbiopsien.*

Alle Tumoren (mit Ausnahme der 5 Biopsien der Scalenusknoten) wurden auf eine mögliche Association mit Asbestkörpern überprüft. In keinem Falle wurden Asbestkörper gefunden. Andere mögliche kausale Faktoren wurden untersucht, jedoch ohne eindeutigen Erfolg.

In den Nekropsieserien ist das durchschnittliche Alter 63, 3 *Jahre, die Häufigkeit bei Männern* 19/23 *Fälle und histologisch der anaplastische Typ der häufigste* (16/23 *Fälle*).

Bei den Operationsproben war das durchschnittliche Alter 53, 8 *Jahre, die Häufigkeit bei Männern* 10/15 *und des anaplastischen Types* 8/15.

Bei den Bronchialbiopsien war das durchschnittliche Alter 59, 5 *Jahre, die Häufigkeit bei Männern* 13/15 *Fälle und des squamösen Types* 11/15 *Fälle.*

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Examination of Lung Smears for Asbestos Bodies in 109 Consecutive Necropsies in Perugia

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INTRODUCTION

IN VIEW of accumulating evidence that asbestos bodies are present in approximately 30% of male and 20% of female adult lungs examined *post mortem*, regardless of the cause of death and in different parts of the world, first reported by Thomson and associates [1], it was decided to make a similar survey in Perugia.

Perugia is not an industrial city and does not suffer from the atmospheric pollution which it is usual to observe in large industrial cities.

About 350 necropsies are performed annually in the Institute of Pathological Anatomy and Histology, University of Perugia, on cases from the general hospital in Perugia and from neighbouring towns and villages in Umbria.

MATERIAL

This investigation was carried out on 109 necropsies; all but 13 of these 109 necropsies were on persons resident in Perugia or in neighbouring small towns and villages in Umbria (Table 1).

Smears were prepared by incising the bases of both lungs and scraping the cut surfaces with the edge of a clean microscope slide, which collects enough blood and fluid with which to make thick smears on other clean slides, as described by Thomson [2]. Each smear covered approximately 6×2 cm, and two such slides were made from each lung, four slides for each case.

METHOD

The smears were allowed to dry under cover, to avoid contamination by airborne dust, and were then fixed by heating on a warm stage. They were allowed to cool under cover, cleared with xylol under a coverslip and immediately scanned completely under high power magnification. High power scanning is much more time-consuming than low power, but was performed in every case where the latter proved negative for asbestos bodies. It was found that a 24×32 mm coverslip would hold a film of xylol long enough for this area to be scanned, and each slide was examined in two stages, each corresponding with the area covered by such a coverslip. Xylol was found to be better than oil or permanent mounting media because it evaporates, leaving the slides clean, and does not contain birefringent contaminants which could confuse the picture, particularly when slides remounted in water are to be photographed under polarised light.

RESULTS

In the first 80 cases examined no asbestos bodies were found, but in the eighty-first case two morphologically typical asbestos bodies were found in smears from the right lower lobe (Figs. 1 and 2). The smaller body was 70μ in length with rounded ends and a delicate

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Table 1. 109 *Consecutive necropsies examined for asbestos bodies in the lungs*

Sex	Age	Occupation	Town	Cause of death	Asbestos bodies
M	69	Retired	Umbertide	Accident	None
M	72	Retired	Perugia	Myocardial infarct	None
M	63	Retired	Gubbio	Hodgkin's disease	None
M	67	Retired	Vienna	Haemorrrhage; pyloric ulcer	None
M	76	Retired	Orvieto	Cancer of prostate	None
M	72	Farmer	Perugia	Cancer of lung	None
M	73	Retired	Perugia	Abscess in liver	None
F	62	Teacher	Assisi	Acute leukaemia	None
F	47	Housewife	Perugia	Acute hepatitis	None
M	15	Student	Perugia	Suicide	None
F	79	Housewife	Terni	Arteriosclerotic myocardiopathy	None
F	44	Housewife	Deruta	Suicide	None
M	56	Dealer	Roccantica	Cerebral thrombosis	None
M	75	Retired	Perugia	Cerebral haemorrhage	None
M	24	Clerk	Terni	Tuberculous meningitis	None
M	60	Retired	Perugia	Cirrhosis of liver	None
F	61	Housewife	Foggia	Cancer of breast	None
M	65	Retired	Umbertide	Subdural haemorrhage	None
M	72	Retired	Torgiano	Myocardial infarct	None
M	78	Retired	Umbertide	Arteriosclerosis	None
M	84	Retired	Perugia	Senile marasmus	None
M	47	Clerk	Cannara	Renal calculosis	None
F	65	Housewife	Marsciano	Hepatitis	None
F	78	Retired	Umbertide	Myocardial infarct	None
F	75	Housewife	Perugia	Pulmonary tuberculosis	None
F	71	Retired	Ariccia	Bronchitis & emphysema	None
M	61	Businessman	Terni	Cirrhosis of liver	None
M	68	Dealer	Valfabbrica	Myocardial infarct	None
F	70	Retired	Perugia	Cancer of lung	None
F	71	Teacher	Reggio di Calabria	Myocardial infarct	None
M	55	Retired	Castiglione del Lago	Cancer of lung	None
M	63	Disabled	Assisi	Chronic bronchitis and emphysema	None
F	67	Housewife	Castiglione del Lago	Lymphatic leukaemia	None
M	56	Retired	Gubbio	Cancer of prostate	None
F	76	Housewife	Perugia	Arteriosclerosis	None
M	72	Retired	Perugia	Fracture of skull	None
F	58	Teacher	Valfabbrica	Subarachnoid haemorrhage	None
F	61	Farmer	Bevagna	Fracture of spine	None
M	53	Disabled	Perugia	Pulmonary phthisis	None
M	50	Clerk	Citta di Castello	Heart failure	None
F	67	Retired	Porto Recanati	Mycosis fungoides	None
F	86	Retired	Perugia	Arteriosclerosis	None
F	54	Housewife	Perugia	Chronic nephritis	None
M	14	School boy	Magione	Congenital heart disease	None
M	42	Farmer	Passignano	Tumour of brain	None
F	71	Retired	Sarnano	Arteriosclerosis	None
M	73	Retired	Bettona	Agranulocytosis	None
F	81	Housewife	Umbertide	Pneumonia; gastric carcinoma	None
F	74	Housewife	Perugia	Arteriosclerosis	None
M	60	Retired	Matelica	Traumatic shock	None
M	52	Farmer	Cortona	Pulmonary emphysema	None
F	63	Worker	Bologna	Myocardial infarct	None
M	69	Retired	Marsciano	Cancer of stomach	None
M	71	Retired	Torgiano	Aortic aneurysm	None
M	47	Dealer	New York	Acute haemorrhagic pancreatitis	None
M	80	Retired	Turin	Arteriosclerosis	None
F	40	Farmer	Leonessa	Cancer of ovary	None
M	49	Unemployed	Spoleto	Chronic Pachymeningitis	None
F	33	Housewife	Gubbio	Homicide	None

Table 1.—cont.

Sex	Age	Occupation	Town	Cause of death	Asbestos bodies
F	69	Retired	Perugia	Bronchopneumonia	None
M	69	Farmer	Perugia	Fracture of skull	None
M	57	Retired	Tuoro	Acute leukaemia	None
F	90	Housewife	Magione	Cerebral haemorrhage	None
F	80	Housewife	Perugia	Arteriosclerosis	None
F	58	Housewife	Brescia	Cerebral haemorrhage	None
M	61	Miller	Sassari	Pemphigus	None
F	79	Housewife	Perugia	Arteriosclerotic myocardiopathy	None
M	69	Dealer	Paciano	Traumatic shock	None
M	61	Retired	Perugia	Pulmonary embolism	None
M	71	Retired	Perugia	Cerebral hamorrhage	None
F	76	Housewife	Piegaro	Fracture of skull	None
M	49	Teacher	Porto Recanati	Myocardial infarct	None
M	48	Clerk	Ferrara	Cancer of stomach	None
M	59	Road-man	Piegaro	Heart failure	None
F	75	Retired	Perugia	Peritonitis	None
F	55	Retired	Perugia	Cerebral embolism	None
F	18	Domestic servant	Gubbio	Carbon monoxide poisoning	None
M	28	Farmer	Perugia	Fracture of skull	None
F	62	Farmer	Magione	Cancer of stomach	None
M	74	Retired	Perugia	Purulent cholangeitis	None
M	81	Retired	Perugia	Cancer of stomach	Present
F	65	Housewife	Marsciano	Haemorrhage from duodenal ulcer	None
F	83	Retired	Perugia	Cancer of lung	None
F	45	Housewife	Perugia	Cancer of uterus	None
M	74	Retired	Perugia	Myocardial infarct	None
M	18	Labourer	Perugia	Traumatic shock	None
M	65	Labourer	Perugia	Traumatic shock	None
F	17	Student	Perugia	Cerebral haemorrhage	None
M	48	Clerk	Monte Gabbione	Mesenteric neoplasm	None
M	70	Retired	Perugia	Cerebral thrombosis	None
M	47	Disabled	Perugia	Pulmonary phthisis	None
M	31	Clerk	Perugia	Traumatic shock	None
F	42	Housewife	Perugia	Cirrhosis of liver	None
F	78	Housewife	Bastia	Heart failure	None
M	57	Farmer	Perugia	Heart failure	None
M	70	Retired	Piegaro	Chronic bronchitis	None
M	44	Labourer	Umbertide	Traumatic shock	None
M	70	Retired	Buenos Aires	Myocardial infarct	None
M	41	Labourer	Perugia	Traumatic shock	None
F	74	Retired	Umbertide	Carbon monoxide poisoning	None
M	82	Retired	Perugia	Arteriosclerosis	None
F	56	Housewife	Bevagna	Cancer of breast	None
F	77	Housewife	Bastia	Acute pulmonary oedema	None
M	23	Student	Czecho-Slovakia	Fracture of skull	None
F	57	Housewife	Monte Gabbione	Mitral defect	None
M	76	Retired	Assisi	Arteriosclerotic myocardiopathy	None
F	83	Retired	Perugia	Senile marasmus	None
M	45	Shepherd	Nuoro	Arteriosclerosis	None
F	80	Housewife	Modena	Pulmonary tuberculosis	None

slightly beaded shaft with a kink, one third of the shaft showing slight lateral displacement, possibly caused in making the smear. Both rounded ends or "heads" had been engulfed by macrophages, which also contained amorphous pigment granules (soot) (Fig. 1).

The larger body measured 88μ in length and appeared to contain two separate fibrous cores terminating in a large common head at one end and in two distinct heads, one much smaller than the other at the opposite end (Fig. 2). The shaft of the smaller body showed

a faintly birefringent fibrous core, that of the larger body was defective in just over half its length revealing a delicate fibre which was not birefringent. Attempts to reveal the fibrous cores in more detail by micro-incineration and ultrasonication failed because the bodies were lost during the manipulation. The remaining cases in this small series had no demonstrable asbestos bodies, though several showed "curious bodies" associated with irregularly-shaped dust particles, and most had abundant macrophages with variable amounts of carbon and refractile dust particles.

DISCUSSION

Perugia is not an industrial city and this fact, together with its favourable situation 500 m above the valley of the Tiber, renders it almost free from atmospheric pollution. Moreover, the surrounding countryside is mainly agricultural. These considerations may account for the striking difference between the smears obtained from the lungs of the hospital population in Perugia and those similarly obtained in other centres where a search has been made for asbestos bodies in such material.

However, modern construction methods employed in the many new buildings which have been and are being built around this ancient walled city involve the handling of asbestos products, and the possibility that airborne asbestos fibres may be inhaled more frequently than in the past must be anticipated.

It is interesting to note that no case of mesothelioma has been reported from this centre so far, though primary tumours of the lung are seen about as frequently as in other comparable large departments of pathology.

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RESUME

Une étude microscopique des frottis prélevés des poumons, dans une série de 109 *nécropsies* (63 *hommes et* 46 *femmes décédés entre* 23 *et* 90 *ans), n'a donné qu'un seul cas dans lequel deux corps d'asbeste seulement ont été trouvés. Chez* 5 *adolescents* (3 *hommes et* 2 *femmes de* 14 *à* 18 *ans), les résultats ont été négatifs. Cette faible fréquence* (1%) *est en contraste frappant avec les* 20%, *ou plus, observés dans de nombreux Centres, où les conditions d'hospitalisation sont comparables aux nôtres.*

SUMMARY

Microscopic examination of smears prepared from the lungs of 109 *consecutive adult necropsies* (63 *male and* 46 *female) aged between* 23 *and* 90 *years yielded only one case* (*an* 81*-year-old man) in whom two asbestos bodies were detected. Five adolescents* (3 *male and* 2 *female) also gave negative results for asbestos bodies. This low incidence* (1%) *is in striking contrast with the* 20% *or more incidence reported from many other parts of the world with otherwise comparable hospital populations and laboratory facilities.*

ZUSAMMENFASSUNG

Bei der mikroskopischen Untersuchung von 109 *Ausstrichen von Lungen* (*Nekropsis*) 60 *erwachsener Männer und* 46 *erwachsener Frauen im Alter zwischen* 23 *und* 90 *Jahren wurden nur in einem Falle* (*eines* 81*-jährigen Mannes) zwei Asbestkörper entdeckt. Bei* 5 *Jugendlichen* (3 *männlich,* 2 *weiblich) wurde auch ein negatives Ergebnis erhalten. Diese geringe Häufigkeit* (1%) *steht im Gegensatz zu den* 20% *oder mehr, die aus anderen Ländern berichtet werden.*

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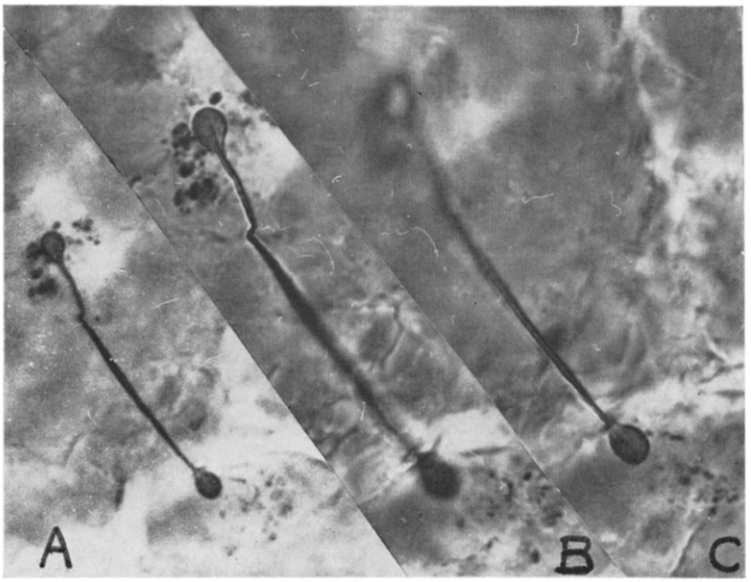

Fig. 1. *Serial photomicrographs of unstained smear mounted in distilled water.*

(*A*) *Asbestos body* 70μ *long with spherical heads each within a macrophage, which also contains particles of soot and dust. Note kink in shaft of body.* × 1000.

(*B*) *Upper head critically focused under oil immersion, showing the end of the fibrous core visible within the head and double right-angle kink in the shaft at is leaves the macrophage.*

(*C*) *Lower head critically focused under partly crossed polaroids to show faintly birefringent fibrous core visible within head just below transverse shadow at point where the shaft leaves the macrophage.* × 1500.

Fig. 2. *Unstained smear mounted in distilled water. Asbestos body* 88μ *long with partly defective shaft containing two fibrous cores terminating in one large and one very small head (marked by arrow); the fibres were not birefringent, possibly due to their orientation on the slide.* × 1000

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Analyse Chromosomique d'une Tumeur Cancéreuse Humaine en Culture Organotypique

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Les études cytogénétiques des tumeurs solides se heurtent encore à d'importantes difficultés, probablement techniques. Elles ne sont, en outre, possibles qu'une seule fois au cours de la vie de la tumeur, lors de l'intervention chirurgicale. Une fois prélevées, les tumeurs ne peuvent, en effet, être maintenues en culture que pendant des temps généralement très courts.

La culture organotypique, mise au point par Et. et Em. Wolff, permet d'entretenir *in vitro* des tumeurs cancéreuses douées d'un potentiel de multiplication quasi indéfini [1, 2]. Elle offre, de ce fait, un intérêt considérable pour les études caryotypiques.

Dans la présente note, nous rapportons les observations cytogénétiques, faites à deux ans d'intervalle, à partir d'une telle culture organotypique.

MATERIEL ET METHODES

La tumeur

La tumeur Z 200 provient d'une métastase hépatique d'un cancer d'origine gastrique [3, 4]. Elle a été prélevée directement sur une malade du sexe féminin, âgée de 34 ans, au cours d'une intervention chirurgicale. Elle a été maintenue en culture par Et. et Em. Wolff depuis janvier 1962, soit depuis sept ans, selon des modalités techniques précédemment décrites [3, 4]. Elle est constituée par de petits nodules massifs et lisses, dépourvus de stroma, et ayant conservé la même structure organisée que dans l'organisme.

Les études caryotpyiques

Elles ont été faites à deux ans d'intervalle, en 1966 et en 1968. La technique employée est une modification des techniques habituelles d'étude chromosomique utilisées pour les fibroblastes ou les leucocytes sanguins [5, 6].

Environ quatre jours après un repiquage (les repiquages se font en moyenne tous les 7 jours), les divisions cellulaires sont bloquées par l'addition à la culture d'une solution de colchicine à 0,4%. La culture est replacée à 37°C, pendant 5 heures.

Les tumeurs sont alors placées dans une solution de trypsine à 0,5% et dissociées par agitation magnétique à 37°C, pendant 30 à 60 minutes, le degré de dissociation étant apprécié à la vue.

La suspension est centrifugée pendant 10 minutes à 800 tours/min. Le culot est soumis à un choc hypotonique dans de l'eau distillée à 37°C, pendant 20 minutes.

Après centrifugation (10 minutes, 800 tours/min.) le culot est fixé dans le mélange alcool: acide acétique, 3:1, pendant 20 minutes à 4°C.

Après une dernière centrifugation, on aspire le surnageant à la pipette, et le culot est remis

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Tableau 1. Distribution des nombres chromosomiques. De A à G, première série d'examens faits en 1966. De H à J, examens faits en 1968. Dans un but de simplification, seuls figurent les nombres chromosomiques qui ont été observés

	44	46	54	57	58	59	60	61	62	63	64	65	66	67	68	69	71	73	77	82	88	90	91	92	5N	6N
A					1		1	1	1	1	7	2	1	1				1								1
B	1	1	1	1		1		4		2	4		1							1	2	1	1	2		
C							2	2		1	2	2	1	2									1			1
D								1				1		1		3	1						1			
E																			1							
F													1	1												
G											1	2														
Total	1	1	1	1	1	1	3	8	1	4	14	7	4	5		3	1	1	1	1	2	1	3	2		2
H													1	1		3										
I											1	3		3												
J	1		1	2		1				1			4	2	3	5									1	
Total	1		1	2		1				1	1	3	5	6	3	8									1	

en suspension dans la goutte de fixateur restant dans le fond du tube.

On étale sur des lames préalablement refroidies à 4°C dans de l'eau distillée, et on sèche à la flamme éclairante d'un bec Bunsen.

Les lames sont colorées, après hydrolyse (HCL N, 6 minutes et demie à 60°C), par la solution de Giemsa R.

RESULTATS

La première série d'examens, pratiqués en 1966, a permis d'examiner 7 séries d'explants provenant de repiquages échelonnés sur quelques mois. La seconde série (en 1968) a porté sur 3 examens.

Les études caryotypiques ont montré d'importants remaniements de nombre et de structure, ces derniers ayant conduit à la formation de chromosomes marqueurs.

Aberrations numériques

La distribution des nombres chromosomiques apparaît dans le Tableau 1. Les deux séries d'examens y sont nettement séparées. Chaque examen est défini par une lettre (A–J).

Dans la première série, le mode de la distribution est à 64. Dans la seconde il est déplacé vers 69.

En raison de la mauvaise qualité de certaines d'entre elles, seule une partie des métaphases a pu être caryotypée avec une sûreté suffisante. Les analyses chromosomiques de ces métaphases apparaissent dans le Tableau 2.

On note une aneuploïdie importante caractérisée par: 1° la perte constante de chromosomes du groupe D; 2°un excès général de chromosomes dans les autres groupes, excès auquel participe surtout le groupe X, C.

La fluctuation du nombre de surnuméraires, dans les divers groupes, demeure pourtant extrêmement limitée.

Aberrations de structure: chromosomes marqueurs

Outre les aberrations de nombre, on note la présence constante de chromosomes marqueurs. Dans la mesure où l'on peut les identifier, c'est-à-dire où ils ne sont pas confondus avec des chromosomes normaux, ces marqeurs sont au maximum au nombre de 7. Ils ont été arbitrairement classés comme suit:

Mar. 1. Grand médiocentrique presque deux fois plus long qu'un 1.

Mar. 2. Grand submétacentrique de la même taille que Mar. 1. Il apparaît souvent comme un acentrique. Ces deux chromosomes ressembleraient à deux "grands" 1 et 2 normaux.

Mar. 3. Dicentrique plus grand qu'un 1–2.

Mar. 4. Subtélocentrique de la taille d'un 1.

Mar. 5, 6, 7, Subtélocentriques de la taille d'un C.

La fréquence de ces différents marqueurs figure dans le Tableau 2.

DISCUSSION

L'aneuploïdie semble stéréotypée. Les caryotypes apparaissent, en effet, remarquablement constants, tant d'une cellule à l'autre dans un même examen, que d'un examen à l'autre, ou que d'une série d'examens à l'autre, alors que ces dernières sont espacées de deux ans.

Le nombre chromosomique paraît toutefois varier quelque peu, au cours du temps, vers la surnumération. Le mode est, en effet, d'abord à 64, puis, deux ans plus tard, aux environs de 69. Cette variation est dans le sens opposé à celui qu'on observe dans les cellules de souches HeLa par exemple [7].

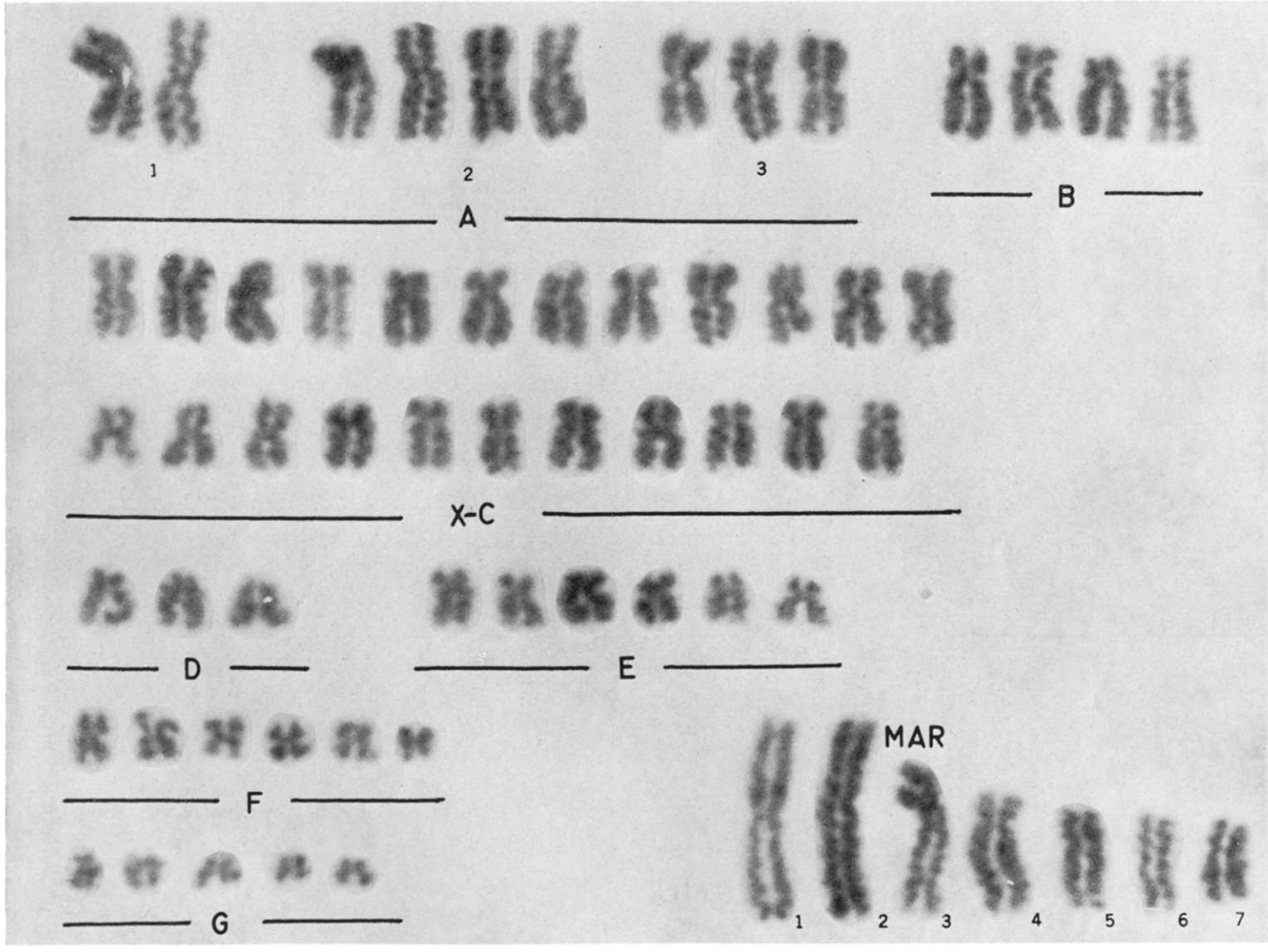

Fig. 1. Caryotype organisé d'une cellule à 63 chromosomes. Noter la présence de 7 marqueurs.

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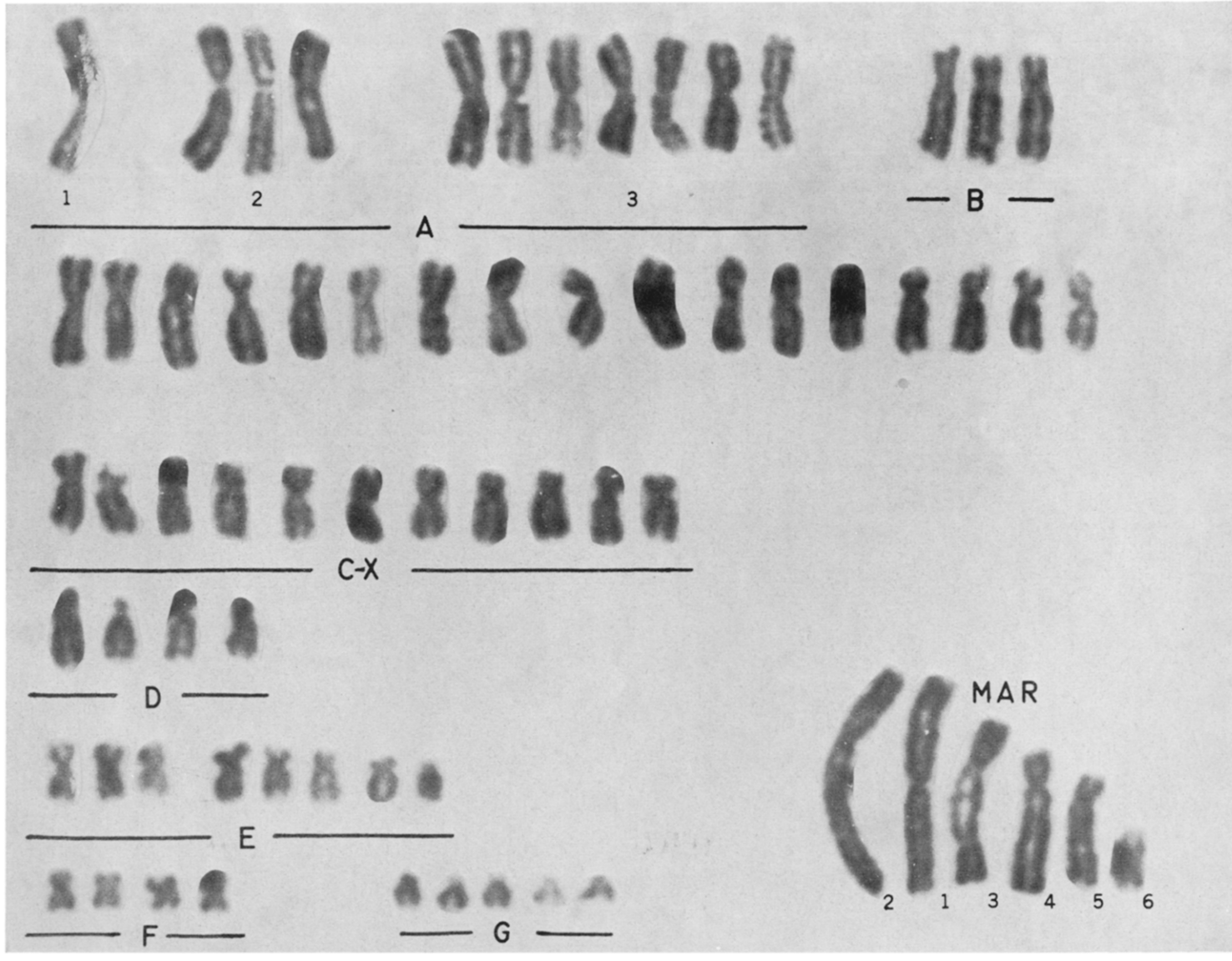

Fig. 2. Caryotype organisé d'une cellule à chromosomes. Noter la présence de 6 marqueurs.

Tableau 2a. Analyses caryotypiques effectuées lors de la première série d'examens

Cel.	No.	A	B	XC	D	E_{16}	E_{17-18}	F	G	M_1	M_2	M_3	M_4	M_5	M_6	M_7
1	61	7	5	26	2	1	6	5	5	+	+	+	+			
2	62	6	4	32	3	4	3	2	4	+	+		+	+		
3	63	6	4	30	3	5	3	4	5		+	+	+			
4	64	7	5	27	2	2	5	7	5	+	+	+	+			
5	64	6	4	26	3	4	7	5	6	+			+	+		
6	64	6	5	27	4	2	3	4	6	+	+	+	+	+		
7	65	5	5	24	2	2	7	8	5	+	+	+	+		+	
8	65	9	5	25	3	3	5	6	5	+	+	+	+			
9	65	5	6	26	4	3	6	6	6	+	+	+				
10	66	3	3	28	4	3	7	6	8	+		+	+	+		

Cette surnumération, à laquelle participent tous les groupes, sauf le groupe D, est remarquable du fait qu'elle tend vers un nombre égal à 3N, sans que l'on puisse vraiment observer une trisomisation nette à l'intérieur de chaque groupe.

Il existe deux "variants communs" [8] évidents qui sont pratiquement inchangés au cours du temps. Ce sont la perte de chromosomes du groupe D et la présence de chromosomes marqueurs.

La perte de chromosomes du groupe D est étonnamment constante; jamais le nombre de D n'a dépassé 4.

Les chromosomes marqueurs montrent la même constance. On les retrouve identiques à chaque examen, au cours d'une période de deux ans. En réalité, lors des premiers examens, ce sont surtout les chromosomes Mar. 1, Mar, 2, Mar, 3 et Mar 4 qui sont présents. Puis s'ajoutent les marqueurs Mar. 5, Mar. 6 et Mar. 7 (Tableau 2).

La persistance du dicentrique démontre clairement la stabilité d'une telle structure à travers les mitoses successives, alors même que les deux centromères sont très éloignés l'un de l'autre. On sait combien ce fait est discuté, par exemple à propos des remaniements radio-induits [9]. Une autre observation intéressante est la fréquence avec laquelle Mar. 2 paraît être un fragment acentrique. Cela pourrait être la conséquence d'une séparation précoce du centromère.

En définitive, la faible fluctuation, tant des nombres chromosomiques totaux que des nombres de chromosomes dans les différents groupes, ainsi que les deux "variants communs" qui viennent d'être définis, confèrent aux caryotypes un "air de famille" indiscutable.

Cette relative constance caryotypique est très importante, eu égard à la théorie chromosomique de la carcinogénèse. Celle-ci stipule, en effet, que les anomalies chromosomiques représentent la voie d'action commune des différents facteurs oncogènes, et sont elles-mêmes responsables du déclenchement du processus malin [8, 10].

Dès l'abord, il est difficile de discuter les liens

Tableau 2b. Analyses caryotypiques effectuées lors de la deuxième série d'examens

Cel.	No.	A	B	XC	D	E_{16}	E_{17-18}	F	G	M_1	M_2	M_3	M_4	M_5	M_6	M_7
1	44	6	1	18	1	4	3	4	3	+	+	+			+	+
2	54	6	3	20	4	1	5	5	4	+	+	+	+	+	+	
3	57	7	4	24	3	2	5	4	4	+		+		+	+	
4	57	7	3	25	3	3	4	4	4	+		+		+	+	
5	59	6	1	23	3	3	8	6	4	+	+	+	+		+	
6	63	9	4	20	3	3	6	6	5	+	+	+	+	+	+	+
7	63	11	3	28	4	3	5	4	5	+	+	+	+	+	+	
8	66	6	5	29	4	4	3	5	5	+	+	+	+	+		
9	66	5	4	29	4	3	6	5	5	+	+	+		+	+	
10	66	8	3	27	3	2	4	7	6	+	+	+	+	+	+	
11	66	6	4	17	3	4	13	6	7	+	+	+	+	+	+	
12	67	12	5	26	3	2	4	5	5	+	+	+	+		+	
13	67	5	5	26	3	3	5	7	6	+	+	+	+	+	+	+
14	67	8	2	23	2	4	7	8	6	+		+	+	+	+	+
15	68	4	6	34	4	2	4	5	6			+	+		+	
16	68	7	4	32	4	4	2	4	6	+	+	+			+	+
17	69	9	7	26	3	2	5	4	7	+	+	+	+	+	+	+
18	69	6	4	30	3	5	5	5	6	+	+	+	+		+	

qui unissent l'aneuploïdie observée et l'évolution de la tumeur elle-même, étant donné que nous ne savons pas ce qu'était le caryotype lors de la mise en culture organotypique et surtout lors du début du processus malin.

On peut néanmoins penser que les caryotypes observés représentent un stade optimum pour les cellules tumorales. A ce niveau d'évolution les éventuelles modifications compatibles avec la survie cellulaire demeurent sans doute limitées.

On peut également se demander si l'établissement et le succès de la culture organotypique ne sont pas fonction du caryotype. D'autre part, la stabilité de la structure tumorale s'accorde bien avec la stabilité du caryotype.

Ainsi que Lejeune l'a suggéré, le concept d'évolution caryotypique repose sur un certain nombre de "lois" [8].

La première est celle d'une évolution clonale progressive. Des modèles précis ont été décrits, surtout au cours des leucémies myéloïdes chroniques [11, 12]. On sait, en effet, combien il est difficile d'étudier les évolutions caryotypiques dans les tumeurs solides. Dans le cas présent, ainsi que nous l'avons dit, nous voyons sans doute l'aboutissement de cette évolution.

La deuxième loi est celle du "variant commun". Le meillleur exemple en est le chromosome Ph^1 témoin inchangé de toute l'évolution de la leucémie myéloïde chronique. Mais le variant commun peut être un événement beaucoup plus complexe faisant intervenir des anomalies numériques et de structure. Les "variants communs", observés dans la tumeur Z 200, sont à coup sûr une illustration parfaite de cette notion. Leur stabilité, démontrée sur une période de deux ans, est un argument de poids en faveur de l'hypothèse.

Une troisième loi est la tendance à la duplication des surnuméraires normaux et remaniés. Il est remarquable que, dans notre étude, aucun des marqueurs ne semble être à l'état disomique. Il est bien difficile d'interpréter cette constatation.

L'ensemble des observations préliminaires décrites ici démontre clairement l'intérêt des études chromosomiques dans les cultures organotypiques qui représentent probablement un reflet relativement fidèle de la tumeur *in vivo*. A l'inverse de ce qui se passe dans les cultures de cellules dites de souche, il y a ici maintien d'une structure organisée, et donc maintien d'un jeu complexe de facteurs de sélection comparable à celui qui existe *in vivo*. Et on sait l'importance du rôle joué par la sélection dans l'évolution caryotypique.

RESUME

Deux séries d'études cytogénétiques ont été entreprises à deux années d'intervalle (en 1966 *et en* 1968*) sur une tumeur cancéreuse entretenue* in vitro *par Et. et Em. Wolff grâce à la culture organotypique décrite par ces auteurs. La tumeur est la tumeur Z*200*, provenant d'une métatastase hépatique d'un cancer d'origine gastrique, et maintenue en culture depuis* 1962*. Ces études ont montré une aneuploïdie de nombre et de structure remarquablement constante (à des détails près), tant d'une cellule à une autre, que d'un examen à l'autre, et qu' enfin d'une série d'examens à l'autre. Le mode de la distribution des nombres chromosomiques est de* 64 *en* 1966*. Il tend ensuite vers un nombre égal à* 3N*. Tous les groupes, sauf le groupe* D*, participent à cette hyperploïdie, mais le groupe X, C y contribue le plus. Il existe deux "variants communs" inchangés pendant la période qui a été étudiée. Ce sont (a) la perte de chromosomes dans le groupe D; (b) la présence de 7 chromosomes marqueurs, dont un dicentrique. L'ensemble de ces observations semble être en accord avec la théorie chromosomique de la carcinogénèse.*

SUMMARY

Two series of cytogenetic studies have been performed, at two years interval (in 1966 *and in* 1968*), on a cancerous tumour growing* in vitro *by organotypic culture as described by Et. and Em. Wolff. The tumour Z*200*, derived from a liver metastasis of a gastric cancer, has been maintained in culture since* 1962*. These studies showed a numerical and structural aneuploidy remarkably constant (with minor variations) from one cell to another, as well as from one study to another, or from one series of studies to the other. The mode of the distribution of chromosome numbers is* 64 *in* 1966*. It tends towards* 69 *in* 1968*. All groups, except group D, participate in this hyperploidy but group X, C contributes more than the other groups. Two " common variants" appear unchanged during the period under study, namely (a) loss of chromosomes in group D; (b) the*

presence of 7 marker chromosomes, one of which being a dicentric. These observations are discussed with respect to the chromosomal theory of carcinogenesis.

ZUSAMMENFASSUNG

Es wurden in einem Zeitabstand von 2 Jahren (1966 *und* 1968) *zwei Reihen cytogenetischer Untersuchungen an einem Tumor durchgeführt, der* in vitro *nach Et. und Em. Wolff in organotypischer Kultur wächst. Der tumor Z* 200 *stammt von einer Lebermetastase eines Magencarcinoms ab und wird seit* 1962 *in Kultur gehalten. Diese Untersuchungen ergaben eine zahlenmässig und strukturell bemerkenswert konstante Aneuploidie (nur geringe Abweichungen), und zwar von Zelle zu Zelle als auch von Untersuchung zu Untersuchung und zwischen den einzelnen Untersuchungsreihen. Die Auswahl der Chromosomen betrug* 64 *im Jahr* 1966. *Für das Jahr* 1968 *tendiert die Chromosomenzahl gegen* 69. *Alle Chromosomengruppen, ausser D, tragen zu dieser Hyperploidie bei, jedoch trägt die Gruppe X, C mehr bei als die anderen Gruppen. Zwei generelle Varianten erscheinen im Verlauf der Untersuchung unverändert, nämlich: (a) Verlust von Chromosomen in Gruppe D (b) Anwesenheit von 7 Marker-Chromosomen, von denen eines dizentrisch ist. Diese Beobachtungen werden im Rahmen der Chromosomentheorie der Carcinogenese diskutiert.*

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Tumorigenesis with Isonicotinic Acid Hydrazide and Urethan in the Syrian Golden Hamsters*

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INTRODUCTION

ISONICOTINIC acid hydrazide (INH) has been reported to induce pulmonary tumors in several strains of mice [1–9] but failed to produce neoplasms in other species, including hamsters and rats. In particular, in Albino rats repeated intraperitoneal injections [10], in Wistar rats subcutaneous, intraperitoneal injections and the oral administration in the drinking water [9] and also in Desert rats the oral administration in the drinking water [9] were without carcinogenic effect. In the Syrian golden hamsters repeated intraperitoneal injections and the administration of INH in the drinking water exerted no neoplastic action [9]. In our laboratory, the Syrian golden hamsters were also treated at three different dose levels with INH in the drinking water for life without any detectable carcinogenic effect [11].

The reasons behind the current experimental set up were the following: It is known that INH is able only to induce lung tumors in mice where this spontaneous lesion appears frequently but not in hamsters, where such tumors have been rarely seen [12]. Urethan on the other hand, was shown to be capable to induce lung tumors in hamsters [13].

It was, therefore, considered of interest to investigate the carcinogenic actions of the separate and concurrent administration of INH and urethan in the Syrian golden hamster in order to determine whether these chemicals may, under these conditions, act synergistically. The presently used dose of urethan was aimed essentially to induce a limited number of lung tumors, while with the administration of INH we hoped to increase the development of urethan induced neoplasms.

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MATERIALS AND METHODS

Syrian golden hamsters were used from the colony originally obtained from Abrams Small Stock Breeders, Chicago, Illinois, and bred randomly in our laboratory since 1959. They were housed in plastic cages with sterilized granular cellulose bedding, separated according to sex in groups of 5 and were given Rockland diet in pellets (A. E. Staley Mfg., Decatur, Illinois) and tap water or the test solution was given *ad libitum*.

The drugs used were isonicotinic acid hydrazide (isoniazid, INH, Eastman Organic Chemicals, Rochester, N.Y.); and ethyl carbamate (urethan, U, Fisher Scientific Co., Chicago, Illinois). The solutions were prepared twice weekly, and the total consumption of water containing the chemicals was measured at the same intervals during the treatment period. The experimental and control groups and the treatments are described as follows:

Group 1. U was dissolved in the drinking water as a 0·1% solution and was given continuously for the life span of 48 female and 52 male golden hamsters, which were 5-week old at the beginning of the experiment.

Table 1. *Survival rate in urethan (U) and isonicotinic acid hydrazide (INH) treated and control golden hamsters*

Group	Treatment	Initial No. and sex of hamsters	No. of survivors (age in weeks) 10	20	30	40	50	60	70	80	90	100	110	120	130	140	150
1	0·1% U in drinking water daily	48 ♀	46	46	45	44	41	33	20	7	1	—	—	—	—	—	—
		52 ♂	51	51	51	49	43	40	27	16	12	3	—	—	—	—	—
2	0·1% INH in drinking water daily	50 ♀	48	48	46	45	42	36	32	22	18	8	5	2	—	—	—
		50 ♂	48	48	48	48	45	45	39	29	24	17	11	4	1	—	—
3	0·1%U and 0·1% INH in drinking water daily	60 ♀	60	60	60	57	51	40	29	15	5	—	—	—	—	—	—
		50 ♂	50	49	49	48	43	38	25	19	12	9	3	1	—	—	—
4	Control	100 ♀	89	86	86	84	79	72	66	54	30	17	5	—	—	—	—
		100 ♂	94	91	90	88	83	79	68	60	52	43	26	8	4	1	—

The average daily U consumption per animal was 15·4 mg for the females and 15·1 mg for the males.

Group 2. INH was dissolved in the drinking water as a 0·1% solution and was given continuously for the life span of 50 female and 50 male golden hamsters which were 5-week old at the beginning of the experiment. The average daily INH consumption per animal was 14·7 mg for the females and 17·4 mg for the males.

Group 3. U and INH were dissolved in the drinking water each as a 0·1% solution and was given continuously for the life span of 60 female and 50 male golden hamsters which were 5-week old at the beginning of the experiment. The average daily consumption of both U and INH per animal was 12·9 mg for the females and 12·7 mg for the males.

Group 4. As a control, 100 female and 100 male golden hamsters were kept untreated.

The experimental and control animals were carefully checked and weighed at weekly intervals, and the gross pathological changes were recorded. The animals were either allowed to die or were killed with ether when found in poor condition. Complete necropsies were performed on all animals. All organs were examined macroscopically and were fixed in 10% buffered formalin. Histological studies were done on the liver, kidney, spleen, and at least 4 lobes of the lungs of each hamster, as well as on those organs which showed gross pathological changes. Sections from these tissues were stained routinely with hematoxylin and eosin and with additional special methods when necessary.

RESULTS

The survival rate at 10-week intervals is recorded in Table 1. It is evident from this data that the treatments with urethan, INH,

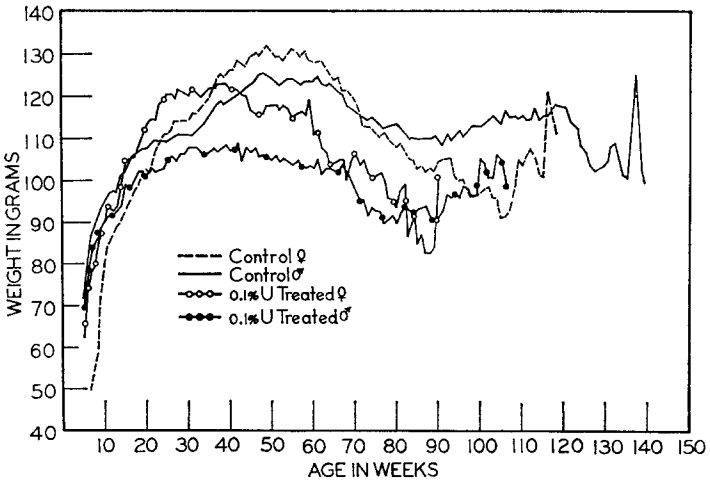

Fig. 1. *Average weekly weights of U-treated and control golden hamsters.*

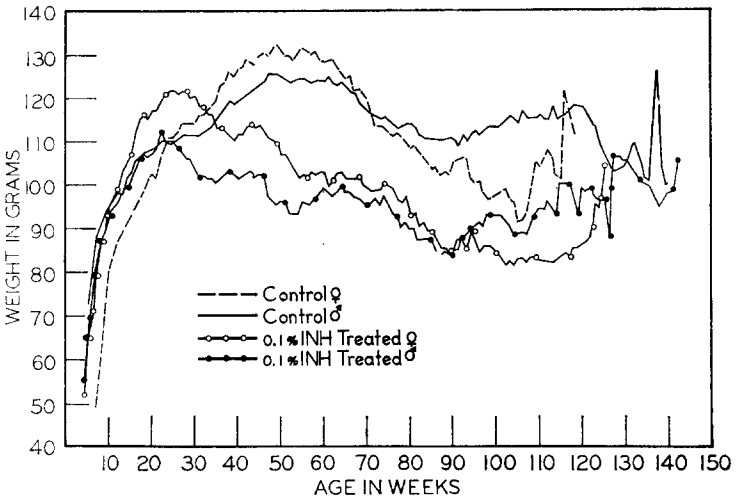

Fig. 2. Average weekly weights of INH-treated and control golden hamsters.

and urethan plus INH reduced the survival rate. The average weekly weight curves of the treated and control hamsters are illustrated in Figs. 1–3. It is apparent that the body weights of hamsters treated with urethan, INH and urethan plus INH were reduced.

Tumor incidences. Table 2 presents the number, incidences, and latent periods of the different type of tumors in the treated and control golden hamsters. As can be seen from these data, a significant number of dermal melanocytomas, papillomas, and carcinomas of forestomach, adenomatous polyps of caecum, pulmonary adenomatoses, adrenal cortical and thyroid tumors were observed in the U and U plus INH treated animals. In addition there were other types of tumors. Their incidences were slightly higher than in the controls, nevertheless, from statistical view point they do not appear to be significant. The tumor incidences in the INH-treated groups were, however, similar to those in the untreated controls. It is clear, therefore, from these data that INH alone or in combination with U had no significant effect on tumorigenesis.

DISCUSSION

The main object of this investigation was to study the possible carcinogenicity of INH in the hamsters. INH is primarily a lung tumor producer in mice [1–9], although it was reported to be capable to induce various other types of tumors by some investigators [1, 2, 14], not yet confirmed by others, however. In hamsters, nevertheless, INH was found to be without any carcinogenic effect [9, 11]. On the other hand, U is one of the most powerful substance in evoking a variety of tumors in the different tissues including the lungs in mice [15–17]. In hamsters, it also induced lung lesions. The administration of 0·2 and 0·4% solutions in the drinking water produced a considerable number of pulmonary adenomatoses [13]. Therefore, from the biological

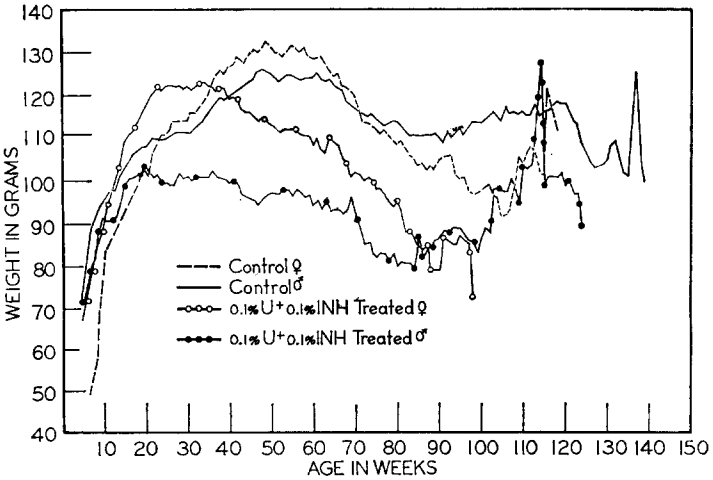

Fig. 3. Average weekly weights of U plus INH-treated and control golden hamsters.

Table 2. Distribution of tumors in urethan (U) and isonicotonic acid hydrazide (INH) treated and control golden hamster

Group	Treatment	Effective No. and sex of hamster	Number of Animals with: Dermal melanocytomas (no.)	(%)	Latent period*	Papillomas of forestomach (no.)	(%)	Latent period*	Carcinomas of forestomach (no.)	(%)	Latent period*	Malignant lymphoma (no.)	(%)	Latent period*	Adenomatous polyps of cecum (no.)	(%)	Latent period*
1	0·1 % U in drinking water daily	48 ♀	25	52	73 (56–90)	35	72	69 (42–90)	7	14	73 (56–84)	3	6	75 (59–89)	7	14	74 (68–86)
		52 ♂	26	50	76 (44–100)	36	69	75 (44–107)	18	34	82 (61–103)	5	9	78 (60–95)	4	7	81 (63–99)
2	0·1% INH in drinking water daily	50 ♀	—	—	—	5	10	103 (93–123)	—	—	—	—	—	—	—	—	—
		50 ♂	1	2	126	5	10	105 (92–125)	—	—	—	1	2	112	2	4	99 (87–111)
3	1·0%U and 0·1 % INH in drinking water daily	60 ♀	20	33	73 (29–91)	37	61	71 (42–98)	—	—	—	3	5	66 (58–80)	8	13	73 (58–86)
		50 ♂	21	42	82 (53–124)	35	70	81 (46–124)	4	8	95 (84–103)	—	—	—	6	12	82 (53–110)
4	Control	100 ♀	—	—	—	2	2	93 (83–103)	—	—	—	2	2	82 (75–89)	—	—	—
		100 ♂	1	1	109	6	6	106 (92–116)	—	—	—	7	7	94 (76–114)	—	—	—

*Age in weeks

Table 2—continued.

		Number of Animals with:		
		Other tumours (latent period in weeks)		
1		1 Adrenal cortical adenoma (67)	3 Pulmonary adenomatosis (55, 57, 86)	1 Adenocarcinoma of breast (90)
		6 Adrenal cortical carcinomas (65, 68, 69, 73, 73, 81)	4 Papillomas of gall baldder (59, 63, 70, 77)	1 Hemangioma of spleen (78)
		2 Adenomas of ovary (59, 78)	4 Carcinomas of vagina (70, 73, 75, 85)	2 Hemangiosarcomas of spleen (57, 75)
	♀	4 Carcinomas of ovary (57, 70, 71, 90)	1 Lung adenoma (73)	1 Carcinoma of anal gland (56)
		2 Adenomas of thyroid (73, 86)	1 Adenomatous polyp of colon (77)	1 Carcinoma of skin appendage (57)
		3 Carcinomas of thyroid (57, 78, 80)	1 Cholangiocarcinoma (83)	1 Mal. mesenchymoma (57)
		2 Pulmonary adenomatosis (91, 99)	1 Carcinoma of thyroid (91)	1 Hemangiosarcoma of spleen (66)
	♂	2 Adrenal cortical adenomas (91, 103)	2 Adenomatous polyps of colon (92, 93)	1 Hemangiosarcoma of liver (100)
		1 Adrenal cortical carcinoma (91)	1 Gall baldder papillomas (92)	1 Hemangiosarcoma of liver and spleen (49)
		1 Lung adenoma (92)	1 Hemangioma of liver (77)	2 Mal. mesenchymomas (84, 90)
2	♀	1 Hemangioma of spleen (78)		
		1 Adrenal cortical adenoma (70)		
	♂	1 Adrenal cortical carcinoma (111)		
		1 Pheochromocytoma (98)		
3		3 Pulmonary adenomatosis (42, 56, 80)	2 Adrenal cortical carcinomas (77, 87)	1 Carcinoma of uterus (73)
		4 Adenomas of thyroid (60, 67, 73, 91)	2 Hemangiomas of spleen (74, 87)	1 Carcinoma of anal gland (88)
		2 Carcinomas of thyroid (85, 88)	1 Hemangioma of liver (86)	1 Mal. plasmacytoma (67)
	♀	2 Adenomatous polyps of colon (86, 88)	2 Hemangiosarcomas of spleen (54, 56)	1 Carcinoma of salivary gland (81)
		3 Papillomas of vagina (75, 77, 86)	3 Hemangiosarcomas of liver (47, 71, 73)	1 Papilloma of urinary bladder (65)
		1 Carcinoma of vagina (61)	2 Carcinomas of ovary (67, 76)	1 Hemangiosarcoma of spleen, liver and lungs (67)
		1 Papillomas of gall bladder (75)	1 Adenoma of uterus (67)	1 Adenocarcinoma of breast (67)
		3 Adenomatous polyps of colon (89, 99, 110)	1 Harderian gland adenoma (88)	3 Hemangiosarcomas of liver and spleen (69, 92, 116)
		1 Adrenal cortical adenoma (100)	1 Adenoma of anal gland (76)	1 Hemangiosarcoma of liver, spleen and pancreas (114)
	♂	2 Adrenal cortical carcinomas (80, 109)	1 Hemangioma of spleen (103)	
		3 Gall bladder papillomas (92, 110, 116)	1 Hemangioma of liver and spleen (102)	
		1 Lung adenoma (103)	2 Hemangiosarcomas of liver (55, 89)	
4		2 Cholangiomas (90, 113)	1 Adrenal cortical carcinoma (106)	1 Leiomyosarcoma of uterus (104)
	♀	2 Cholangiocarcinomas (88, 115)	1 Adenocarcinoma of kidney (118)	1 Mal. schwannoma of facial nerve (106)
		1 Adrenal cortical adenoma (69)	1 Adenoacanthoma of uterus (88)	
		2 Hemangiomas of liver (114, 138)	1 Adrenal cortical adenoma (108)	
	♂	1 Adenoma of thyroid (128)	1 Adrenal cortical carcinoma (84)	
		1 Carcinoma of thyroid (128)		

point of view, the hope for their additive effect appeared to be reasonable, since one of their main target tissues was the lung.

The present results show that 0·1% solution of urethan administered in the drinking water to 5-week-old Syrian golden hamsters for the remainder of their lifetime resulted in the induction of dermal melanocytomas, papillomas and carcinomas of forestomach, adenomatous polyps of cecum, pulmonary adenomatoses and various other types of tumors. Similar administration of 0·1% INH alone was without any apparent carcinogenic effect while 0·1% INH given simultaneously with 0·1% urethan gave rise to similar incidences of tumors than what had resulted by either compounds alone.

In view of the fact that INH alone and in combination with U have not produced any tumors or gave rise to similar incidences than what had resulted by either compounds alone mean that summation or synergism did not occur. Such results might be interpreted either as evidence of a failure of INH to influence the development of lung tumors (or other types) or, alternatively, as a reflection of inadequate conditions operating in the particular experimental set-up. It is our belief that this later possibility cannot be excluded until further studies with modified experimental techniques clarify this field of interest.

The tumor yield in the urethan-treated groups is noteworthy. Particularly that an unusual variety of neoplasms were evoked by the treatment. Some of these, dermal melanocytomas, forestomach tumors were often induced in this species also by other treatments. Others, such as tumors of cecum, thyroid, lungs, ovary were produced in relatively fewer instances [18]. Their appearance, therefore, presents a valuable information for carcinogenesis studies.

At present, it is difficult to explain why INH is carcinogenic only in one species and not in others. Such a phenomenon is not too often reported in the cancer research literature, and it seems now too early to comment on this, because metabolic studies are obviously needed which could hopefully provide explanation based on chemical grounds for the various biological results.

Acknowledgments—We wish to thank Mrs. Harriet Lukas for her technical assistance in the experimental work.

RESUME

Des solutions à 0,1% *d'hydrazide de l'acide nicotinique et d'uréthane furent administrées, seules ou conjointement, dans l'eau de boisson de hamsters dorés de Syrie, depuis l'âge de* 5 *semaines, et pour toute la durée de leur vie. L'administration d'uréthane provoqua l'apparition de mélanocytomes dermiques, de papillomes et de carcinomes du ventricule succentérique, de polypes adénomateux du caecum, d'adénomatoses pulmonaires, et de divers autres types de tumeurs. Le traitement par l'hydrazide de l'acide nicotinique seul était sans effet cancérogène apparent, tandis que l'administration simultanée d'hydrazide de l'acide nicotinique et d'uréthane ne produisait pas plus de tumeurs que l'uréthane seul.*

La cancérogénicité sélective de l'hydrazide de l'acide nicotinique dans diverses espèces est discutée en fonction des résultats obtenus.

SUMMARY

Solutions of 0·1% *of isonicotinic acid hydrazide and urethan were given alone and also jointly in the drinking water to* 5*-week old Syrian golden hamsters for the remainder of their lifetime. The urethan-administration resulted in the induction of dermal melanocytomas, papillomas and carcinomas of forestomach, adenomatous polyps of caecum, pulmonary adenomatoses and various other types of tumors. Isonicotinic acid hydrazide-treatment alone was without any apparent carcinogenic effect, and the simultaneous isonicotinic acid hydrazide and urethan treatments gave rise to no significantly higher incidences of tumors than what resulted by either chemicals alone.*

The selective carcinogenicity of isonicotinic acid hydrazide in the various species are discussed in the light of the results obtained.

ZUSAMMENFASSUNG

Lösungen von 0·1%*igen Isonicotinsäure-hydrazid und Urethan wurden einzeln und zusammen* 5 *Wochen alten syrischen Goldhamstern für den Rest ihrer Lebensdauer im*

Trinkwasser verabreicht. Die Urethangaben verursachten Melanocytome der Haut, Papillome und Carcinome des Vormagens, adenomatöse Polypen des Coecums, Adenomatosen der Lunge und verschiedene andere Typen von Tumoren. Die Behandlung mit Isonicotinsäure-hydrazid war ohne ersichtlichen carcinogenen Effekt. Eine Kombination von Isonicotinsäure-hydrazid und Urethan erhöhte die Tumorrate nicht wesentlich im Vergleich zur Wirkung der einzelnen Substanz. Die selektive carcinogene Wirkung von Isonicotinsäure-hydrazid in den verschiedenen Spezies wird im Zusammenhang mit den erzielten Resultaten diskutiert.

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Changes of Cell Proliferation Characteristics in a Rat Rhabdomyosarcoma Before and After X-Irradiation

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INTRODUCTION

THE GROWTH of a solid tumour is mainly determined by cell production and cell loss. The rate of cell production is a function of the fraction of proliferating cells and the cell cycle time, both of which can be studied by autoradiographic methods [1]. The rate of cell loss can at present only be derived from the difference between the rate of cell production and the rate at which the total cell population increases [2]. In a number of experimental solid tumours the relationship between the volume increase and cell proliferation has been shown to depend on tumour size [3–6]. The results of these studies indicate that with increasing tumour volume, the fraction of proliferating cells may decrease, while in addition the cell cycle time may change. Furthermore it was shown that the fraction of proliferating cells as well as the cell cycle time are not constant parameters throughout a tumour, but that these parameters may vary at different locations in the tumour [7].

Irradiation may induce changes in tumour growth rate, which must be due to changes in cell proliferation characteristics. Knowledge of the cell cycle parameters before and after irradiation is required to interpret the effect of this treatment. Primarily, irradiation of a tumour induces loss of the capacity for unlimited proliferation in a fraction of the tumour cell population. Many of these cells can still divide a few times before they die and are eliminated. Consequently, the tumour volume does not decrease immediately after irradiation. If the dose of radiation is large enough, the rate of production of new cells eventually becomes smaller than the rate of elimination of cells from the tumour, and as a result the tumour volume will decrease. If no cure is attained, the rate of cell production by cells, which have retained the capacity for unlimited proliferation, will finally exceed the rate of removal of dead cells and the tumour will resume growth. The sequence of changes occurring in a tumour after irradiation during regression and recurrence can only be interpreted through studies of all the factors involved, including the fraction of cells which have retained the capacity for unlimited proliferation, the fraction of cells which divide only a limited number of times, the distribution of cell cycle times and the mean life time of cells which no longer proliferate.

The studies described in this paper were undertaken to obtain information about the relative importance of these factors in an experimental rhabdomyosarcoma transplantable in an inbred strain of rats.

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MATERIALS AND METHODS

(a) *Tumours*

The solid tumours employed, are transplantable rhabdomyosarcoma's, derived by a special selection procedure. These tumours have very reproducible growth properties and can be investigated by various techniques to be described. The rhabdomyosarcoma originated in December 1962 in an irradiated rat of the inbred WAG/Rij strain. The tumour was found to be transplantable in this strain. From the ninth passage, in December 1963, a tumour was excised and with a cell dispersion technique, a mono-cellular suspension was prepared [8]. These cells were cultured *in vitro* in a medium consisting of Hanks' balanced salt solution, Eagle's amino acids and vitamins (MEM), 6% newborn calf serum, inactivated by heating to 56°C during one hour, and 100 i.u. penicillin per ml. Subsequently, a single clone of cells was removed from a culture dish and these cells were implanted into the flank of a rat, where they grew into a tumour. From this tumour a cell suspension was prepared again and the cells cultured *in vitro*. A selected clone from one of these cultures was then implanted subcutaneously into the flank of a rat to grow a tumour. This selection procedure was repeated several times and in July 1966 resulted in a tumour, denoted R-1, which can be transplanted reproducibly from the *in vitro* system to the *in vivo* system and conversely. The cells derived from this R-1 tumour have been propagated in tissue culture since 1966 and are denoted R-1 cells.

In the present investigation R-1 cells cultured *in vitro* were trypsinized in order to make a cell suspension which was used to inoculate female WAG/Rij rats, 7–8 weeks old. Each animal received one inoculation, containing 10^6 cells, per flank. The tumours arising from the inoculated cells were allowed to grow to a volume of 1·0–2·0 cm^3. Subsequently the tumours were irradiated with a dose of 2000 rad of X-rays or assigned to control groups.

During the experiments all animals were kept under the same conditions and supplied with food pellets and water *ad libitum*. During the interval between labelling of the animals and excision of the tumours, the room temperature was kept at 20°C and the light intensity at 600 lx.

(b) *Assay of the capacity of tumour cells for unlimited proliferation*

The selection procedure described in the previous section has yielded a tumour which can be assayed with respect to the proliferative capacity of the constituent cells by a direct plating technique. From unirradiated and irradiated tumours, cell suspensions can be prepared and known numbers of cells can be plated in culture dishes. With cells from unirradiated tumours, plating efficiencies of $35 \pm 10\%$ were obtained. From a comparison of the plating efficiency of cells derived from an irradiated tumour with that of controls, the 'surviving fraction' of clonogenic cells present in the treated tumour can be calculated. Details of the experimental procedure have been described elsewhere [9, 10].

(c) *Irradiations*

Irradiations were carried out with a 300 kV, 10 mA Philips Müller X-ray generator. The half value layer of the radiation was 2·0 mm Cu and the dose rate at the centre of the tumour was approximately 500 rad/min. Prior to irradiation the animals were anaesthetised by injection of 0·045 mg of Veterinary Nembutal* per g body weight. The animals were shielded in a lead box from which the tumour protuded through a slit. The slit was made wide enough to prevent obstruction of the blood supply of the tumour. Nevertheless, as discussed later, measurements of surviving fractions of cells and of growth delay after a dose of 2000 rad of X-rays indicate that the fraction of anoxic cells in tumours irradiated in these conditions may have been larger as compared with tumours which were irradiated with a collimated beam.

(d) *Measurements of tumour volumes*

Tumour volumes before and after irradiation were derived by measuring with vernier callipers three dimensions of the tumour, which can easily be palpated through the skin. From these data tumour volumes were calculated for a sphere or a spheriod, depending on the shape of the tumour. Relative volumes are computed from the ratio of the volume at a given time and the volume on the day of irradiation. For all points of growth curves, relative volumes of at least five tumours were averaged.

(e) *Autoradiography*

In order to study the proliferation parameters of cells in tumours, animals were injected intraperitoneally with a single dose of 1 μc

*Pentobarbital Natrium (Veterinair) was obtained from Abbott S.A., Brussels, Belgium.

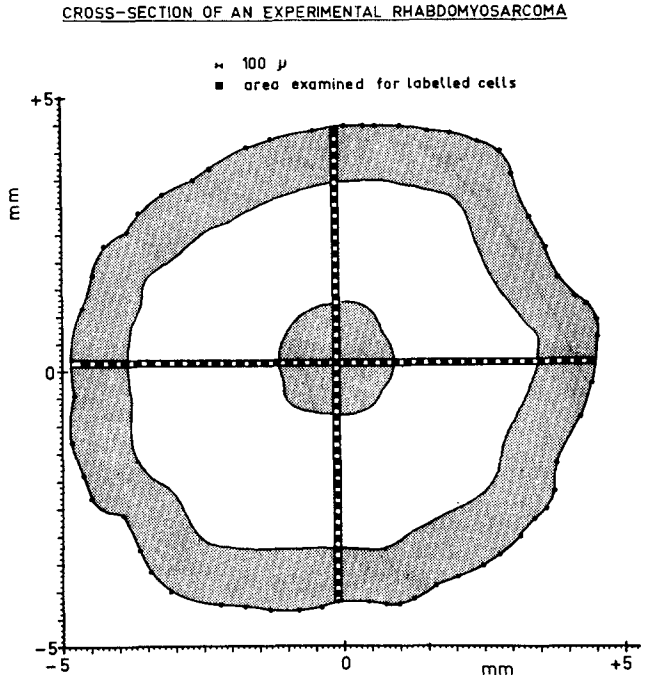

Fig. 1. Cross section of a tumour. Labelled and non-labelled cells in mitosis were counted in the shaded areas parallel to the circumference and in the centre of the tumour cross section respectively. Labelled and non-labelled cells in interphase were scored in areas indicated by the small black squares, localized in two zones across the tumour centre.

^{3}H-thymidine* per gram body weight. The animals of control groups were injected at time $t=0$, where as the animals with the irradiated tumours were injected at four days ($t=4$) after irradiation, when the tumours, after an initial continued growth and subsequent regression, have attained volumes equal to those of the controls at time $t=0$ (Fig. 2).

Subsequently, at regular time intervals of up to 40 hr after injection of ^{3}H-thymidine, the tumours were excised from animals anaesthetised with ether. After excision the tumours were fixed in Bouin's solution, and by standard histological techniques, serial cross sections of 3μ thick were prepared and used to make autoradiographs by the dipping film emulsion technique [11]. Two types of film emulsions have been used, the G-5 and the K-2 Nuclear Research Emulsions.†. The second type of emulsion has finer grains and is therefore more suitable for the study of histological aspects of cells in mitosis, especially in the irradiated tumours. The exposure time of the autoradiographs was 6 weeks for the G-5 emulsion and 8 weeks for the K-2 emulsion. The autoradiographs were processed by a standard photographical processing technique and stained with hematoxylin and eosin immediately after fixation and washing in distilled water. In these autoradiographs the numbers of labelled and non-labelled cells in interphase were scored in individual areas measuring 100×100 μ^2 each, localized at intervals of 100 μ, in two zones perpendicular to each other, which pass through the centre of the tumour cross section (Fig. 1). Interphase cells which morphologically showed signs of pyknosis, lysis or severe nuclear distortion were excluded. This fraction did not exceed 10% in the control tumours and 20% in the irradiated tumours.

For each tumour the numbers of labelled and non-labelled cells in mitosis (metaphases+anaphases) were scored separately in an area of about 500–1000 μ wide, parallel to the circumference of the tumour cross section and in a circular area with a diameter of about 1000–2000 μ, located in the centre of the tumour (Fig. 1). Per tumour cross section at least 100 cells in mitosis were scored in each of the areas.

The cell cycle time of tumour cells proliferating *in vitro* was estimated from R-1 cell cultures grown on cover slips in Petri dishes containing 4 ml of culture medium. Single cells were plated and allowed to grow for 6 or 10 days in an incubator flushed with a mixture of air and 2·5% CO_2 at 37°C. After 6 or 10 days colonies had developed with an average number of 80 or 1200 cells respectively.

*^{3}H-thymidine with a specific activity of 1·9 c/m.mole was obtained from Schwarz Bioresearch Inc., Orangeburg, N.Y., U.S.A.

†Obtained from Ilford Nuclear Research, Essex, England.

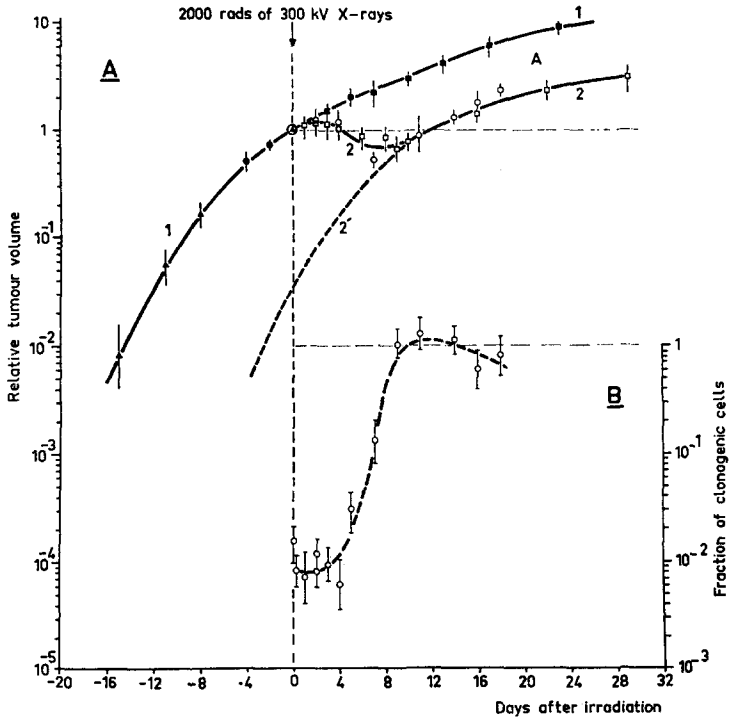

Fig. 2. Growth curves of R-1 rhabdomyosarcoma's and the recurrence after a dose of 2000 rad of 300 kV X-rays.

Part A, curve 1: Growth curve of control tumours. The relative tumour volume of 1·0 corresponds to an average tumour volume of 1·5 cm³.

Part A, curve 2: Growth curve of tumours irradiated at t=0.

Part A, curve 2': Hypothetical curve extrapolating the growth of the recurrent tumour parallel to the growth of an untreated tumour.

Part B: Variation of the fraction of clonogenic cells as a function of the time after irradiation.

^{3}H-thymidine was then added to the medium to a final concentration of 0·1 μc ^{3}H-thymidine per ml medium. After 15 min incubation the radioactive medium was discarded. The cells were then first washed with medium at 37°C containing 10 μg/ml non-radioactive thymidine and subsequently with standard medium at 37°C. Finally the cells were supplied with standard medium at 37°C and incubated for time intervals of up to 36 hr. During this period of incubation, coverslips were taken at regular intervals from the Petri dishes and dipped in Bouin's solution to fix the cells. Autoradiographs were then prepared by coating the coverslips with a G-5 Nuclear Research Emulsion and exposure for 10 days.

Cells were considered to be labelled, if the number of reduced silver grains over the nucleus was larger than four in the case of the K-2 emulsion and larger than five in the case of the G-5 emulsion.

RESULTS

(a) *Growth of the tumour and survival of its constituent cells*

Some of the morphological properties of the tumour which are of importance with respect to the tumour growth, have been reported previously [7]. In particular it may be noted that in tumours with a volume of less than 3 g, massive necrosis is generally not observed.

In Fig. 2A, the growth curves for unirradiated and irradiated tumours are presented. Curve 1, obtained for unirradiated tumours, shows that the growth is not exponential. The growth rate decreases with increasing tumour volume. At the time of irradiation, at $t=0$, the tumour volume doubling time (T_d) is about 6 days, while at $t=-15$, $T_d=1·5$ days and at $t=+15$, $T_d=10$ days. Curve 2 was obtained by irradiating at $t=0$, a number of tumours with a dose of 2000 rad 300 kV X-rays. During the first two days after irradiation the tumours continue to grow slowly and subsequently from $t=2$ on, a decrease in volume is observed. The minimum relative volume, attained at 6–8 days after irradiation, is equal to 0·75, and at day $t=12$, the volume is equal to the volume at $t=0$. The growth rate of the recurrent tumour does not differ significantly from the unirradiated controls of the same volume. It can be con-

cluded that the dose of 2000 rad of X-rays results in only a relatively small decrease of the tumour volume and in a short growth delay of 12 days, i.e. only two times the volume doubling time of 6 days of unirradiated tumours of this size.

In Fig. 2B, fractions of clonogenic cells in tumours are presented, which were measured at various intervals after irradiation with a dose of 2000 rad of 300 kV X-rays. From each tumour a cell suspension was prepared and plated in four Petri dishes, according to techniques described in more detail elsewhere [10]. After 12 days the clones were fixed and stained. The fraction of clonogenic cells in an irradiated tumour was calculated in relation to the plating efficiency of cells from unirradiated control tumours of the same volume. Each point represents the value for a single tumour. The vertical bars indicate the confidence intervals calculated from the variation between the numbers of clones consisting of more than 50 cells on the four dishes. The results show that a dose of 2000 rad X-rays reduces the fraction of clonogenic cells to a value of about 0·8 % relative to unirradiated tumours. The term 'clonogenic cells' is more appropriate here than 'surviving cells' because at time intervals exceeding a few hours after irradiation, cell proliferation and cell loss may modify the fraction of cells, capable of unlimited proliferation in the tumour.

The results presented in Fig. 2B show further that during three days after irradiation the fraction of cells capable of unlimited proliferation remains approximately constant. Starting after day 4, a rapid increase is observed and at day 9, the fraction of clonogenic cells in the irradiated tumours is equal to that in an unirradiated tumour. Neither the small increase beyond 100% between $t=11$ and $t=14$, nor the small decrease between $t=16$ and $t=18$, can be considered as significantly different from the 100% value.

Comparison of the growth curve 2 of Fig. 2A and the curve of Fig. 2B shows that, although the effect of 2000 rad X-rays on the tumour volume is relatively small, important changes occur in the cell population with respect to cell proliferation and cell loss.

(b) *Labelling patterns and cell cycle characteristics*

Fractions of labelled cells were calculated from the numbers of labelled and non-labelled cells in interphase, counted in alternating areas of $10^4\mu^2$ in tumour cross sections (Fig. 1). The mean number of cells scored per area of $10^4\mu^2$ is equal to about 150 cells for untreated

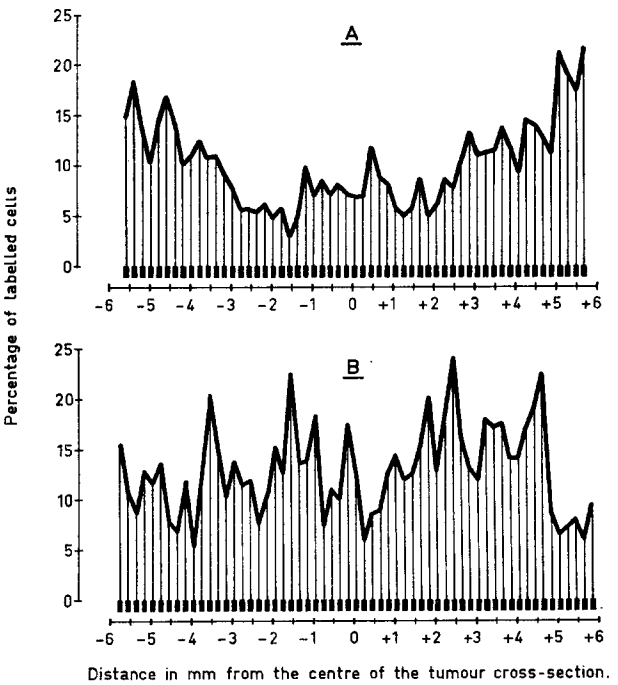

Fig. 3. *Patterns of labelled cells* 2 *hours after pulse labelling. Fractions of labelled cells, indicated by vertical bars, are plotted as a function of distance from the centre of the tumour cross section. Each black area in the figure corresponds to one of the areas examined for labelled and non-labelled cells as indicated in Fig.* 1.

Part A is an example of the pattern of labelled cells in untreated tumours.

Part B is an example of the pattern of labelled cells in tumours, 4 *days after treatment with* 2000 *rad of X-rays.*

Table 1. *Fractions of labelled cells in the periphery and the centre of unirradiated and irradiated tumours excised at different times after pulse labelling*

Time of excision after pulse labelling (hr)	Percentage labelled cells*			
	Unirradiated tumours		Tumours labelled 4 days after irradiation	
	Periphery	Centre	Periphery	Centre
2	14·2±1·4	8·6±3·2	17·5±6·4	14·3±2·9
3	17·3±3·2	8·1±2·1	—	—
4	20·9±5·1	11·0±6·8	14·7±2·5	14·6±4·7
8	18·4±3·9	8·4±4·3	17·3±3·6	13·5±3·7
10	20·7±5·8	8·2±3·3	18·7±8·3	13·6±4·3
12	25·6±6·6	9·0±5·8	21·6±6·0	20·5±6·2

*Standard errors have been calculated from the variations between values obtained for at least 6 areas of 100 μ × 100 μ.

tumours and equal to about 100 cells for irradiated tumours labelled at $t=4$. These data were used to construct labelling patterns of the tumour cross sections by plotting the fraction of labelled cells as a function of the location in the tumour.

In Fig. 3, examples are presented of labelling patterns of unirradiated tumours and irradiated tumours, labelled at $t=0$ and $t=4$ respectively and excised two hours after injection of ^{3}H-thymidine. It can be concluded that in untreated tumours, excised 2 hr after labelling (Fig. 3A), large fractions of labelled cells occur more frequently near the periphery, whereas generally smaller fractions are found in the centre of the tumour. A similar pattern, but somewhat less pronounced, has been described previously for smaller tumours [7].

In Table 1, column 2 and 3, mean values of fractions of labelled cells, computed from data obtained for the periphery and the centre respectively, are tabulated for *unirradiated tumours* excised at different time intervals after pulse labelling. It can be concluded that in the periphery the fractions of labelled cells increase with time after pulse labelling, whereas no significant increase is observed in the centre. The increase of the fraction of labelled cells in the periphery with the time interval after labelling may be due to two phenomena namely proliferation of labelled cells and elimination of non-labelled cells. Further evaluation of these factors depends on results to be obtained from experiments in which continuous labelling is employed.

The labelling pattern of an *irradiated tumour*, injected with ^{3}H-thymidine at $t=4$ and $t\pm4$ and excised 2 hr post-labelling, is shown in Fig. 3B. It can be seen that, although considerable variations in fractions of labelled cells are observed the systematic difference between the centre and the periphery of the tumour shown in unirradiated tumours, is not present in the irradiated tumour.

Mean values of fractions of labelled cells for the periphery and the centre of tumours, irradiated at $t=0$ and excised at $t=4$ at different time intervals after pulse labelling, are presented in column 4 and 5 of Table 1. It can be concluded that the differences between the values for the periphery and the centre are much smaller for the irradiated tumour as compared with the controls. Furthermore no significant increase of fractions of labelled cells as a function of time after pulse labelling is shown either for cells present in the periphery or for the centre of the irradiated tumours.

In order to derive the fractions of proliferating cells and the rates of cell proliferation from labelling indexes (fractions of labelled cells), cell cycle parameters were estimated for cells proliferating in the centre and near the periphery of unirradiated and irradiated tumours, according to the method described by Quastler and Sherman [12]. For this purpose fractions of labelled cells in mitosis were determined as a function of the time after pulse labelling. The results obtained for unirradiated tumours, labelled at $t=0$, are presented in Fig. 4.

The fraction of cells in the population which are in mitosis is 0·65%. The maximum percentages of labelled cells in mitosis observed in the first wave of the curves A and B of Fig. 4 are equal to 97% and 89%, respectively. The maximum values for the corresponding second waves are equal to 70% and 52%. From these curves mean values of the period of DNA synthesis (T_s), the time which the cells spend in G_2+M, and of the cell cycle time (T_c) can be estimated. These estimations involve uncer-

Table 2. Proliferation parameters of R-1 rhabdomyosarcoma cells in unirradiated and irradiated tumours and in culture

	Symbols	Units	Small unirradiated tumours	Large unirradiated tumours labelled at $t=0$			Large irradiated tumours labelled at $t=4$			R-1 cells in culture	
				Centre	Periphery	Mean	Centre	Periphery	Mean	small clones	large clones
A. Measured parameters											
Mean tumour volume	V	(cm^3)	0·3	0·5	1·0	1·5	0·5	1·0	1·5	—	—
Total number of cells	$\mathcal{N}$		$1·5\times10^8$	$2·5\times10^8$	5×10^8	$7·5\times10^8$	$1·4\times10^8$	$2·7\times10^8$	$4·1\times10^8$	80	1200
Tumour doubling time	T_d	(hr)	60	×	×	144	×	×	−96	17	40
Fraction of labelled cells ($N_s/\mathcal{N}$)	LI		0·195	0·086	0·142	0·123	0·143	0·175	0·164	0·55	0·20
Cell cycle time	T_C	(hr)	20·4	25·5	18·5	×	12·5	11·5	×	16·0	16·5
Pre-synthesis time	T_{G1}	(hr)	7·9	13·5	7·0	×	0	0	×	3·5	3·5
DNA-synthesis time	T_S	(hr)	9·9	8·5	9·0	×	7·0	8·0	×	8·5	8·5
Post-synthesis time+mitosis	T_{G2+M}	(hr)	2·6	3·5	2·5	×	5·5	3·5	×	4·0	4·5
B. Derived parameters											
Potential doubling time	T	(hr)	40	73·6	48·5	54·5	40	36·6	38	16·0	35·0
Proportionality constant ($T.LI)/T_S$	λ		0·79	0·75	0·76	0·76	0·81	0·80	0·80	1·00	0·82
Number of P-cells produced per division $\ln\alpha=\ln 2.T_C/T$	α		1·42	1·27	1·30	1·29	1·24	1·24	1·24	2·0	1·39
Growth fraction $f=\alpha-1$	f		0·42	0·27	0·30	0·29	0·24	0·24	0·24	1·0	0·39
Fraction of non-proliferating cells (Q-cells)	1−f		0·58	0·73	0·70	0·71	0·76	0·76	0·76	0	0·61
Rate constant production new cells ($k_{P+Q}=\ln2/T$)	k_{P+Q}	(hr^{-1})	0·0173	0·0094	0·0143	0·0127	0·0173	0·0189	0·0183	0·043	0·020
Rate constant growth $k=\ln 2/T_d$	k	(hr^{-1})	0·0115	×	×	0·0048	×	×	−0·0069	0·041	0·017
Rate constant cell loss ($k_L=k_{P+Q}-k$)	k_L	(hr^{-1})	0·0058	×	×	0·0079	×	×	0·0252	0·002	0·003
Cell loss factor $\phi=k_L/k_{P+Q}$	ϕ		0·34	×	×	0·62	×	×	1·37	0·05	0·15
Mean life span Q-cells, assuming random loss: $\overline{T}(Q)=(1-f)/k_L$	$\overline{T}(Q)$	(hr)	100	×	×	90	×	×	30	×	200

*Not applicable

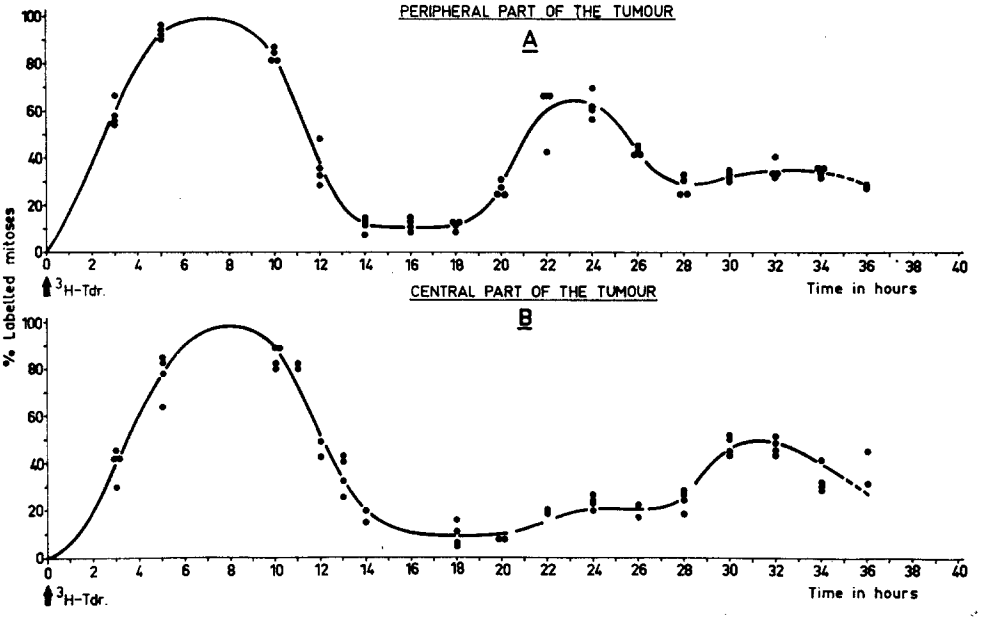

Fig. 4. Variations of the fractions of labelled cells in mitosis as a function of time after pulse labelling in untreated tumours. Each point represents the fraction of labelled mitoses computed from a total of 100 *metaphases+anaphases observed. For each time interval slides of at least two tumour cross sections were used.*
The curve of part A represents data obtained from peripheral areas of tumour cross sections.
The curve of part B represents data obtained from central parts of tumour cross sections.

tainties, due to the fact that the cell cycle parameters are subject to variations, as evidenced by the observation that, after the first wave, the percentage of labelled mitoses does not decrease to zero and that the second wave does not reach values close to 100%. The values of T_{G_2+M}, T_S and T_C are presented in Table 2. T_{G_2+M} has been derived as the time interval between labelling and the midpoint of the ascending part of the first wave. T_S has been derived as the time interval between the midpoints of the ascending and descending parts of the first wave. T_C has been derived as the time interval between the midpoints of the ascending parts of the first and the second wave of labelled mitoses. T_{G_1} has been derived from $T_{G_1}=T_C-T_S-T_{G_2+M}$. From the values presented in Table 2, it can be concluded that T_C in the centre of the tumour is about 7 hours longer than T_C in the periphery, mainly due to a longer G_1 period. As discussed by Quastler [13] and Steel [4], the cell cycle parameters derived from this analysis, apply to cells observed in mitosis. It is not certain that these cells represent a random sample of the population of proliferating cells present in the tumour at any one time. The cell cycle parameters presented in Table 2 are therefore weighted towards those of cells which have short cycle times.

In Fig. 5 the fractions of labelled mitoses as a function of the time interval after labelling, are presented for tumours irradiated with 2000 rad of X-rays on $t=0$ and excised at $t=4$. The fraction of cells of the total tumour cell population which are in mitosis, is 0·62%.

Table 3. Fractions of surviving cells measured in tumours irradiated with 2000 *rad X-rays in different exposure conditions*

Exposure conditions	Fraction of surviving cells (%)	Fraction of anoxic cells* %	Growth delay
Tumour in dead animal	2·5	100	—
Anaesthetized animal, tumour protruding through slit in lead shield	0·8	32	12–13 days
Anaesthetized animal, tumour irradiated with a collimated beam	0·25	10	18 days

*Calculated from the ratio of surviving fractions relative to completely anoxic tumours as described by Hewitt and Wilson [17] and applied for the rhabdomyosarcoma by Reinhold [18] and Barendsen [9].

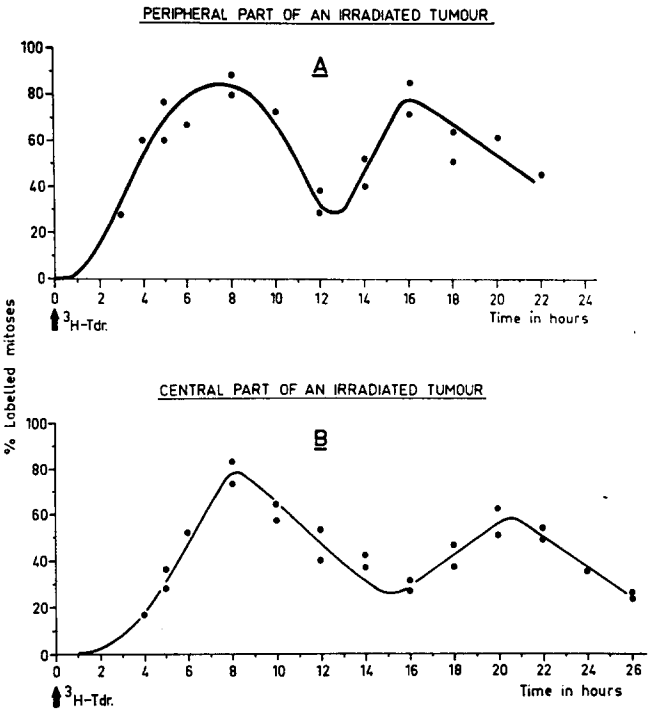

Fig. 5. *Variations of the fractions of labelled cells in mitosis as a function of time after pulse labelling of tumours irradiated four days earlier with* 2000 *rad* 300 *kV X-rays. Each point represents the fraction of labelled mitoses computed from a total of* 100 *metaphases+anaphases observed.*

The curve of part A represents data obtained from peripheral areas of irradiated tumours.

The curve of part B represents data obtained from central areas of irradiated tumours.

The maximum percentages of labelled cells in mitoses for the first and the second wave in the periphery are equal to 88% and 83% respectively and in the centre 83% and 62% respectively. The cell cycle parameters summarized in Table 2 have been derived in the same way as described for unirradiated tumours.

It can be concluded that the cell cycle times, for cells in the periphery of the irradiated tumours as well as for cells in the centre, are considerably shorter than corresponding values for unirradiated tumours. The differences are mainly due to variations in the values of T_{G_1}. The time which the cells in the irradiated tumour spend in G_2+M phase is slightly longer as compared with cells in unirradiated tumours.

(*c*) *Cell cycle parameters of R-*1 *cells cultured* in vitro

In Fig. 6 the fractions of labelled cells in mitosis are presented as a function of the time after pulse labelling *in vitro*. Each point represents the mean value derived from counting all mitoses in a number of clones containing between 1000 and 1400 cells each, growing on a Petri dish. Similar data were measured for clones containing an average of 80 cells. The values derived for T_C, T_{G_1}, T_S and T_{G_2+M} have been summarized in Table 2.

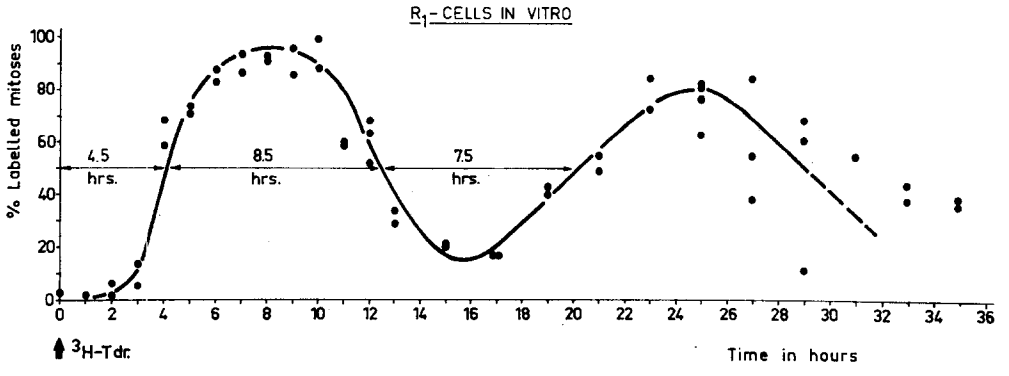

Fig. 6. *Variations of the fractions of labelled cells in mitosis as a function of time after pulse labelling of cells cultured* in vitro. *Each point represents the data of mitoses scored in large clones containing between* 1000 *and* 1400 *cells, computed from a total of at least* 100 *metaphases+anaphases per individual slide.*

DISCUSSION

(a) *Cell survival and growth delay after irradiation*

The work presented in this paper is part of a comprehensive study concerning the reactions of the experimental rhabdomyosarcoma and its constituent cells, to various treatment regimes of fast neutrons and X-rays. The purpose of the present study is to find an interpretation of the apparent discrepancy between two effects induced by a dose of 2000 rad X-rays, namely, a decrease of the surviving fraction of cells to 0·8% and a decrease in tumour volume by only a factor of 0·7, followed by a rapid recurrence [9]. Theoretical aspects of the influence of irradiation on cell proliferation and tumour growth have been discussed by various authors, in particular with respect to changes in the growth fraction [14–16]. The results presented in this paper demonstrate that the changes induced by irradiation are more complex than has been anticipated.

In our experiments effects of a single dose of 2000 rad were investigated, because a dose of 1000 rad causes only a temporary decrease in growth rate, but no distinct volume decrease, while a dose of 3000 rad produces a very large decrease in volume and results in such a high frequency of cell death, that studies of proliferation parameters are very difficult. The time interval of four days post-irradiation was investigated first, because at this time the tumour volume has started to decrease significantly from its maximum at two days post-irradiation and has reached a volume equal to that at $t=0$. Results pertaining to other time intervals will be reported in a subsequent paper.

The growth delay of 12 days, induced by 2000 rad. X-rays in the present experiments, is smaller than the value of 18 days measured for the same R-1 rhabdomyosarcoma, irradiated in a different experimental arrangement, described elsewhere [10]. The value of 12 days was derived as the time interval required for the tumour to regrow to the volume at the time of irradiation. In the experiments which yielded a value of 18 days, growth delay was estimated as the time interval required for the irradiated tumor to regrow to twice the volume at the time of irradiation, from which the doubling time of unirradiated tumours was subtracted. From curve 2 of Fig. 2A it can be deduced that the latter method applied to the present data, results in an estimate of the growth delay of 13 days, which is not significantly different from the value of 12 days derived by the first method. The difference between the two values for growth delay of 13 and 18 days respectively, is presumably due to a difference between the oxygenation conditions of the tumours in the different irradiation arrangements. Although the slit in the lead shield used, was wide enough, the blood flow in the tumour protruding through the slit may have been impaired as a result of a decrease in temperature of the tumour below the rat body temperature. The assumption of an increased tumour hypoxia, relative to tumours irradiated without shielding of the rat, is supported by the results of measurements of surviving fractions of cells for tumours irradiated in three different conditions shown in Table 3. The level of hypoxia in the tumour at the time of irradiation does not cause uncertainties in the interpretation of the results however, because the actual number of cells capable of unlimited proliferation, is known from the measurements.

As noted earlier the growth delay of 12 to 13 days, caused by 2000 rad X-rays in the present experiments is equal to only twice the doubling time of 6 days for unirradiated tumours of the same volume. If the very simple assumptions are made that before irradiation all cells in the tumour contribute to the growth with a cell cycle time of 6 days, and that the irradiation only reduces the fraction of cells capable for unlimited proliferation and finally that cells in which this capacity is impaired are lost from the tumour very rapidly, then it might be concluded that 2000 rad has reduced the fraction of surviving cells to a fraction of 0·25. Figure 2B shows that these assumptions are not valid because the surviving fraction is reduced to 0·008.

If the assumption is made that the cells in the tumours irradiated at $t=0$, start to multiply at a rate corresponding to that of unirradiated tumours of smaller size, e.g. as indicated by curve 2′, which is drawn parallel to curve 1, then the conclusion might be drawn that the surviving fraction of cells is equal to about 4%. Comparison of this value with the curve of Fig. 2B, shows that this assumption is not valid either, and it must be concluded that the recurrence of the irradiated tumour is a more complex phenomenon. This curve shows that the fraction of cells capable for unlimited proliferation, remains constant during about 3 days after irradiation. This does not imply of course that no proliferation of cells occurs, but rather that, after an unknown period of mitotic delay, a large fraction of the cells that have lost the capacity for unlimited proliferation, may divide a few times and consequently the ratio of the fractions of clonogenic and

non-clonogenic cells remains approximately constant. This proliferation of cells that have lost the capacity for unlimited proliferation is also reflected in the fact that growth of the tumour continues for 2 days after irradiation.

The curve of Fig. 2B shows further that, starting at approximately the fourth day after irradiation, the fraction of clonogenic cells increases very rapidly and at 9 days after irradiation, this fraction has returned to a value which is equal to that of unirradiated tumours. This rapid increase is due to two factors, namely elimination of non-clonogenic cells from the tumour and proliferation of clonogenic cells. These data clearly show that the measurements of changes in tumour volume alone provide very little information about the phenomena occurring inside the tumour at the cellular level.

(b) *Cell proliferation characteristics before and after irradiation*

The suggestion that proliferation of cells in an irradiated tumour during regression and recurrence may occur at a rate which is larger than the rate of the proliferation in an unirradiated tumour of the same volume, has been made before, based on a comparison of the growth rate of the tumour with measured or assumed survival curves of the cells [9, 16, 19]. The data concerning changes of fractions of clonogenic cells with time, provide experimental evidence of this phenomenon but they do not show whether the small volume decrease and the rapid recurrence of this tumour after irradiation is due to an increase in the growth fraction, defined by Mendelsohn [3] and discussed by Tubiana [16], or whether it is due to a shortening of the cell cycle.

In principle, changes in growth fraction can only cause a relatively rapid repopulation of the tumour after irradiation if in the untreated tumour the growth fraction was considerably smaller than 1. Furthermore, changes in cell cycle times can cause a relatively rapid repopulation only if the cycle times of cells in unirradiated tumours were longer than the minimum cell cycle time possible under the most favourable conditions. For instance, in case the cells in the untreated tumour are all engaged in proliferation and if all these cells have a cell cycle time which is as short as possible under optimal conditions, then no accelerated repopulation of the tumour by surviving cells can be expected after irradiation. These conditions appear to be approximately met by rapidly growing experimental tumours [20, 21]. On the other hand, if a tumour contains many non-proliferating cells, i.e. 'resting but viable' cells, and if the cell cycle times of the proliferating cells show large variations and are relatively long compared with the minimum cell cycle time possible under optimal conditions, then after irradiation the surviving cells may repopulate the tumour at a rate which is larger than anticipated from the proliferation characteristics before irradiation. These conditions appear to prevail in slowly growing solid tumours in animals and man. The cause for such an increase in proliferation of surviving cells may be complex and might depend on improvement of the supply of nutrients or oxygen [22] or on changes in spatial relationships with other cells due to cell loss.

In order to obtain further insight in the cellular phenomena occurring after irradiation in the R-1 rhabdomyosarcoma, the cell proliferation characteristics, which were determined from the results of labelling experiments, will be analysed.

(b 1) *Unirradiated tumours*

Labelling experiments with small tumours of 0·3 cm^3 have been described previously and the results are summarized in Table 2, together with those of tumours of 1·5 cm^3 [7]. As noted earlier, considerable variations in fractions of labelled cells are observed, depending on the location in the tumour. Especially in the large tumours, the fractions of labelled cells are larger in the periphery than in the centre. In order to assess the importance of these differences for the growth of the tumour, an estimate of the relative volumes of the peripheral and the central part of the tumour must be made. From Fig. 3 and many similar data for both small and large tumours, it can be derived that the peripheral part with a high labelling index extends to approximately 2 mm from the capsula of the tumour. The histological cross sections of the tumours investigated, have a mean diameter of about 12 mm. For a spherical tumour, calculation shows that a centre with a diameter of 8 mm comprises about one third of the total volume. This value is presented in the first row of Table 2. For a small tumour with a volume of 0·3 cm^3, the central part comprises less than 10% of the total volume. For these small tumours the growth rate will mainly be determined by cells localized in the peripheral part and in Table 2, separate cell cycle parameters for the central part of small tumours have not been included [6].

The total number of cells in a tumour was estimated from a comparison of its DNA content with the DNA content per cell deter-

mined in a suspension containing a known number of cells [8]. These values are presented in the second row of Table 2. The numbers of cells present per unit volume in small and large tumours did not differ significantly. Consequently the decrease of the tumour volume doubling time with increasing tumour volume, as shown in Fig. 2A and presented in the third row of Table 2, is not due to a change in the mean volume per cell.

In order to evaluate in more detail the cause of the decrease in growth rate with tumour volume and its relation to the growth fraction and the cell cycle parameters, it is necessary to calculate a number of characteristics of the cell proliferation. These calculations are based on a mathematical analysis described in detail by Steel [2]. In this analysis a potential doubling time T is defined as the 'expected doubling time of the tumour in the assumed absence of cell loss' [2, 23]. T can be calculated from

$$T=\frac{\lambda \,.\, T_S^*}{LI} \tag{1}$$

and

$$\lambda=\frac{T}{T_S}\left[\exp\left\{\frac{\ln 2}{T}\,(T_{G_2+M}+T_S)\right\}-\exp\left\{\frac{\ln 2}{T}\,.\,T_{G_2+M}\right\}\right]. \tag{2}$$

Employing an approximation method outlined by Steel [2], λ and T can be calculated if T_{G_2+M}, T_S and LI are known. In Table 2, the values of T and λ, calculated by this method, are presented for the different tumours in the central and the peripheral parts respectively. From the values of T and T_C it is then possible to calculate a factor α according to:

$$\ln\alpha=\frac{T_C}{T}\,(\ln 2) \tag{3}$$

where α is the number of proliferating cells (denoted P-cells) produced per division. If a number of assumptions, specified by Steel [24] are made, the fraction of proliferating cells or growth fraction f is equal to:

$$f=\alpha-1. \tag{4}$$

These assumptions pertain to identical phasing of the cell cycle and the influence of starting conditions. If these assumptions are met, growth of the tumour is exponential. Since the R-1 rhabdomyosarcoma does not grow exponentially, the values of the various calculated characteristics must be considered as approximations. Consideration of possible variations in the measured parameters shows however, that the uncertainties in the estimated growth fractions do not exceed 10%. Accordingly, the difference between the growth fractions in small and large tumours respectively is sufficiently large to conclude that a significant reduction has occurred during growth of the tumour from 0·3 cm³ to 1·5 cm³.

Estimates of α and f are presented in Table 2. The value of $f=0{\cdot}42$ for the small tumours differs little from the value of 0·40 quoted earlier, derived by a different method [7]. When f is known, the fraction of non-proliferating cells (Q-cells) can be calculated as equal to $(1-f)$.

For the large tumours, estimates of T, α, f and $(1-f)$ can be made for the centre and the periphery separately. By considering the relative volumes of the centre and periphery, it is possible to calculate mean values of α, f and $(1-f)$ for the whole tumour.

From the values of T for the centre and periphery another parameter can be calculated namely the rate constant k_{P+Q} for the production of new cells:

$$k_{P+Q}=\ln 2/T. \tag{5}$$

From the values of k_{P+Q} for the different parts of the tumour, it is possible to calculate mean values for each tumour, taking into account the relative contributions of the centre and the periphery to the total volume. From the mean k_{P+Q} value derived in this way, a mean value for T can be calculated. The rate constant for cell loss k_L can subsequently be calculated from

$$k_L=k_{P+Q}-k \tag{6}$$

where k is the rate constant for growth and is equal to

$$k=\ln 2/T_d \tag{7}$$

(see Table 2). The cell loss factor has been defined by Steel [24] as

$$\phi=k_L/k_{P+Q}. \tag{8}$$

A mean life span of non-proliferating cells can be derived if the assumption is made that only Q-cells are eliminated from the tumour and that this loss occurs randomly distributed in time:

$$T_{(Q)}=\frac{1-f}{k_L}\,. \tag{9}$$

From the calculated parameters summarized in Table 2, a number of conclusions can be drawn with respect to the growth of unirradiated tumours.

*For the definitions of the symbols compare Table 2.

Comparison of the parameters for small and large unirradiated tumours shows that the mean cell cycle times of the proliferating cells in the small tumours and in the peripheral part of the large tumours respectively, are approximately equal, whereas in the central part of the large tumours, the cell cycle is longer due to an increase of the G_1-period. Furthermore the slow growth of the large tumours as compared with the small tumours is due to a lower factor α, and consequently a lower growth fraction f, in both the central and peripheral areas. This implies that the number of new proliferating cells (P-cells) produced per division is lower in large tumours, resulting in a larger fraction of non-proliferating cells (Q-cells) and a larger cell loss factor. The mean life span of the Q-cells has decreased only slightly, however. It is of interest to note that relatively small changes in the growth fraction f, which depends on the factor α, and in the cell cycle parameters, which determine the potential doubling time T, cause a much larger change in tumour doubling time T_d. Variations in the fractions of proliferating cells with tumour volume have been reported for other solid tumours as well [3, 5]. Variations in cell cycle parameters have been reported for an ascites tumour transplantable in mice [20]. In the R-1 rhabdomyosarcoma both factors contribute about equally to the decrease in growth rate.

(b 2) *Irradiated tumours*

A similar analysis as described for the proliferation characteristics of unirradiated tumours can be made from the data measured for irradiated tumours, labelled at four days after irradiation. As noted earlier, the cell cycle time measured in these irradiated tumours during the period of regression, is shorter as compared with the controls. The values for T_{G_2+M} and T_S are not considerably different from those of cells in unirradiated tumours, but the G_1-period is virtually absent. Similar reductions of cell cycle times have been reported by others for normal tissues [25, 26]. Furthermore, as shown in Table 1, Table 2 and Fig. 3, the differences between the labelling indexes in the periphery and in the centre are much smaller in the irradiated tumours as compared with the controls, due to a markedly larger labelling index in the centre of the irradiated tumour.

The rate of decrease in volume of the irradiated tumour can be characterized by a negative doubling time of -96 hr, determined from the tangent to the growth curve 2 of Fig. 2A at $t=4$. This value is only an approximate measure of the rate at which the total number of cells in the tumour decreases. The number of cells/cm^3 is equal to $5{\cdot}0\times10^8$ in unirradiated tumours, whereas in the irradiated tumours at four days after irradiation this number is equal to $2{\cdot}7\times10^8$, i.e. a factor of 0·54 lower as compared with unirradiated tumours. However, between day 4 and 5 after irradiation, the number of cells/cm^3 does not change significantly. It can be concluded that a decrease in cell density has occurred during the first few days after irradiation, but at $t=4$, the rate of decrease of the volume is a good measure of the rate at which the number of cells decreases.

The results of calculations of growth characteristics of irradiated tumours are presented in Table 2. As noted earlier, it should be emphasized that the results of the calculations must be regarded as approximations, because the assumptions discussed by Steel [2, 24] with respect to the applicability of the mathematical analysis are not met. However, these uncertainties do not preclude some qualitative conclusions to be drawn.

The potential doubling time of the irradiated tumour is equal to 38 hr. This value is smaller than the corresponding value of large unirradiated tumours and is very close to the value of small unirradiated tumours. It can be concluded that the rate of cell production in these tumours at four days after irradiation is not much smaller as compared with unirradiated tumours. It is important to note that this cell production in the irradiated tumours is mainly due to proliferation of cells, which are not capable for unlimited proliferation. Figure 2B shows that at four days after irradiation the fraction of clonogenic cells is still less than 1%. This small fraction of 1% of cells, capable of unlimited proliferation, cannot be distinguished from the 99% of cells, which still proliferate but will eventually die.

The cell loss factor in the irradiated tumour is much larger than in the control tumours and the calculated mean life span of the Q-cells is much shorter. It may be concluded that in the irradiated tumour at four days after irradiation cells die at a high rate, but the volume decreases not very rapidly and not to a large extent because cell production by cells which will die eventually, continues at a relatively high rate. Repopulation of the tumour by cells which have retained the capacity for unlimited proliferation becomes important only after the fourth day post-irradiation and this will be the subject of a subsequent publication.

(c) *Cells cultured* in vitro

The cell cycle parameters and growth characteristics of R-1 rhabdomyosarcoma cells in small and large clones in culture were determined in order to relate the changes observed *in vitro* with changes occurring in growing tumours. The data obtained for small clones, containing an average of 80 cells, show all characteristics of an exponentially growing population, in which all cells proliferate ($f=1$) and very little cell loss occurs ($\phi=0{\cdot}05$). The doubling time of 17 hr of the cell population is approximately equal to the cell cycle time, which is equal to the potential doubling time of 16 hr. In the large clones, containing an average of 1200 cells, the cell cycle parameters are not significantly different from those of cells in small clones, but the growth fraction is only 39% as compared with 100% in the small clones. Similar observations have been reported by Frindel [27]. It is of interest to note that this decrease with increasing clone size, in the growth fraction of cells cultured *in vitro* cannot be due to inadequacy of the nutrient or oxygen supply, because the medium of the cultures was changed frequently. Other factors, e.g. lack of space, must be assumed to cause the reduction in growth fraction with increasing population volume. The cell loss factor is slightly larger in large clones as compared with small clones and the mean life time of *Q*-cells is approximately 200 hr.

It can be concluded that the decrease in growth fraction and the increase in the potential doubling time, which is inversely proportional to the rate of cell production, observed during tumour growth, is also observed in cultured cell populations. This may be considered as an indication that neither in the tumour nor in culture it is the supply of oxygen or nutrients which reduces the growth rate, but that the lack of physical space is the limiting factor.

The similarity in the decrease in the growth fraction between cell populations in culture and cell populations in the tumours is also of interest because in culture it has been shown that cells in plateau phase of growth fail to repair sub-lethal damage [28]. The problem of whether *Q*-cells in tumours can repair sub-lethal damage awaits further investigation.

Investigation of variations in α-values are in addition of great interest because, as Bresciani [29] has discussed in detail, this factor determines to a large extent whether a cell population will grow indefinitely.

(d) *Concluding remarks*

The results presented in this paper suggest a tentative interpretation of the phenomena occurring at the cellular level after irradiation of the R-1 rhabdomyosarcoma with a single dose of 2000 rad of X-rays. This dose produces a relatively small decrease of the tumour volume by 25% and a rapid recurrence, notwithstanding the fact that more than 99% of the cells have lost the capacity for unlimited proliferation.

During the first two days after irradiation tumour growth continues, but the growth rate decreases, while starting on the third day the tumour volume decreases as well. At the fourth day after irradiation, the cell loss factor was found to be much larger as compared with unirradiated tumours. This increase in cell loss must have started before the fourth day. Fractions of damaged mitoses, measured as a function of the time after irradiation, show a rapid increase to a maximum of about 90% between 24 and 48 hr after irradiation, with a subsequent decrease at later intervals. This indicates that the cell loss factor increases rapidly during the first two days after irradiation. As a consequence the cell density of the tumour will decrease, followed by a decrease in tumour volume starting on day 3 after irradiation. The extent of this decrease in tumour volume is relatively small however, due to the fact that all *P*-cells, including those which have lost the capacity for unlimited proliferation, start to reproduce with a cell cycle time which is much shorter than that of *P*-cells in unirradiated tumours. Thus a large decrease in tumour volume is prevented by a temporary rapid proliferation of cells which are mainly capable of only a *limited* number of divisions. The shortening of the cell cycle of these cells might result from the increase in space available per cell, caused by the increased cell loss.

Starting on day 4 after irradiation, the small fraction of cells capable of *unlimited* proliferation was found to increase rapidly. This results in a resumption of growth of the tumour at about day 8 after irradiation and a regrowth to the pre-irradiation volume at about day 12. Further investigations of cell cycle times and cell loss factors on day 8 and 12 are in progress in order to evaluate whether in the recurrent tumour these parameters and the growth fraction show important changes.

The changes in cell cycle parameters observed after a single dose of 2000 rad, invite the speculation that responses of these cells to subsequent treatments with either radiation or chemotherapeutic agents, might be altered as

compared with untreated tumours and that these changes might provide a possibility to achieve improved treatment schemes in radiotherapy combined with chemotherapy.

RESUME

Les paramètres de la prolifération cellulaire ont été étudiés dans un rhabdomyosarcome transplantable, chez le rat WAG/Rij. La durée des différentes phases du cycle cellulaire est mesurée par la méthode des mitoses marquées, après injection in vivo *de thymidine marquée au tritium, pendant la croissance des tumeurs, et après irradiation. Les résultats sont comparés avec les phénomènes observés sur les mêmes cellules, cultivées* in vitro.

On observe que le temps de doublement T_d des tumeurs témoins augmente de 2,5 *jours pour les petites tumeurs* (0,3 cm^3) *à* 6,0 *jours pour les grosses tumeurs* (2,5 cm^3). *Cette augmentation est causée partiellement par l'allongement de la durée du cycle des cellules, mais surtout par le fait que le coefficient de prolifération diminue et que le nombre des cellules éliminées augmente. Le pourcentage des cellules proliférantes (growth fraction) diminue de* 42% *à* 29%.

Le volume des grosses tumeurs (1,5 cm^3) *irradiées par une dose de* 2000 *rad de rayons X, diminue, et, dix jours après l'irradiation, elles atteignent un volume représentant* 75% *du volume originel. Quatre jours après l'irradiation, le pourcentage de cellules capables de multiplication illimitée n'est plus que* 0,8%. *La durée du cycle des cellules proliférantes est réduit à* 12 *heures. Le nombre de cellules éliminées dépasse le nombre de cellules produites, et c'est là la cause de la réduction de volume.*

La durée moyenne du cycle des cellules du rhabdomyosarcome cultivées in vitro *est de* 16 *heures pour les cellules proliférant dans de petites colonies (de* 80 *cellules) et de* 16,5 *heures pour les cellules proliférant dans de vastes colonies (de* 1200 *cellules). Le pourcentage des cellules proliférantes (growth fraction) est de* 100 *pour les petits colonies et atteint une valeur de* 39% *pour les grandes colonies.*

SUMMARY

Proliferation characteristics of cells in an experimental rhabdomyosarcoma, transplantable in an inbred strain of WAG/Rij rats, have been studied for unirradiated and irradiated tumours by autoradiography. The cell cycle parameters have been compared with equivalent data for the same strain of cells cultured in vitro. *Fractions of labelled cells and labelled mitoses were counted as a function of time after pulse labelling with tritiated thymidine. The changes in proliferation characteristics of cells in the tumours are discussed in relation to the volume variations of the tumours before and after irradiation.*

The results show that the tumour growth rate decreases with increasing tumour volume. The volume doubling time T_d is 2·5 *days for a tumour volume V of* 0·3 cm^3 *and* $T_d =$ 6 *days at* $V = 1{\cdot}5$ cm^3. *This decrease is due to a change in cell cycle parameters, including a lengthening of the mean cell cycle from* 20 *to* 25 *hr in parts of the tumour corresponding to a decrease in the rate of production of new cells, as well as to an increase in the rate of cell loss. The growth fraction in small tumours is equal to* 0·42 *and in the large tumours to* 0·29.

After irradiation with 2000 *rad X-rays of tumours with a volume of* 1·5 cm^3, *tumour volume decreases and on the tenth day it reaches a value of about* 0·75 *of the pre-irradiation volume. However, the fraction of cells which after this radiation dose have retained the capacity for unlimited proliferation is equal to only* 0·008. *Measurements of cell cycle parameters on the fourth day after irradiation showed that cells present in the tumour at that time proliferate with a shorter cell cycle time of about* 12 *hr. Cell loss at four days after irradiation exceeded cell production however and caused the volume to decrease.*

Cell cycle parameters of the same cell line of rhabdomyosarcoma cells cultured in vitro *showed that in small clones containing about* 80 *cells, the mean cell cycle time is* 16 *hr, the growth fraction is* 1·0 *and no cell loss occurs, whereas in large clones containing about* 1200 *cells, the mean cell cycle time is* 16·5 *hr, the growth fraction is* 0·39 *and the cell loss factor is small.*

ZUSAMMENFASSUNG

Die Charakteristiken der Zellproliferation wurden mittels ^{3}H-Thymidin und Autoradiographie bei bestrahlten und unbestrahlen experimentellen Rhabdomyosarkomen untersucht, welche auf Inzucht-WAG/Rij-Ratten transplantiert waren. Ebenfalls wurden der Generationszyklus und seine Teilphasen von den in Gewebekultur gezüchteten Rhabdomyosarkomzellen ermittelt.

Die Wachstumskurve des Rhabdomyosarkoms weicht vom exponentiellen Typ ab, und zwar so, daß die Volumenverdoppelungszeit (T_d) zunimmt von T_d=60 Stunden für kleine Tumoren (0,3 cm^3) bis zu T_d=144 Stunden für grössere Tumoren (1,5 cm^3). Die zeitliche Änderung der Volumenverdoppelung ist nicht nur zum Teil zurückzuführen auf eine Zunahme der Zellzyklusdauer von 20 Stunden in kleinen Tumoren bis zu 25 Stunden in grösseren Tumoren, sondern auch auf wichtige Faktoren wie die Abnahme der Fraktion proliferierender Zellen von 42% bis 29% und die Zunahme der Fraktion eliminierter Zellen.

Nach Behandlung mit 2000 rad Roentgenstrahlen nimmt das Tumorvolumen ab und erreicht nach 10 Tagen einen Mindestwert von 75% des ursprünglichen Volumens. Dieser anscheinend geringe Effekt ist in scharfem Kontrast zu dem Befund, daß der Prozentsatz überlebender Zellen mit unbegrenzter Proliferationsfähigkeit, bestimmt mittels der Zellklonbildungstechnik, nur um 0,08% beträgt. Zellgenerationszeitenmessungen zeigen, daß die Zellproliferation ab 4 Tagen nach der Bestrahlung mit verkürzter Zellzyklusdauer stattfindet. Die Elimination abgetöteter Zellen überschreitet 4 Tage nach der Bestrahlung dennoch die Zellproduktion.

Die Zellgenerationscharakteristiken von in vitro *gezüchteten Zellen, studiert in kleinen Zellklonen (80 Zellen pro Klon) und großen Zellklonen (1200 Zellen pro Klon) zeigen, daß die respektiven Zellgenerationszeiten nicht wesentlich voneinander abweichen, und daß die Werte der respektiven Wachstumsfraktionen von 100% bis 39% abnehmen.*

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Enhancement by Hypophyseal Hormones on the Malignant Transformation of Transplanted Hyperplastic Nodules of the Mouse Mammary Gland

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IN THE development of mammary carcinoma in mice two distinct phases can be distinguished, first the development of hyperplastic nodules and second the transformation of these nodules into carcinoma. It can be considered as established, that all known causative factors in the genesis of mammary carcinoma as hormones and viruses are involved in the development of hyerplastic nodules [1]. Less certainty exists if these factors are also instrumental in the transformation of these nodules into carcinoma.

In the present investigation an attempt has been made to determine if hypophyseal hormones enhance the transformation from nodule to carcinoma.

MATERIAL AND METHODS

(♀C_3H × ♂0_{20})F_1 females were used. These females have the mammary tumour virus from their C_3H-mothers. They have a high incidence of mammary cancer; after force-breeding 95% have a mammary tumour at an average age of 254 days. When these females have a palpable tumour in one mammary gland the hyperplastic nodules are usually abundant in the other mammary glands. In untreated females they are recognizable with the naked eye. Owing to their yellowish pigmentation they are clearly distinct from the surrounding normal mammary gland tissue and appear as "spots" about 0·5 mm in diameter. Only these yellowish nodules have been used for transplantation. Besides these yellowish nodules similar nodules with a pink colour or a greyish colour can be recognized in the mammary glands of older females. The nodules with a pink colour are small carcinomas, the nature of the greyish nodules has still to be determined. For the transplantation the nodules are cut out with a pair of fine scissors and freed as much as possible from the surrounding normal mammary gland and fat tissue.

The host animals are 21 days old female (♀C_3H × ♂0_{20}) F_1 hybrids. The fat pads of the fourth mammary glands on both sides are freed from mammary gland tissue according to the method of DeOme [2]. The nodules are transplanted into the remaining part of the cleared fat pads.

The host animals were kept three in a cage with food pellets and tapwater *ad libitum*. Tumours were noted when palpable. Tumours

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appearing in one of the transplanted nodules were extirpated together with the whole fat pad, so that the development of the tumour on the other side could be awaited. When the second tumour appeared, the animal was sacrificed. In some cases mammary tumours developed in the own glands of the host, before tumours appeared in the transplanted nodules. These tumours were also extirpated to prolong the life of the animals. A histological diagnosis of the mammary tumours was made; the outgrowth of the nodule was studied in a "whole mount" preparation, also in animals in which no tumour appeared, to ascertain the fate of the transplanted nodule.

The host animals with the transplanted nodules were divided in two groups. One group of animals received no further treatment; in the second group of animals a hypophysis was transplanted together with the nodule into the fat pad. It is known that an additional hypophysis producing prolactine is a strong hormonal stimulus, which leads to the development of mammary carcinoma even in the absence of the mammary tumour virus [3]. The fat pad of the mammary gland has been proved as one of the most efficient sites for the isograft of the hypophysis. As donors of the hypophysis male and female adult (♀C_3H × ♂0_{20}) F_1 hybrids were used.

RESULTS

The fate of the transplanted nodules

After transplantation the hyerplastic nodules show a characteristic growth pattern which has been described as hyperactive outgrowth [4]. This outgrowth is in structure similar to a prelactating mammary gland or a mammary gland stimulated by prolactine. Mammary tumours can develop in any part of these outgrowths.

In group I without further treatment 90 nodules were transplanted in 50 animals. No data are available from 2 transplants, no tissue was found in 4 cases and in 10 cases the transplant was still surviving but no outgrowth occurred. The remaining 74 nodules showed well developed outgrowth into the fat pad. In 53 of the 74 transplants a further analysis of the outgrowth could be made. In 23 cases the outgrowth was hyperactive, whereas the outgrowth in 21 cases was showing normal gland structure the remaining 9 cases showed a mixture between hyperactive and normal outgrowth. It should be noted however that this analysis could only be done in a part of the outgrowth in case the tumour covered a greater part of the transplanted nodule.

Group II with hypophyseal isografts

One hundred and four nodules have been transplanted in 53 animals. Eighty-five transplants showed well developed outgrowth. No data are available from 5 transplants, no tissue was found in 5 cases and 9 transplants showed only stunted growth. From the 85 well developed transplants 71 were available for further analysis. Because of the hormonal stimulation by the hypophyseal isograft no outgrowth of normal gland structure could be found in this group. Only in 2 transplants part of the outgrowth had normal gland structure. Thirty-two of the 68 transplants had an outgrowth comparable to the outgrowth in group 1. The remaining 36 transplants showed a hyperactive outgrowth with a much stronger hormonal activation: the lobular development was more massive and the ducts were filled with a milky fluid.

The development of mammary tumours in the transplanted nodules

In the control group 52 mammary tumours developed (70%) in the 74 well transplanted nodules at a mean age of 180 days. In the experimental group with an additional hypophysis implanted into the fat pad 56 mammary tumours (66%) developed in the 85 well transplanted nodules at a mean age of 150 days. The difference in the average tumourage is statistically significant. The additional hypophyses transplanted into the fat pad have an enhancing effect on the development of a tumour from the nodule.

A comparison made on the tumour development in the different forms of outgrowth from the transplanted nodule shows an earlier tumour development in the hyperactive outgrowth than in the normal outgrowth. A tumour can, however, also develop in the normal outgrowth of a nodule. No differences were found in the morphological appearance of the tumours in both groups.

DISCUSSION

From the results of this investigation it can be concluded that a hypophyseal isograft in the fat pad next to a transplanted nodule has a growth stimulating effect and enhances the transformation of a nodule into a mammary carcinoma. Remarkable is the spread of the time at which after a transplantation of the nodule a tumour develops. Probably this has to be interpreted as a sign that the nodules are in different stages of transformation to tumours. There are, however, no morphological criteria to recognize the different stages of nodules.

Table 1. *Transplantation of nodules into the cleared fat pad without and with hypophyseal isografts*

	Nodules transplanted	
	Without hypophys. isograft	With hypophys. isograft
Number of transplants per number of animals	90/50	104/53
Evaluation of the transplanted nodule		
positive	74=84%	85=86%
stunted growth	10=11%	9= 9%
negative	4= 5%	5= 5%
no data	2	5
Outgrowth in a positive nodule transplant	total 53	total 71
++=hyperactive + lact. cells		++=24
+=hyperactive outgrowth		++/+=12
n=normal outgrowth		++/*n*=1
	+=23	+=31
	+/*n*=9	+/*n*=1
	n=21	+/*n*=2
Number of tumours	50=70%	56=66%
Average tumour age	180 days	150 days
Range	72–384 days	60–257 days
Number of tumours in the different forms of outgrowth	+=19 (148 days)	++=19 (138 days)
	+/n=5 (204 days)	++/+=4 (176 days)
	n=7 (249 days)	++/*n*=–
		+=7 (161 days)
		+/*n*=1 (190 days)
		n=0

There is also no absolute correlation with the form of outgrowth. After transplantation the nodule shows partly hyperactive outgrowth and normal outgrowth. With transplantation of a hypophysis into the fat pad at the same time practically, no normal outgrowth is found; all nodules show hyperactive outgrowth (Table 1).

There is convincing evidence that the formation and the maintenance of nodules in the mammary gland is dependent on hormones [5]. After hypophysectomy the nodules disappear if the hypophysectomy is done two months before the appearance of a mammary tumour. When one mammary tumour in a mammary gland has appeared and the hypophysectomy is done at this moment other mammary tumours in the other glands do appear. This must be interpreted that the transformation of the nodules to carcinoma has already reached a hormone independent stage [6]. In this investigation the nodules were taken from the donors when they had already a mammary tumour in one mammary gland. This probably explains why in many cases the tumour development occurred so rapidly in the transplanted nodules.

It can be assumed that the higher amount of hormones present and produced by the hypophyseal isograft especially of prolactin is the accelerating factor.

The tumour-formation is distinctly enhanced in a hyperactive outgrowth after intensive hormonal stimulation.

In a recent publication [7] it was reported that the tumour potential of a nodule cell population can also be increased by exposure to the Bittner mammary tumour virus. Both factors—hormonal and viral—which are instrumental in the first phase of mammary carcinogenesis are therefore also operative in the second phase of malignant transformation of the hyperplastic nodule.

RESUME

L'apparition d'un nodule hyperplastique constitue la première manifestation du carcinome mammaire chez la souris. On démontre que des nodules transplantés se transforment plus vite en carcinomes quand une isogreffe hypophysaire est placée à côté du transplant. La stimulation hormonale plus forte accélère la transformation du nodule hyperplastique.

SUMMARY

The first step in the development of mammary carcinoma in mice is the formation of a hyperplastic nodule. Hormonal and viral factors are instrumental in the formation of the nodule. It is shown that transplanted nodules transformed earlier into a carcinoma when a hypophyseal isograft next to the transplant was present. The stronger hormonal stimulation accelerates the tumour formation from a hyperplastic nodule.

ZUSAMMENFASSUNG

Der erste Schritt in der Entwicklung eines Mammacarcinoms bei der Maus ist die Bildung eines hyperplastischen Knotens. Hormonale und virale Faktoren sind bei der Entstehung dieses Knotens beteiligt. Es wird jetzt gezeigt dass transplantierte Knoten früher in ein Carcinom transformiert werden, wenn ein Hypophysentransplantaat gleichzeitig gegeben wird. Die grössere hormonale Stimulation beschleunigt die Carcinombildung aus dem hyperplastischen Knoten.

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Surface Properties of Normal Human Circulating Small Lymphocytes and Lymphocytes in Chronic Lymphocytic Leukaemia: Separation, Adhesiveness and Electrokinetic Properties

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INTRODUCTION

THE HETEROGENEITY and abnormal properties of circulating lymphocytes in patients with chronic lymphocytic leukaemia (C.L.L.) have been discussed recently [1, 2]. The column of polystyrene beads (PSB) used during blood fractionation to remove (by retaining) the polymorphonuclear neutrophils (PMN) retained also significantly large numbers of C.L.L. lymphocytes in certain cases. This suggested the presence in the circulation of these patients of lymphocytes with an abnormal adhesiveness. These could later [3] be characterized as also abnormal by their ultrasensitivity *in vitro* to the cytocidal action of colchicine, as could the majority of lymphocytes in populations isolated from each case. Colchicine-ultrasensitive C.L.L. lymphocytes, adhesive or non-adhesive, are here termed 'abnormal' cells. Populations of both types could display the abnormal surface property of decreased membrane fragility (see reference 2, Fig. 6).

In the present work the technique of cell electrophoresis was applied to lymphocytes from normal blood, adhesive abnormal C.L.L. lymphocytes and non-adhesive abnormal C.L.L. lymphocytes from leukaemic blood in order to investigate the physico-chemical properties of their surface regions. Factors affecting adhesiveness on PSB have been investigated.

Lack of knowledge on the electrophoretic properties of human lymphocytes is surprising but perhaps a reflection of difficulties attending reported methods for separating lymphocytes from other blood cells. The method for preparing lymphocytes here had to be varied to establish reliable means for collecting electrokinetic data. In particular, those red cells remaining after bulk sedimentation with a macromolecular reagent had to be destroyed in order to ensure recognition of lymphocytes once placed in the electrophoresis chamber. A preliminary report on one aspect of this work has recently been published [4].

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PATIENTS, MATERIAL AND METHODS

Patients

Thirteen patients were investigated (seven for electrokinetic studies), in whom the diagnosis of C.L.L. had been established for maximum periods of between 1 month and 10 years. None had ever received radiotherapy. Six (cases 1, 2, 3, 5, 8 and 10) had received or were receiving various forms of chemotherapy at the time of any investigation and one of these (case 5) had been splenectomized. The other seven patients (cases 4, 6, 7, 9, 11, 12 and 13) had never received any chemotherapy. The clinical and haematological state of all these untreated patients and of treated patient 6 was stable at the time of every investigation whereas that of the other untreated patients was generally unstable.

Materials

Gelatin (batch 213 or 277) was provided by the Galtine and Glue Research Association, Birmingham, U.K. and carbonyl iron powder (type SF) by Fine Dyestuffs & Chemicals Ltd., Manchester, U.K. For electrokinetic studies 0·145M NaCl, made 10^{-4}M with respect to $NaHCO_3$, pH7·2±0·2 [5], was used. Adjustments of this "standard saline" to other pH values were made with 0·145 N HCl or 0·145 N-NaOH. The buffered BSS (B.B.S.S.) used in preparation and elution of polystyrene columns and for trypsin incubations was a modified Tyrode medium containing 30 mM tris/HCl, pH 7·4. For adhesion experiments requiring lower pH values and for incubations with neuraminidase 0·05 M acetate buffers were prepared

Fig. 1. *Summary of experimental procedure prior to electrokinetic studies*

Venous blood
(ca.50 ml; freshly withdrawn)

Heparinixed blood
(2·5 units/ml.)

Defibrinated blood
(Obtained mechanically, by rotation with glass beads on suspension mixer for 15 min)

PMN removal (first stage)
Exposure to carbonyl iron powder (100 mg./10 ml. blood) on rotating suspension mixer at 37°C for 30 min (cf. ref. 7)

Leukaemic donors: Usually unnecessary owing to high blood-lymphocyte count

Normal donors: Alternative to exposure of GS to column of polystyrene beads (PSB)

Gelatin sedimentation
Heparinized/Defibrinated/Iron-treated defibrinated blood
plus
One-third its volume of gelatin solution (3% in saline).
Maintained at 37°C for 30 min

Gelatin supernatant
(Red cell-depleted upper layer)

Gelatin plasma (GP)

Normal donors: Where no iron treatment performed above, PMN removed by exposure of GS at 37°C for 30 min to polystyrene beads (PSB) housed in a column and pre-equilibrated in cell-free GS.

Gelatin serum (GS)

PMN removal
Normal or leukaemic donors: Where iron treatment performed above, GS stood over magnet and PMN-depleted supernatant aspirated off.

(i) *Standard adhesion test* (on GS lymphocytes from leukaemic donors): To assess the proportion of adhesive/non-adhesive lymphocytes in constituent abnormal lymphocyte population, sample of GS exposed for 30 min. at 37°C to column of PSB pre-equilibrated in cell-free GS conditions under which normal lymphocytes largely non-adherent (see text). Non-adhering cells removed by displacing GS with two column volumes of buffered balanced salt solution (B.B.S.S.) at a flow rate of 1 ml/min.

(ii) *Standard survival test* (on GS and GP lymphocytes from leukaemic donors): To determine the abnormal lymphocyte content from 20-hour cultures run in the presence of 10^{-7} M colchicine (cf. ref. 3).

(iii) *Electrokinetic studies* (on GS- and GP-lymphocytes resuspended in saline after washing in saline and with or without prior removal of residual red cells).

Residual red cell removal

(A) *Hypotonic shock*: 30-second exposure of cells to water, restoration of isotonicity, washing and resuspension in standard saline (cf. ref. 6).

(B) *Exposure to ammonium chloride:* Incubation of cells in 0·75% NH_4Cl at room temperature (ca.22°C) (cf. ref. 8). Centrifugation (70**g**) commenced after 7 min and resuspension in saline for first washing accomplished after a total (optional) exposure of 16–18 min (determined from haemolysis rate curve). Lymphocytes resuspended in saline for electrophoretic mobility determination after a second wash in saline.

Normal donors: Method (B) only employed
Leukaemic donors: Methods (A) and (B) compared

in 0·65% saline. Crude trypsin ('1:250') and cryst, trypsin ('Tryptar') were supplied by Difco Laboratories Detroit, Michigan and Armour Pharmaceutical Co. Eastbourne, U.K. Neuraminidase (500 units per ml.), a Behringwerke preparation from *Vibrio cholerae*, was obtained from Koch-Light Laboratories, Slough, U.K. The polystyrene beads (styrene divinylbenzene copolymers with 8% cross-linking, 40–60 mesh; dia. 0·25–0·42 mm) were provided by the Dow Chemical Co. (U.K.) Ltd, and were prepared for use in glass columns as described previously (6).

Methods

Separation and characterization of lymphocytes for electrokinetic studies. In Fig. 1 a summary is given of the various ways used to prepare lymphocytes for electrokinetic studies and of the methods for measuring the abnormal cell content and adhesive abnormal cell content of C.L.L. lymphocyte populations.

Counting and cytologic analysis. The procedure of Trowell [9, 10] involving wet fixation in susa and staining with Mayer's haemalum was used extensively to prepare smears for differential cell-counting and for cytologic examination of lymphocytes after culture and after haemolytic or other treatments. All cell counting in the adhesion studies was performed by haemocytometer.

Electrophoretic mobilities. These were determined at 25°C using an electrophoresis chamber of circular cross section with some modifications [4].

Some of the data were programmed for a digital computer (TITAN) and the values of electrophoretic mobilities and standard deviation were rounded off to the second place of decimal, since calculation of these values to the third place of decimal is beyond the scope of the experimental technique.

RESULTS

ADHESIVE STUDIES

Adhesiveness of normal and C.L.L. lymphocytes on PSB at 37°C.

Normal lymphocytes suspended in gelatin-serum (GS) were largely unretained by columns of PSB at 37°C (range of recovery for 13 healthy donors 52–92%; median, 73%). Recoveries were consistently higher (80–100% for 7 donors; median 91%) when the column had been pre-equilibrated in cell-free GS in place of B.B.S.S. (Table 1). The GS populations from 13 C.L.L. patients were tested under these conditions (standard adhesion test, Fig. 1). The populations contained 70% or more abnormal (colchicine-ultrasensitive) cells. Serial investigations showed that in each of four cases (treated patients 1 and 2; untreated patients 6 and 9) two-thirds or more, on average, of the abnormal lymphocytes were retained on the columns (Table 1) compared to one-third or less for the other nine cases. Beads removed and stained revealed adhering lymphocytes (see reference 7, Fig. 7). Subsequent tests were limited to certain patients.

In protein-free media (NaCl or B.B.S.S.) GS lymphocytes from patients (Cases 1, 3 and 6) and normal donors were retained (Table 1). Retention of normal cells in this situation was largely prevented when beads had been pre-exposed to cell-free GS and rinsed twice with B.B.S.S., implying that beads had been coated with GS proteins. Retention was not prevented using B.B.S.S. containing gelatin, implying that serum proteins were responsible for lack of adherence of normal cells in GS.

Attempts to detach adhering normal and C.L.L. lymphocytes

C.L.L. lymphocytes adhering on PSB in protein-free media were not detached by persistent elution of the column with media at decreasing pH (7·4 down to 5·5) (patient 6), by incubation and elution with 0·02% EDTA (disodium salt) (patient 2), by incubation with neuraminidase (200 units per ml.,

Table 1. Temperature, serum and trypsin treatment as factors affecting adherence of GS lymphocytes (Fig. 1) on columns of polystyrene beads

Column conditions*			Percentage non-adhesive	
Medium†	Temp (°C)	Pre-treatment of lymphocytes	Normal Donors	Leukaemic donors‡
Serum present	37	None	80–100	30
Serum (homologous, normal) present	37	None	—	30
Serum present	37	Incubated with crude or cryst. trypsin and washed	—	80
Serum present	4	None	—	90
Serum absent	37	None	10	10
Serum absent	4	None	10	10

*First-noted conditions, those of standard adhesion test (Fig. 1).

†Each serum-containing medium was gelatine-serum as arising in blood fractionation (Fig. 1), except at 4°C where serum saline used to avoid gelation.

‡Average values for experiments with certain patients with C.L.L. (see text).

Table 2. Characteristics and treatment of lymphocytes from two C.L.L. patients for electrophoretic mobility determination in NaCl at pH 7·2±0·2 at 25°C (cf. Fig. 1.)

Case No.	Starting blood sample			Resultant red cell-depleted 'GP' or 'GS' suspension						
				Cells for culture with colchicine (10^{-7}M)	Cells for polystyrene column	Saline-washed cells for electrophoreisis				
						Destruction of residual red cells		Description of resultant NaCl-washed cells		
	Nature	White cells (per mm³)	Lymphocytes (%)	Pyknotic lymphocytes after 1 day (%)	Lymphocytes adhering (%)	Agent	Lymphocyte mortality induced %	Ratio of lymphocytes to red cells	Pyknotic lymphocytes (%)	Average mobility of lymphocytes* (μ/sec/V/cm)
2	Heparinized	124,000	99	—	—	None	—	12:1	1	−1·01 (20)
								170:1	2	−1·05 (28)
						None	—	16:1	2	−1·02 (20)
	Defibrinated	112,000	99	96	83	NH_4Cl	2	170:1	2	−1·01 (22)
						H_2O	3	90:1	2	−1·02 (36)
5						None	—	16:1	1	−1·07 (20)
	Heparinized	75,000	95	80	—	NH_4Cl	2	500:1	2	−1·09 (20)
						H_2O	13	500:1	2	−1·07 (20)
						None	—	16:1	1	−1·06 (20)
	Defibrinated	73,000	95	80	5	NH_4Cl	1	500:1	1	−1·10 (20)
						H_2O	14	500:1	1	−1·07 (20)

*Number of lymphocytes in parentheses. Standard deviation: ±0·08.

45 min., pH 5·5) (patient 1), by incubation with 0·2% or 2% Difco '1:250' trypsin (30 min, pH 7·4) (patients 3 and 4, respectively), or by reducing the temperature to 4°C (patients 1 and 2).

Lymphocytes from normal donors adhering in protein-free media were likewise not detached by enzymic treatment or reduction in pH. They were detached progressively by repeated incubation and elution with a medium containing autologous serum (30%).

Attempts to affect abnormal adhesiveness

Abnormal adhesiveness of C.L.L. lymphocytes was not affected when GS cells (patient 2) were washed twice in NaCl before exposing to the column resuspended in cell-free GS or when the washed cells (patients 1 and 2) were resuspended in cell-free GS prepared from fresh homologous serum obtained from healthy individuals (Table 1). Adhesiveness in GS was not affected significantly by the presence of 0·02% (patient 2) or 0·2% (patients 1 and 2) EDTA, or after GS cells (patient 1) had been incubated in protein-free medium with neuraminidase (200 units per ml per 5×10^6 lymphocytes, 45 min). Adhesiveness in GS was, however, markedly altered by pre-incubation in protein-free medium with 0·2% Difco '1:250' trypsin (Table 1). In one experiment (patient 1) the proportion of non-adhesive cells increased from 14% without to 65% with treatment and in two other experiments (patient 2) from 11% to 75% and 95%, respectively. An equivalent concentration (0·03% in B.A.E.E. units) of crystalline trypsin was also effective and a sticky, mucous-like material was produced (cf. reference 11).

When C.L.L. lymphocytes (patient 1) were applied to a column in protein-free medium at 4°C they behaved like normal by adhering, as at 37°C (Table 1). In contrast, when cells were applied at 4°C in the presence of serum (Table 1) abnormal adhesiveness manifested at 37°C was lost as shown by recoveries increasing to 86% (patient 2) and 96% (patient 1). Abnormal adhesiveness was restored by rewarming to 37°C.

ELECTROKINETIC STUDIES

Electrophoretic mobility at pH 7·2 ±0·2

Certain base-line observations (Table 2) were made possible using blood samples from Patients 2 and 5. The samples contained similar, high proportions of abnormal lymphocytes and contrasting proportions of adhesive abnormal lymphocytes. The losses of lymphocytes from trapping during defibrination were small. The GS and GP populations could thus be presumed equally representative of their respective circulating populations and investigations could be made of effects on mobility from the use of heparin. Combination of high peripheral count and efficient action by gelatin led to a proportion of residual red cells low enough to permit measurement of lymphocyte mobility without additional haemolytic treatment. This facilitated the investigation for effects of heparin and also permitted investigation of any effects on mobility from the heamolytic treatments. This was in turn facilitated by the fact that negligible lymphocyte mortality was induced by either treatment.

Electrophoretic data on lymphocytes from five other patients is given in Table 3. The loss of lymphocytes during defibrination varied from patient to patient and was excessive (75%) for patient 1. The GS populations

Table 3. Electrophoretic mobility (μ/sec/V/cm) of lymphocytes from C.L.L. patients and normal donors in NaCl *at pH* 7·2±0·2 *at* 25°

	Lymphocytes from heparinized blood (GP cells)			Lymphocytes from defibrinated blood (GS cells)		
Case No.	Before use of haemolytic agent	After use of NH_4Cl	After use of H_2O	Before use of haemolytic agent	After use of NH_4Cl	After use of H_2O
1	—	−1·13 (34)	−1·09 (42)	—	−1·16 (30)	−1·09 (19)
6	−1·08 (30)	−1·09 (33)	—	—	−1·05 (30)	−1·01 (20)
9	—	—	—	—	−1·05 (30)	−1·06 (26)
12	−1·08 (20)	−1·08 (20)	−1·08 (18)	−1·09 (20)	−1·10 (20)	−1·07 (20)
10 (22 Aug. 67)	—	−1·27 (32)	—	—	−1·25 (30)	1·21 (41)
10 (23 Oct. 67)	—	−1·05 (20)	−1·00 (22)	—	−1·08 (20)	−0·97 (36)
NORMAL a	—	—	—	—	−1·09 (30)	—
NORMAL b	—	—	—	—	−1·11 (22)	—
NORMAL c	—	—	—	—	−1·17 (30)	—
NORMAL d	—	−1·04 (15)	—	—	—	—

Note: Number of lymphocytes in parentheses. S.D. ±0·08.

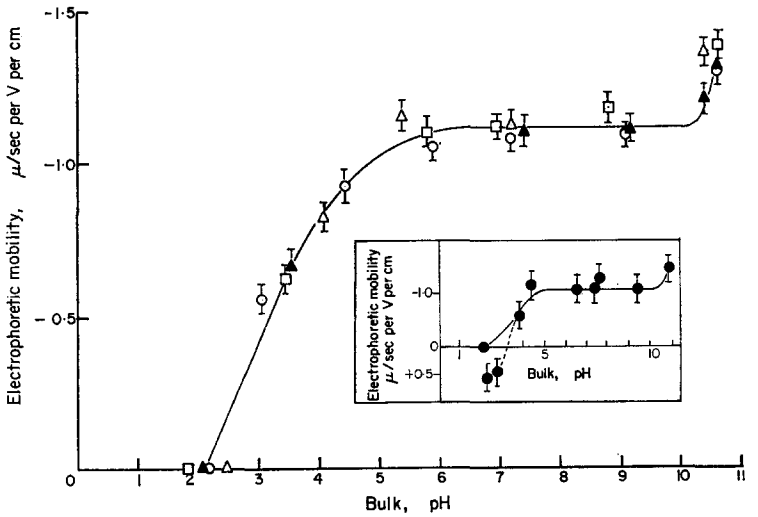

Fig. 2. pH-electrophoretic mobility relationships for saline-washed lymphocytes resuspended in 0·145 M *saline determined at* 25°C. 0·145N *HCl or* 0·145N *NaOH were used to alter the pH values and the ionic strength was kept constant at* 0·145. *The inset shows a pH-mobility relationship of C.L.L. lymphocytes (Case* 1) *together with the effect of prolonged exposure to low pH in producing positive values of electrophoretic mobilities (stippled portion of curve). (The inset is reproduced from reference* 4 *with permission of the Editor, Nature).*

○ *Case* 5: *C.L.L. lymphocytes (G.P. cells, isolated from heparinized blood).*
□ *Case* 12: *C.L.L. lymphocytes (G.P. cells, isolated from heparinized blood).*
△ *Case* 1: *C.L.L. lymphocytes (G.P. cells, isolated from heparinized blood exposed to NH_4Cl and saline-washed).*
▲ *Case* 1: Different experiment: *C.L.L. lymphocytes (G.P. cells, isolated from heparinized blood exposed to NH_4Cl and saline-washed).*
● *Case* 1: *C.L.L. lymphocytes (GS cells—isolated from defibrinated blood).*

Standard deviations are shown. Some of the data programmed for a digital computer (TITAN) to work out the mean and the standard deviation.

contained varying proportions of adhesive and non-adhesive abnormal cells (see above). Exceptionally high mortality (50%) was induced in the population from patient 9 by water treatment which was important to note since this leads to selective loss from normal populations [6].

Within experimental error, and with the exception of one experiment (patient 10; 22.8.67) the individual average values of mobility for C.L.L. lymphocytes (Tables 2 and 3) and lymphocytes from normal donors (Table 3) are not significantly different from one another.

Electrokinetic stability and viability

No changes in mobility resulted from lymphocytes being kept in NaCl awaiting measurement for several hours or after incubation at 37°C when losses of intracellular K^+ ions were detected by flame photometry. Pyknosis induced by NH_4Cl in normal or C.L.L. populations never exceeded 3%. Normal-lymphocyte populations exposed to this haemolytic agent were further shown by cytologic analysis of 3-day cultures to have retained their capacity for survival *in vitro* but not their proliferative potential, as shown by 10% less of a population becoming transformed in the presence of phytohaemagglutinin (cf. reference 6).

Mobility as a function of pH

Electrophoretic mobilities were determined in the pH range 1·8–10·7 at constant ionic strength (0·145). Only in a few experiments were enough lymphocytes available to permit sufficient measurements for establishing a pH-mobility relationship. The relationships for 4 such experiments concerning three different cases (Patients 1, 5 and 12) could be described by a single curve, which is shown in Fig. 1.

A gradual discharge over the pH range 4·8–2·0 is observed, suggestive of more than one type of ionogenic group contributing to the electrokinetic charge. The isoelectric points (majority of cells) in the pH range 1·8–2·2 indicate the presence of a strongly acidic group such as a phosphoric acid ester group or a sialic acid carboxyl group, the pK of which is estimated to be approximately 3. To ascertain whether a sialic acid carboxylic group was contributing to the anodic mobility,

Table 4. Electrophoretic mobility (μ/sec/V/cm) at 25°C of C.L.L. lymphocytes† following neuraminidase treatment and saline wash*

	pH 7·2–7·4		pH 10·5–10·7		pH 2·1‡	
Case No.	Control	Exptl.	Control	Exptl.	Control	Exptl.
1	−1·10 (10)	−0·29 (7)	−1·32 (10)	−1·21 (10)	0	0
5	−1·01 (10)	−0·29 (7)	−1·15 (10)	−0·58 (10)	0	0
12	−1·12 (10)	−0·51 (10)	−1·37 (10)	0·77 (10)	0	+0·35 (10)

In each experiment 100 units neuraminidase incubated with $50\text{-}60 \times 10^6$ lymphocytes at pH 5·8 for 45 min at 37°C in 0·7 ml reactiom mixture made 0·005M with respect to $CaCl_2$.
*S.D.±0·08 No. of lymphocytes measured in parentheses.
†GP cells (Fig. 1) treated with NH_4Cl and saline-washed.
‡Whether or not mobility zero was observed within 1 min of adjusting pH.

electrophoretic mobilities of lymphocytes were determined after treatment with neuraminidase. The data are given in Table 4.

The electrokinetic behaviour of lymphocytes in the pH region 6–9·5 (Fig. 2) shows the complete discharge of the acidic groups and in the pH region 9–10·8 suggests the presence of a basic group(s) with a pK of 10·5.

Lymphocytes isolated from a healthy individual (case 'Normal d') behaved like abnormal lymphocytes by exhibiting a mobility of −1·39±0·11 μ/sec/V/cm (10 cells) at pH 10·7 and zero mobility at pH 2·0.

Electrokinetic response

Determinations of electrophoretic mobility at non-physiological pH values were made in awareness of possible effects of time. The time required to reduce bulk pH (to 1·8–2·2) and to observe that the mobility of the majority of the lymphocytes was zero was less than 2 minutes. The insert in Fig. 2, reference 4 illustrated an experiment where the mobility was initially zero at pH 2·0 but where positive mobilities were recorded after the lymphocytes had remained at low pH (2–3) for more than 10 min.

Determination of mobility of 10 cells at other non-physiological pH values could be completed by the time 4 minutes had elapsed after adjusting the pH. In three experiments when pH was restored to 7·2±0·2 from between 10 and 1 after 4, 5 and 7 min, respectively, the original mobility of the lymphocytes was also restored. Reversible electrokinetic behaviour of lymphocytes which had been held at the isoelectric point for 4 min was also observed.

Cytologic analysis

All lymphocytes appeared intact when examined by oil immersion in wet fixed smears of neutralised suspensions prepared at times varying between 2·5 and 4·5 min after altering bulk pH to a value in the range 2·0–2·5, or at times varying between 3·5 and 6 min after altering pH to 10·5–10·6. No striking changes were observed in nuclei after exposure to the high pH but in lymphocytes exposed to acid, a redistribution of chromatin was suggested by prominent staining of the nuclear membrane and loss of staining between chromatin blocks. This effect was similar to the primary changes seen in cultured lymphocytes in the process of turning pyknotic but did not result in altered electrophoretic mobility at physiological pH.

DISCUSSION

The fact that normal and abnormal lymphocytes were retained on polystyrene columns at 4°C as well as at 37°C (Table 1) in the absence of serum suggests that adhesion was not dependent upon cellular behaviour but upon surface composition alone as a result of some form of bonding between the cells' surface regions and polystyrene. The observed reversal or prevention of adhesion of normal cells observed at 37°C using serum was probably partly the result of lack of affinity for a new surface produced by adsorption of serum proteins on polystyrene (cf. ref 12). Shortman [13] showed that serum could reduce the retention of lymphoid cells on siliconised glass beads at 4°C. Application of serum as a desorbing agent has already been proposed for trial as a means of sub-fractionating small lymphocytes into different classes [2]. However, it has since been observed (unpublished) that a normal population could not be sub-fractionated when progressively desorbed in this way at 37°C according to tests made on lymphocytes in the eluates for differences in

membrane fragility or modified ability to respond to the action of PHA [6].

Lack of affinity for serum-coated polystyrene would also explain why abnormal cells failed to adhere in serum at 4°C and leads to possibility that the adhesiveness displayed at 37°C was the result of abnormal cellular activities. Schrek's observation of abnormal motile behaviour of lymphocytes in C.L.L. [14] lends support to this idea. Moreover, the loss of capacity for adherence in serum at 4°C observed in the present experiments applied to the PMN (from normal donors or leukaemic patients) whose adhesiveness on glass surfaces is probably affected by reduced temperature concomitantly with loss of glycolytic activity and motility [15]. A different mechanism would, however, appear to operate for the adhesion of abnormal lymphocytes since, unlike PMN [15, 16] the use of EDTA as a chelating agent for bivalent cations (Ca^{2+} and Mg^{2+}) did not reduce adhesiveness. At the same time, the action of trypsin in abolishing adhesiveness would seem to implicate surface composition in addition to abnormal behaviour patterns of the lymphocytes.

Hedeskov and Esman [17] have pointed out that no great differences exist between the glycolytic activity of normal lymphocytes and lymphocytes in C.L.L. and no clear cut effect has been noted here from preliminary attempts to affect adhesiveness by inhibition of glycolysis with iodoacetamide (4×10^{-3}M). Whilst it might be argued that adhesiveness is a property of abnormal lymphocytes in early stages of degeneration *in vitro*, the standard column did not retain abnormal cells where these had first been allowed to turn completely pyknotic. It is also of interest that PMN characterized as dead on account of exhibiting nuclear pyknosis had lost their capacity to adhere on the standard column.

The fact that the same retention of abnormal rather than ostensibly-normal lymphocytes occurred at 37° in the two cases (patients 1 and 2) tested when homologous serum from healthy people replaced autologous serum, further supported the view that adhesiveness was due to abnormalities in the leukaemic cells rather than the leukeamic serum. But the involvement of serum factors in the adhesion process cannot yet be excluded. Lack of such factors may have prevented the detection of all adhesive cells, particularly in those cases (patients 5, 11, 12, 13) where the degree of retention did not exceed the range for populations from normal people (up to 20%). On the other hand, it is striking that the retention of abnormal cells from patient 2 was observed (unquoted experiment) to *increase* as the concentration of serum in suspension of GS cells applied to separate columns was diminished from a standard concentration of 60% to 15%. An outcome of this effect was a separation of ostensibly normal from abnormal cells improved from six-fold to nine-fold such that the proportion in the post-column populations increased from 50% to 70%. If this were a general phenomenon, such a procedure could prove important for obtaining ostensibly normal cells free from abnormal cells for separate study.

Electrokinetic properties

The present studies do not suggest differences in surface composition of adhesive abnormal, non-adhesive abnormal or normal lymphocytes.

Enzyme treatment. A sialomucopeptide has been demonstrated at the surface of EAT cells [18–20]. The electrokinetic data on nerauminidase-treated, saline-washed lymphocytes could be taken as possible evidence for the presence of bound sialic acid (cf. reference 21). The reduction noted in one experiment (unquoted) following treatment with trypsin may be consistent with sialic acid-bound protein having been removed (cf. also reference 5, 22, 23). However the effects of neuraminidase should be treated with extreme caution due to possible action or adsoprtion of impurities in the preparation and/or adsorption of products leaking from the cells [24, 25].

Amino and sulphydryl groups. The electrokinetic behaviour of lymphocytes at alkaline pH (Fig. 2) suggests the presence of amino (18) and sulphydryl groups [4, 26, 27], the high pK (10·5) resulting from their interaction as in the case of cysteine [28]. The indication for SH groups is of further particular interest because of its possible bearing upon the ultraradiosensitivity of lymphocytes but will require confirmation with the use of specific reagents.

Heterogeneity. An indication of heterogeneity in the sets of lymphocytes providing each average value of electrophoretic mobility was given by the deviation of mobilities of individual cells from the mean ($\pm0\cdot08$ μ/sec/V/cm) being greater than that observed for red cells ($\pm0\cdot02$ μ/sec/V/cm), calculated for each experiment separately but under identical conditions.

Lymphocyte separation. The lack of influence of method of preparation or treatment with heparin, water, or ammonium chloride upon

electrokinetic properties noted from the studies at pH 7·2±0·2 was confirmed in several experiments where comparisons were made of the average mobility at non-physiological pH values. Whilst no selective loss of abnormal or ostensibly-normal lymphocytes would seem to occur during defibrination [29], the use of heparin as anti-coagulant might be recommended for future use in electrokinetic studies since it ensures that the lymphocyte population isolated is fully representative of that in the blood sample. It should be stressed however that higher concentrations than 2·5 units per ml of blood will be needed to prevent the delayed clotting effects sometimes observed.

Ruhenstroth-Bauer [30] obtained the buffy layer by oblique sedimentation of citrate-treated blood and examined the cell mixture (containing erythrocytes, lymphocytes and granulocytes) in an electrophoresis chamber equipped with copper/copper sulphate electrodes and prepared haemocytopherograms. He reported that the electrophoretic mobility of lymphocytes from a healthy man was −1·009±0·035 μ/sec/V/cm, but in twenty cases of lymphatic leukaemia the normally moving lymphocytes were almost missing and a fast-moving fraction, near the erythrocyte band with a mobility of −1·202 μ/sec/V/cm with a much higher value of standard deviation (±0·150 μ/sec/V/cm) than for the normal sample, was observed. Unfortunately, the details of the heterogeneity of the lymphocyte population in the cell mixture from the buffy layer, the composition of the suspending medium and its ionic strength are not given in reference 30 and direct comparison with the present data on characterised lymphocytes is difficult.

Acknowledgements—The authors thank Miss Ann Reid for skilled technical assistance, Mrs. Mary Robinson for help during the early part of the work and Professor G. Wetherley-Mein, Department of Haematology, St. Thomas's Hospital, London for providing the patients for study and for the great interest he has shown. The authors are grateful to Dr. G. F. Marrian, F.R.S. and Professor J. S. Mitchell, F.R.S. for supporting this collaborative work. Financial support to J. N. M. from the British Empire Cancer Campaign and Professor Mitchell's Research Fund is gratefully acknowledged.

RESUME

(1) *En l'absence de sérum, les lymphocytes de patients atteints de leucémie lymphoïde chronique (L.L.C.) se comportent comme les lymphocytes d'individus normaux, en adhérant aux colonnes de grains de polystyrène, tant à 37°C qu'à 4°C. Chez 4 patients sur 13 (2 traités et 2 non traités), les deux tiers des lymphocytes anormaux, très sensibles à la colchicine, manifestent une anomalie supplémentaire en adhérant en présence de sérum à 37°C. Ces cellules sont appelées 'cellules anormales adhérentes'. Cette adhésivité peut être abolie par le traitement à la trypsine, ou, de façon réversible, en opérant à 4°C. Ces observations semblent mettre en évidence, pour ces cellules, une motilité différente de celle des cellules normales.*

(2) *A la suite des résultats d'une expérience, on suggère d'utiliser des concentrations plus faibles de sérum, de façon à retenir une plus forte proportion de cellules anormales sur les grains de polystyrène, et de façon à obtenir, en vue d'expériences ultérieures, des populations de lymphocytes de L.L.C. ayant, vis-à-vis de la colchicine, un comportement normal (lymphocytes sensibles résistant à la colchicine). La désadsorption des petits lymphocytes normaux des colonnes ne contenant pas de protéines peut être réalisée grâce à l'addition de sérum, et peut être recommandée comme un procédé capable de fractionner les populations normales en plusieurs types fonctionnels.*

(3) *Chez des donneurs normaux et chez des patients atteints de L.L.C. (4 traités et 3 non traités), les lymphocytes isolés par diverses méthodes, lavés deux fois dans le sérum physiologique (solution 0, 145 M de NaCl, pH 7,2±0,2), et remis en suspension dans le sérum physiologique, avaient une mobilité électrophorétique d'une valeur moyenne de −1,09±0,08 μ/sec./V/cm. L'utilisation de l'héparine comme anticoagulant (2, 5 unités par ml de sang), de même que l'expositions des lymphocytes au NH_4CL, ou à un choc hypotonique, en vue de détruire les globules rouges contaminants, n'a pas d'effet sur la mobilité électrophorétique. En dehors d'une seule exception, on n'a pas observé de différence de mobilité entre lymphocytes normaux et lymphocytes de L.L.C., ou entre lymphocytes de leucémie lymphoïde chronique chez divers patients, en dépit de variations de l'état clinique, hématologique ou thérapeutique, et malgré la proportion variable de cellules adhérentes normales dans les différentes populations de cellules étudiées.*

(4) *Les variations de la mobilité en fonction du pH ont été étudiées chez 4 patients atteints de L.L.C. Le comportement électrocinétique des lymphocytes semble résulter de la présence*

de plusieurs groupes ionisables (acides et basiques) à la surface des cellules adhérentes anormales et des cellules anormales non adhérentes. Le pK d'un groupe acide est évalué à 3, *et est probablement dû à un groupe carboxyl de l'acide sialique. Les groupes basiques sont probablement des groupes aminés et/ou sulfhydryles, avec un pK de* 10,5. *Dans une expérience, ces groupes paraissent aussi présents à la surface des petits lymphocytes normaux.*

SUMMARY

(1) *In the absence of serum, lymphocytes from patients with chronic lymphocytic leukaemia (C.L.L. lymphocytes) behaved like lymphocytes from healthy individuals (normal lymphocytes) by adhering to columns of polystyrene beads at* 37°C *and/or at* 4°C. *Two-thirds or more of the abnormal (colchicine-ultrasensitive) cells contained in the populations isolated from* 4 *of the* 13 *patients* (2 *treated,* 2 *untreated) displayed further abnormality by adhering in the presence of serum at* 37°C *and are termed 'adhesive abnormal cells'. This type of adhesiveness could be abolished by treatment with trypsin or, reversibly, by operating columns at* 4°C, *and it is suggested that it reflected motile behaviour different to normal cells.*

(2) *The results of one experiment suggest the future trial of reduced serum concentrations, leading to higher retention of abnormal cells on polystyrene beads, as a means of obtaining ostensibly-normal (colchicine-resistant) members of C.L.L. lymphocyte populations for separate study. Desorption of normal small lymphocytes from protein-free columns with the aid of serum is recommended for trial as a possible means of subfractionating normal populations into different functional types.*

(3) *The lymphocytes which had been isolated in different ways from the blood samples (normal donors and* 7 *C.L.L. patients;* 4 *treated,* 3 *untreated) washed twice in the standard saline* (0·145 M *NaCl; pH:* 7·2±0·2) *and resuspended in saline were observed to have average electrophoretic mobility values of* −1·09±0·08 μ/sec./*V*/*cm. No effects upon electrophoretic mobility were noted from the use of heparin* (2·5 *units per ml. of blood) as anticoagulant, or after exposing lymphocytes to* NH_4Cl *or hypotonic shock treatment in order to remove residual red cells. With one exception, no differences were noted between the mobilities of normal lymphocytes and C.L.L. lymphocytes, or C.L.L. lymphocytes from different patients, despite variations in clinical, haematological and therapeutic status, and despite the presence of contrasting proportions of adhesive abnormal cells in the different populations.*

(4) *The pH-mobility relationships for* 4 *C.L.L. patients were established and suggest that the electrokinetic behaviour of the lymphocytes arises as a result of the presence of several ionizable (acidic and basic) groups on the surface of adhesive abnormal and non-adhesive abnormal cells. The pK of one acidic group is estimated to be* 3 *and it is probably due to a sialic acid carboxyl group. The basic groups are probably amino and/or sulphydryl with a pK of* 10·5 *and from one experiment these would appear to be present also on the surface of the normal small lymphocyte.*

ZUSAMMENFASSUNG

(1) *In der Abwesenheit von Serum verhielten sich die Lymphozyten von Patienten mit chronisch lymphatischer Leukämie (C.L.L. Lymphozyten) beim Anhaften an einer Säule von Polystryolperlen bei* 37°C *und/oder* 4°C *wie Lymphozyten von gesunden Personen. Zwei Drittel oder mehr der abnormalen (colchicinultrasensitiven) Zellen, welche in der Menge enthalten waren und von* 4 *bis* 14 *Patienten isoliert wurden, zeigten eine weitere Abnoramalität beim Anhaften in Gegenwart von Serum und bei* 37°C. *Sie werden als abnormal haftende Zellen bezeichnet. Diese Art von Anhaftungsfähigkeit konnte durch die Behandlung mit Trypsin aufgehoben werden oder sie wurde umgekehrt durch die Benutzung der Säulen bei* 4°C. *Es wird angenommen, daß es das Beweglichkeitsverhalten widerspiegelt, welches anders als das normaler Zellen ist.*

(2) *Das Ergebnis eines Experimentes läßt annehmen, daß für einen künftigen Versuch die Serumkonzentration herabgesetzt werden muß, welche zu einer höheren Retention von abnormalen Zellen an Polystryrolperlen führt, um 'ostensibly' normale (colchicinresistente) Zellen von C.L.L. für andere Versuche zu erhalten. Die Ablösung von normalen kleinen*

Lymphozyten von proteinfreien Säulen mit Hilfe von Serum wird als Versuch empfohlen, als eine Möglichkeit, eine normale Zellpopulation in andere funktioneller Art zu unterteilen.
(3) *Die Lymphozyten, welche man auf verschiedene Weise aus Blutproben isolierte (normale Spender und 7 C.L.L. Patienten; 4 behandelt, 3 unbehandelt) wurden zweimal in der Standardsalzlösung* (0,145 M—*NaCl; pH:* 7,2±0,2) *gewaschen und wieder in Salzlösung aufgeschwemmt. Es wurde beobachtet, daß diese Zellen eine durchschnittliche elektrophoretische Beweglichkeit von* −1,09±0,08 μ/*sec./V/cm haben. Bei der Anwesenheit von Heparin als Antikoagulum konnte kein Effekt auf die elektrophoretische Beweglichkeit festgestellt werden* (2,5 *Einheiten Heparin/1 ml Blut*), *desgleichen nicht nachdem die Lymphozyten mit* NH_4Cl *behandelt wurden oder bei einer hypotonischen Schockbehandlung, um die restlichen roten Blutzellen zu entfernen. Nur mit einer Ausnahme wurden keine Unterschiede zwischen der Beweglichkeit von normalen und C.L.L. Lymphozyten oder C.L.L. Lymphozyten von verschiedenen Patienten beobachtet, trotz Variation im klinischen, hämatologischen und therapeutischen Status und trotz der Anwesenheit von gegensätzlichen Proportionen von in der Anhaftung abnormalen Zellen in den verschiedenen Populationen.*
(4) *Das Verhältnis von pH-Wert zu Beweglichkeit für 4 C.L.L. Patienten wurde ermittelt und dahin gedeutet, daß das elektrokinetische Verhalten der Lymphozyten als Ergebnis der Gegenwart von verschiedenen ionisierbaren (sauren und basischen) Gruppen an der Oberfläche von abnormal haftenden und nicht haftenden abnormalen Zellen erscheint. Der pK von einer sauren Gruppe wird mit 3 geschätzt, es kommt wahrscheinlich durch eine CarboxylGruppe der Sialsäure. Die basischen Gruppen sind wahrscheinlich Amino- und/oder Sulfhydrylgruppen mit einem pK von* 10,5. *Nach einem Experiment ist anzunehmen, daß sie auch an der Oberfläche von normalen kleinen Lymphozyten anwesend sind.*

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Announcements

International Symposium of the C.N.R.S. on White Cell Transfusions

Centre National de la Recherche Scientifique
15, quai Anatole-France, Paris 7e
Wednesday 18th and Thursday 19th June 1969

Chairmen: C. GORDON ZUBROD, National Cancer Institute (Bethesda) and GEORGES MATHÉ, Institut de Cancérologie et d'Immunogénétique (Villejuif) and Institut Gustave-Roussy (Villejuif).

Secretary: LÉON SCHWARZENBERG, Institut de Cancérologie et d'Immunogénétique, Hôpital Paul-Brousse, 14, avenue Paul-Vaillant-Couturier, 94 Villejuif, France.

I. Wednesday 18th June

A. Morning session: 9 a.m.

1. Introduction:
   G. MATHÉ (Villejuif)
2. Physiological problems:

Chairman: S. FARBER (Boston)

(a) Stem cell problems in the use of white cell transfusions:
E. MCCULLOCH (Toronto)

(b) Kinetics problems in the use of white cell transfusions:
T. M. FLIEDNER (Ulm)

(c) Preservation of cells at low temperatures:
P. J. PEGG (London)

(d) White cell histocompatibility:
J. DAUSSET (Paris)

(e) Discussion

Moderator: G. HAMILTON FAIRLEY (Sutton)

B. Afternoon session: 2 p.m.

3. Granulocyte transfusions:

Chairman: E. FREI (Houston)

(a) Prevention of septicemia by granulocyte transfusion in the irradiated dog:
D. THOMAS *et al.* (Seattle)

(b) Haematopoietic graft after irradiation and buffy coat cell transfusion:
D. THOMAS *et al.* (Seattle)

(c) White cell transfusions in the curative treatment and the prevention of infections:
E. HENDERSON *et al.* (Bethesda)
L. SCHWARZENBERG *et al.* (Villejuif)
J. TANZER (Paris)

(d) The side effects of white blood cell transfusions on recipients:
Immunization:
J. L. AMIEL *et al.* (Villejuif)
Graft vs. host:
G. MATHÉ *et al.* (Villejuif)
M. J. DE VRIES (Rijswijk)

(e) The effect of leucophoresis on donors:
E. FREIREICH (Houston)
E. HENDERSON (Bethesda)
L. SCHWARZENBERG (Villejuif)
(f) Discussion:
Moderator: H. E. M. KAY (London)

II. Thursday 19th June

A. Morning session: 9 a.m.

4. Lymphocyte transfusions and cancer adoptive immunotherapy
Chairman: J. BURCHENAL (New York)
(a) Introduction: Small and large lymphocytes:
J. G. HALL (London)
(b) Lymphocyte paralysis:
by incubation at 37°C:
J. F. DORE *et al.* (Villejuif)
by antilymphocyte serum:
D. W. VAN BEKKUM *et al.* (Rijswijk)
(c) Use of lymphocytes in cancer therapy:
Transfusions of lymphocytes from donors not immunized against the tumour:
Experimental:
M. BORANIC (Rijswijk and Zagreb)
G. MATHÉ *et al.* (Villejuif)
Clinical: Adoptive immunotherapy of leukemic patients:
L. SCHWARZENBERG (Villejuif)
E. FREIREICH (Houston)
Transfusions of lymphocytes from donors immunized against the tumour:
P. ALEXANDER (London)
(d) Discussion:
Moderator: D. BERGSAGEL (Toronto)

B. Afternoon session: 2 p.m.

5. Continuous flow blood cell separator:
Chairman: J. F. HOLLAND (Buffalo)
(a) The use of the separator to obtain granulocytes:
R. GRAW (Bethesda)
(b) The use of the separator to obtain lymphocytes:
E. FREIREICH (Houston)
L. SCHWARZENBERG (Villejuif)
(c) The use of the "separator lymphocytes" for A.L.S.:
J. CHOAY *et al.* (Paris)
(d) The use of the separator for plasma exchange:
D. THOMAS (Seattle)
(e) Other uses
(f) Future prospects in the development and utilisation of the cell separator:
Engineering aspects:
G. T. JUDSON (I.B.M., New York)
Medical aspects:
S. PERRY (Bethesda)
(g) Discussion:
Moderator: P. CARBONE (Bethesda)
6. General conclusions:
C. G. ZUBROD (Bethesda)

Plenary Session of the European Organisation for Research on Treatment of Cancer (EORTC) and its Cooperative Groups

Conference Hall of the
Centre National de la Recherche Scientifique
15, quai Anatole-France, Paris 7e
Friday 20th and Saturday 21st June 1969

First day: Recent advances in the treatment of acute leukaemia

A. Morning session: 9 a.m.
Chairman: S. FARBER (Boston)

1. Introduction:
   S. FARBER (Boston)
2. New drugs in chemotherapy:
   Rubidomycin:
   J. BERNARD (Paris)
   The "Leukaemia and Hematosarcoma" group of the EORTC
   P. STRYCKMANS (Bruxelles)

   L. Asparaginase:
   J. BURCHENAL (New York)
   G. HAMILTON FAIRLEY (Sutton)
   M. HAYAT (Villejuif)

   Other drugs:
   E. HENDERSON (Bethesda)
   other speakers

3. Induction of remissions by chemotherapy:
   E. FREI (Houston)
   D. A. G. GALTON (London)
   other speakers

4. Complementary chemotherapy:
   J. F. HOLLAND (Buffalo)
   other speakers

B. Afternoon session: 2 p.m.
Chairman: D. BERGSAGEL (Toronto)

5. Active immunotherapy:
   G. MATHÉ (Villejuif)
   G. HAMILTON FAIRLEY (Sutton)

6. Adoptive immunotherapy:
   (a) Summary of the white cell transfusion meeting:
   P. ALEXANDER (Sutton)
   (b) J. L. AMIEL (Villejuif)
   E. FREIREICH (Houston)

7. Acute leukaemia secondary to:
   (a) chronic myeloid leukemia and polycythemia vera:
   P. CARBONE (Bethesda)
   J. BOUSSER (Paris)
   P. STRYCKMANS (Bruxelles)
   M. SCHNEIDER (Villejuif)
   (b) lymphosarcoma and reticulosarcoma:
   J. R. SCHLUMBERGER (Villejuif)
   J. F. HOLLAND (Buffalo)

8. Protected environments and new antibiotics:
   M. SCHNEIDER and D. SELAYA (Villejuif)
   H. KAY (London), Sister LYNCH and Sister BERN (Sutton)
   E. FREI (Houston)
   T. M. FLIEDNER (Ulm)

9. Long term survivors:
   (a) without intensive treatment:
   J. BURCHENAL (New York)
   (b) after intensive treatment:
   J. L. AMIEL (Villejuif)

10. Who must treat acute leukaemia patients?:
    J. F. HOLLAND (Buffalo)

11. General conclusions:
    C. G. ZUBROD (Bethesda)
    G. MATHÉ (Villejuif)

Second day: Free communications on experimental cancer treatment

These communications can be presented by members of the EORTC or its cooperative groups or by non-members introduced by a member of the EORTC.

The titles and abstracts of the proposed communications must be sent to Professor G. Mathé before 1st May 1969, who will submit them to a selection committee and let the authors know if they have been accepted.

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Der Einfluss von Methylcholanthren auf das Schrumpfungsverhalten der Mausehaut

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EINLEITUNG

SOWOHL in der Embryologie [1] wie auch bei der Regeneration [2] ist die steuernde Wirkung des Mesenchyms bzw. der Dermis auf Proliferation und Differenzierung der Epidermis bekannt. Transplantationsversuche, bei denen die Differenzierung der adulten Epidermis von der unterlagernden Dermis gesteuert worden war [3], zeigten eine solche Wirkung auch für die physiologische Regeneration der Epidermis. Es liegt deshalb die Vermutung nahe, daß Differenzierungsrückgang und unkontrollierte Proliferation, wie sie bei malignem Wachstum der Epidermis zu beobachten sind, auf Defekte der Dermis zurückzuführen sind. Untersuchungen über Wundheilung haben gezeigt, daß Zerstörung der Dermis zu einer verstärkten Proliferation der Epidermis führt, welche schließlich—wird sie nicht durch Granulationsgewebe gestoppt—in invasives und zuletzt malignes Wachstum übergeht [4, 5]. Trotzdem histologisch deutliche Veränderungen der Kollagenstruktur einer mit cancerogenen Chemikalien behandelten Dermis nachweisbar sind [6–8], konnten bei chemischen Analysen keine wesentlichen Veränderungen des Kollagens festgestellt werden [9, 10].

In dieser Arbeit soll untersucht werden, ob cancerogene Chemikalien ein beschleunigtes Altern der Dermis hervorrufen. In der Humanpathologie wird parallel den Altersveränderungen der Dermis eine Zunahme von Spontancarcinomen beobachtet [11]. Das Altern der Dermis beruht in der Hauptsache auf einer Zunahme der Vernetzung durch kovalente Brücken zwischen den einzelnen Kollagenmolekülen [12]. Parallel mit der Zunahme der Vernetzung geht das Kollagen von einem leicht löslichen Zustand über schwer lösliche Stadien in eine Form über, in der es ohne Zerbrechen der primären Molekülstruktur nicht gelöst werden kann [13]. Auch dieses unlösliche Kollagen zeigt verschiedene Grade der Vernetzung [14]. Bei der Hitzedenaturierung von Kollagenfasern hindert zunehmende Vernetzung die Moleküle in steigendem Maße an der Aufgabe ihrer langgestreckten Struktur [15]. Deshalb erfolgt die bei Erhitzung beobachtete Schrumpfung der Fibrillen mit steigendem Vernetzungsgrad bei immer höherer Temperatur [15, 16]. Parallel mit der Zunahme der Schrumpfungstemperatur nimmt die nach einiger Zeit eintretende Dehnungsfähigkeit ab. Wie Verzár [15] gezeigt hat, können mit Hilfe solcher Schrumpfungs- und Dehnungsuntersuchungen an Mäuse- und Rattensehnen genaue Altersbestimmungen durchgeführt werden. Wenngleich sich die Haut wegen des ungleichmäßigen Verlaufs der Kollagenfasern nicht im selben Maß zu diesen Untersuchungen eignet, sind auch bei ihr altersbedingte Unterschiede im Schrumpfungsverhalten nachweisbar [17]. Dies bildet die Voraussetzung für die Untersuchung über den Einfluß von cancerogenen Chemikalien an Kollagenfasern.

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Akzeptiert am 18. November 1968.

TECHNIK

Die Versuche wurden an je 10 männlichen

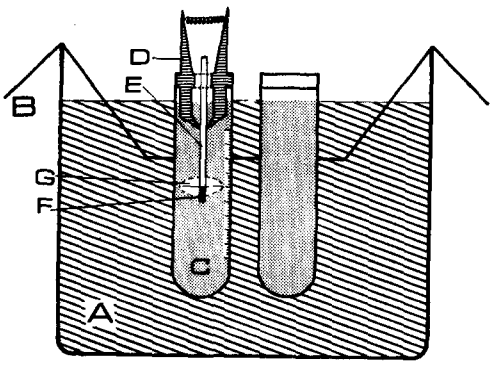
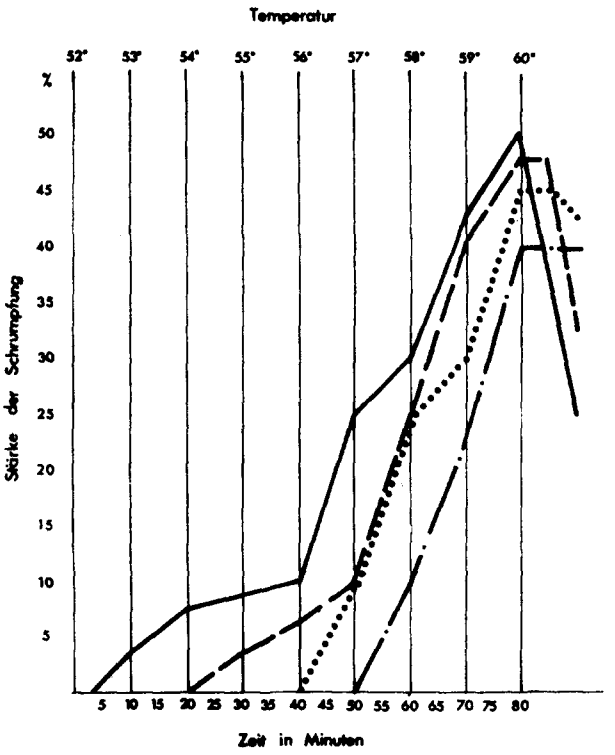


Abb. 1. *Schema der Apparatur zur Messung der Hautschrumpfung.*

A *Thermostatiertes Wasserbecken*
B *Einsatz für Meßröhrchen*
C *Meßröhrchen mit mm-Graduierung gefüllt mit Tyrode pH* 8·2
D *Verstellbare Haltevorrichtung*
E *Hautstreifen von denen* 2 *cm zwischen Haltevorrichtung und*
F *Hebegewicht gespannt sind. Hebegewicht mittels Haken an Hautstreifen befestigt.*
G *Meßplättchen, welches bei Schrumpfung längs der Skala wandert.*

Tabelle 1



Schrumpfungsverhältnisse der Rückenhaut von GP-Mäuseweibchen während laufender Temperaturerhöhung und bei verschiedenem Alter sowie nach Behandlung mit Methylcholanthren. Die in % angegebene Schrumpfungsstärke bezieht sich auf ein Hebegewicht von 0·08 g, erwärmt in Tyrode pH 8·2.

——— Rückenhaut einer 1½ Monate alten Maus
– – – – Rückenhaut einer 7 Monate alten Maus mit oder ohne Benzolbehandlung
– · – · – · Rückenhaut einer 7 Monate alten Maus nach Methylcholanthrenbehandlung
····· Rückenhaut einer 12 Monate alten Maus

Versuchstiere: GP-Weibchen.

und 10 weiblichen GP-Mäusen im Alter von 6 Wochen, 8 und 12 Monaten durchgeführt. Weiters sind je 10 Stück 8 Monate alte Männchen innerhalb 10 Wochen 30× mit Methylcholanthren, gelöst in Benzol und mit Benzol allein gepinselt worden. Verwendet wurden gleich breite Streifen (1–5 mm) der Rückenhaut mit einer Länge von 2 cm. Die Hautstreifen wurden einzeln freihängend in einem Glasröhrchen an einem Ende fixiert. An das andere Ende wurden verschiedene Gewichte von 0,03 bis zu 0,8 g befestigt. Beim Schrumpfungsvorgang zog die Haut das Gewicht längs einer Skala des Röhrchens. Die Glasröhrchen waren mit Tyrodelösung pH 8,2 angefüllt, und diese wurden je nach Versuchsreihe gleichzeitig im Wasserbad erwärmt. (Abb. 1). Untersucht wurden vor allem das Einsetzen der Schrumpfung, die wiedereinsetzende Dehnung und die Schrumpfungsstärke. Letztere zeigte größere Schwankungen und kann bei einer Altersbestimmung durch die Haut nicht herangezogen werden. Die Hebekraft, vor allem bei Verzár als Alterskontrolle angegeben, läßt sich bei der Haut nicht anwenden. Wegen der verschiedenen Dehnbarkeit verschieden alter Haut, ist eine Normierung der Hautbreite nicht möglich. Die Hebekraft nimmt aber mit der Hautbreite zu. Die besten Ergebnisse lassen sich mit niederen Gewichten (0,08 g) erreichen. Gemessen wurden die Temperaturbereiche von 52–75°. In diesem Temperaturbereich wurde die Messung der Hautveränderung sowohl bei gleichbleibender Temperatur, als auch bei laufender Temperaturerhöhung untersucht. Temperaturen über 60° zeigten keine zusätzlichen Schrumpfungen. Die meisten Messungen erfolgten daher von 52–60°.

ERGEBNISSE

Hautstreifen des Rückens, der Breite oder der Länge nach geschnitten, zeigten keinen Unterschied in ihrem Schrumpfungsverhalten.

Kurze Trypsinierung (1% 5 Minuten), führt zum völligen Verlust der Schrumpfungsfähigkeit.

Dermis ohne Epidermis zeigt eine verringerte Schrumpfungstemperatur, sowie eine vergrößerte Schrumpfung.

Hautstreifen verschiedener Breite zeigen gleiche Schrumpfungstemperaturen, gleiche Schrumpfungsgröße, jedoch verschieden große Hebekraft. Letztere nimmt mit Breite des Hautstreifens zu.

In der Haut nimmt, wie die Tabelle zeigt, mit dem Alter die Schrumpfungstemperatur zu, während die Schrumpfungsstärke abnimmt. Diese Unterschiede sind bei den Weibchen stärker ausgeprägt.

Die mit Methylcholanthren behandelte Rückenhaut zeigte eine sehr spezifisch erhöhte Schrumpfungstemperatur. Sie erreichte bis 3°, gegenüber den gleichalten Tieren, die nur mit Benzol behandelt worden waren. In 80% der Fälle konnte auch an der mit Methylcholanthren behandelten Haut eine Verringerung der Schrumpfungsstärke under Der Dehnungsfähigkeit beobachtet werden.

DISKUSSION

Die Ergebnisse sprechen für einen sehr spezifischen Alterungsprozeß der Dermis unter dem Einfluß des cancerogenen Chemikals. Die Erhöhung der Schrumpfungstemperatur spricht für eine rasch zunehmende, intermolekulare Vernetzung des Kollagens [15]. Der durch Methylcholanthren festgestellte Alterungsprozeß der Dermis könnte Beziehungen haben zu der beobachteten Zunahme von Spontancarcinomen mit steigendem Alter in der Humanpathologie. Es besteht allerdings auch die Möglichkeit, daß andere Veränderungen im Mesenchym, vor allem im Kollagengehalt bei gleichzeitiger Hyperplasie der Epidermis, für die Erhöhung der Schrumpfungstemperatur verantwortlich sind.

RESUME

Les expériences sur le rétrécissement de la peau ont montré que le méthylcholanthrène change le comportement de la peau du dos chez les animaux traités, par comparaison avec celle des animaux non traités ou badigeonnés au benzol, utilisé comme solvant.

(1) *La température du rétrécissement s'élève en moyenne de* 3°*C.*

(2) *L'intensité du rétrécissement s'abaisse d'environ* 10%.

(3) *La réextensibilité de la peau rétrécie diminue considérablement.*

Ces faits semblent indiquer que les cancérogènes renforcent les liaisons intermoléculaires du collagène dermique.

D'autres suggestions sont envisagées.

SUMMARY

Methylcholanthrene was found to alter the shrinkage properties of mouse back skin:

(1) *the shrinkage temperature is raised by about* 3°C;

(2) *the degree of contraction is decreased by almost* 10%;

(3) *the capacity for relaxation after thermal contraction is greatly reduced.*

These observations argue for an increased intermolecular linkage of dermal collagen under carcinogenic influence. Other possible explanations are discussed.

ZUSAMMENFASSUNG

Unter dem Einfluß von Methylcholanthren treten Veränderungen im Verhalten der Rückenhaut bei Schrumpfungsversuchen gegenüber unbehandelten oder mit dem Lösungsmittel Benzol gepinselten Tieren auf.

(1) *Die Schrumpfungstemperatur steigt um durchschnittlich* 3°C.

(2) *Die Schrumpfungsstärke sinkt um nicht ganz* 10%.

(3) *Die Fähigkeit, nach der Schrumpfung wieder zu dehnen, geht stark zurück.*

Diese Vorgänge würden für eine verstärkte intermolekulare Vernetzung des Collagens der Dermis unter Cancerogeneinfluß sprechen.

Andere Möglichkeiten werden erörtert.

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Der Unterschied in der Induzierbarkeit von Carcinomen zwischen Rücken- und Schwanzhaut der Maus in Beziehung zu ihrem Schrumpfungsverhalten

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EINLEITUNG

SPENCER konnte bei Mäusen an der Schwanzhaut im Gegensatz zum Rücken durch Cancerogene kein malignes Wachstum induzieren [1]. Untersuchungen von Orr [2], Gillman [3] und Seilern-Aspang [4, 5] legen es nahe, malignes Wachstum als Versagen jener steuernden Wirkung aufzufassen, welche die Dermis auf die Proliferation und Differenzierung der Epidermis ausübt. Die Steuerung dieser Vorgänge durch die Dermis ist aus der Embryologie und von Untersuchungen über die Regeneration bekannt. Transplantationsversuche mit der Epidermis haben gezeigt, daß auch im adulten Tier eine Steuerung der Epidermis durch unterlagernde Bindegewebselemente stattfindet [6]. Die Dermis des Mäuserückens macht ihrem Schrumpfungsverhalten nach unter dem Einfluß von Cancerogen einen stärkeren Alterungsprozeß durch [7]. Die Resistenz der Schwanzhaut gegen Carcinombildung macht es notwendig, den Alterungsprozeß und die Cancerogeneinwirkung im Vergleich zum Rücken mit Hilfe der Schrumpfungsvorgänge zu untersuchen.

TECHNIK

Die Alterungsuntersuchungen wurden an je 10 GP Mäusemännchen im Alter von 6 Wochen, 8 Monaten und 12 Monaten durchgeführt. Weiters wurden 100 Männchen mit 0,5% Methylcholanthren, gelöst in Benzol, 30× innerhalb von 10 Wochen gepinselt. Der gleichen Behandlung wurden 100 Tiere mit Benzol allein ausgesetzt. Schrumpfungsvorgänge wurden nach der früher beschriebenen Methode [7] untersucht.

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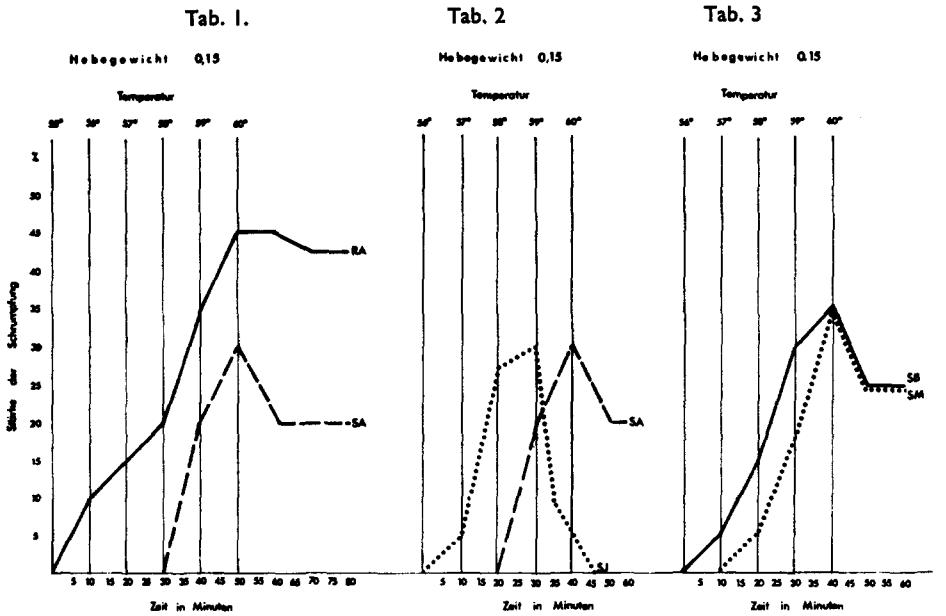
Akzeptiert am 18. November 1968.

ERGEBNISSE

An den mit Cancerogen behandelten Tieren traten am Rücken zu 100% Papillome und zu 90% Carcinome auf. Am Schwanz konnten in keinem Fall Papillome oder Carcinome beobachtet werden. Der Schwanz hat dem gleichalten Rücken gegenüber eine bis zu 3° höhere Schrumpfungstemperatur, eine etwas größere Dehnungsfähigkeit und eine wesentlich geringere Schrumpfungsgröße (Tabelle 1). Wie der Rücken zeigt der Schwanz eine parallel dem Alter zunehmende Schrumpfungstemperatur und abnehmende Dehnungsfähigkeit. Die junge Schwanzhaut erreicht ihre maximale Schrumpfungsstärke bei niedrigerer Temperatur als die alte. Die Schrumpfungsstärke variiert von Tier zu Tier und erscheint nicht altersspezifisch (Tabelle 2). Bei Temperaturen über 60° tritt ein spiraliges Einrollen der Schwanzhaut auf. Dies wurde an der Rückenhaut nicht beobachtet. Es nimmt mit dem Alter und gegen die Schwanzspitze zu. Nach Behandlung mit Methylcholanthren steigt die Schrumpfungstemperatur gegenüber den Benzolkontrollen um *ca.* 1°.

Tabellen 1–3



Schrumpfungsverhältnisse der Rücken- und Schwanzhaut von GP-Mäusemännchen während laufender Temperaturerhöhung und bei verschiedenem Alter. Die in % angegebene Schrumpfungsstärke bezieht sich auf ein Hebegewicht von 0·08 g, erwärmt in Tyrode pH 8·2.

RA = Rückenhaut einer 12 Monate alten Maus
SA = Schwanzhaut einer 12 Monate alten Maus
SJ = Schwanzhaut einer 1½ Monate alten Maus
SB = Schwanzhaut einer 8 Monate alten Maus nach 30 Pinselungen mit Benzol
SM = Schwanzhaut einer 8 Monate alten Maus nach 30 Pinselungen mit Methylcholanthren gelöst in Benzol

Schrumpfungsstärke und Dehnungsfähigkeit zeigen keine Veränderungen (Tabelle 3).

DISKUSSION

Das Steigen der Schrumpfungstemperatur beim Schwanz gegenüber dem Rücken könnte auf eine verstärkte intermolekulare Vernetzung des Kollagens zurückgeführt werden. Da jedoch die Dehnungsfähigkeit gegenüber dem Rücken nicht verringert ist, besteht die Möglichkeit, daß andere Kräfte, wie eine veränderte fibrilläre Vernetzung oder eine stärkere Zementierung der Kollagenfibrillen durch Mucopolysaccharide, ähnlich einem größeren Hebegewicht, die Schrumpfungstemperatur erhöhen. Für eine verstärkte Quervernetzung der Fibrillenstruktur spricht auch die bei 60° einsetzende Eindrehung der Schwanzhaut. Die veränderten Schrumpfungsvorgänge bei Methylcholanthrenbehandlung, die den Veränderungen des Alterungsprozesses entsprechen, deuten auf einen ähnlichen Alterungsprozeß unter Cancerogeneinfluß hin, wie beim Rücken beobachtet wurde [7]. Der Alterungsprozeß ist jedoch gering. Er liegt bei 8 Monate alten Tieren, welche mit Cancerogen behandelt wurden, unter dem eines 12 Monate alten. Im Gegensatz dazu zeigt der Rücken nach Cancerogenbehandlung Alterserscheinungen, die wesentlich die eines 12 Monate unbehandelten übersteigen [7]. Der Unterschied zwischen Rücken und Schwanz im Verhalten ihrer Epidermis gegenüber dem Cancerogen könnte also auf den geringeren durch Cancerogene ausgelösten Alterungsprozessen bei der Schwanzdermis beruhen. Das völlig andere Verhalten der Schwanzdermis gegenüber der des Rückens bei Schrumpfungsuntersuchungen gibt weiters die Möglichkeit, daß Strukturunterschiede der Dermis bei der Resistenz gegen malignes Wachstum beteiligt sind. Die Bildung eines Haarbalges durch epidermale Invasion in die Dermis stellt jeweils einen dem Beginn des malignen Wachstums vergleichbaren Vorgang dar [3]. Die Haararmut des Schwanzes könnte anzeigen, daß die im Schrumpfungsverhalten aufscheinende Veränderung der Struktur der Schwanzdermis ein invasives Wachstum der Epidermis ganz allgemein erschwert.

RESUME

On confirme les résultats obtenus par Spencer [1] *sur l'inefficacité des cancérogènes quant à l'induction de cancers sur la peau de la queue des souris.*

La température du rétrécissement de la peau de la queue est beaucoup plus élevée que celle du dos, toutefois l'intensité du rétrécissement est moindre, et l'extensibilité peu augmentée.

En comparaison avec la peau dorsale, le traitement de la queue par les cancérogènes provoque des modifications de la température du rétrécissement, moins marquées qu'au niveau du dos, et ne change pas l'intensité du rétrécissement et l'extensibilité de la peau.

SUMMARY

It was found that chemical carcinogens fail to induce carcinomas in mouse tail skin. This confirms the results previously obtained by Spencer [1].

Tail skin is characterized by a considerably higher shrinkage temperature, a lower degree of contraction, and a slightly better subsequent relaxation, as compared to back skin. Treatment with carcinogens alters the shrinkage temperature of tail skin to a lesser degree than that of back skin. The degree of contraction and of relaxation is not affected at all.

ZUSAMMENFASSUNG

Es werden die Ergebnisse von Spencer [1] *über die Nichtinduzierbarkeit von Carcinomen mit Cancerogenen in der Haut des Mäuseschwanzes bestätigt.*

Die Schrumpfungstemperatur der Schwanzhaut ist gegenüber der Rückenhaut viel höher, die Schrumpfungsstärke jedoch geringer und die Dehnungsfähigkeit höher.

Die Veränderungen nach Cancerogenbehandlung des Schwanzes sind in der Schrumpfungstemperatur gegenüber dem Rücken viel geringer und fehlen in der Schrumpfungsstärke und Dehnungsfähigkeit.

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Cancer du Sein

Association Cobaltothérapie à Hautes Doses-Chirurgie. Confrontation des Résultats Histologiques, Cliniques et Evolutifs; à propos de 105 Cas

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PARMI les associations thérapeutiques offertes pour traiter le cancer du sein, le traitement mixte cobaltothérapie à hautes doses, puis chirurgie, peut offrir des perspectives nouvelles.

La mise en service, dans notre pays, de sources de cobalt radioactif, remontant à 1958, il est possible aujourd'hui d'évaluer les résultats de cette association thérapeutique.

L'examen anatomique de la pièce opératoire fournit, dans ces conditions, des informations très précises sur l'action locale de la radiothérapie, certains cancers disparaissant, d'autres survivant à l'action des rayons.

Le but de ce travail est moins d'analyser les indications et les mérites éventuels de cette méthode que d'établir des relations entre la survie de ces malades et l'efficacité locale de la radiothérapie.

Une première approche de ce problème a été faite (F.B.) en 1966 [1]. Les publications traitant de ce problème particulier sont peu nombreuses et, toutes, récentes: Rudowski [2], Petrov [3]; Widow [4] et Lysakowska [5].

MATERIEL ET METHODES

Notre expérience se fonde aujourd'hui sur l'étude de 105 dossiers, dans lesquels nous disposons, après une cobaltothérapie homogène, donnée par le même radiothérapeute (F.B.), de l'analyse anatomo-pathologique de la pièce opératoire et de la surveillance clinique et évolutive de la malade.

Toutes nos observations, à l'exception d'une seule, concernent des cancers mammaires féminins.

L'âge des malades s'échelonne entre 26 et 75 ans. Dans cette série, les cancers, pour la plupart, sont des tumeurs déjà importantes par leur volume et leur extension; ils se placent, selon les critères de la classification internationale, dans les catégories suivantes.

T_1	6 cas
T_2	37 cas
T_3	43 cas
T_4	3 cas

Deux formes bilatérales ont été incluses dans ce lot.

Des adénopathies de nature cliniquement métastatique sont découvertes chez 58 malades, alors que le stade clinique T.N.M. est seulement précisé 89 fois.

Une poussée évolutive est relevée de manière certaine dans 39 cas.

Parmi eux, il est 4 PEV_1 et 35 PEV_2 selon les critères distinctifs définis dans les travaux de Sarrazin [6].

Quatorze malades ont été examinées à l'oc-

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casion de récidivies. Le protocole thérapeutique a, dans l'ensemble, peu varié.

(I) Dans les cas les plus simples et les plus nombreux (79 dans cette série), le traitement par le cobalt est entrepris d'emblée après confirmation du diagnostic clinique et mammographique par un examen cytologique ou une drill-biopsie. L'irradiation est effectuée suivant le schéma suivant (Fig. 1). Elle est faite en 6 champs:

—deux champs mammaires; l'un interne, l'autre externe prenant tout le sein, la malade étant strictement de profil, calée par des sacs de sable (la dimension des champs mammaires est réduite progressivement à partir de 5.000 r ou 6.000 r/tumeur et l'irradiation est centrée sur la tumeur).

—deux champs axillaires: l'un direct le bras relevé, l'autre antérieur, transpectoral.

—Un champ pour la chaîne ganglionnaire mammaire interne, le rayon étant incliné de 40° du côte à irradier.

—Un champ sus-claviculaire direct.

La durée de l'irradiation est, en moyenne, de 7 à 8 semaines.

Les doses tumeurs s'échelonnent entre 7.300 rads et 9.700 rads, la dose moyenne étant de 8.500 rads. Les doses reçues par les ganglions axillaires variant de 4.850 rads à 8.700 rads, le chiffre moyen étant de 7.700 rads.

(a) *La décision opératoire* a été prise sur les arguments suivants:

—disparition incomplète clinique (50 fois) ou mammographique (14 fois) de la tumeur.

—réapparition d'une tumeur palpable (16 fois) ou d'une image mammographique suspecte (8 fois).

—sécurité supplémentaire et confort psychologique offerts à la malade (17 cas).

(b) Les types d'intervention ont été très variables et n'ont pu être précisés que dans 100 observations. Dans 35 cas, il s'agissait d'une mammectomie simple complémentaire, laissant en place les muscles pectoraux. Dans 65 cas, l'intervention effectuée fut une mammectomie élargie, comportant 54 fois un curage axillaire.

(c) Le traitement cobalto-chirurgical a été complété 16 fois par une castration radiothérapique ou chirurgicale, et 14 fois par une chimiothérapie.

(II) A côté de cette série de 79 malades, existe un groupe moins homogène de 26 malades, chez qui l'irradiation a été effectuée, soit après une exérèse chirurgicale, partielle, incomplète ou jugée insuffisante (12 cas), soit, longtemps après le début réel de la maladie, à l'occasion d'une récidive par exemple (14 cas).

Ces cas comportent néanmoins le même schéma de traitement et le même examen anatomique de la pièce opératoire.

Analyse de la pièce opératoire

L'analyse macroscopique est le temps à la fois le plus important et le plus difficile. En effet, après l'irradiation, la glande apparaît très dure et homogène et il est assez souvent difficile de retrouver dans cette sclérose l'ancien foyer tumoral. La persistance, sur les plans cutanés, de repères dermographiques, le concours du radiothérapeute qui a suivi l'évolution tumorale, constituent une aid précieuse dans cette enquête. Le procédé suivant est utilisé: la pièce de mammectomie est systématiquement retournée, les plans musculaires, s'ils font partie de la pièce opératoire, sont enlevés et la lame celluleuse interpectorale systématiquement prélevée, de même que la graisse du curage axillaire.

Des coupes macroscopiques sont réalisées de 5 mm en 5 mm perpendiculairement au plan cutané, sans entamer celui-ci, afin de conserver à la pièce son homogénéité. Ainsi la zone tumorale peut être repérée, à la vue et au toucher. Parfois, elle est évidente, marquée par un territoire nécrotique jaunâtre, à limites incertaines. Parfois elle est douteuse ou peu typique et se présente, au mieux, sous forme d'une petite cicatrice de moins d'un centimètre carré, blanchâtre, stellaire, dure et concave à la coupe (Fig. 2).

Les prélèvements soumis à l'étude histologique sont multiples et complétés si un doute subsiste. L'ensemble des relais ganglionnaires est systématiquement prélevé.

Les préparations histologiques sont effectuées suivant les procédés classiques et colorées par l'hématéine-éosine-safran. Les colorations spéciales n'ont paru d'aucune aide dans cette étude. Aucune coloration mitochondriale ou enzymatique n'a été faite.

RESULTATS

L'exploitation des informations recueillies par cette enquête se place sur deux plans différents:

—d'une part, un plan histopathologique où seront jugées et estimées les modifications apportées par l'irradiation à la tumeur;

—d'autre part, un plan clinique et évolutif où, suivant l'usage, seront établis le taux de survie des malades, ainsi que les récidives et les métastases.

La juxtaposition de ces deux types de renseignements peut donner lieu à des recoupements et fournir des précisions sur des liens

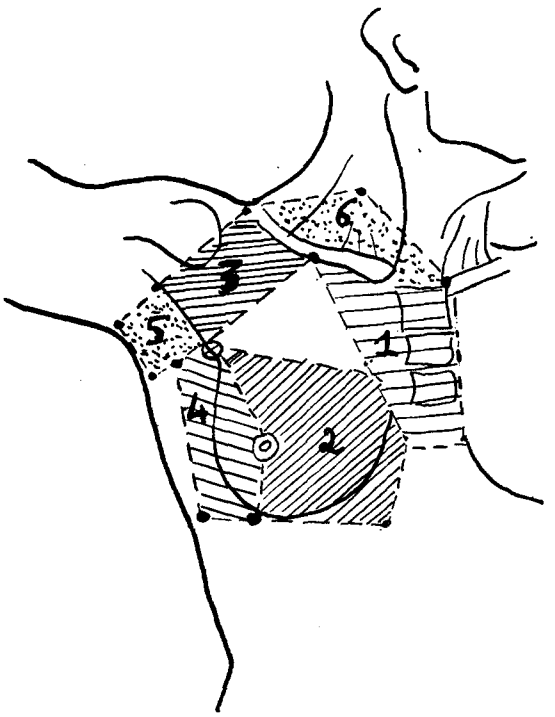

Fig. 1. *Champ d'irradiation. Technique du Docteur Baclesse.*

éventuels entre la radiosensibilité et l'évolutivité de telle ou telle tumeur mammaire.

Résultats anatomo-pathologiques

Cent cinq pièces ont été étudiées.

Deux éventualités sont rapidement apparues (Tableau 1).

(1) Aucune lésion cancéreuse n'est reconnaissable, sur les prélèvements effectués. Ces cas sont dits 'stérilisés'. Il sont au nombre de 22 et représentent 21% de notre matériel.

Les coupes histologiques ne sont pas totalement muettes. Elles montrent (Fig. 3), tranchant sur le fond fibro-atrophique de la glande des foyers fibro-hyalins, nodulaires, acellulaires ou pauci-cellulaires, dans lesquels ne persiste aucune cellule tumorale et ne subsiste aucun canal galactophore. La découverte de ces cicatrices (Fig. 4) est importante, car elle permet d'affirmer que les prélèvements ont bien intéressé le territoire tumoral et que ce dernier est bien 'stérilisé'.

Ont été exclus de ce groupe 2 cas où la tumeur était détruite, mais où subsistaient des métastases ganglionnaires.

(2) Deuxième éventualité: le cancer mammaire est encore identifiable. Il apparaît plus ou moins modifié par l'action des rayons, d'où l'individualisation dans ce groupe de 4 catégories.

(a) La première répond à des tumeurs qui semblent n'avoir nullement souffert dans leur morphologie. Ce sont des tumeurs totalement radiorésistantes. Elles sont dites "florides". Elles sont au nombre de 6/105 (5,7%).

(b) Dans la deuxième catégorie, ont été réunies des tumeurs dans lesquelles persistent des éléments carcinomateux au sein de zones de nécrose et de fibrose cicatricielles. La présence d'importantes lésions vasculaires, l'existence d'une sclérose élastigène, signent l'action de la radiothérapie. Il serait, là encore, possible de distinguer les tumeurs profondément modifiées de celles qui le sont peu, mais nous n'avons pas retenu cette distinction afin de ne pas compliquer l'analyse statistique des résultats. La découverte de quelques noyaux tumoraux (Fig. 5) survivant au milieu de la sclérose, même s'ils sont peu nombreux,

Tableau 1. *Repartition des résultats histologiques*

	'Stérilisés'	Douteux	Modifiés	'Florides'	Reprise évolutive	Total
nombre absolu	22	3	65	6	9	105
%	21	3	62	6	8	100

Tableau 2. Répartition des résultats histologiques en fonction de la therapeutique

	'Stérilisés'	Douteux	Modifiés	'Florides'	Reprise évolutive	Total
I. Cobalt+ Chirurgie	17	2	49	5	6	79
II. Chirurgie+ Cobalt+ Chirurgie	3		8		1	12
III. Récidives irradiées	2	1	8	1	2	14
Total nbre absolu	22	3	65	6	9	105
%	21	3	62	6	8	100

a été jugée suffisante pour classer une tumeur dans cette catégorie.

Il n'est généralement pas possible d'évaluer, morphologiquement, la vitalité des cellules tumorales persistantes. La basophilie du cytoplasme, la présence de mitoses, donnent à penser que ces cellules, même modifiées, conservent leur capacité de prolifération.

Ces tumeurs, modifiées plus ou moins profondément par l'irradiation, sont les plus nombreuses 65/105 (61,9%).

(c) Une troisième catégorie est réservée aux "reprises évolutives". Ce diagnostic repose sur la découverte d'un et souvent de plusieurs foyers carcinomateux proliférant activement, développés autour et souvent au voisinage immédiat d'une cicatrice tumorale, généralement parfaite et acellulaire (Figs. 6 et 7). Ces cas sont peu nombreux 9/105 (8,5%), mais ils illustrent la vitalité persistante des foyers tumoraux, même s'ils paraissent, un moment, très profondément altérés par la radiothérapie.

(3) Enfin, il est quelques cas dans lesquels l'étude histopathologique, pour des raisons diverses, est incapable de juger de la nature tumorale ou non des cellules persistantes. Ces cas sont peu nombreux; 3/105 (2,85%) et sont classés comme 'douteux'.

De telles constatations ne sont pas nouvelles; l'un de nous [1, 7], dès 1962, obtenait un pourcentage de destruction tumorale de 25%; Lumb [8], utilisant le 200 KV, affirme, mais sans contrôle histologique, obtenir une destruction tumorale dans 1/3 des cas.

Les méthodes de contrôle et les chiffres donnés par Lysakowska [5] sont plus proches des nôtres: sur 200 tumeurs irradiées (la méthode n'est pas précisée), cet auteur obtient une stérilisation dans 12% des cas et des dégénérescences cellulaires dans 48%.

Les chiffres de Petrov [3] sont plus faibles; sur une série de 58 cas, 9% seulement ont été détruits.

L'analyse des résultats (Tableau 1) montre que l'efficacité locale de la cobaltothérapie, à doses égales, est difficilement prévisible.

Il paraît utile d'étudier les facteurs locaux ou généraux susceptibles d'influencer éventuellement ces résultats et de modifier peut-être la sensibilité d'une tumeur mammaire aux rayons. Dans la série actuelle, différents paramètres ont été analysés:

(1) *La chronologie de traitement* (Tableau 2).

Que le traitement habituel soit une cobaltothérapie suivie de chirurgie (79 cas), ou qu'il

Tableau 3. Résultats histologiques en fonction du stade clinique

	'Stérilisés'	Douteux	Modifiés	'Florides'	Reprise évolutive	Total
Stade I	2		6	2	1	11
Stade II	7	1	16	3	5	32
Stade III	10	2	32		2	46
Stade non précisé	3		11	1	1	16
Total	22	3	65	6	9	105

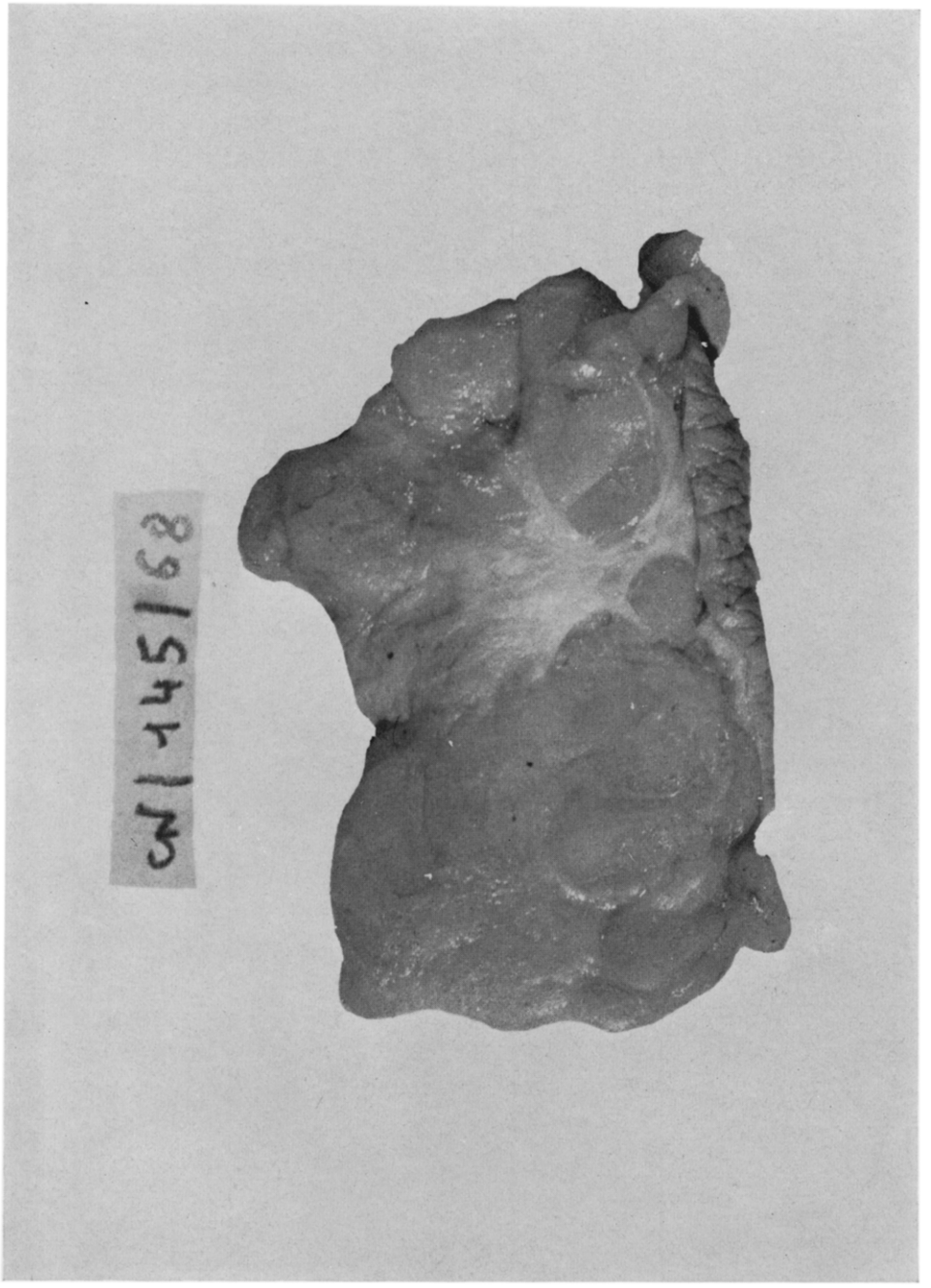

Fig. 2. Aspect macroscopique de la cicatrice tumorale.

(to face p. 222)

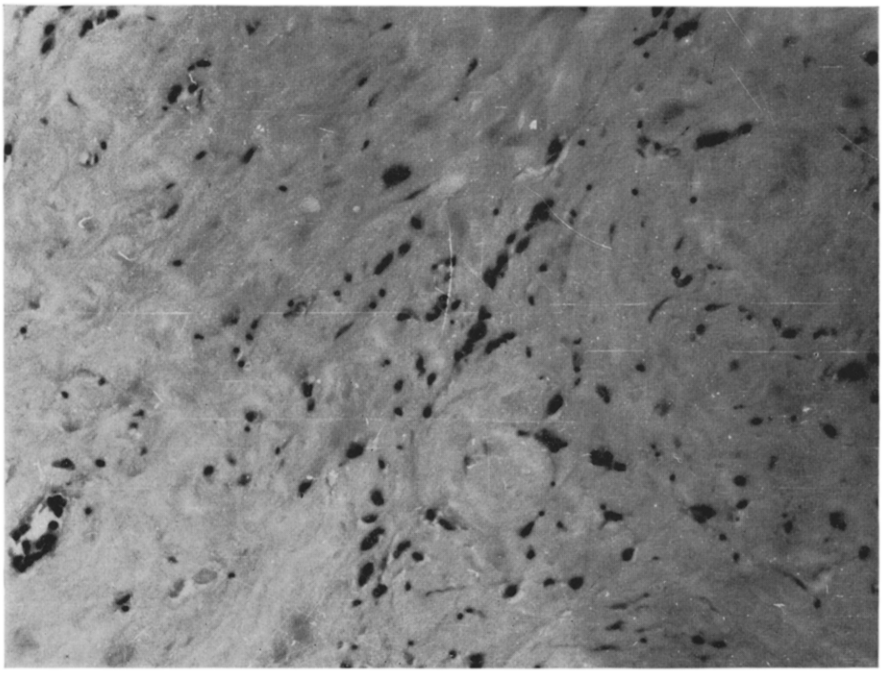

Fig. 3. *Tumeur 'stérilisée; cicatrice fibro-hyaline, paucicellulaire.*

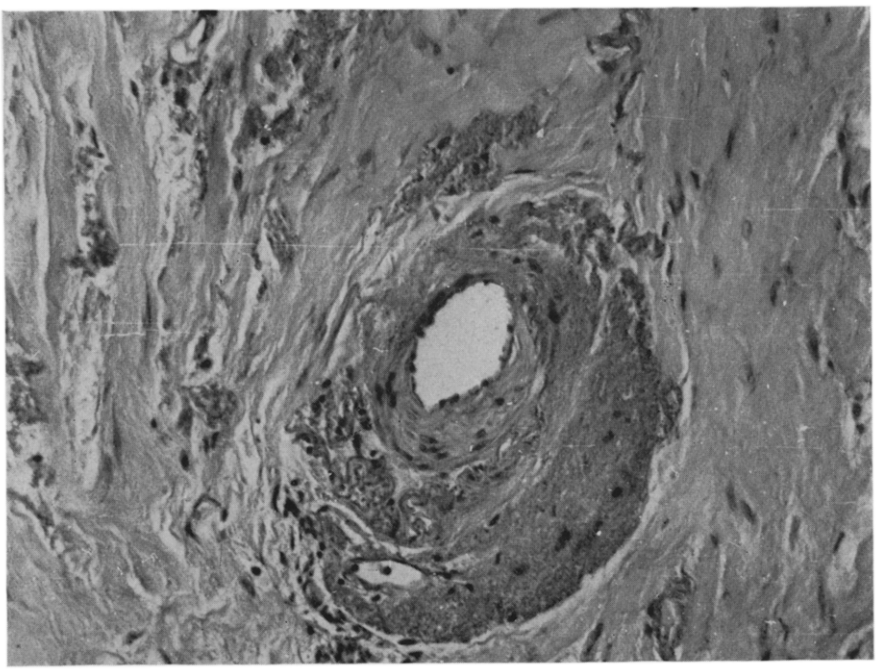

Fig. 4. *Tumeur 'stérilisée'; foyer nécrotique signant l'emplacement de la tumeur 'stérilisée' par le cobalt*-60.

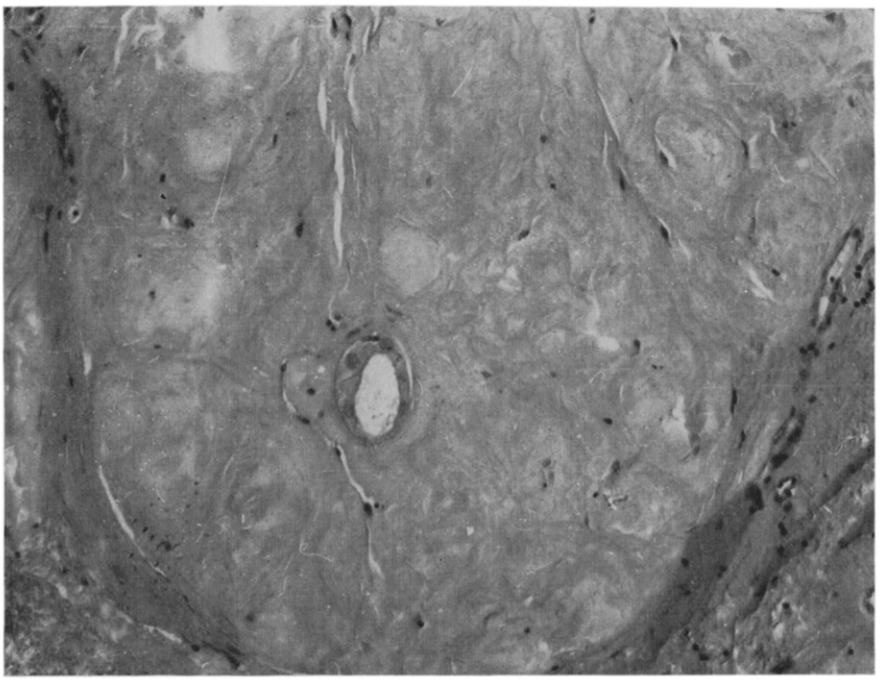

Fig. 5. *Tumeur modifiée par l'irradiation; au milieu de la fibrose persistent quelques cellules tumorales.*

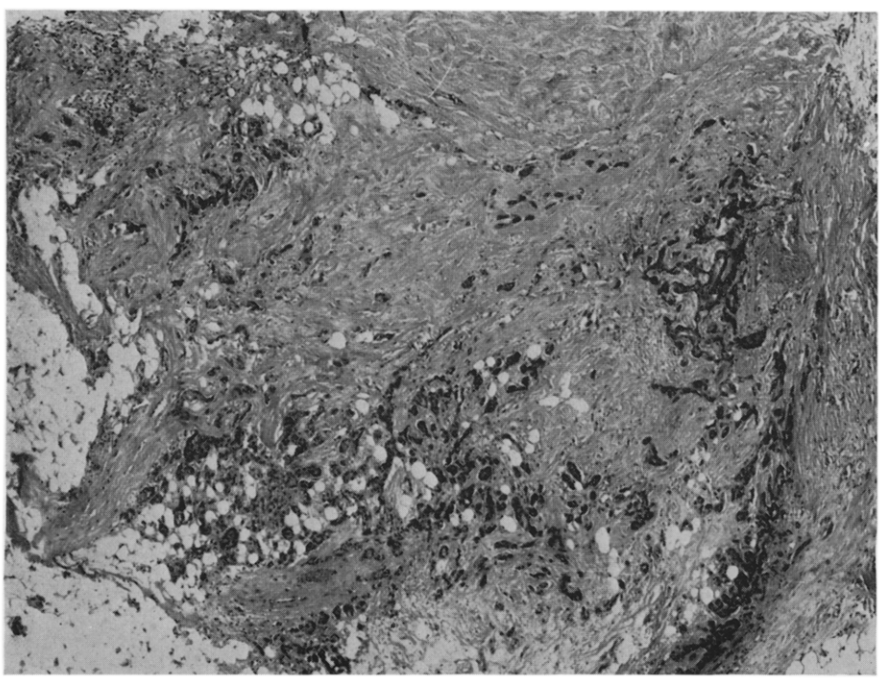

Fig. 6. *'Reprise évolutive'; noter les travées tumorales en périphérie de la cicatrice évoquant une reprise évolutive.*

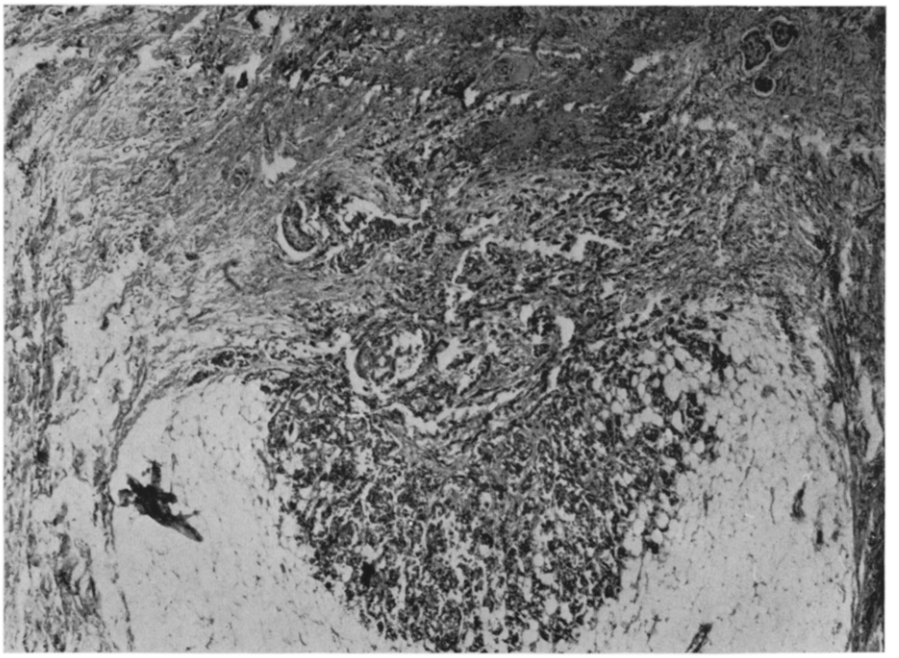

Fig. 7. *'Reprise évolutive'; extension dans la graisse d'une tumeur en reprise évolutive.*

Tableau 4. *Résultats histologiques en fonction du volume de la tumeur*

	'Stérilisés'	Douteux	Modifiés	'Florides'	Reprise évolutive	Total
T_1	2		1	2	1	6
T_2	7	1	21	3	5	37
T_3	9	2	30		2	43
T_4	1		2			3
T non précisé	3		11	1	1	16
Total	22	3	65	6	9	105

soit entrepris après une tumorectomie imparfaite (12 cas), ou à l'occasion d'une récidive post-chirurgicale (14 cas), la répartition des formes stérilisées, douteuses ou modifiées, est sensiblement la même.

(2) *Le stade clinique* (Tableau 3).

Le stade clinique évalué avant tout traitement, suivant la classification internationale, a pu être précisé 89 fois; il n'a pu l'être 12 fois, en raison d'une tumorectomie incomplète antérieure; il a été omis 4 fois.

Le Tableau 3 souligne que près de 50% des malades appartiennent au stade III, et que les résultats locaux de la cobaltothérapie ne semblent pas liés au stade clinique.

(3) *Le volume de la tumeur*

Le volume de la tumeur a pu être précisé dans 89 cas. Le Tableau 4 montre que les T_2 et T_3 représentent 80% environ de notre matériel. Là encore, confirmant les résultats du chapitre précédent, l'efficacité locale de la cobaltothérapie semble indépendante du volume initial de la tumeur.

(4) *Le délai écoulé entre la fin de l'irradiation et l'acte opératoire*

Ce délai (Tableau 5) est variable puisqu'il s'étend de 1 mois à 5 ans. Cependant, la plupart des mammectomies complémentaires sont effectuées dans l'année qui suit l'irradiation. On note en outre que plus de la moitié des tumeurs détruites par la radiothérapie ont été enlevées dans les 6 premiers mois.

(5) *L'âge de la malade*

Le Tableau 6 montre bien qu'il n'existe aucun lien entre l'âge de la malade et l'action locale de l'irradiation.

En conclusion, l'ensemble de ces documents tend à montrer que l'efficacité locale apparente de la cobaltothérapie est indépendante du volume, du stade clinique de la tumeur, de l'âge de la malade, et même du délai séparant l'irradiation de l'acte opératoire.

L'analyse morphologique des tumeurs ainsi irradiées a permis d'individualiser 4 catégories de résultats: les formes stérilisées, les formes modifiées, les cas douteux et les reprises évolutives.

Tableau 5. *Résultats histologiques en fonction du délai séparant cobaltothérapie et chirurgie*

	1–6 m	6–12 m	12–18 m	18–24 m	>24 m	Délai non précisé	Total
'Stérilisés'	12	5	3	1	1		22
Douteux	1	2					3
Modifiés	33	21	2	1	5	3	65
'Florides'	1	2	2		1		6
Reprise évolutive	1	5	1		2		9
Total	48	35	8	2	9	3	105

Tableau 6. *Résultats histologiques en fonction de l'âge*

Age (ans)	'Stérilisés'	Douteux	Modifiés	'Florides'	Reprise évolutive	Total
25–30			2			2
30–40	3		11		1	15
40–50	6	2	18	2	4	32
50–60	6		15	4	1	26
60–70	6	1	15		3	25
>70	1		4			5
Total	22	3	65	6	9	105

DONNEES EVOLUTIVES

Deux questions sont posées:

—quelle est la survie des malades ainsi traitées?

—quels sont les rapports entre la durée de la survie et les résultats locaux obtenus par la cobaltothérapie?

Avant d'envisager les résultats cliniques et évolutifs, il est bon de rappeler que les cas appartenant à cette série répondent à une sélection de formes à priori défavorables, comprenant 88% de stades II et III, ainsi qu'un pourcentage élevé de tumeurs, soit évolutives, soit volumineuses, soit récidivantes, homo- ou controlatéralement.

(1) *La survie globale* de l'ensemble de ces cas.

Le Tableau 7, établi sur 103 malades suivies sur une période échelonnée de 1 an à 9 ans, montre le taux de survie.

A cinq ans, il est de 53% (34/64).

Tableau 7. *Survie de l'ensemble des malades*

Recul (ans)	Survie nbre absolu	Survie (%)
1	103/103	100
2	81/92	88
3	60/80	75
4	45/73	62
5	34/64	53
6	26/58	45
7	20/52	38,5
8	9/41	(22)
9	3/31	(10)

A neuf ans, le taux de survie tombe à 10% (3/31). Le chiffre n'est probablement pas significatif car il répond au faible échantillon

Tableau 8. *Survie en fonction de l'image histologique*

	1 an	2 ans	3 ans	4 ans	5 ans	6 ans	7 ans	8 ans	9 ans
'Stérilisés'	19/19	18/18	13/13	12/12	6/7	4/5	4/5	1/2	
(%)	100	100	100	100	(86)	(80)	(80)	(50)	
Douteux	3/3	2/3	2/3	2/3	2/3	2/3	2/3	2/3	1/2
(%)	(100)	(67)	(67)	(67)	(67)	(67)	(67)	(67)	(50)
Modifiés	65/65	50/59	39/54	23/47	19/43	15/41	11/37	7/33	2/28
(%)	100	85	72	49	44	36,5	30	21	7
Florides	6/6	5/6	4/6	4/6	4/6	4/6	2/4	1/3	
(%)	(100)	(83)	(67)	(67)	(67)	(67)	(50)	(33)	
Reprise évolutive	9/9	6/6	3/5	3/5	2/4	1/3			
(%)	(100)	(100)	(60)	(60)	(50)	(33)			

Tableau 9. *Survie en fonction de l'atteinte ganglionnaire histologique*

		1 an	2 ans	3 ans	4 ans	5 ans	6 ans	7 ans	8 ans	9 ans
No	nbre	72/72	53/63	44/54	33/47	25/40	18/35	13/30	7/24	
	(%)	100	84	81	70	62, 5	51	43	29	
N+	nbre	27/27	19/26	14/25	11/25	8/21	7/20	7/20	2/15	
	(%)	100	73	56	44	38	35	35	13	

des premiers cas traités lesquels représentaient une sélection de très mauvaises formes.

(2) *Durée de survie comparée à l'efficacité locale de la cobaltothérapie*

Le Tableau 8 résume l'essentiel des résultats fournis par cette étude. Son interprétation est rendue difficile par la dispersion en 5 catégories des résultats histologiques et par le faible nombre des malades survivant au-delà de 5 ans. Néanmoins, il permet de souligner les points suivants:

Dans le groupe des cancers stérilisés, on ne relève qu'un seul décès, survenu 5 ans après la fin du traitement. Bien que 4 d'entre elles souffrent de métastases, toutes les autres malades sont en vie. Dans cette catégorie, le taux de survie de 5 ans est de 86%.

Dans les autres groupes, les résultats sont nettement moins bons. Ainsi, dans la catégorie des formes modifiées qui regroupent le plus grand nombre de cas, sur 43 malades suivies, 19 seulement (soit 44%) sont en vie après 5 ans.

Le pronostic des formes dites florides ou en reprise évolutive ne paraît pas plus mauvais que celui des formes dites modifiées puisqu'à cinq ans, le taux de survie est respectivement de 66% et 50%. En fait, le faible nombre de cas étudiées retire à ces pourcentages leur signification.

La courbe (Fig. 8) souligne bien la différence de pronostic existant entre les cancers stérilisés histologiquement par la cobaltothérapie et ceux qui ne le sont pas. Rudowski *et al.* [2] avaient fait, dès 1959, des constatations analogues puisqu'ils ne comptaient aucun décès à 5 ans dans le groupe des tumeurs entièrement détruites par la radiothérapie. De son côté, Lysakowska [5] signale un taux de survie de 5 ans de 75% dans les formes stérilisées, alors qu'il n'est que de 55% dans les tumeurs modifiées et 36% dans les tumeurs non modifiées.

Les faits mettent en évidence une relation

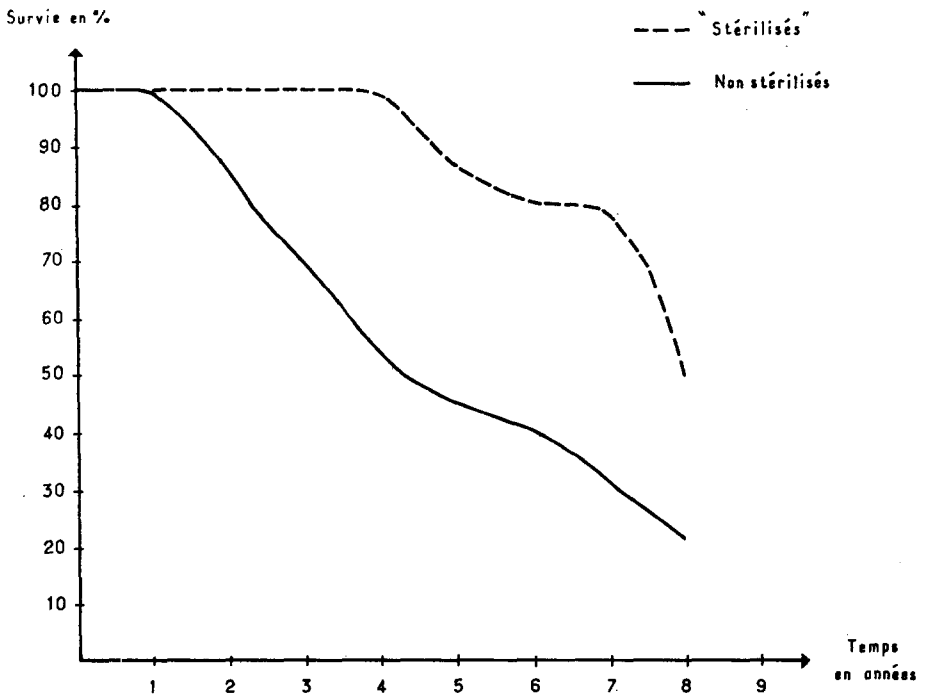

Fig. 8. *Taux de survie.*
Noter la différence du taux de survie entre les formes 'stérilisées' par la cobaltothérapie et les formes non stérilisée (ligne pleine).

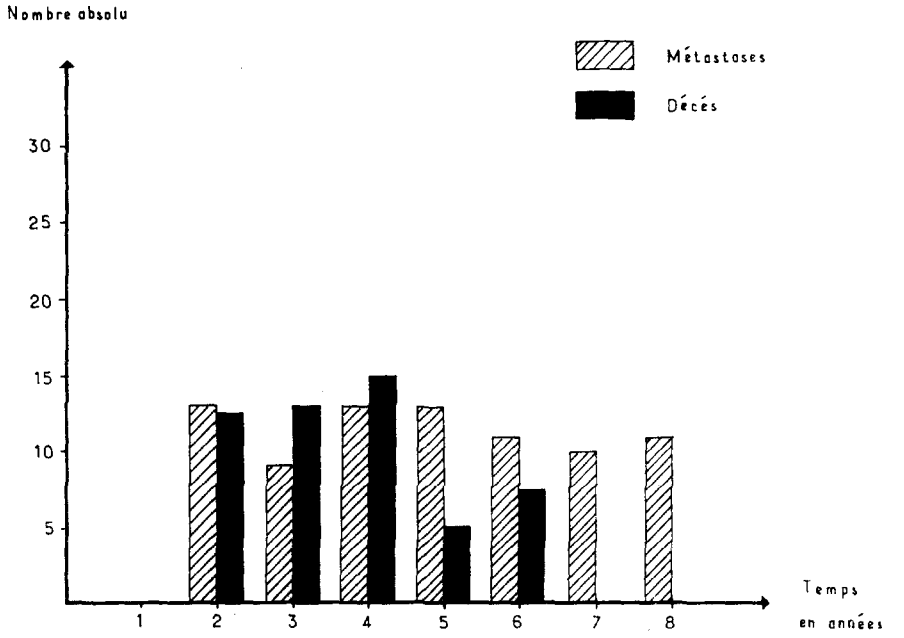

Fig. 9. Fréquence des métastases et des décès en fonction de la durée évolutive, après le traitement associé cobaltothérapie–chirurgie.

précise et jusqu'ici peu connue, entre la radiosensibilité d'une tumeur mammaire et son pronostic.

(3) *L'atteinte ganglionnaire*

Établie sur document histologique apporte là également un élément capital dans l'évaluation du pronostic. Dans 99 cas les chaînes ganglionnaires mammaires externes et axillaires ont pu être vérifiées (Tableau 9).

Dans 72 cas, les ganglions se sont révélés indemnes d'envahissement tumoral, soit qu'ils aient été épargnés, soit qu'ils aient été stérilisés par l'action du cobalt. Cette catégorie regroupe bien entendu des tumeurs mammaires stérilisées, modifiées ou florides.

Il existait, dans 27 cas, une extension ganglionnaire histologiquement évidente. La différence de pronostic est, là encore, flagrante, puisque le taux de survie de 5 ans passe, dans notre série, de 62% dans le premier groupe à 38% dans le deuxième groupe.

Les *résultats locaux* de ce traitement mixte sont en général bons. Les incidents relevés sont: un clivage opératoire difficile (2 fois), une hémorragie post-opératiore (1 cas), une sclérose axillaire (2 cas), une cicatrice dystrophique (3 cas), un gros bras (4 cas). Une fibrose pulmonaire post-radiothérapique a été notée une fois. Aucun accident sanguin n'a été relevé. Les chiffres sont beaucoup moins pessimistes que ceux fournis par Rudowski [3] qui déplore une nécrose cutanée dans 27% des cas, et ceux donnés par Sarrazin *et al.* [9] qui relèvent des complications imputables à la cobaltothérapie dans 54% des cas, dont 7,5% d'oedème du membre supérieur.

Les récidives locales sont rares. Neuf ont été comptées dans notre série; 6 d'entre elles sont survenues dans l'année qui a suivi l'intervention chirurgicale.

Ce taux faible de récidives loco-régionales est retrouvé dans la plupart des séries associant la radiothérapie à la chirurgie: 9% dans les statistiques différentes de Lysakowska [5] d'une part, et Lavigne [10] d'autre part. Petrov [3] relève seulement 2,3% de récidives dans une série de 613 cas irradiés avant l'intervention. Möldovan et Petrosan [11], comparant deux groupes de malades, l'un traité par la chirurgie seule, l'autre par l'association radio-chirurgicale, signalent un pourcentage égal de métastases, mais un taux moindre de récidives (7%) dans les deuxième groupe.

Les *métastases* (Fig. 9) sont beaucoup plus fréquentes et sont généralement responsables de l'évolution mortelle.

La Fig. 9 les situe dans l'évolution de la maladie cancéreuse et montre que leur pourcentage de survenue reste généralement le même d'une année à l'autre (de 8 à 13% des malades suivies au cours d'une année).

Le but de ce travail était essentiellement de confronter le délai de survie et les effets locaux de la cobaltothérapie sur la tumeur mammaire. Néanmoins, les données accumulées ici permettent de comparer en outre les résultats de l'association cobalt-chirurgie avec ceux donnés

par d'autres méthodes. Ces tentatives de comparaison thérapeutique se heurtent cependant toujours à l'hétérogenéité du matériel étudié et aux classifications utilisées.

Cependant, on estime grossièrement que le taux de *survie de cinq ans* de cancers non traités oscille entre 18 et 22% [12]. Les différents traitements utilisés ont globalement élevé ce pourcentage à 40 et 55%.

Les résultats varient beaucoup à l'intérieur et même à l'extérieur de ces limites puisque, parmi d'autres auteurs, Urban [13] fait état d'un taux de survie de 70% par mastectomie radicale étendue.

Watson [14] d'une part, et Lewison [15] d'autre part obtiennent un taux de survie de 5 ans de 52% par mastectomie radicale suivie de radiothérapie.

Le chiffre donné par Mac Whirter est de 42% par mastectomie simple ou tumorectomie suivie de radiothérapie.

Ces différences tiennent probablement moins à la qualité du traitement qu'au stade clinique, au volume de la tumeur, et à l'existence d'une poussée évolutive.

Comparant des formes semblables, des stades C (Columbia Clinical Classification), Haagensen [16, 17] obtient un taux de survie de 42,9% par action chirurgicale et F. Baclesse de 41% par radiothérapie isolée.

Le taux de survie global de 5 *ans obtenu ici est de* 53%. Il se place dans les limites définies plus haut.

La comparaison des malades traités par radiothérapie seule [18, 19] et ceux traités par cobaltothérapie plus chirurgie est en faveur du deuxième groupe, puisque, dans le premier groupe, on enregistre un taux de survie de 5 ans de 42% (contre 53%), et on déplore un pourcentage plus élevé de récidives loco-régionales (38% contre 9%), alors que les métastases se répartissent de façon égale (37% dans chacun des deux lots).

Il est important de rappeler que les malades ainsi traitées répondaient globalement à une sélection des mauvaises formes puisque, dans le groupe actuel, près de la moitié des cas étaient des stades III et des T3.

Remerciements—Nous tenons à remercier très vivement:

Monsieur Zajdela qui a bien voulu faire et interpréter pour nous la plupart des examens cytologiques préopératoires;

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ainsi que Mademoiselle S. Dankaert pour son aide technique.

RESUME

Ce travail est fondé sur l'analyse clinique, histologique et évolutive de 105 *cancers du sein, suivis durant une période de* 9 *ans, et traités par l'association cobaltothérapie-chirurgie. La confrontation des documents histologiques et des données cliniques et évolutives montre, à doses d'irradiation égales, l'inégalité des cancers du sein devant l'action des rayons.*

Le plus souvent, dans 4 *cas sur* 5 *environ, les rayons donnés à doses élevées, ou modifient plus ou moins profondément le volume et l'image histologique de la tumeur, ou ne semblent pas l'altérer, ou n'empêchent pas une reprise évolutive. Le pourcentage de survie de* 5 *ans, dans ce groupe, compte tenu qu'il sélectionne des formes fréquemment volumineuses ou évolutives, est de* 47%.

Dans un faible nombre de cas (1 *cas sur* 5 *environ), la cobaltothérapie, quel que soit l'âge de la malade, le stade clinique, et le volume initial de la tumeur, détermine la disparition histologique du cancer. Ce groupe a un pronostic nettement meilleur puisque le taux de survie de* 5 *ans est de* 86%.

Il semble qu'il existe un lien entre la radiosensibilité d'un cancer et son pronostic.

Enfin, l'association cobaltothérapie-chirurgie diminue, de façon significative, la fréquence des récidives loco-régionales (9% *dans la série actuelle*).

SUMMARY

This study is based on the clinical analysis, the histology and the evolution of 105 *cases of breast cancer, followed for a* 9*-year period, and treated by a combination of cobalt therapy and surgery. The confrontation of the histological documents with the clinical and evolutive factors demonstrate that breast cancers are differently influenced by equal doses of irradiation.*

Most often, in about 4 *out of* 5 *cases, irradiation given in high doses either modify more or less profoundly the volume and histological picture of the tumour, or do not apparently alter the cancer, or do not prevent an evolutive recurrence. The* 5*-year survival rate of this group is* 47% *because voluminous or evolutive forms are frequently selected.*

In a small number of cases (about 1 out of 5) cobalt therapy induced histological disappearance of cancer regardless the patient's age, the clinical stage and the initial volume of the tumour. The prognosis of this group is clearly better since the rate of the 5-year survival period is up to 86%.

The prognosis of breast cancer is apparently related to its radiosensibility.

Finally the association of cobalt therapy and surgery considerably diminishes the frequence of local-regional recurrences (9% in the present series).

ZUSAMMENFASSUNG

Diese Arbeit basiert auf der Verlaufsanalyse sowie auf der klinischen und histologischen Analyse von 105 Mammacarcinomen. Die 9 Jahre lang unter Beobachtung stehenden Patienten wurden gleichzeitig mit Kobalt und durch Chirurgie behandelt. Der Vergleich der histologischen Dokumente und der Verlaufsdaten und klinischen Daten zeigt bei gleichen Bestrahlungsdosen ein ungleiches Verhalten der Carcinome unter der Wirkung der Strahlen.

Hohe Strahlendosen bewirkten in etwa 4 von 5 Fällen eine mehr oder weniger grosse Veränderung des Volumens und des histologischen Bildes des Tumors, oder schienen eine Wiederaufnahme des Wachstums nicht zu beeinflussen oder zu verhindern. Die Überlebensdauer von 5 Jahren wurde in dieser Gruppe von 47% erreicht, unter Berücksichtigung der Tatsache, dass es sich mehrfach um voluminöse oder fortschreitende Formen handelt.

Bei einer geringen Zahl von Fällen (ca. 1 auf 5) bewirkte die Kobalttherapie unabhängig vom Alter des Patienten, dem klinischen Stadium und dem ursprünglichen Volumen des Tumors das histologische Verschwinden des Carcinoms. Diese Gruppe weist eine weit bessere Prognose auf, denn die Überlebensrate bei 5 Jahren beträgt 86%.

Es scheint also, dass ein Zusammenhang besteht zwischen der Strahlenempfindlichkeit eines Carcinoms und seiner Prognose.

Es sei noch hinzugefügt, dass die Verbindung Kobalttherapie—chirurgische Behandlung deutlich die Zahl der lokalen und regionalen Rezidive vermindert (bei unserer Reihe 9%).

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Ultrastructural Alterations of the Cytomembranes of Rat Pancreatic Exocrine Cells Induced by Treatment with 2-Acetylaminofluorene

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INTRODUCTION

ALTHOUGH the compound, 2-acetylaminofluorene (2-AAF) has been studied mainly in terms of its hepatocarcinogenic activity, its ability to induce neoplasms at a variety of other sites has been long established [1] and attributed to the action of its metabolites, in particular the *N*-hydroxy derivative [2, 3]. However, the action of 2-AAF on pancreatic tissue has received little attention hitherto. It was demonstrated previously [4] that the prolonged feeding of 2-AAF to the rat resulted in permanent alterations in the ultrastructure of its hepatocytes. Among these alterations were modifications of the granular endoplasmic reticulum and Golgi apparatus, as well as of the cell surface. In view of these findings it was considered to be desirable to study the effect of 2-AAF on the fine structure of an alternative cell type. The pancreatic acinar cell was chosen for this purpose, owing to its possession of an extensive granular endoplasmic reticulum. The aim of this investigation was to discover whether 2-AAF would also produce permanent ultrastructural changes in the pancreatic acinar cells of treated rats and whether any changes produced would resemble those observed previously in preneoplastic liver cells [4].

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MATERIALS AND METHODS

A standard diet, containing 0·05% by weight of 2-acetylaminofluorene (2-AAF), was fed *ad libitum* to male rats of the Leeds strain. Control animals received the same standard diet but without added 2-AAF, but were maintained under otherwise identical conditions. Three different groups of 2-AAF-fed rats were used in this study, each group containing from 4–6 surviving animals at the time of autopsy. The first group was sacrificed after 3–4 weeks of treatment and the second after 8–10 months. The third group of rats was fed the carcinogen-containing diet uninterruptedly for 10 months and was then returned to the standard diet without 2-AAF. Animals of the third group were sacrificed at intervals between 2 and 6 months after the end of 2-AAF-feeding. All animals were killed, by stunning, at the same time of day, in order to minimize any influence of diurnal variation of pancreatic activity upon the fine structure of the acinar cells. At autopsy portions of pancreatic tissue were removed rapidly and immediately placed in ice-cold fixative. The tissue was minced finely and fixed for 4 hours in 4% glutaraldehyde in 0·067 M cacodylate buffer, pH 7·2, at 0–4 C [5, 6]. It was then rinsed overnight in cold cacodylate-buffered 0·25 M sucrose and postfixed in 1·0% phosphate-buffered os-

mium tetroxide [7]. The tissue was dehydrated and embedded in Epon 812 according to the method of Luft [8]. Thin sections were cut with GeFeRi diamond knives, on a Sorvall MT1 ultramicrotome, and stained with Millonig's lead tartrate [9]. The sections were examined in an RCA EMU-3G electron microscope, using an accelerating voltage of 100 kV.

Portions of pancreas, adjacent to those which were processed for electron microscope studies, were also removed for routine histological examination. These were fixed in 10% formaldehyde-saline and embedded in paraffin wax. Sections were stained with haematoxylin and eosin.

RESULTS

At autopsy, macroscopical examination failed to reveal any differences in appearance between the pancreases of the three groups of 2-AAF-treated rats, or between these and those of untreated control rats. Microscopical examination of the pancreatic tissues from the 2-AAF-treated rats showed that their structure was apparently normal in all respects. After both brief and prolonged exposure to 2-AAF, and also, in the latter case, after the cessation of 2-AAF-feeding, the pancreatic tissue contained a normal system of acinar and ductural structures arranged in typical lobular formations. The microscopic structure of the islets of Langerhans also appeared to be unaffected by the administration of 2-AAF.

Electron microscopy

The fine structure of the pancreatic acinar cells of the normal rat has been described in detail by other workers [10] and therefore no attempt will be made here to present any further description. Electron microscope study of pancreatic acinar cells from control animals used during the present experiment served mainly to confirm these previous observations and to establish the non-pathological state of these cells.

Electron microscopy of the pancreatic acinar cells obtained from the carcinogen-treated rats revealed the presence of a conspicuous lesion of their granular endoplasmic reticulum. With the occasional exception of the Golgi apparatus, all other subcellular components were apparently unaffected. The lesion was present in approximately half of the acinar cells of rats which had been fed for 3–4 weeks on the 2-AAF-containing diet. After 8–10 months of 2-AAF-feeding, a larger proportion of the pancreatic acinar cells were affected, amounting to about three quarters of the total number observed. The changes persisted in animals which had been fed 2-AAF diet for 10 months and had then been returned to a normal diet and therefore appeared to be permanent in character. Up to 6 months after the rats had been returned to a normal diet there were no signs of recovery of normal pancreatic fine structure. Pancreatic fine structure also appeared to be unaffected by the presence of hepatic tumours in some of the treated animals at the time of sampling. At all stages of the experiment apparently normal acinar cells were observed, adjacent to those exhibiting an altered ultrastructural organization. There was no evidence of any preferential localization of the transformed cells within the pancreatic tissue.

The 2-AAF-induced pancreatic lesion consisted principally of a dilatation of the granular endoplasmic reticulum, varying in its degree of severity from cell to cell. In those cells which were only mildly affected, this dilatation was confined mainly to the basal and juxtanuclear regions of their cytoplasm. Where the changes were more pronounced, however, the apical cytoplasm was usually also involved. The extent of cisternal dilatation at all stages of treatment ranged, from cell to cell, between that depicted in Figs. 1 and 2 and the more extreme examples which are illustrated in Figs. 6 and 7, where the ground cytoplasm was reduced to thin septae subdividing the intracisternal spaces into large intercommunicating compartments. Even where the cisternal membranes were widely separated, however, they bore numerous ribosomes attached to their outermost surfaces. There was no evidence, at any stage of the experiment, that any significant detachment of ribosomes from the granular endoplasmic reticulum had taken place.

The perinuclear cisternae behaved in a similar manner to the surrounding granular endoplasmic reticulum. They became irregularly distended, as shown in Figs. 2, 3 and 6, their outer membranes generally being displaced outwardly. Continuity between the outer and inner membranes of the perinuclear cisternae was maintained, however, at the sites of nuclear pores (Figs. 2 and 6). Although the inner membranes of the perinuclear cisternae remained closely applied to the nuclear contents, increasing cisternal dilatation was accompanied by increasing irregularity of the nuclear profiles. There was no evidence, however, that this was related to any change in nuclear volume, which appeared to remain constant and within normal range.

The lumina of the distended granular endo-

Fig. 1. *Portion of a binucleate pancreatic acinar cell from a rat fed on 2-AAF-containing diet for 3–4 weeks. The granular endoplasmic reticulum and perinuclear cisternae are irregularly dilated. Two lysosomes* (*) *are present in the cytoplasm.* (×23,000)

(to face p. 232)

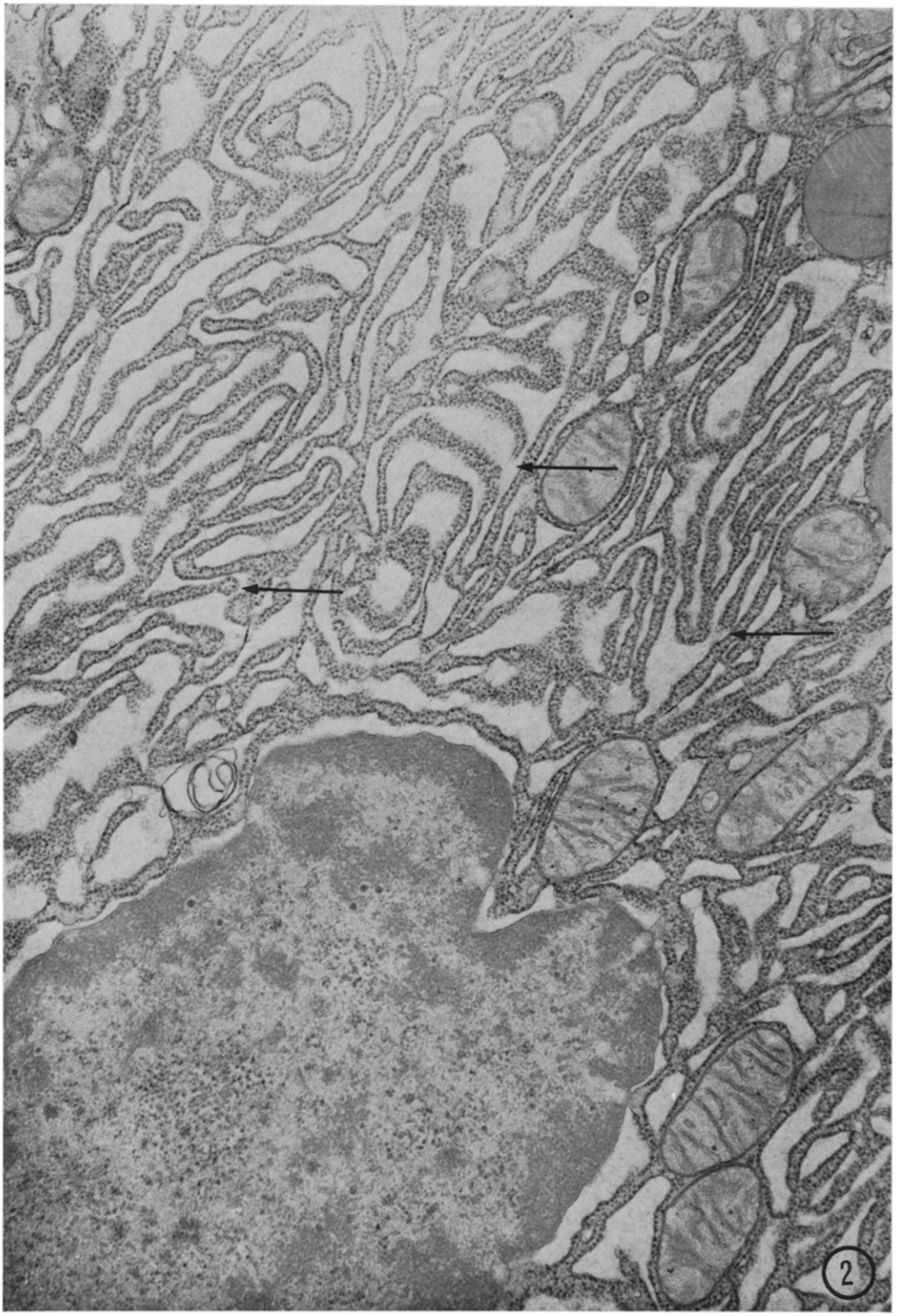


Fig. 2. Pancreatic acinar cell from rat which had received 3 to 4 weeks of 2-AAF treatment. A more advanced stage of the granular endoplasmic reticulum lesion is illustrated here. The cisternal spaces are shown to intercommunicate freely (arrows). (×23,000)

Fig. 3. *Pancreatic acinar cells from* 2-*AAF-treated rat, sampled after withdrawal of treatment. The granular endoplasmic reticulum of the upper cell shown is widely dilated and contains dense lamellar inclusions* (*arrows*), *while that of the lower, adjacent cell is normal in appearance.* (×23,000).

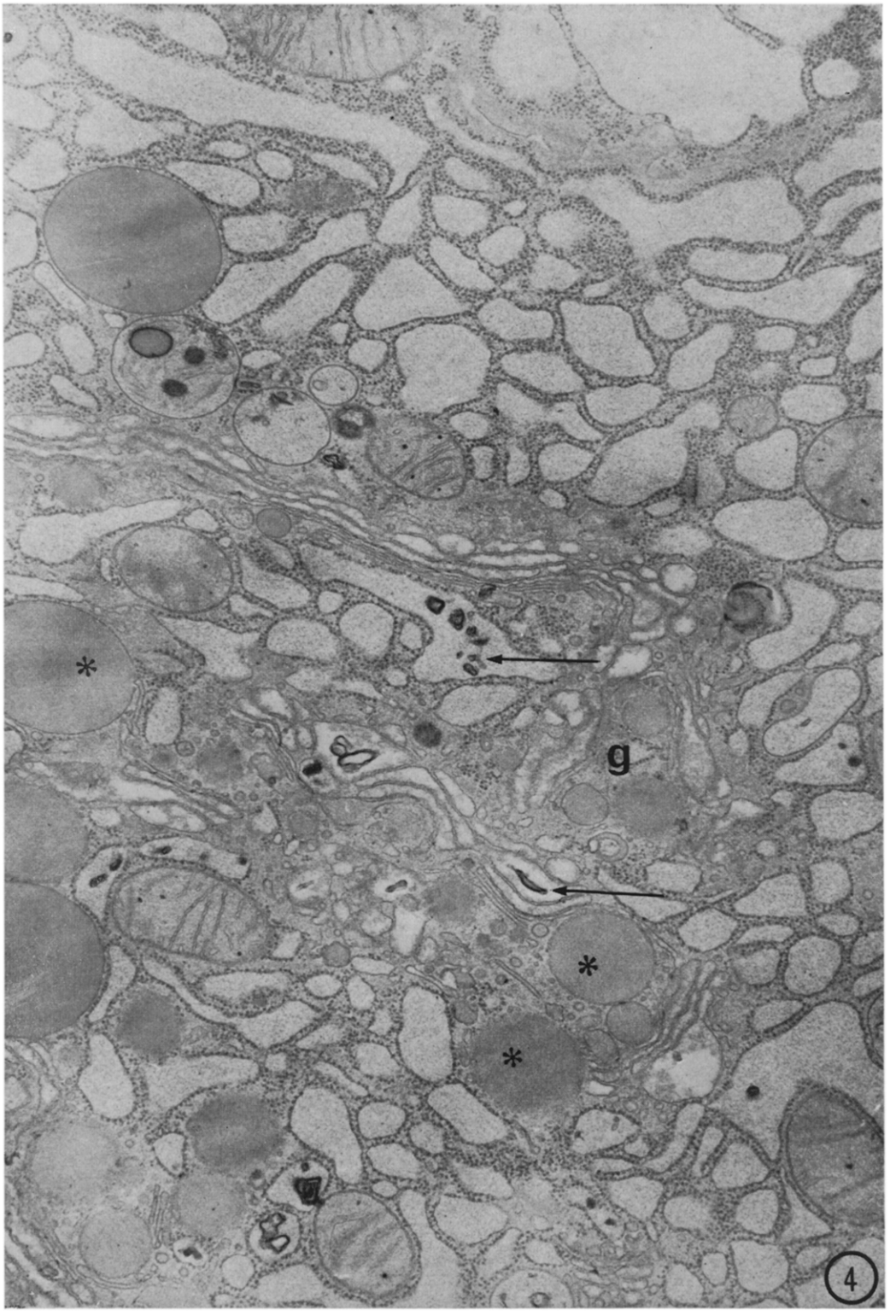


Fig. 4. Pancreatic acinar cell from 2-AAF-treated rat, sampled after withdrawal of treatment. The presence of lamellar inclusions (arrows) within the Golgi apparatus (g) *as well as the diluted granular endoplasmic reticulum are demonstrated. A number of prozymogen granules are also present* (*). (×23,000)

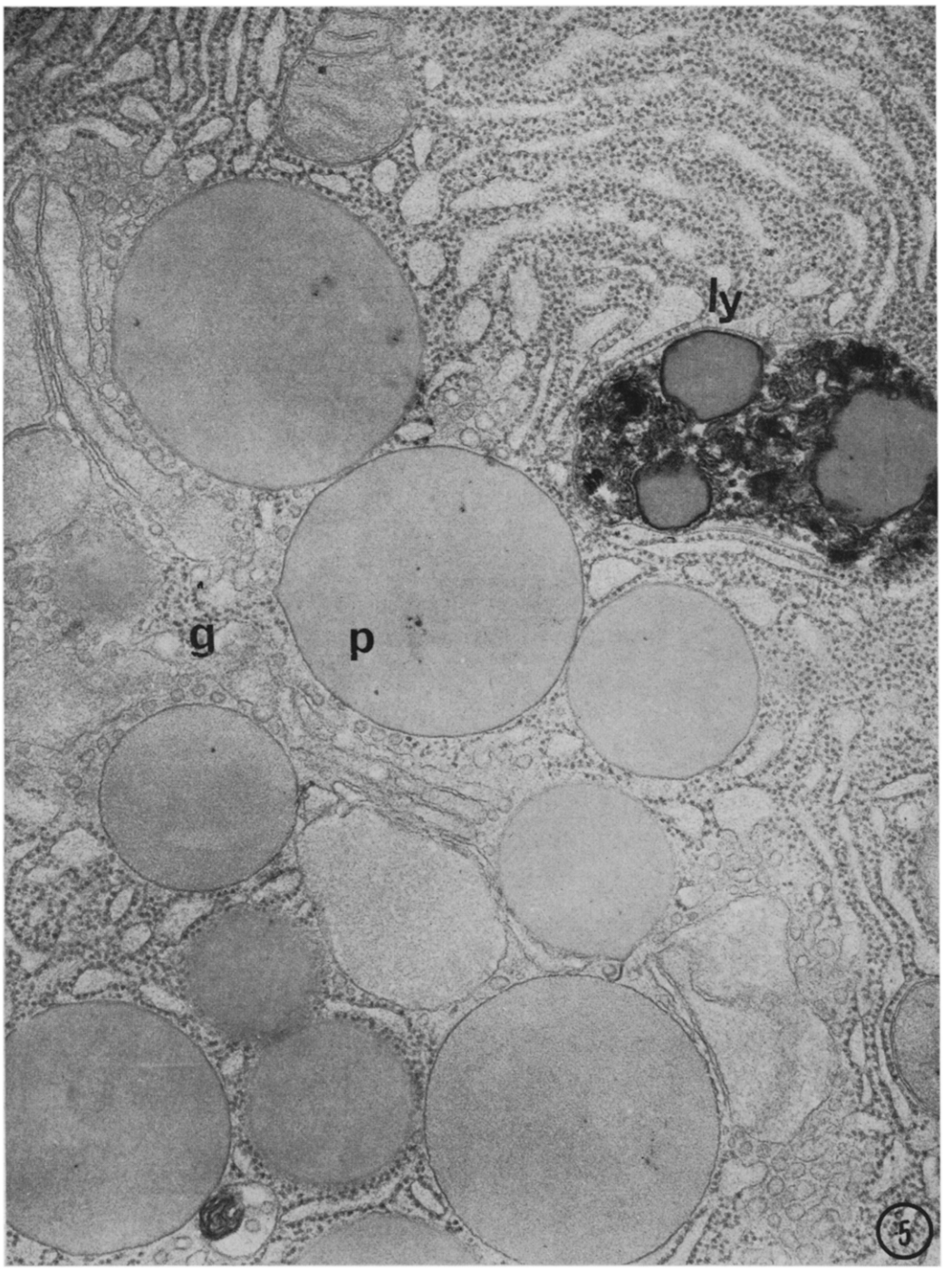


Fig. 5. Pancreatic acinar cell from 2-AAF-treated rat, sampled after withdrawal of treatment. The Golgi zone shown (g) *contains an abundance of finely granular material and is surrounded by prozymogen granules* (p) *which possess smooth-surfaced limiting membranes. An autophagic vacuole* (ly) *is present near the Golgi zone.* (×30,000)

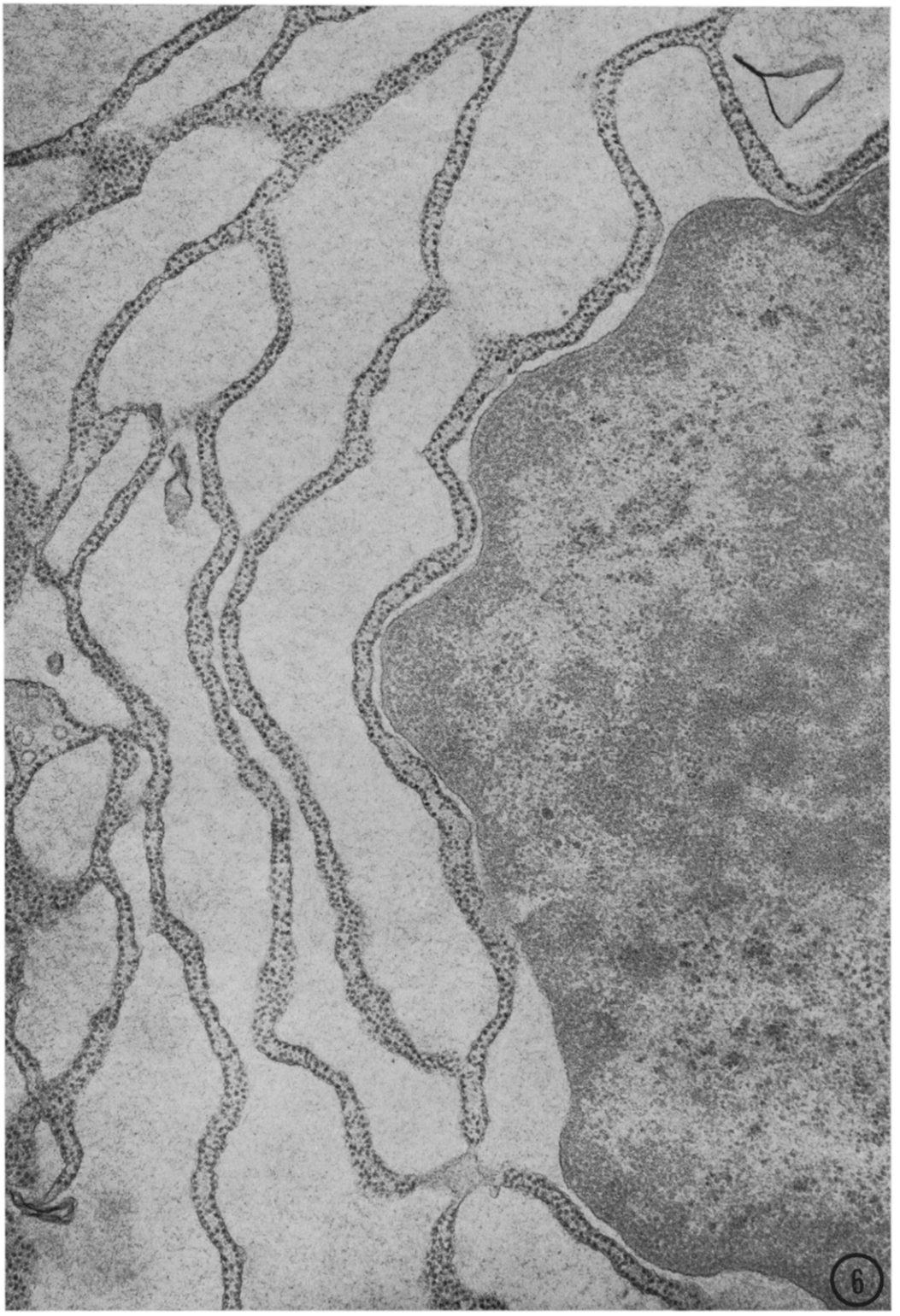


Fig. 6. *Pancreatic acinar cell from* 2-*AAF-treated rat, sampled after withdrawal of treatment. Note the irregularly distended perinuclear cisterna surrounding the indented nucleus. Both the perinuclear cisternae and the dilated endoplasmic reticulum contain finely granular material.* (×33,000)

Fig. 7. Pancreatic acinar cell from 2-AAF-treated rat, sampled after withdrawal of treatment. The ground cytoplasm is reduced to thin septae owing to the extreme distension of the granular endoplasmic reticulum. The cisternae contain dense granules in addition to the loose fine granular contents. (×30,000)

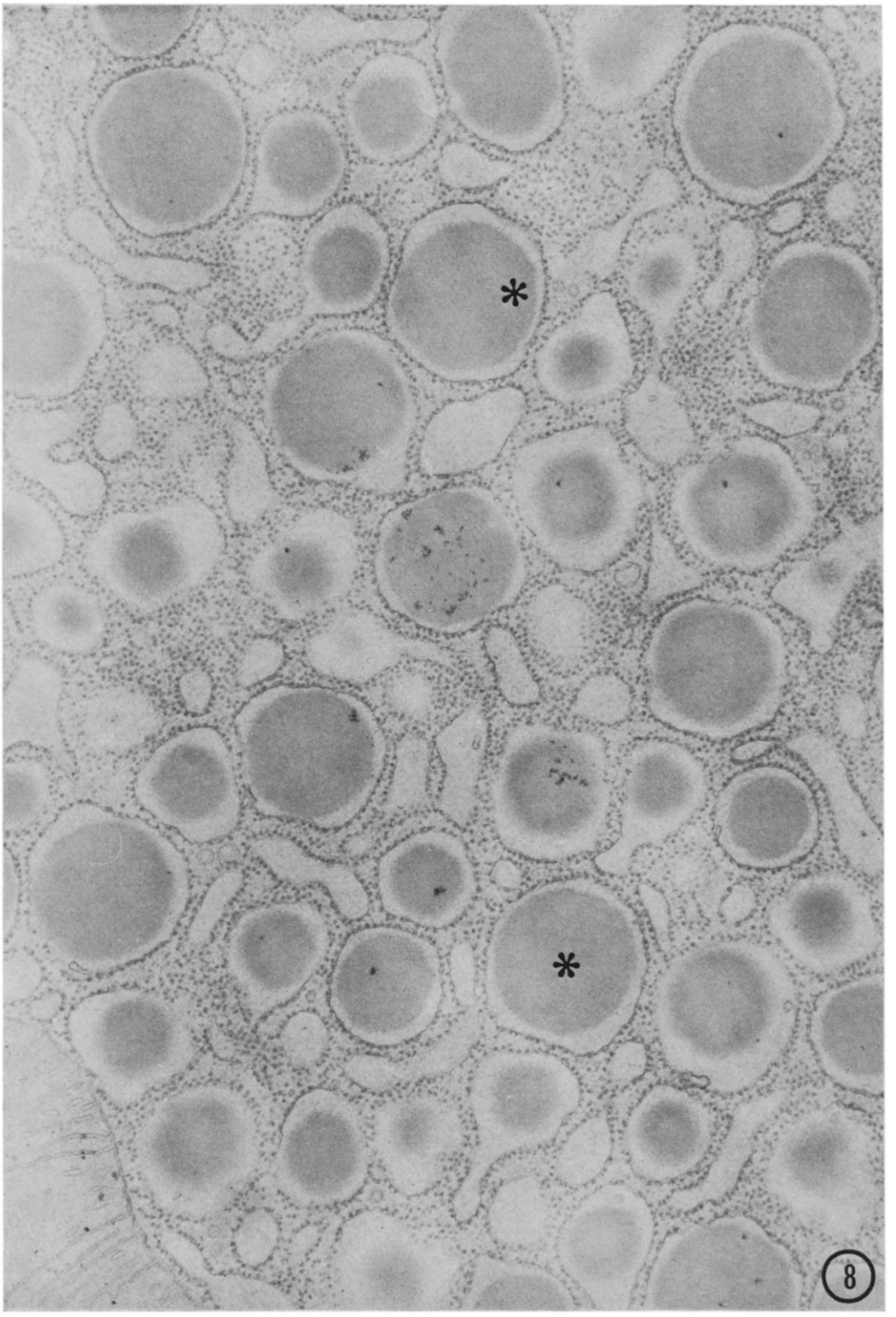
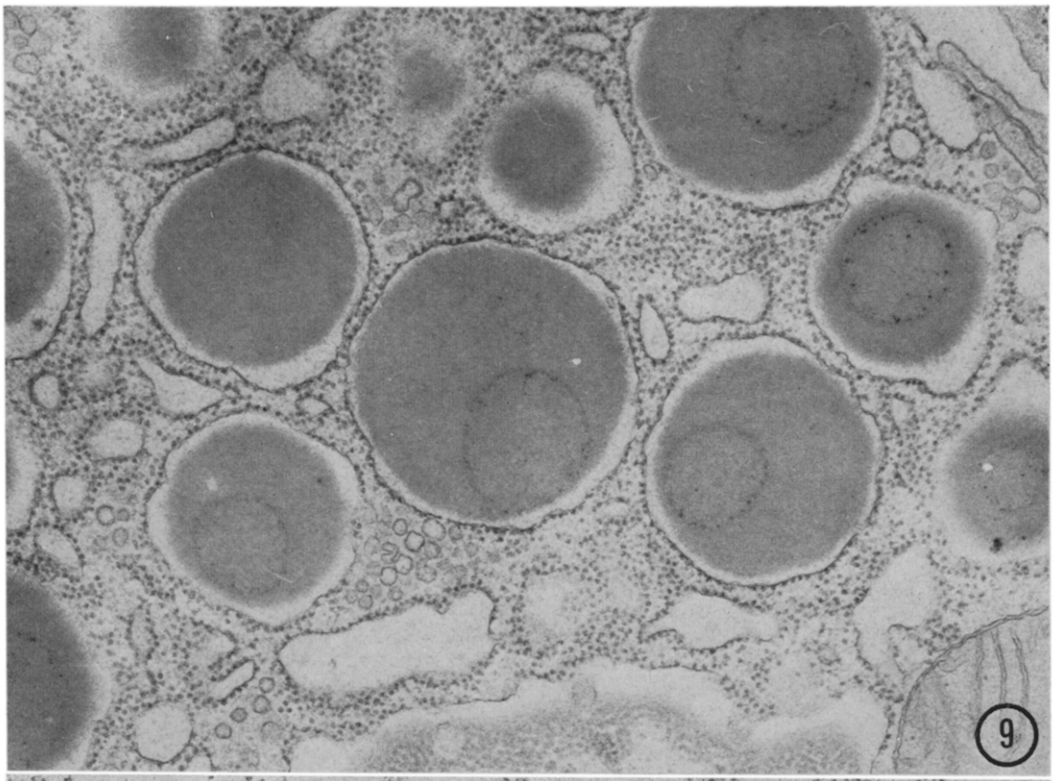


Fig. 8. *Pancreatic acinar cell from* 2-*AAF-treated rat, sampled after withdrawal of treatment. The presence of larger intracisternal granules* (*) *within the rough-surfaced cisternae is shown.* (×30,000)



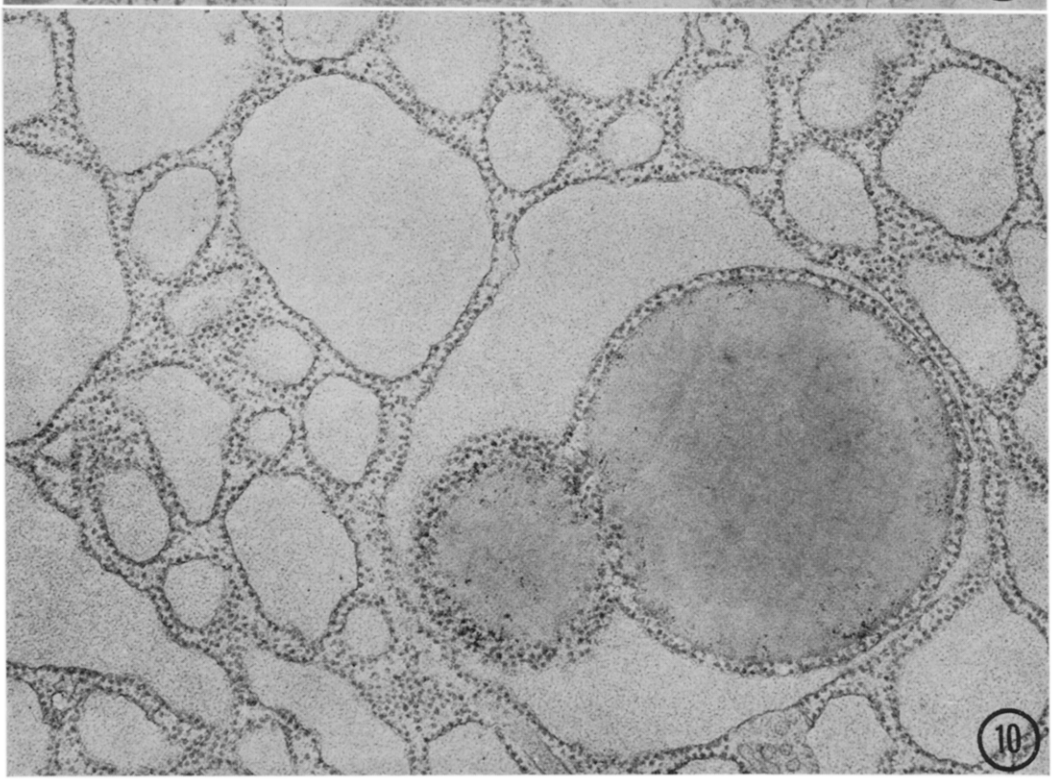


Fig. 9. *Pancreatic acinar cell from* 2-*AAF-treated rat, sampled after withdrawal of treatment. The granular internal structure of the large intracisternal granules is illustrated here.* (×30,000)

Fig. 10. *Pancreatic acinar cell from* 2-*AAF-treated rat, sampled after withdrawal of treatment. The intracisternal granules present have come to fill the lumen of the granular endoplasmic reticulum, thereby acquiring rough-surfaced limiting membranes.* (×30,000)

plasmic reticulum and perinuclear cisternae were filled with a finely granular material, resembling in appearance that which was observed within the saccules of the Golgi apparatus in both normal and experimental animals.

A further development of the endoplasmic reticulum lesion was observed in many of the pancreatic acinar cells of those rats which had been sacrificed during the post-treatment period. This was characterized by the appearance of granules within the rough-surfaced cisternae of the affected cells. These granules, which apparently consisted of condensation of the cisternal contents, varied considerably in size (Figs. 7–10) and in some cases the surrounding rough-surfaced membranes were closely applied to their surfaces (Fig. 10). The general appearance of these intracisternal granules closely resembled that of prezymogen granules except for the closely-applied smooth-surfaced limiting membranes surrounding the latter, the finely granular contents being very similar in both cases (compare Figs. 5 and 9). The presence was noted, within many of the intracisternal granules, of dense particulate accumulations, frequently annular in arrangement (Fig. 9). These were not found in other sites and their localization appeared to be specific to these granules, but they probably represented stain contamination of the surface of the sections.

In a substantial proportion of these pancreatic acinar cells which possessed a dilated granular endoplasmic reticulum, observations were made of dense, highly osmiophilic inclusion bodies within the intracisternal spaces. In these cells apparently identical inclusions were frequently also found within the distended perinuclear cisternae and inside the saccules of the Golgi system (Fig. 4). The dense bodies appeared to consist of closely apposed concentric membraneous lamellae, although fusions of many of the adjacent membranes were common, with consequent loss of lamellar ultrastructure. The bodies were similar in appearance to the inclusions which were frequently observed within the autophagic vacuoles or cytolysosomes of the acinar cells of 2-AAF-treated animals (see for example, the lower part of Fig. 5). No evidence was obtained during the present study to suggest the origin of these intracisternal inclusion bodies, although they may have been artefacts produced during fixation with glutaraldehyde.

The Golgi zones contained an abundance of granular material within their membraneous elements (Fig. 5) and were, in general, normal in size, thus presenting a normally "active" appearance. In some of the affected pancreatic cells, however, the Golgi apparatus appeared to be somewhat reduced in size and extent. This was observed rather irregularly and infrequently and was therefore apparently only characteristic of a minority of cells. There did not appear to be any direct or constant relationship between the degree of (relative) atrophy of the Golgi apparatus in a cell and the extent of dilatation of its endoplasmic reticulum.

The pancreatic acinar cells from the experimental animals at all stages of treatment contained variable numbers of autophagic vacuoles (Figs. 1 and 5), which were most frequently observed in close proximity to the Golgi elements. They appeared to be more abundant than in normal acinar cells and they were also usually larger. They often contained membraneous material at various stages of degeneration, which sometimes resembled the lamellar inclusion bodies of the intracisternal spaces.

Apart from the changes which have been described above, the pancreatic acinar cells of 2-AAF-treated rats appeared to be normal, at any rate on the basis of electron microscopical evidence. Zymogen granules in the process of being discharged were observed at the apices of these cells. The fine structural appearance of the cell surfaces was also normal. Although, as has been already noted, the nuclei of the more severely affected cells frequently possessed irregularly indented surfaces, their internal structure in general, and that of the nucleoli in particular, was apparently unaffected by exposure to 2-AAF.

DISCUSSION

From consideration of the results obtained during the present study, it is evident that the most significant alterations in the ultrastructure of the pancreatic acinar cells, following 2-AAF-administration, were those involving the granular endoplasmic reticulum and the structurally related perinuclear cisternae. On the basis of an abundance of biochemical, autoradiographic and electron microscopical evidence [11–19], it is now generally accepted that the production of secretory proteins by the pancreatic acinar cell involves the synthesis of these proteins on ribosomes attached to the granular endoplasmic reticulum their passage into the lumen of this organelle and their subsequent transfer to the Golgi apparatus. Here they may be further elaborated, concentrated and formed into prezymogen granules, which subsequently

become mature zymogen granules in the apical cytoplasm, before their contents are finally released into the acinar lumen. Any lesion affecting the granular endoplasmic reticulum might therefore have been expected to bring about some degree of disruption of this system of secretory protein production. However, the presence of mature zymogen granules, in apparently normal abundance and having an apparently normal ultrastructure, in the apical cytoplasm of even those acinar cells which exhibited the most extensive alterations of their granular cytomembranes, indicated that the terminal stages of the process were not significantly affected by 2-AAF. It was also evident that the normal process of discharge of zymogens from the apices of the acinar cells was unimpaired, while the majority of Golgi zones presented a normally "active" appearance. Thus there was no evidence that the functional capacity of the exocrine pancreas of the rat had been materially altered by either short or long term administration of 2-AAF. In addition, the apparent absence of discernable nuclear or nucleolar changes and the presence of numerous ribosomes attached to the granular endoplasmic reticulum in the abnormal pancreatic acinar cells suggested that the initial stages of secretory protein synthesis may also have been unimpaired by 2-AAF treatment. This view was reinforced by the presence of large quantities of finely granular material within the distended rough-surfaced cisternae, probably representing the accumulation of newly synthesized proteins.

It was apparent, therefore, that the 2-AAF-induced lesion of the granular membranes was most probably due to some alteration of the membrane structure itself. Incorporation of 2-AAF into the membrane structure might be expected to occur in the pancreatic acinar cells, since it is known to be incorporated in this way in the hepatocytes of 2-AAF-treated animals [20–22]. The stability of the resulting altered membranes, as well as their gross conformation, may differ from that of the normal structures. It is also possible that the presence of such altered membranes may itself affect the rate of synthesis of new membranes, perhaps by interfering with the controlling cellular feed-back mechanism. It has been shown that in the liver cell, and therefore, by inference, probably in other highly differentiated cells, the biosynthesis of new cytomembranes takes place by the extension of the existing granular endoplasmic reticulum membranes [23, 24]. It seems probable, therefore, that the existing membrane may act as a template for this biosynthesis [25]. Following the incorporation of 2-AAF this template may be altered in such a way that future membrane synthesis results in the formation of a new, but abnormal type of membrane, either in part or as a whole. The operation of such a mechanism would be consistent with the persistence of the endoplasmic reticulum lesion after the cessation of exposure to 2-AAF, which was observed during these experiments. The alterative possibility, that the observed lesion was due merely to the continuing presence of membranes which had been altered by their direct exposure to 2-AAF (or its metabolites), would require the assumption that there is no turn-over of the endoplasmic reticulum membranes in the pancreatic acinar cell during a period of six months or more. Since membrane biosynthesis and the synthesis of the constitutive enzymes of the cytomembranes have been shown to occur at different rates [24] and are therefore not necessarily directly linked, the 2-AAF lesion may not have been accompanied by any significant derangement of membrane-linked enzyme activity. However, it is possible that changes in the molecular conformation of the endoplasmic reticulum membranes produced by the incorporation of 2-AAF may result in changes in the stability of the attached polyribosome-mRNA complexes [25]. This could lead either to abnormal ribosomal detachment from the granular endoplasmic reticulum or to failure of the ribosomes to become detached at the appropriate time. There is evidence [23, 24] that the smooth-surfaced cytomembranes, together with their constitutive enzymes, are synthesized initially as part of the granular endoplasmic reticulum and then acquire their final form by the detachment of ribosomes from the latter and by membrane flow. It is probable that the membranes, as well as the contents, of the Golgi apparatus are derived in this way from the granular endoplasmic reticulum. A decrease in the normal rate of ribosomal detachment may lead to a partial blockage of this process. The abnormal presence of large intracisternal granules in the pancreatic acinar cells of rats which had received 2-AAF for a prolonged period suggested that such a partial blockage may indeed have taken place, resulting in the partial retention of secretory protein in the granular endoplasmic reticulum, which at all times bore numerous attached ribosomes. The formation of these granules was probably due to a process of concentration analogous, if not identical, to that occurring mainly, if not solely, in the Golgi apparatus of normal rat pancreatic

cells. It would seem, therefore, that the granular endoplasmic reticulum may have been able to assume the functions of the Golgi apparatus, as a consequence of 2-AAF treatment. This view was supported by the presence, in some cells, of structures which closely resembled prezymogen granules except insofar as they bore ribosomes, attached to their enclosing membranes (Fig. 10).

Similar intracisternal granules have been observed by previous workers in normal pancreatic acinar cells of the guinea pig [12, 26] and dog [27], in the cells of murine hepatomata [28] and in the pancreatic acinar cells of the rat following dietary administration of β-3-furylalanine [29] or ethionine [30]. Various degrees of cisternal dilatation have been shown to be produced in pancreatic acinar cells by a number of chemical agents other than 2-AAF [29–37] as well as by protein deficiency [38]. At least one of these, ethionine, resembles 2-AAF in that it has been shown to be incorporated into cellular proteins [39] and to be hepatocarcinogenic in the rat [40]. Many of these agents also produce broadly similar ultrastructural changes in the hepatocyte of treated animals, including disorganization of the granular endoplasmic reticulum [29, 36 and 41–43]. However, the lesions produced by these various chemical agents in pancreatic acinar cells differ, in several important respects, from those produced by 2-AAF. Perhaps the most striking of these differences is the apparent absence, in the acinar cells of 2-AAF-treated rats, of the marked inhibition of the synthesis of secretory proteins which is characteristic of the action of ethionine [33], β-3-thienylanine [35], azaserine [36], one-aminocyclopentane carboxylic acid [37] and to a less extent β-3-furylalanine [29] and which is also caused by dietary protein deficiency [38]. In addition, during the present study no observations were made of ribosomal detachment from the endoplasmic reticulum, such as is produced by treatment with ethionine [30, 31, 33] or protein deficiency [38], nor was there any evidence that 2-AAF induces either hypertropy of the Golgi zones or nucleolar hypertrophy, which have, however, been shown to result from the action of some of the chemicals mentioned above [29, 35, 37]. Furthermore, although the administration of any of the chemical agents mentioned above to the rat results in cytolysis in the pancreatic acinar cells, frequently involving large areas of cytoplasm, this was much less extensive in the acinar cells of 2-AAF treated rats. In this negative respect the effect of 2-AAF feeding resembled that of protein deprivation [38]. The extent of autophagic activity might be expected to reflect the degree of intracellular damage due to chemical treatment and, in this context, it is therefore of interest to record that the rate of feeding of 2-AAF used in the present experiment was sufficiently high to cause a high initial mortality rate during the first month of treatment. A second peak of mortality was found among those animals which were fed 2-AAF for 8–10 months. More evidence of degenerative changes might therefore have been expected during the present study than was actually obtained, in view of the observations of previous workers, cited above.

The variation in experimental procedures used by different workers, involving the use of various strains of rats and various modes of administration of chemicals, does not facilitate direct comparison between the effects of different chemical agents upon cellular ultrastructure. However, it appears that 2-AAF does produce an ultrastructural lesion of the pancreatic acinar cells of the rat which differs qualitatively from that produced by many other chemical agents. The 2-AAF lesion appears to be more specific in nature and thus less likely to be due to a non-specific toxic effect. The evidence presented here is not inconsistent with the suggestion that the primary effect of 2-AAF may be to alter the molecular conformation of the cytomembranes of the pancreatic acinar cell, probably by being incorporated into these membranes. However, direct biochemical evidence of incorporation of 2-AAF into the membranes in the present case is not available, and the argument is therefore based on the assumption that it occurs in the pancreatic acinar cell as in the liver cell. These changes appear to be compatible with the continuing viability and functional activity of the affected cells. It is also clear that they are not dependent on the continued presence of 2-AAF in the cellular environment, as is evidenced by their persistence and further development after the withdrawal of 2-AAF treatment. They may therefore be considered to be permanent in character.

It was demonstrated previously [4] that prolonged feeding of 2-AAF to the rat results in permanent changes in the ultrastructure of the hepatocytes, also involving the protein-synthetic apparatus of these cells. These changes included dilatation of the granular endoplasmic reticulum. The liver cell changes were considered to be associated with subsequent

hepatocarcinogenesis, since some of them were shown to persist in the cells of hepatomata arising in the treated animals [44]. Although there is no evidence that the pancreatic cell lesion which is induced by 2-AAF is in any way related to carcinogenesis in this tissue, it is nevertheless possible that this type of non-lethal damage may represent an important feature of the action of at least some carcinogens. The ability to produce cellular lesions which are limited to specific cytoplasmic loci may provide a valuable research tool for future studies of cell pathology.

RESUME

Une alimentation contenant 0,05% *de* 2-*acétylaminofluorène* (2-*AAF*) *fut donnée à des rats mâles de la lignée Leeds albinos. Les rats furent tués après* 3 *à* 4 *semaines et* 8 *à* 10 *mois de traitement. Certains animaux furent tués* 6 *mois après la fin d'un traitement d'une durée de* 10 *mois. Chez tous ces animaux, les cellules des acini du pancréas furent examinées au microscope électronique. Le* 2-*AAF provoque, dans ces cellules, une lésion relativement spécifique, localisée, dans une large mesure, au réticulum endoplasmique granulaire et aux citernes périnucléaires. Cette lésion consiste principalement en une dilatation des citernes et en l'apparition de granules à l'intérieur de celles-ci; elle semble permanente puisqu'on la retrouve* 6 *mois après l'arrêt du traitement. L'activité fonctionnelle des cellules acineuses du pancréas ne semble pas modifiée par le* 2-*AAF. La nature de la lésion produite par cette substance est discutée.*

SUMMARY

Male rats of the Leeds albino strain were fed a diet containing 0·05% *of* 2-*acetylaminofluorene* (2-*AAF*). *The rats were killed after* 3–4 *weeks and* 8–10 *months of treatment, and also up to* 6 *months after the end of a* 10 *month period of* 2-*AAF-feeding, and their pancreatic acinar cells studied by electron microscopy.* 2-*AAF appeared to produce a relatively specific lesion within these cells, being largely confined to the granular endoplasmic reticulum and perinuclear cisternae. This consisted mainly of cisternal dilatation and the appearance of intracisternal granules and appeared to be permanent in character, since it persisted up to* 6 *months after treatment had been withdrawn. The functional activity of the pancreatic acinar cells appeared to be unaffected by* 2-*AAF. The possible nature of the* 2-*AAF lesion is discussed.*

ZUSAMMENFASSUNG

Männliche Albino-Ratten ('Leeds'-Zucht) wurden mit einer Diät gefüttert, die 0,05% 2-*Acetylaminofluoren* (2-*AAF*) *enthielt.*

Die Ratten wurden 3 *bis* 4 *Wochen und* 8 *bis* 10 *Monate nach der Behandlung getötet, ebenso* 6 *Monate nach dem Ende einer* 10 *monatlichen periodischen Fütterung mit* 2-*AAF. Das pankreatische Zellgewebe wurde mit dem Elektronenmikroskop untersucht.* 2-*AAF scheint eine relativ spezifische Läsion der Zellen hervorzurufen, die weitgehend gegenüber das endoplasmatische Retikulum und den Zellkern umgebende Cysternen gerichtet ist. Es entstehen hauptsächlich erweiterte Hohlräume und intracisterne Granula. Dies erscheint als eine bleibende Veränderung, da sie bis zu* 6 *Monate nach Absetzen der Behandlung weiterbestand.*

Die funktionelle Aktivität von pankreatischem Zellgewebe erscheint mit 2-*AAF unverändert. Die mögliche Natur der durch* 2-*AAF bedingten Veränderungen wird diskutiert.*

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Conjugated and Unconjugated Dehydroepiandrosterone, Aetiocholanolone and Androsterone in the Peripheral Plasma of Patients with Cancer of the Breast, Ovary, Uterus or Prostate

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BÉNARD *et al.* [1] have recently summarized their extensive studies on the amounts of free (unconjugated) 17-oxosteroids in the peripheral plasma of normal men and women, and on patients suffering from various cancers. In these studies they have shown that free 17-oxosteroids are rarely detectable in the plasma of normal adults but are often present in elevated concentrations in plasma from patients with cancer. In their exhaustive review of the literature it is apparent that there is some disagreement concerning the amounts of free 17-oxosteroid present in plasma in various pathological conditions.

Gas–liquid chromatography is a method which has some advantages in terms of sensitivity and specificity over many colorimetric methods for the determination of 17-oxosteroids in biological fluids [2, 3]. This technique was used, therefore, to study the levels of free dehydroepiandrosterone, aetiocholanolone and androsterone in the peripheral plasma of subjects with cancer of the breast, ovary, uterus or prostate, and with benign breast tumours.

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METHOD

Reagents

All chemicals were of analytical reagent grade and all solvents were redistilled before use.

Silica gel (Kiesel G, Merck) was washed with ethanol before use in thin-layer chromatography. The reagents used to prepare the steroid chloromethyldimethylsilyl ether have been described by Thomas and Walton [4].

4-^{14}C Dehydroepiandrosterone (27·5mC/mM) was obtained from the Radiochemical Centre, Amersham. 1,2-^{3}H Aetiocholanolone (1·0c/mM) was purchased from New England Nuclear Corporation. 9,11-^{3}H Androsterone (40·2c/mM) was prepared by reduction of 3α-hydroxy-5α-androst-9,11-en-17-one with tritium.

Radioactivity was determined in a Packard Tricarb liquid scintillation counter, model No. 3003; efficiency for tritium was 50% and 60% for ^{14}C. Resolution of ^{3}H and ^{14}C counts was achieved using the method outlined by Okita, Kabara, Richardson and LeRoy [5].

Plasma was obtained from patients with cancer of various sites and stored at −20° before analysis. These patients were 7 women

Table 1. *Benign breast disease*

Age (years)	Androsterone	Dehydroepi-androsterone	Aetiocho-lanolone	Androsterone sulphate†	Dehydroepi-androsterone sulphate†
43	0·9	n.m.*	n.m.	—	—
79	0·5	0·5	n.m	64	100
47	0·5	0·4	n.m	—	—
29	0·4	0·7	n.m.	59	208
36	0·7	1·3	n.m.	60	131
52	0·8	0·4	0·1	16	77
53	n.m.	n.m.	n.m.	63	88

*n.m.=not measurable.
†Expresssed as free steroid after solvolysis.
All values are expressed as μg/100 ml plasma.

with benign breast disease, 14 women with early breast cancer and 14 with advanced breast cancer. A further 6 subjects had cancers of the ovary, uterus or prostate. Diagnosis was confirmed histologically. The patients' ages and plasma steroid levels are given in Tables 1–4.

Method of analysis

Plasma levels of dehydroepiandrosterone sulphate and androsterone sulphate were determined by the method of Wang *et al.* [3] with the modification that Sephadex LH20 was used in place of celite 535.

Unconjugated dehydroepiandrosterone, aetiocholanolone and androsterone plasma levels were determined using the following method. To 5 ml plasma was added 0·6 ml 2N NaOH, 5 ml water and 10,000 counts/min of 4-^{14}C dehydroepiandrosterone and 1,2-^{3}H aetiocholanolone (or 9,11-^{3}H androsterone). The plasma was extracted (3×) with 10 ml ether. The pooled ether was washed with 2 ml water, 2 ml 5% (v/v) acetic acid and 2 ml water and the ether evaporated. The dried ether extract was subjected to silica gel thin-layer chromatography using the system ethyl acetate and cyclohexane (equal volumes). The areas containing free 17-oxosteroids were located by comparison with standard compounds. The standard compounds could be seen under u.v. illumination after spraying with Rhodamine G. The relevant areas were scraped from the thin-layer plates and the silica gel inactivated with 0·5 ml water and extracted twice with 3 ml hexane. The pooled hexane extract was taken to dryness and the chloromethyldimethylsilyl ether derivative of the steroids were prepared [4]. The chloromethyldimethylsilyl ethers, after drying *in vacuo*, were dissolved in 0·05 ml hexane and then 0·45 ml cyclohexane. This solution was then added to

Table 2. *Early breast cancer*

Age (years)	Androsterone	Dehydroepi-androsterone	Aetiocho-lanolone	Androsterone sulphate†	Dehydroepi-androsterone sulphate†
38	1·3	0·1	n.m.*	50	25
55	0·9	0·3	n.m.	49	69
35	0·5	0·8	n.m.	97	181
65	n.m.	0·2	n.m.	—	—
75	0·8	n.m.	n.m.	23	46
76	0·8	n.m.	n.m.	46	41
40	0·3	0·2	n.m.	—	—
34	0·8	n.m.	n.m.	9	38
59	0·8	0·4	n.m.	11	57
60	1·0	n.m.	n.m.	9	24
33	1·1	0·7	n.m.	19	41
42	0·8	0·1	n.m.	86	118
65	0·4	n.m.	n.m.	18	37
47	1·2	1·9	n.m.	46	84

*n.m.=not measurable.
†Expresssed as free steroid after solvolysis.
All values are expresssed as μg/100 ml plasma.

Table 3. Advanced breast cancer

Age (years)	Androsterone	Dehydroepi-androsterone	Aetiocho-lanolone	Androsterone sulphate†	Dehydroepi-androsterone sulphate†
57	0·3	n.m.*	n.m.	—	—
40	n.m.	n.m.	n.m.	15	13
52	n.m.	0·9	n.m.	46	129
56	0·4	0·5	n.m.	13	40
53	2·3	n.m.	n.m.	51	89
40	0·7	1·1	n.m.	47	135
37	1·4	0·5	n.m.	83	137
52	1·33	0·1	n.m.	34	63
41	0·6	2·2	0·8	29	75
81	n.m.	n.m.	n.m.	0	38
68	0·4	n.m.	n.m.	0	47
47	0·3	0·3	0·1	31	81
53	n.m.	0·3	n.m.	39	91
60	n.m.	n.m.	n.m.	101	112

*n.m.=not measurable.
†Expresssed as free steroid after solvolysis.
All values are expressed as μg/100 ml plasma.

0·1 g alumina (containing 8% water) and shaken for 15 min, centrifuged and the supernatant discarded. The alumina was washed by shaking with 0·5 ml cyclohexane for 15 min and discarding the supernatant after centrifugation. The chloromethyldimethylsilyl ether derivatives were removed from the alumina by shaking for 15 min with 10% (v/v) acetone in cyclohexane. After centrifugation this solution was transferred to a tube and dried. The steroid derivatives were then redissolved in 0·1 ml hexane containing a known amount of 5α-dihydrotestosterone chloromethyldimethylsilyl ether (about 1 μg). The radioactivity in 20 μl was determined to estimate recovery and 20 μl used for gas chromatography. The gas chromatographic conditions are those described previously [3] and outlined in the legend to Fig. 1. The amount of steroid in the original plasma was calculated using recovery of radioactivity and comparison of peak height ratios of standard solutions. These values were also corrected for blank values using radioactive tracers alone. These corrections were negligible for aetiocholanolone and 0·7 and 1·5 μg/100 ml plasma for androsterone and dehydroepiandroesterone, respectively.

Levels of dehydroepiandrosterone were corrected to 100% recovery on the basis of recovery of ^{14}C-dehydroepiandrosterone. Levels of androsterone and aetiocholanolone were both corrected to 100% on the basis of recovery of whichever tritiated standard was added.

RESULTS

The levels of unconjugated dehydroepiandrosterone, aetiocholanolone and androsterone in the peripheral plasma of women

Table 4

Clinical status	Age (years)	Andro-sterone	Dehydroepi-androsterone	Aetiochol-anolone	Andro-sterone sulphate†	Dehydroepi-androsterone sulphate†
Normal male	48	0·1	0·7	0·7	105	350
Normal female	44	0·1	0·8	n.m.	30	98
Ca prostate	62	0·5	0·6	1·0	68	209
Ca prostate	75	n.m.*	n.m.	0·1	45	71
Ca corpus uteri	63	1·1	2·2	1·1	92	192
Ca corpus uteri	57	n.m.	0·6	0·5	35	56
Ovarian Ca	77	n.m.	0·2	0·4	10	13
Ovarian Ca	54	n.m.	n.m.	0·3	16	18

*n.m.=not measurable.
†Expressed as free steroid after solvolysis.
All values are expressed as μg/100 ml plasma.

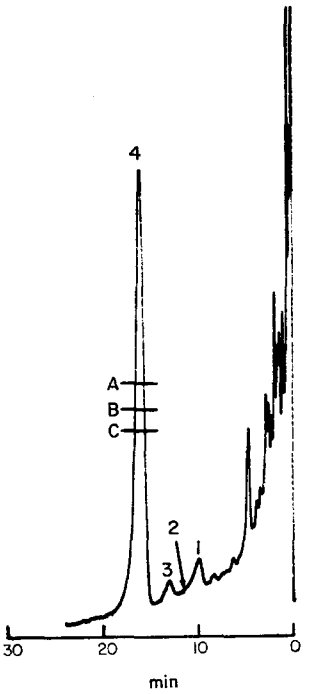

Fig. 1. Gas–liquid chromatographic tracing of the chloromethyldimethylsilyl ether derivatives of plasma steroids. Peaks 1 and 3 are the derivatives of androsterone and dehydroepiandrosterone, respectively. The aetiocholanolone derivative would have a retention time corresponding to 2. Peak 4 is 5α-dihydrotestosterone chloromethyldimethylsilyl ether used as an internal standard.

Conditions: temperature, 210°C carrier gas (N) 100 ml/min; column, 5 ft × 1% XE60 on Gas Chrom Q (100–120 mesh); detector, flame ionisation at a sensitivity of 5×10^{-11}. *A. full-scale.*

A. B and C are the heights of the androsterone, aetiocholanolone and dehydroepiandrosterone peaks, respectively, if they were each present at plasma concentrations of 10 μg/100 ml and with an overall recovery of 50%.

with benign and malignant breast tumours are shown in Tables 1–3 together with the amounts of these 17-oxosteroids released after solvolysis by the method of Burstein and Lieberman [6]. Very small amounts of free steroids were found (the total being less than 5 μg/100 ml plasma); in fact, in all cases the amounts determined were approaching the lower limit of detectability of the method (approximately 0·1 μg/100 ml plasma for any one steroid). A typical gas chromatogram is shown in Fig. 1 and it can be seen that with the amounts of free steroids present in plasma, accurate quantitation is impossible with the small peaks that occur in the regions of the chloromethyldimethylsilyl ethers of dehydroepiandrosterone, androsterone and aetiocholanolone. However, Fig. 1 shows that elevated levels of these steroids would certainly have been detected. The figures in Tables 1–3 are, therefore, semi-quantitative and are more likely to be over-estimates rather than under-estimates of the true values.

The plasma steroid levels in patients with cancers at sites other than the breast are shown in Table 4. It is apparent that no more than trace amounts of free dehydroepiandrosterone, androsterone and aetiocholanolone could be found in any of these subjects.

The amounts of dehydroepiandrosterone and androsterone released by solvolysis for the various clinical groups studied are shown in Tables 1–4. The values for these groups ranged from 13 to 350 μg/100 ml plasma (mean 90 μg) and 0–105 μg/100 ml plasma (mean 43 μg) for dehydroepiandrosterone and androsterone, respectively.

It can be seen that the unconjugated 17-oxosteroids constitute a minor portion of the total plasma 17-oxosteroids.

DISCUSSION

The amounts of free androsterone and dehydroepiandrosterone found in the peripheral plasma of women with benign or malignant breast tumours are very small, the mean levels being 0·7 and 0·4 μg/100 ml plasma for androsterone and dehydroepiandrosterone, respectively. The mean level of aetiocholanolone was indistinguishable from zero. Similar levels were also observed for subjects with cancers of the ovary, uterus and prostate. Such low plasma levels of unconjugated 17-oxosteroids are in general accord with numerous previous reports [7–18]. We were not able to detect raised levels of free 17-oxosteroids as reported by Bénard *et al.* [1, 19], who found appreciable levels (7–65 μg/100 ml.) in more than half their patients with cancers of the breast uterus or cervix.

A possible reason for the differences in levels of free plasma 17-oxosteroids observed by Bénard *et al.* [1] and in this report could be that our method of analysis is different. The former workers, after chromatography on florosil columns, used the Zimmermann reaction for quantitation. We have employed a gas–liquid chromatographic method which has a high degree of specificity. Correction for losses in both cases have been made using radioactive steroids.

One of the methods of choice for measuring low levels of 17-oxosteroids is the double-isotope technique. In normal subjects, the levels of plasma free dehydroepiandrosterone, androsterone and aetiocholanolone obtained with this method are in accord with those in this report [16–18]. It is highly unlikely that elevated levels of free plasma 17-oxosteroids would be missed by this technique since it is

theoretically impossible to underestimate the true level by this method.

Another explanation for the discrepancy between the results of Bénard *et al.* [1] and those reported here is that the clinical condition of the patients was grossly different in the two investigations. In addition, the majority of the free 17-oxosteroids measured by Bénard *et al.* [1] may have contained Zimmermann-reacting chromogens in addition to dehydroepiandrosterone, androsterone and aetiocholanolone.

If patients with cancer had elevated plasma concentrations of free dehydroepiandrosterone, androsterone and aetiocholanolone, their blood production rates required to maintain such levels would be extremely high. The blood production rate of any steroid is equal to the product of the metabolic clearance rate (MCR) and the concentration of that steroid [20]. There does not appear to be any gross difference in the MCR of dehydroepiandrosterone between normal women and those with breast cancer [21, 22]. Thus, with a MCR of 1000l/day and a steroid concentration of 1 μg/100 ml the blood production rate for dehydroepiandrosterone would be 10 mg/day. Levels of free dehydroepiandrosterone of 10 μg/100 ml plasma, observed by Bénard *et al.* [1], would imply a blood production rate of 100 mg/day. The numerous reports on the low levels of urinary 17-oxosteroid in patients with breast cancer do not support such high production rates [23–31]. On the contrary, it seems highly probable that the production rates are lowered [32, 33]. However, even in cases of high steroid output as in adrenal hyperplasia, where the plasma level of solvolysable steroids can be as high as 1 mg/100 ml, the corresponding concentration of free dehydroepiandrosterone was less than 2 μg [17].

In conclusion we have not found elevated plasma concentrations of androsterone, aetiocholanolone and dehydroepiandrosterone in subjects with cancers of the breast, uterus, prostate or ovary. Our results are in marked contrast to those of Bénard *et al.* [1] and an investigation of the reasons for this discrepancy might be instructive.

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RESUME

Les taux plasmatiques des 17-oxostéroïdes conjugués et non conjugués ont été mesurés par chromatographie en phase gazeuse et liquide chez des patients atteints de cancers du sein, de l'ovaire, de l'utérus et de la prostate, et dans des affections bénignes du sein.

Les quantités de déshydroépiandrostérone non conjuguée et d'étiocholanolone étaient toujours faibles (valeur maximale: 2,3 μg/100 ml), ou les traces en étaient indosables. La plus grande partie de la déshydroépiandrostérone et de l'étiocholanolone étaient sous forme conjuguée.

Ces résultats diffèrent de ceux publiés par d'autres auteurs qui avaient trouvé des quantités appréciables de 17-oxostéroïdes libres (7–65 μg/100 ml) dans plus de la moitié des cas de cancer. Cette différence est discutée en termes de vitesse de production, et des explications en sont proposées.

SUMMARY

Plasma levels of conjugated and unconjugated 17-oxosteroids have been measured by gas–liquid chromatography in subjects with cancer of the breast, ovary, uterus or prostate, or with benign breast disease.

The amounts of unconjugated dehydroepiandrosterone, androsterone and aetiocholanolone present in plasma were invariably low (the highest value being 2·3 μg/100 ml) or undetectable. The major portion of the total plasma dehydroepiandrosterone and androsterone were conjugated.

These results differ from those published by other workers [1], in which over half the cancer cases had appreciable levels of free 17-oxosteroids (7–65 μg/100 ml). This difference is discussed in terms of production rates and suggestions are made for possible reasons for the difference.

ZUSAMMENFASSUNG

Der Plasmaspiegel von konjugierten und nicht konjugierten 17-Ketosteroiden wurde

gaschromatographisch gemessen bei Patienten mit Carcinomen der Brust, des Ovars, Uterus oder Prostata oder mit gutartigen Brusterkrankungen.

Die Menge von nicht conjugierten Dehydroepiandrosteron, Androsteron und Aetiocholanolon war im Plasma unverändert niedrig (der höchste Wert betrug 2,3 μg/100 *ml) oder nicht feststellbar. Der größere Teil von dem totalen Plasma-Dehydroepiandrosteron und -Androsteron waren conjugiert.*

Die Resultate unterscheiden sich von denen anderer Autoren [1], *bei welchen über die Hälfte der Carcinomfälle einen beträchtlichen Spiegel von* 17-*Ketosteroid* (7–65 μg/100 *ml*) *besitzen. Dieser Unterschied wird im Zusammenhang mit der Produktionsrate diskutiert und mögliche Gründe des Unterschiedes werden erörtert.*

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The Binding of Steroids to Plasma Proteins in Normal Women and Women with Breast Cancer

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A NUMBER of reports indicate that the clinical course of breast cancer is related to the amounts of androgen and corticosteroid metabolites found in the urine [1–9]. Preliminary results indicate that there is a relation between the amounts of these urinary hormone metabolites in normal women and the subsequent development of breast cancer [10].

Some studies have now been carried out to determine the levels of the plasma precursors of the relevant urinary steroids in patients with breast cancer and to compare these plasma and urinary concentrations [11]. While the levels of plasma and urinary 17-oxosteroids are related, there is no such correlation between plasma and urinary 17-hydroxycorticosteroids [11] and there is tentative evidence of deficient binding of cortisol in some patients with breast cancer [12].

These findings suggested that any study involving the measurement of blood steroid levels should also include measurement of the degree of protein binding of the hormones. The object of this investigation was to determine whether patients with benign breast disease, with early or advanced breast cancer, and normal women showed any differences in the protein binding of a variety of steroids.

METHOD

Subjects

Blood was taken from all subjects between 9 a.m. and noon; plasma was separated from heparinised blood and stored at −20°C. The controls were healthy women resident on the island of Guernsey. The patients were all investigated while in hospital. The early breast cancer group were patients awaiting mastectomy and the advanced group were those awaiting adrenalectomy or hypophysectomy. In these two groups and the group with benign breast lesions, the disease was confirmed histologically.

Chemicals

All reagents were of Analar quality. The radioactive steroids 7-H^3 dehydroepiandrosterone (4·96C/mM), 7α-H^3 androstenedione (1·05C/mM), 6,7-H^3 oestradiol (54·4C/mM) and [7α-H^3] progesterone (2·26C/mM) were obtained from the Radiochemical Centre, Amersham. 1,2-H^3 Aetiocholanolone (1·OC/mM) and 6,7-H^3 oestriol (14·9C/mM) were purchased from New England Nuclear Corporation. 7-H^3 Testosterone (30·8C/mM) was prepared by the method of Coombs and Roderick [13] and 9,11-H^3 androsterone (40·16C/mM) by the catalytic tritiation of 3α-hydroxy-5α-androst-9,11-en-17-one. All these steroids were additionally purified by paper chromatography before use. The sulphate esters of dehydroepiandrosterone and androsterone were prepared and purified as described previously [14].

Equilibrium dialysis

Heparinised plasma was diluted with four

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Table 1. *Percentage of steroid bound to plasma proteins in normal women and women with breast cancer*

Compound		Normal	Benign breast disease	Early breast cancer	Advanced breast cancer
Oestradiol	% Binding	91·9	91·5	91·5	91·9
	±S.D.	1·15	1·51	1·22	0·87
	No.	8	8	8	8
	Mean age	47	47	53	52
	Age range	36–53	21–72	39–68	42–64
Oestriol	% Binding	65·0	64·6	63·5	61·6
	±S.D.	3·89	3·69	2·66	2·67
	No.	7	7	7	7
	Mean age	45	40	51	50
	Age range	36–50	21–72	38–75	39–65
Dehydroepiandro-sterone	% Binding	86·8	85·4	85·5	85·7
	±S.D.	0·82	0·97	0·76	1·59
	No.	7	7	7	7
	Mean age	45	40	51	50
	Age range	36–50	21–72	38–75	39–65
Androsterone	% Binding	90·1	89·2	89·5	89·5
	±S.D.	1·19	0·93	0·96	2·04
	No.	7	7	7	7
	Mean age	45	40	51	50
	Age range	36–50	21–72	38–75	39–65
Aetiocholanolone	% Binding	86·7	87·0	87·1	88·0
	±S.D.	1·65	1·58	1·78	2·48
	No.	7	7	7	7
	Mean age	45	40	51	50
	Age range	36–50	21–72	38–75	39–65
Dehydroepiandro-sterone sulphate	% Binding	95·1	94·7	95·4	94·9
	±S.D.	1·06	1·13	0·44	0·95
	No.	12	8	8	8
	Mean age	46	47	53	51
	Age range	33–55	21–72	39–68	42–60
Androsterone sulphate	% Binding	98·0	97·5	98·1	97·8
	±S.D.	0·68	0·99	0·22	0·52
	No.	12	8	8	8
	Mean age	46	47	53	51
	Age range	33–55	21–72	39–68	42–60
Testosterone	% Binding	94·8	94·5	93·6	94·4
	±S.D.	1·01	1·17	1·47	2·02
	No.	8	8	8	8
	Mean age	47	47	53	51
	Age range	36–53	21–72	39–68	42–60
Androstenedione	% Binding	72·1	71·0	72·4	72·8
	±S.D.	1·51	2·12	1·90	1·98
	No.	8	8	8	8
	Mean age	47	47	53	51
	Age range	36–53	21–72	39–68	42–60
Progesterone	% Binding	93·4	92·9	93·2	92·6
	±S.D.	0·58	1·07	0·59	1·21
	No.	8	8	8	8
	Mean age	47	47	53	52
	Age range	36–53	21–72	39–68	42–64

times its volume of 0·9% NaCl solution (adjusted to pH 7 with NaOH or HCl). The mixture (2 ml) was put into a sac made from 9/16 in. Visking 'Nojax' casing and dialysed at 37°C in 6 ml physiological saline to which the appropriate radioactively labelled steroid (30,000 counts/min) had been added. The steroid–saline solution was prepared by adding

to a vessel the radioactive compound in ethanol and drying; the saline was added and shaken for 15 min. The equilibrium dialysis vessels were gently shaken in a water bath at 37° for 18 hr. Control experiments for each steroid showed that this time was sufficient for equilibrium to be reached.

Multiple equilibrium dialysis was performed as describe previously [15].

Determination of radioactivity

At the end of the period of dialysis the sacs were quickly rinsed with water and dried with paper tissue and the contents emptied into test tubes. Duplicate 0·1 ml plasma solutions and 0·4 ml saline solutions were transferred to counting vials and 15 ml phosphor added (7 g 2,5-diphenyloxazole (PPO), 600 mg 1,4-bis-2-(4-methyl-5-phenyloxzaoyl)-benzene (dimethyl POPOP), 500 ml ethoxyethanol and 500 ml toluene). The vials were counted in a Packard Tricarb liquid scintillation counter, model No. 3003. The efficiency for tritium was 30%. Quenching was determined by the addition of an internal standard (^{3}H-toluene).

The percentage binding was calculated using the formula

$$1-\frac{(S-C)\ (Tp-P)}{4\ (Ts-S)\ (P-C)}\times 100$$

where C is background counts, P and S are counts for plasma and saline solutions respectively, and Tp and Ts are counts for plasma and saline solution after the addition of internal standard, respectively. These counts are all expressed per unit of time.

RESULTS

The data for the ten steroids studied (Table 1) show that the percentage binding varies considerably between steroids; for instance, 62% of the oestriol is bound compared with 92% of the oestradiol-17β. However, for a given steriod there is no significant difference in the mean amount of steroid bound in normal women, patients with benign breast disease, with early breast cancer, or with the advanced disease.

We have previously shown that small differences in percentage binding can be amplified if multiple dialysis is performed [15]. In this technique the plasmas to be compared are dialysed in the same vessel and the radioactive counts bound to the respective plasma proteins compared. Thus, two plasmas binding 98·0% and 98·5% will exhibit a 25% difference in the number of counts bound. Pooled plasma from the four groups of subjects studied were dialysed against each other. The results of these experiments have been expressed in two ways. First, as percentage of steroid bound, calculated in the usual way, and second, as a direct comparison of the number of counts bound to the four plasmas.

Table 2 shows the results for the percentage binding of the ten steroids for each of the four plasma pools. It can be seen that, for a given steroid, there are no differences in percentage binding; this is in accord with the data in Table 1. The second way of expressing results is shown in Table 3 where the number of counts bound to plasma proteins from normal woman is compared with the number of counts bound to one of the other three plasmas.

For convenience the number of counts bound to plasma proteins from normal women is expressed as 100. Table 3 shows that the differences in the number of counts bound for all the steroids was less than 10%, indicating little or no difference in the percentage of radioactivity bound.

Table 2. Percentage bound after multiple equilibrium dialysis

Compound	Mean percentage bound	Fraction of mean percentage bound			
		Normals	Benign breast disease	Early breast cancer	Advanced breast cancer
Oestradiol	90·2	1·00	1·00	1·00	0·99
Oestriol	63·2	1·02	1·00	1·00	0·98
Dehydroepiandrosterone	84·9	1·00	1·00	1·00	1·00
Androsterone	87·7	1·00	0·99	1·00	1·01
Aetiocholanolone	86·7	0·99	1·00	0·99	1·03
Dehydroepiandrosterone sulphate	95·5	1·00	1·00	1·00	1·00
Androsterone sulphate	98·0	1·00	1·00	1·00	1·00
Testosterone	95·0	1·00	0·99	1·00	1·01
Androstenedione	69·7	0·99	0·99	0·99	1·04
Progesterone	91·8	1·00	1·00	1·00	1·01

Table 3. Counts bound to plasma after multiple equilibrium dialysis

Compound	Normals	Benign breast disease	Early breast cancer	Advanced breast cancer
Oestradiol	100*	99	102	93
Oestriol	100*	94	95	93
Dehydroepiandrosterone	100*	99	100	96
Androsterone	100*	95	97	109
Aetiocholanolone	100*	104	95	107
Dehydroepiandrosterone sulphate	100*	97	91	94
Androsterone sulphate	100*	97	101	92
Testosterone	100*	95	97	101
Androstenedione	100*	101	102	109
Progesterone	100*	93	92	105

*For convenience counts bound to 0·1 ml diluted normal plasma has been expressed as 100 and counts bound to the other diluted plasmas as a percentage of this.

Since the percentage binding for the various steroids varied so markedly it was of interest to determine whether these variations were related to any other physiological parameter. One of the obvious parameters which the degree of binding of a steroid could affect is the rate of clearance of that steroid from the peripheral circulation. Tait [16] has formulated a convenient measure of this clearance which he called the metabolic clearance rate (MCR). This is defined as the volume of plasma (or whole blood) which is irreversibly cleared of the steroid in question in a given time (usually 24 hr).

The data for the MCRs has been abstracted from the literature [17–28] and compared with the percentage binding quoted in this paper or other publications from this laboratory [15, 27]. Figure 1 shows the plot of MCR against percentage binding, both expressed logarithmically. It can be seen that there is a relationship between the MCRs and the percentage binding. It is clear that cortisol behaves anomalously compared with the other steroids. Nevertheless, when individual values for cortisol binding and the respective MCRs from controls early or advanced breast cancer subjects are compared [27] a linear correlation was found:

MCR (l/day) = 1072 − 10·0 × percentage binding. This correlation was highly significant ($P<0·001$).

DISCUSSION

It is clear that there are no significant differences in the mean amounts of steroids bound to the plasma proteins in normal women and women with benign breast disease, early breast cancer or metastatic breast cancer. This probably means that plasma levels of the ten steroids studied in the investigation are directly comparable. Although the mean amounts of steroid bound in these groups are similar, there is still the possibility that there are differences in the amounts or proportions of proteins to which the steroid is bound. If this were the case, there might be differences in binding affinities which would necessitate a more intensive investigation.

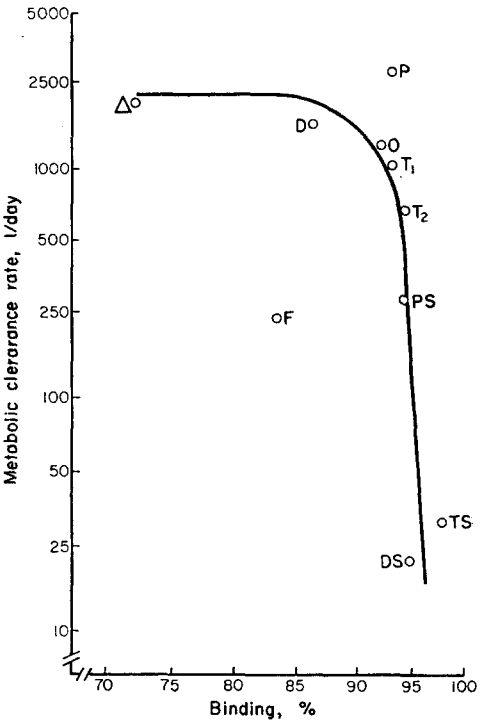

Fig. 1. The relationship of metabolic clearance rate and percent binding.
The ordinate is metabolic clearance rate (l/day) and the abscissa is percentage binding, both are expressed logarithmically.

Δ =androstenedione
P =progesterone
D =dehydroepiandrosterone
O =oestradiol-17β
T_1 =testosterone (male)
T_2 =testosterone (female)
PS =pregnenolone sulphate
TS =testosterone sulphate
DS =dehydroepiandrosterone sulphate
F =cortisol

Bell, Bulbrook and Deshpande [12] found no difference in the mean transcortin levels in four comparable groups of subjects but they noted that a larger variation in the cancer patients compared with the controls. For example, 4 of 13 patients with advanced breast cancer had levels of transcortin below the normal 95% range. With very high rates of binding, small variations of this nature lead to very large changes in the amounts of free steroid. Thus, a 15% fall in transcortin binding would lead to an approximate doubling of free cortisol. In the present study there was no evidence for an increase in the variance of the amounts of steroids bound in the three clinical groups compared with the control.

The functions of the binding proteins is still not entirely clear. Available evidence strongly suggests that only the unbound hormones exert biological effects. Thus, in pregnancy the plasma levels of cortisol, testosterone and thyroxine are increased [29–33], but there are no obvious signs of hyperadrenocorticism, virilization or thyrotoxicosis. It is probable that this is because the plasma proteins which bind these hormones are elevated [31, 34–36]. The changes in binding in these situations may be homeostatic mechanisms to maintain relatively constant levels of unbound hormones.

Another influence of the degree of protein binding is on the MCRs of steroids. From Fig. 1 and the relationship between cortisol binding and its MCR it is seen that there is a correlation between these parameters. It would be an oversimplification to postulate that the degree of protein binding is the sole determinant of the MCR in a complex system in which hepatic and renal factors must presumably participate.

Another feature of the data in Fig. 1 is that the type of protein upon which the hormone is bound does not influence the MCR. Testosterone [37–39], oestradiol-17β [40, 41] and progesterone [42] are bound to globulin-type proteins whereas the steroid sulphates [43, 15] and androstenedione [31] are bound to albumin.

Acknowledgements—The authors thank Miss V. Amor and Miss R. Smith for their skilled technical assistance. They are also grateful to Mrs. V. Jensen for her permission to use her data on cortisol binding.

RESUME

La capacité de fixation des protéines plasmatiques, pour 10 *stéroïdes différents, a été étudiée par dialyse chez des femmes normales, des patientes avec des affections bénignes du sein, et des malades atteintes de cancer du sein à un stade précoce ou avancé.*

La capacité de fixation, pour en stéroïde donné, n'a pas varié selon les groupes. Ceci fut confirmé par des dialyses par équilibration multiple.

Les pourcentages de fixation, pour les divers stéroïdes, étaient les suivants: oestradiol-17β (92%), *oestriol* (64%), *déshydroépiandrostérone* (86%), *androstérone* (98%), *étiocholanolone* (87%), *sulfate de déshydroépiandrostérone* (95%), *sulfate d'androstérone* (98%), *testostérone* (94%), *androsténédione* (72%), *et progestérone* (93%).

SUMMARY

The percentage binding of ten steroids to plasma proteins have been measured by equilibrium dialysis in normal women, women with benign breast disease, and with early or advanced breast cancer.

There were no differences in the mean percentage binding for a given steroid between any of the groups. This finding was confirmed using multiple equilibrium dialysis. The steroids and their mean percentage bindings were: oestradiol-17β (92%), *oestriol* (64%), *dehydroepiandrosterone* (86%), *androsterone* (89%), *aetiocholanolone* (87%), *dehydroepiandrosterone sulphate* (95%), *androsterone sulphate* (98%), *testosterone* (94%), *androstenedione* (72%) *and progesterone* (93%).

ZUSAMMENFASSUNG

Die prozentuale Bindung von 10 *Steroiden an Plasmaproteine wurde mit Hilfe der Verteilungsdialyse bei normalen Frauen, Frauen mit gutartigen Brustkrebserkrankungen und mit frühem oder fortgeschrittenem Brustkrebs gemessen.*

Es wurden keine Unterschiede bei der durchschnittlichen prozentualen Bindung für ein gegebenes Steroid zwischen den Gruppen festgestellt. Diese Befunde wurden durch Anwendung der mehrfachen Verteilungsdialyse gestützt. Die Steroide und ihre durchschnittliche prozentuale Bindung waren:

Oestradiol-17β (92%), *Oestriol* (64%), *Dehydroepiandrosteron* (86 %), *Androsteron*

(89%), *Aetiocholanolon* (87%), *Dehydroepiandrosteron-Sulfat* (95%), *Androsteron-Sulfat* (98%), *Testosteron* (94%), *Androstendion* (72%) *und Progesteron* (93%).

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Studies of Tumour Invasion in Organ Culture

1. Effects of Basic Polymers and Dyes on Invasion and Dissemination

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INTRODUCTION

ALTHOUGH the final justification of studies on invasion *in vitro* is their reproducibility in the whole animal, it is nevertheless clear that some of the underlying cell interactions, e.g. contact inhibition of movement [1], density dependent inhibition of mitosis [2], contact guidance [3] can be investigated more readily in tissue culture. The review of Abercrombie and Ambrose [4] showed clearly that surface properties play an important role in invasion.

The present and following paper describe an attempt to influence invasion by altering cell surface properties in various ways. It was decided to try to develop a quantitative technique for the investigation of invasion *in vitro* using a simpler culture method than those currently available, e.g. the technique first described by Wolff in 1961 [5].

It has been suggested that the increase in surface charge density found in some transformed and tumour cells is due mainly to an increase in the amount of sialic acid in the cell coat [6]. Defendi and Gasic [7] proposed that this material may be responsible for the reduction in contact inhibition often found between these cells. Since a reduction in contact inhibition between tumour and normal cells seems necessary for invasion to take place *in vitro*, it follows that, if the functional component of sialic acid in this situation is the electronegative charge, neutralisation should affect contact inhibition directly, and secondarily, invasion. Neutralisation of the excess charge might also affect invasion by increasing cell adhesions, since some workers [8] have proposed that tumour cells are less adherent because of their abnormally high surface charges.

For these reasons, therefore, cells and cultures were treated with the cationic polyelectrolyte poly-L-lysine, and the basic dye toluidine blue. The enzymatic removal of sialic acid, and the subsequent effect on invasion, will form the substance of the second paper.

METHODS

In the assay method which was eventually evolved for these experiments, small explant cultures were set up on an agar-based medium, and to these was added a constant known number of potentially invading cells. The combined cultures were fixed after five days, sectioned and stained, and the amount and type of invasion, estimated microscopically and semi-quantitatively, were correlated with various types of treatment.

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Details of the tissue cultures

Growth medium consisted of Burroughs Wellcome 'BHK' (Eagle's MEM, modified for BHK cells), 10% fresh chick embryo extract, 10% Burroughs Wellcome heated horse serum (No. 5), 275 mg % $NaHCO_3$, and 100 i.u./ml benzylpenicillin, with a base of 0·6% Difco 'Bactoagar'. The ten times concentrated BHK medium was diluted with tris/HCl buffer (pH 7·4) instead of with water. This helped to maintain the correct pH.

Five ml of this medium were pipetted into a series of 'Esco' (Rubber) disposable 5 cm diameter petri dishes, and 6–8 explant cultures (1 mm^3) of 16–20 day foetal C^- mouse heart muscle were supported on the solidified agar surface by fragments of Millipore filter (RA, 150 μ thick, 1·2 μ average pore size).

BHK cell cultures [9], both untransformed (BHK21) and transformed *in vitro* with polyoma virus (Py) were provided by Professor Stoker, and were maintained to his specification in the authors' laboratory. Two days before setting up the explant cultures, diluted Indian ink was added to the medium of one or two 24 hr monolayer cultures (in 4 oz medical 'flats') as described by Stoker [10]. It was not thought necessary to test the effect of Indian ink on Py cells, since Stoker reported no change in the attachment, spreading or multiplying of ink-labelled Py (and BHK21) cells existing as a monolayer in ink-free medium. On the day an experiment was begun, the cells were detached from the glass with 20 mg %'Versene'. The resulting suspension was washed twice with Hanks saline to remove adsorbed carbon particles, and a cell count was made. The pellet was then resuspended in 0·1 ml saline, and pipetted on to a clean dry sterile glass surface so that it formed a drop.

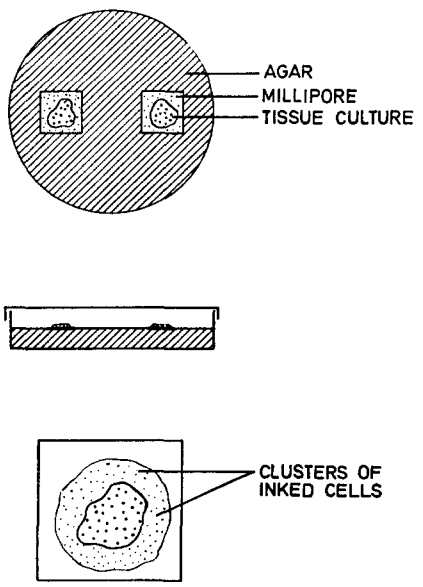

Fig. 1. Surface and side-views of a culture dish, with enlarged diagram of one culture.

The surfaces of the Millipore fragments were first dried with sterile lens paper to prevent the liquid spreading from the edges of the explant, and then drops of approximately 0·4 μml volume containing 40,000–45,000 cells were transferred to each explant in turn, using a very small sterile platinum wire loop. Figure 1 shows several views of a culture at this stage. The culture dishes were then placed in a humidified lunch box, which was permanently fitted with inlet and outlet tubes, and gassed with a mixture containing 5% CO_2, before the box was sealed and incubated at 37°C. Cultures were fixed in Bousins at the end of five days, sectioned at 5μ and stained with haematoxylin (Harris') and eosin.

Substances tested

(a) *Poly-L-lysine.* (Bromide salt, Koch Lights, mol. wt. 50,000).

i. This was added to the growth medium before gelation to give final concentrations of between 1 and 150 μg/ml.

ii. Py cells were incubated at room temperature for 10 min in serum-free BHK medium containing 75 or 150 μg/ml polylysine. The reaction was stopped with 10% serum. Polylysine treatment killed 30% of the cells at the lower, and 50% at the higher concentration. The cells were added to the explants as above, after washing once.

(b) *Toluidine blue.* (Geo. Gurr Ltd., mol. wt. 305·8.)

This was added to the growth medium before gelation, to give final concentration of 1,5 and 10 μg/ml.

(c) *Control.* In each experiment, 6–8 explants received untreated Py cells and were grown on medium containing neither polylysine nor toluidine blue. These were the control cultures: 20% consisted of dead explants at the end of the experimental period, and 1% contained no living Py cells.

Estimation of results

This was carried out by microscopic inspection of one or more sections from each culture, at a magnification of ×480—more rarely at ×1250. Absolute counts of invading cells per field were impossible to obtain because of the difficulty of deciding whether Py cells at the periphery of the cultures were growing directly on the Millipore, or whether they were in that position after replacing normal fibroblasts outgrowing from the original explant, i.e. it

was not possible to recognise invasion in this area. A statistical method based on counts of invading cells in four predetermined areas in the total microscopic field yielded significant results at the beginning, when compared with non-quantitative visual assessment, but this required at least six complete, non-fragmented sections per culture, which could not always be produced. (It was found by one of the authors, M.M.Y., in later experiments, quite practical to prepare suitable sections personally; future investigations will therefore make use of the statistical method outlined above as well.) The results presented here are thus a preliminary survey of the problem.

In default of suitable microscope sections, a less quantitative assay method was devised, by which both the approximate amount and the type of invasion could be estimated. This method consisted of recording deviations from (1) the approximate amount of invasion in untreated explants (termed the 'norm') and (2) the appearance of the invading cells (termed 'alpha') in such cultures.

Normal invasion is defined as that resulting from the addition of between 40,000 and 45,000 Py cells to explants of 16–20 day foetal mouse heart muscle, not more than 1mm^3, cultured under standard conditions as described on p. 256, for five days. The histological picture presented by an explant invaded to the normal degree is of replacement of approximately half the explant with Py cells, this replacement having been carried out by the infiltration of streams of cells, issuing forth from that area of the culture which had already (after five days) been replaced completely with invading cells. In some cases these early replacing cells had entered by way of the top surface of the explant (Fig. 9) upon which they had been deposited originally from the platinum loop. More usually, however, they appeared as the progeny of Py cells deposited on the Millipore, which had invaded by coming in from underneath the explant (Fig. 6). This latter mode tended to cause the death of the normal tissue, if complete, by separating it from its source of nourishment, and was so described by Leighton [11]. Normal invasion was recorded in 178/220 control cultures (81%) under appropriate conditions.

Comparing invasion of BHK21 cells with normal invasion of Py cells, it was noted that at least twice as many cells were required to produce the same amount of invasion, and that infiltration never occurred. BHK21 cells invaded as a solid mass of cells (Fig. 7) and streams of single invading cells, so characteristic of Py invasion, were never observed. The procedure for assaying invasion was as follows. By inspection of a randomly chosen microscope section, the explant was judged alive or dead. This was recorded as A (alive) or B (dead). The invading Py cells were likewise judged alive (I) or dead (II). Cultures consisting of a living explant and live invading cells were then examined to determine the amount of invasion. That corresponding to 'normal' invasion (p. 257) was signified as 1, and the whole section (culture) coded as AI1. If fewer than 10–15 cells were seen to have invaded in the section examined, invasion was judged 'reduced' (AI2) and if more than 3/4 of the explant had been replaced by Py cells, invasion was called 'increased' (AI3). Cultures with dead explants and/or Py cells were scanned in the same way to establish the amount of invasion which had taken place. It will be obvious from this description that only gross deviations from the norm were recorded. This, together with the division of degrees of invasion into three categories only, was done to improve the reliability of the method, subject as it was already to many inherent sources of biological variation.

Finally, the morphological appearance and arrangement of the invading cells was assessed. This fell into four broad categories (Fig. 2).

alpha	Typical of control culture. Cells ovoid in section, with few carbon particles in the cytoplasm.
beta	Cells lying in 'sheaves' as BHK21 cells do in monolayer culture, with long axes parallel, cells and nuclei

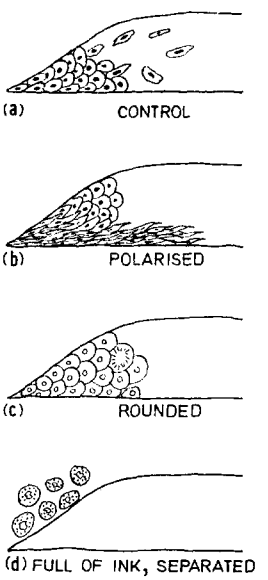

Fig. 2. Diagrammatic representation of (a) alpha, (b) polarised, beta (c) gamma and (d) delta invading cells.

Table 1. *Effects of polylysine and toluidine blue on the viability of standard cultures after* 5 *days*

Control		% Cultures with dead explants 20	dead Py cells only 1	No. cultures 220
Polylysine in culture medium	1–150 µg/ml	23	0	46
Polylysine used to pretreated Py cells	75 µg/ml	32	16	22
	150 µg/ml	45	50	20
Toluidine blue in culture medium	1 µg/ml	14	12	28
	5 µg/ml	45	44	29
	10 µg/ml	81	79	16

flat and elongated, with few carbon particles in the cytoplasm. Also called 'polarised'.

gamma Cells round in section with rounded nuclei, tending to separate, few carbon particles in the cytoplasm.

delta Cells rounded in section and deeply stained, quite separate, many carbon particles in the cytoplasm.

(No gamma cells were observed after treatment with polylysine or toluidine blue.)

Microscopical examination was carried out 'blind'. The histopathological picture presented by an explant normally invaded by alpha cells can be seen in Fig. 3. Results were calculated by expressing as a percentage the number of cultures showing a particular feature within the total number of available cultures, e.g. reduced invasion. Only cultures in which both explant and invading cells were still alive at the time of fixation were considered for the results summarised in Tables 1 and 2. (Values for dead cultures could be obtained in the same way when required.) The results obtained by this method of assessment compared quite satisfactorily with those preliminary results obtained from the statistical method (p. 257).

Mitotic indices were calculated by counting both the cells in mitosis and the total number of Py cells in one section of each culture, and adding together the numbers obtained from each sample in the group, before working out the percentages.

Tests in monolayer cultures

Monolayers were set up in 'Falcon' disposable plastic flasks, 30 ml capacity, by adding 5 ml organ culture medium minus agar, with $0{\cdot}25 \times 10^6$ Py cells, and adjusting the gas phase with CO_2. Substances to be tested were added after 24 hr, with complete medium change. Random counts were made daily on the population, and the final number was counted on the fifth day, after the cells had been removed with trypsin–versene (0·05% trypsin, 20mg% versene). Four test were made with toluidine blue, and two with polylysine.

RESULTS

From preliminary results it was found that the amount of invasion, under standard conditions, varied directly with the number of untreated Py cells deposited on the explants.

(a) *Lethal effects upon explants and invading cells* (*Table* 1)

Polylysine had no adverse effect on either component when present in the medium; toluidine blue, on the other hand, had an increasingly lethal effect on explants and Py

Table 2. *Effects of polylysine and toluidine blue on the amount of invasion and the appearance of the invading cells*

Control		% Cultures with reduced invasion 11	δ cells 0	increased invasion 8	β cells 10	No. cultures 220
Polylysine in culture medium	1–5 µg/ml	4	0	12	54	23
	75–150 µg/ml	7	12	9	12	14
Polylysine used to pretreated Py cells	75 µg/ml	87	35	0	8	15
Toluidine blue in culture medium	1 µg/ml	9	0	30	65	23
	5 µg/ml	7	0	79	63	14

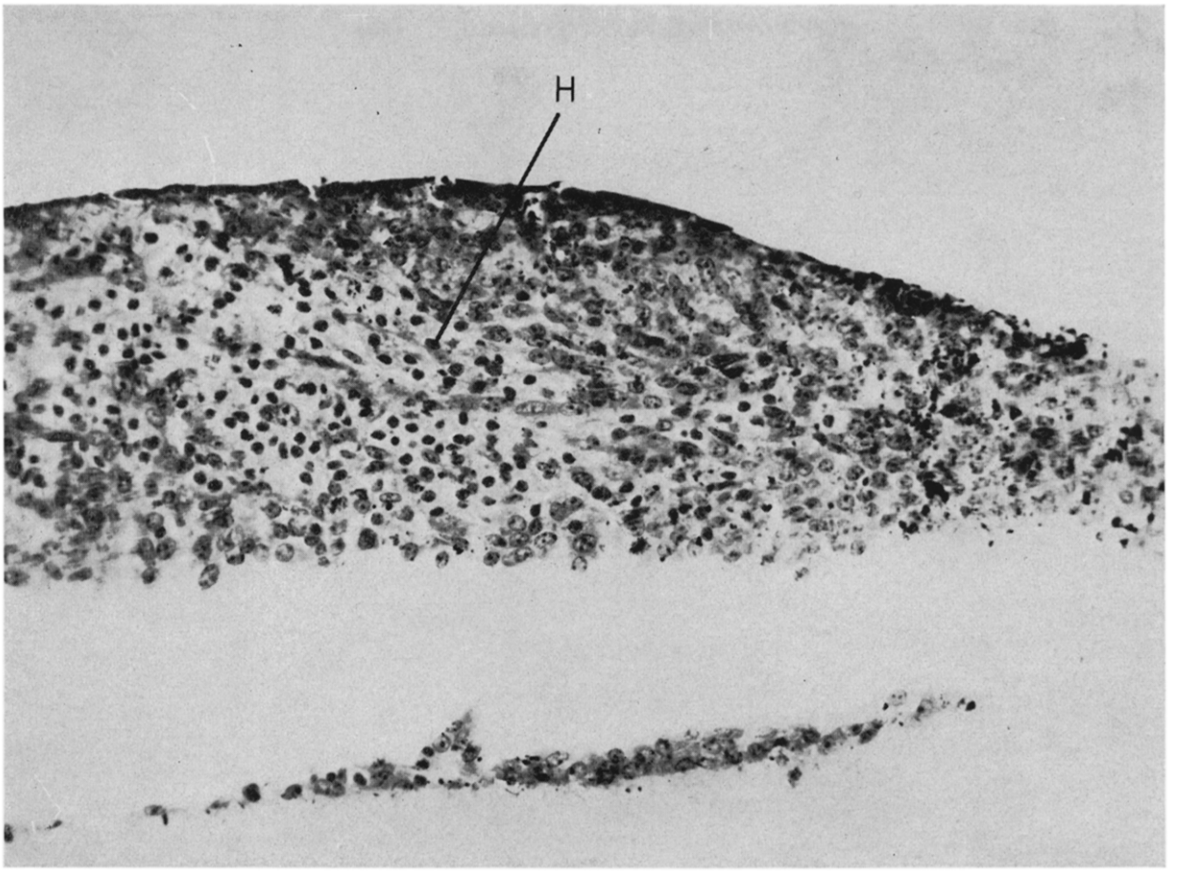

Fig. 3. *The 'normal' amount of invasion of 'alpha' Py cells into foetal mouse heart (H) after* 5 *days. H. and E.* × 300.

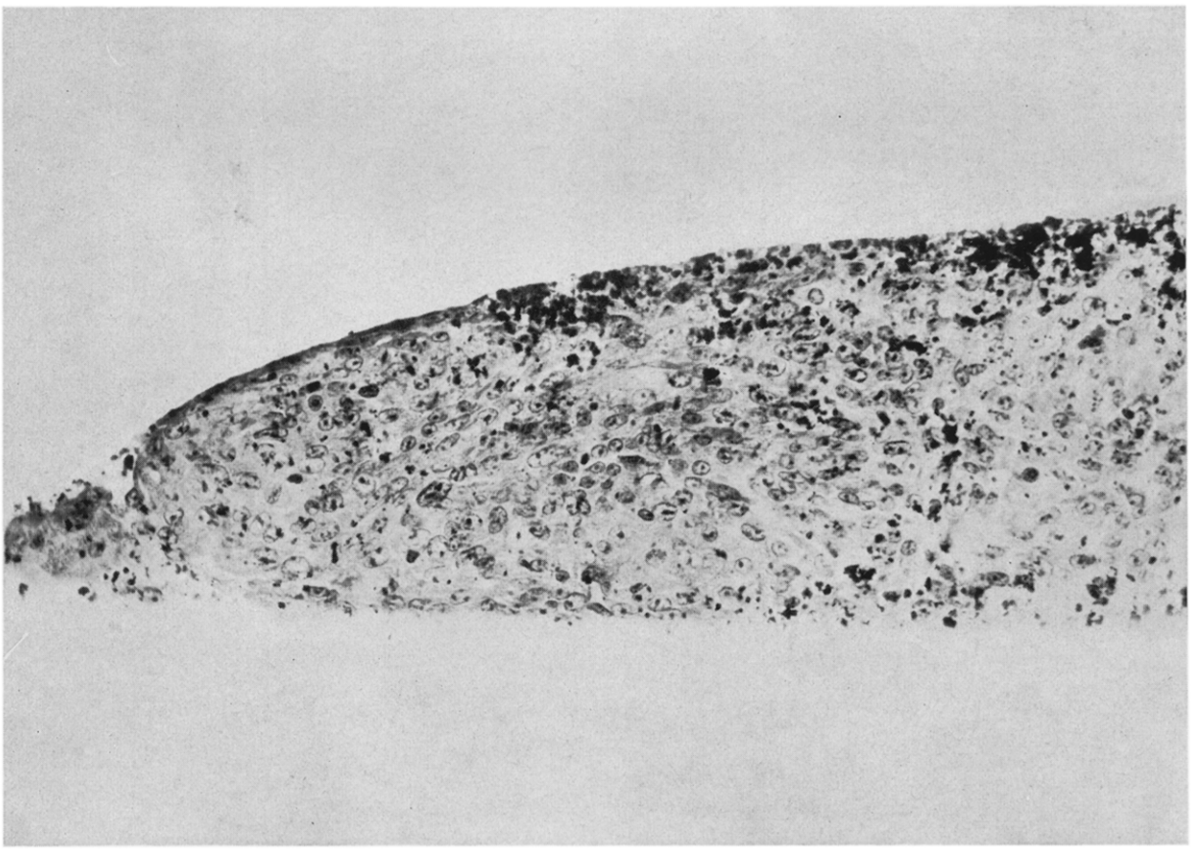

Fig. 4. *The effect of including* 5μg/*ml toluidine blue in the culture medium: increased invasion. H and E.* × 300.

(to face p. 258)

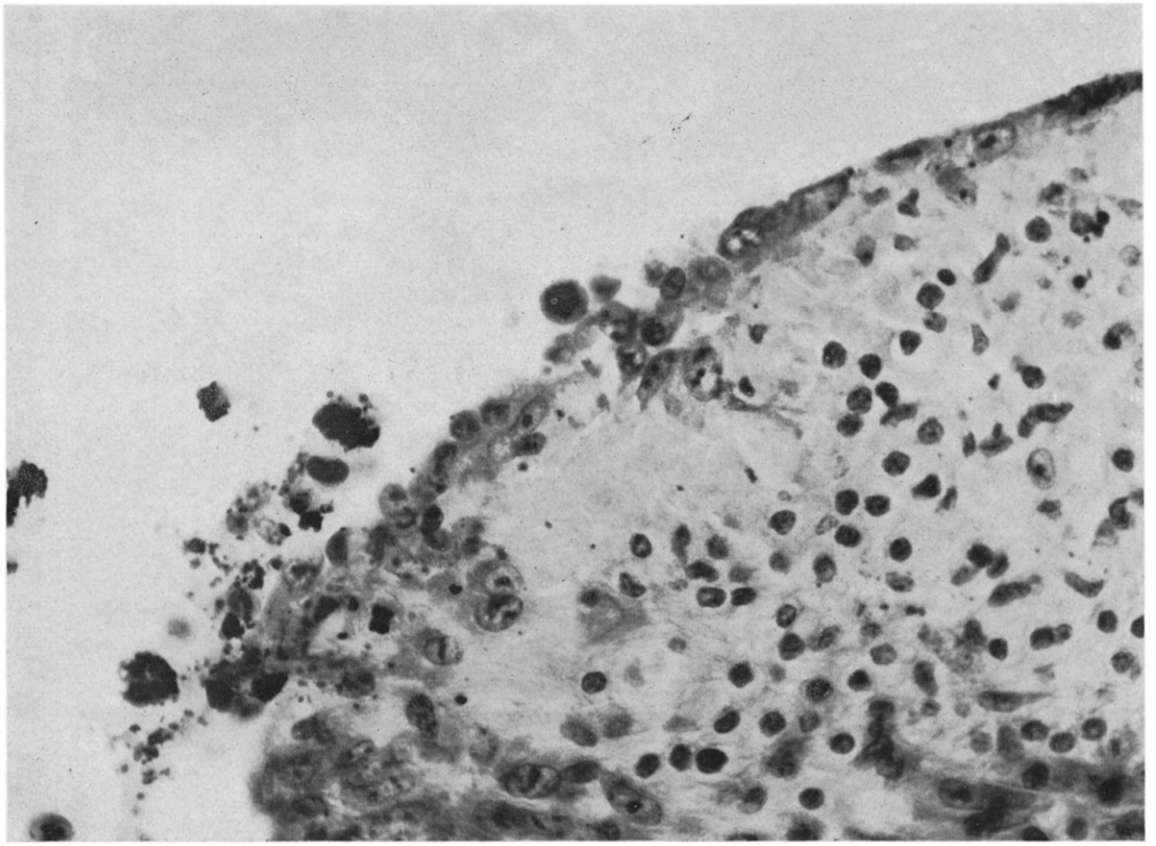

Fig. 5. *The effect of pretreating Py cells with* 75μg/*ml polylysine: delta cells. H. and E.* × 580.

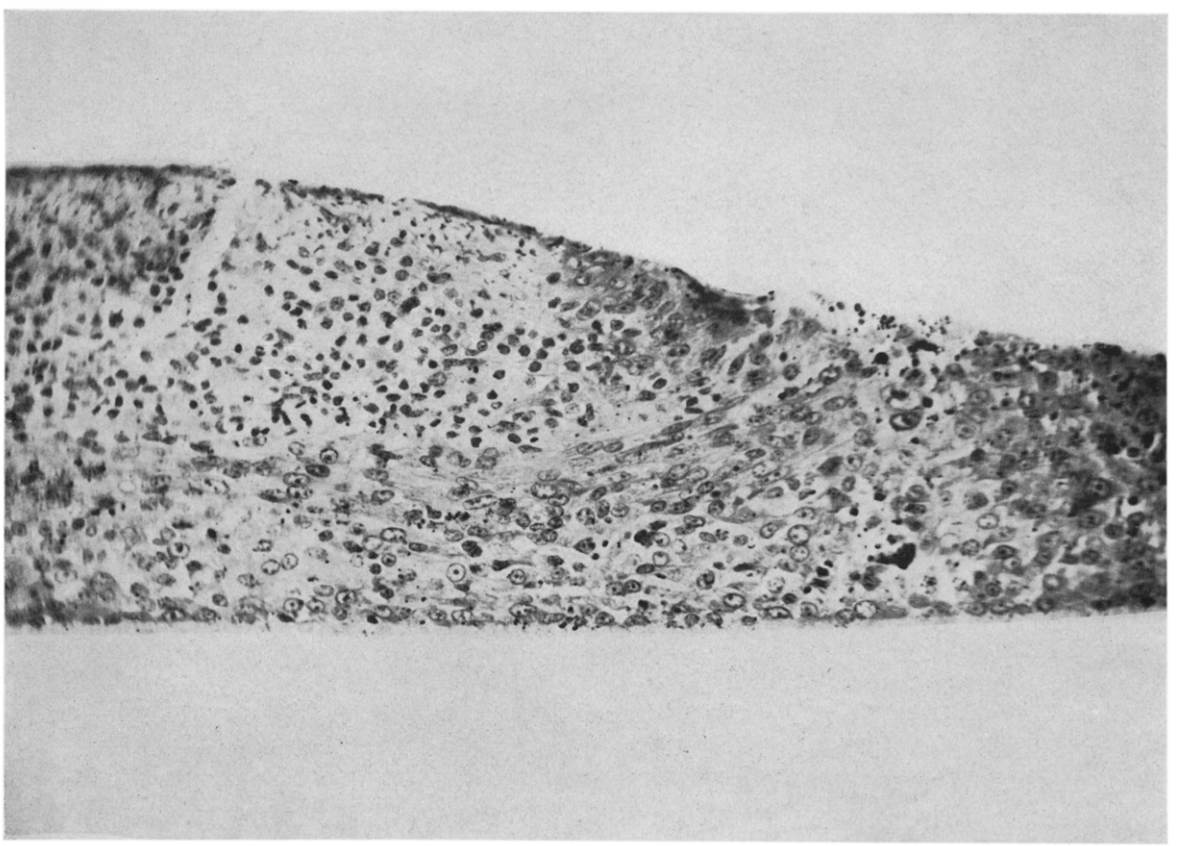

Fig. 6. *The effect of including* 1μg/*ml toluidine blue in the culture medium: polarised Py cells. H. and E.* × 300.

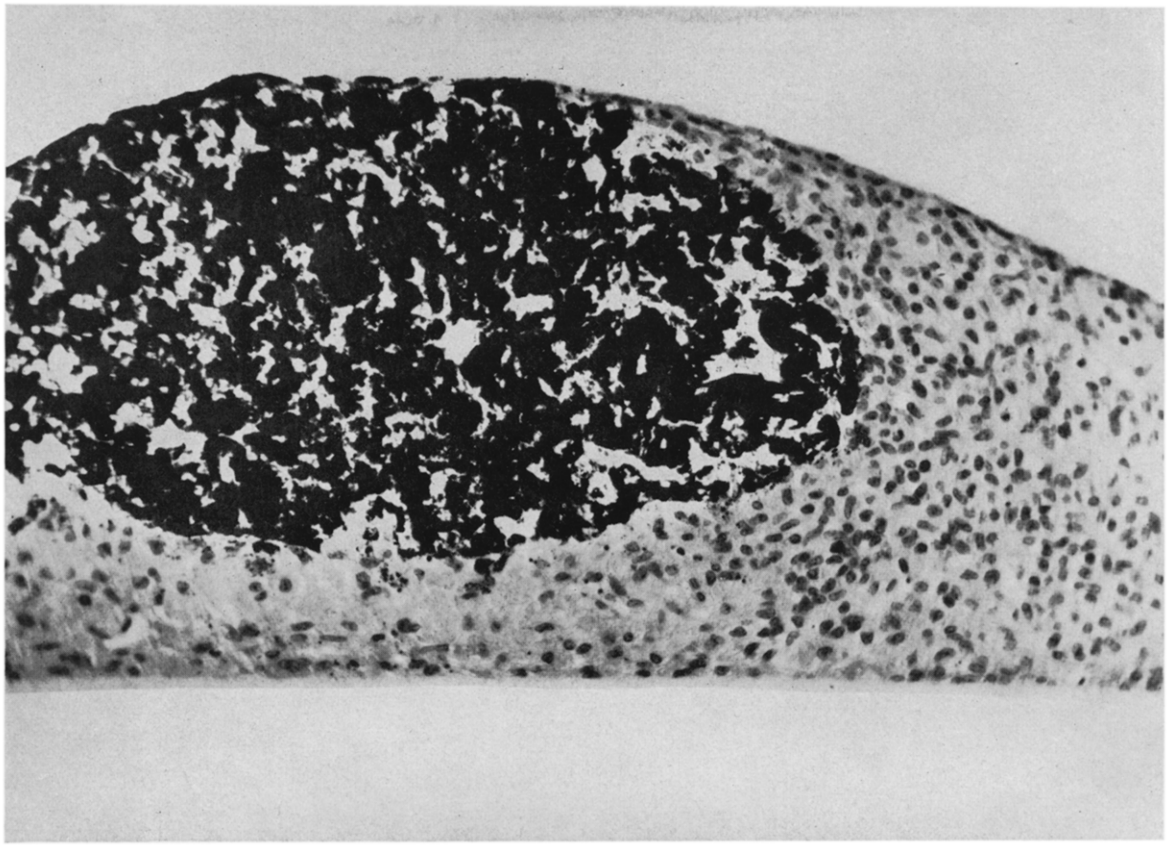

Fig. 7. *Invasion 'en bloc' of BHK*21 *cells labelled with Indian ink into foetal mouse heart after* 7 *days. H. and E.* × 300.

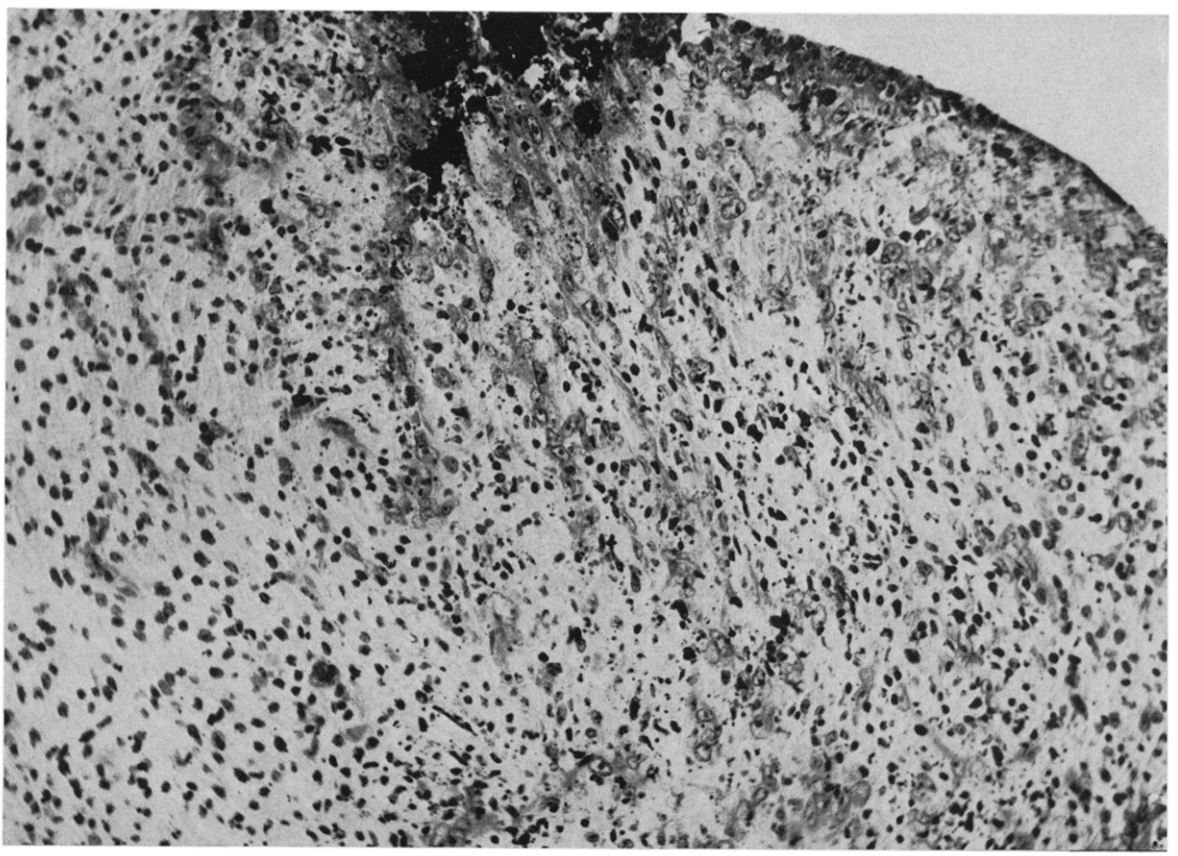

Fig. 8. *Invasion of ink-labelled Py cells into foetal mouse heart showing the parallel arrangement of the invading cells. H. and E.* × 300.

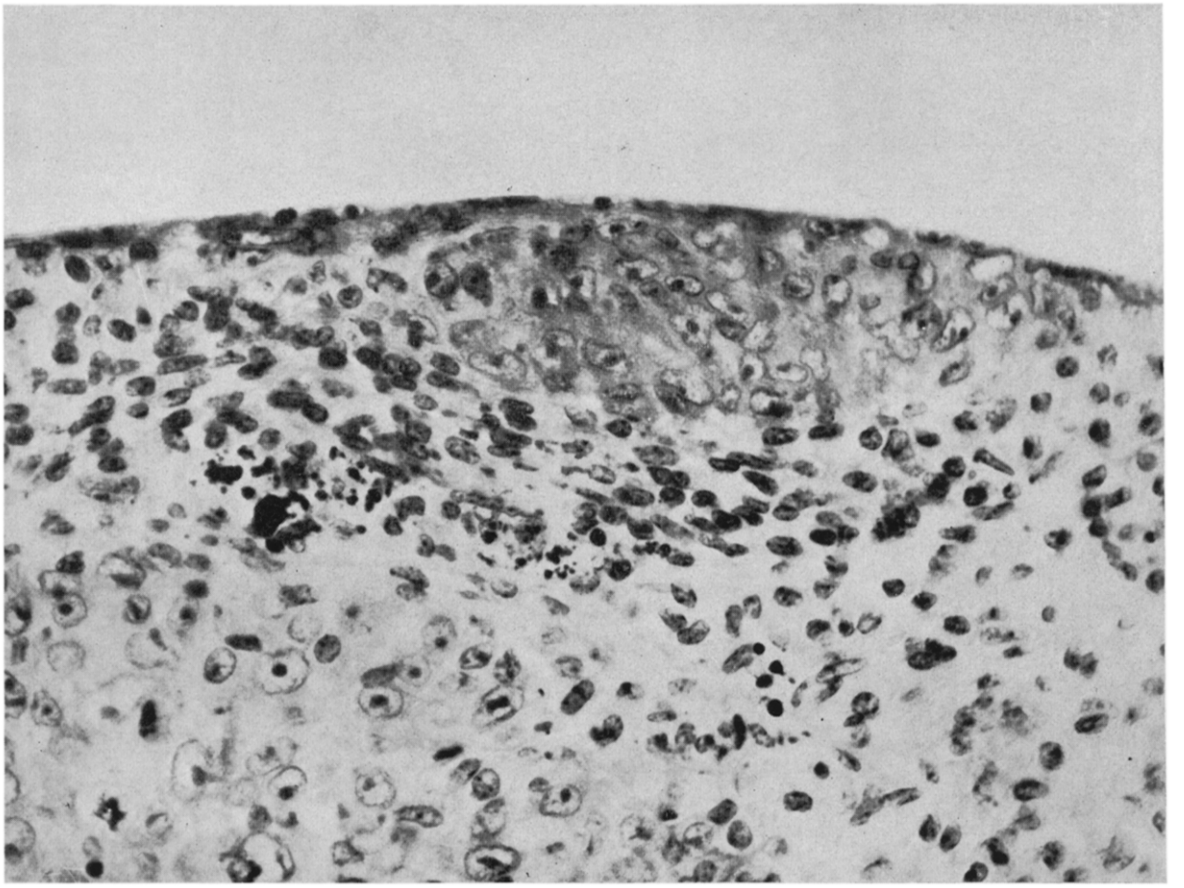

Fig. 9. *Foetal heart muscle fibroblasts exhibiting parallel alignment within an explant. H. and E.* × 580.

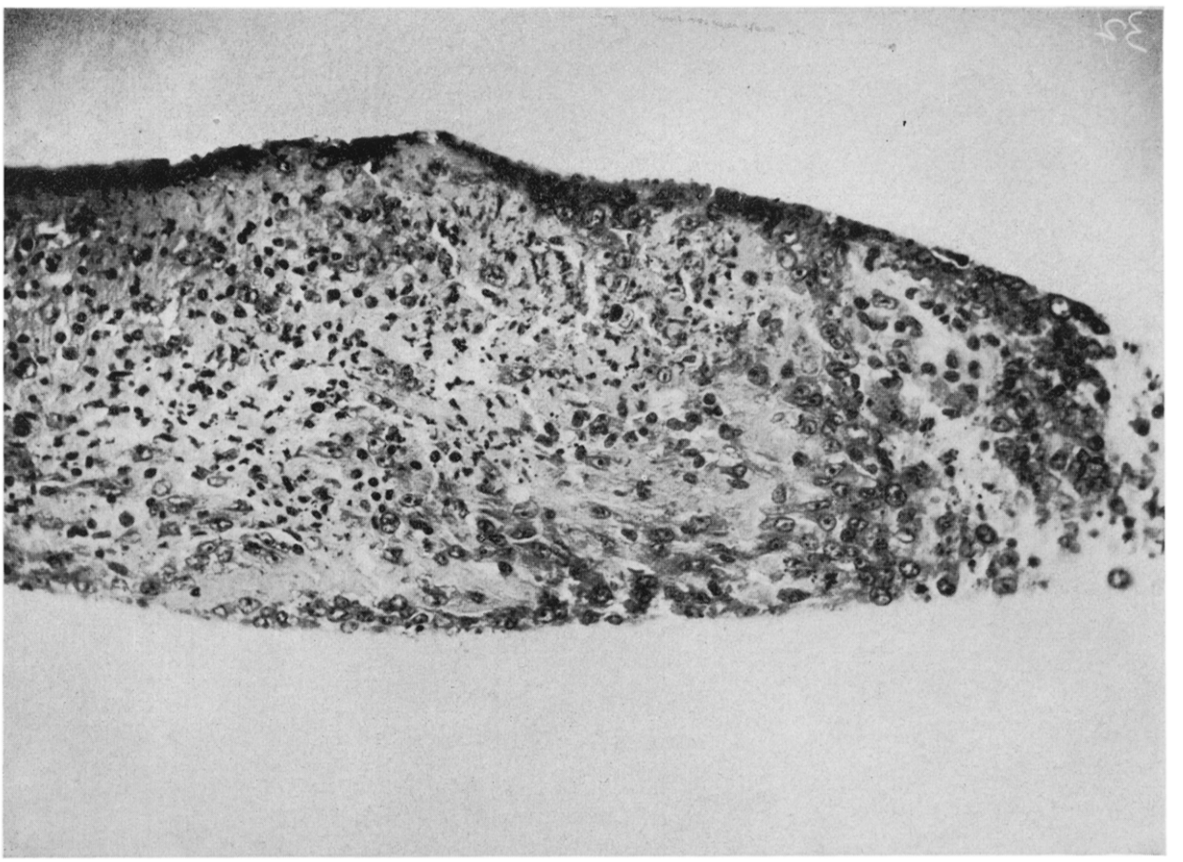

Fig. 10. *Invasion by infiltration of BHK*21 *cells which had spontaneously transformed in monolayer culture to a Py type of growth pattern. H. and E.* × 300.

cells as the concentration in the medium increased from 1 to 10μg/ml. It is interesting to note that toluidine blue was more toxic to the malignant than to the normal element in the cultures.

Invading Py cells, pretreated with 75μg/ml polylysine before combination with the explants, resulted in cultures of which 15% contained no living Py cells and 12% dead explants after five days. Pretreatment with 150μg/ml polylysine was associated with an approximately two-fold increase in mortality of both components.

(b) *Effects upon the amount of invasion* (*Table* 2)

Again, the presence of polylysine in the medium at any of the concentrations tried had no effect on the amount of invasion. Toluidine blue at concentrations of 1 and 5 μg/ml was associated with increased invasion in 22% and 71% (Fig. 4) of the cultures, respectively. At the higher concentration, 19% of explants were completely replaced by invading cells.

Since pretreatment of Py cells with 150 μg/ml polylysine was so lethal to the cultures (Table 1), only the results obtained by the use of 75μg/ml will be considered further. This is necessary because the number of living samples in which the effect of the higher concentration of polylysine could be observed was so reduced.

Pretreatment with 75μg/ml polylysine resulted in reduced invasion in 76% of cultures, though there were no examples of increased invasion.

(c) *Effect upon the appearance and arrangement of Py cells*

The change in appearance in Py cells described as 'delta' did not occur in control cultures. It was seen, however, in 12% of cultures maintained on medium containing up to 150μg/ml polylysine, and 35% when Py cells had been pretreated with 75μg/ml (Fig. 5).

Polarised Py cells were observed in 44% of cultures maintained on medium containing 1–5μg/ml polylysine, and in 55% and 53% of those maintained on 1 (Fig. 6) and 5μg/ml toluidine blue. Polarisation did not occur after pretreatment with polylysine, nor at higher concentrations when present in the growth medium.

(d) *Effect on the type of invasion*

The invasion of polarised Py cells was not of the infiltrating type typical of untreated Py cells (Fig. 3), but rather of the 'en bloc' type seen when BHK21 cells invade (Fig. 7).

(e) *Effect on mitosis in Py cells*

The mitotic index of 25,700 invading non-polarised Py cells maintained on control medium, was 1·1%. That of 2500 polarised Py cells maintained on polylysine-containing medium was 0·6%, and of 8000 similar cells on toluidine blue-containing medium (1 and 5μg/ml) was 0·7%.

DISCUSSION

Lethal effects in general

Considering that Kornguth *et al.* [12] found that polylysine at concentrations less than 200μg/ml had no adverse effect on Ehrlich ascites tumour cells, it was unexpected to find considerable cell death, under similar conditions, with Py cells in the present study. Nevo *et al.* [13] reported that polylysine did not penetrate mammalian red blood cells but, in the absence of serum, was adsorbed on the cell surface. It is clear that polylysine, in the experiments under review, caused no cell damage when serum was present in the medium; from this it may be assumed that the molecule became adsorbed preferentially on the serum molecule. Other results suggest, however, that polylysine did penetrate the treated cells, thereby causing their death when serum was absent. From experiments in monolayer cultures, it was observed that toluidine blue penetrated Py cells even when serum was present, and that this had a lethal effect. This would seem to explain the progressive damage to cultures (p. 258) with increasing concentrations of the dye, and the lack of effect of polylysine under similar conditions.

Effects of polylysine on invasion

If the reduction in invasion which followed pretreatment of invading cells with 75μg/ml polylysine was due to another cause besides the death of 30% of the treated cells; this other cause may be characterised by the presence of cells, since the δ cells were only found after pretreatment. If this is then so, the δ cells have a reduced capacity for invasion. There is an interesting parallel with untreated Py cells during the first 48 hr after deposition on an explant. These cells also have the δ appearance, and invade scarcely at all; the lack of invasion is obviously due to the fact that the cells had not, during the first 48 hr, begun to attach, spread or move about on the explant. This is what happens, of course, when Py cells are seeded on a glass surface and incubated.

It may be that polylysine reduced the capacity of the cells to move. Our cinemato-

graphic observations (unpublished) confirm that polylysine-treated cells exhibit no directional movement at all, in comparison with untreated cells, which move freely. Nevo *et al.* [13] showed that the polylysine molecule is adsorbed on the mammalian cell surface, and that this is associated with a reduction in the surface charge. Korohoda and Ambrose (in press) found that polylysine, in concentrations of 100 μg/ml and above, caused total cessation of movement in the protozoan *Naegleria gruberi*. This paralysis was associated with a decrease in net negative surface charge.

Richardson [14] reported mitotic arrest in prophase in mouse ascites tumour cells after polylysine had been administered intraperitoneally, but no significant increase in the number of treated cells at any stage of the mitotic cycle was observed in the present study.

Polarisation

The appearance and arrangement of invading Py cells which has been termed 'polarised' was observed in 10% of the organ cultures grown under control conditions. An explanation must be found, therefore, not for the development of a new phenomenon, but for the increase of the occurrence of an already existing one, under certain conditions. Not only did the percentage of cultures showing polarisation increase after toluidine blue was included in the medium, but also cultures treated with 5μg/ml had approximately three times as many polarised cells as cultures treated with 1μg/ml. The presence of polarised cells when polylysine was present in the medium will not be considered, since it only occurred at the lowest range of concentrations.

Polarisation could have been caused by the presence of more Py cells than usual within the explant, since it was associated, in many cases, with increased invasion (Table 2). This was due apparently to a stimulation by toluidine blue of mitosis in Py cells; 1μg/ml toluidine blue in the medium of Py monolayer cultures was associated with a two-fold increase in mitotic rate at the end of five days. Since Py cells tend to invade heart muscle in parallel lines (Fig. 8), presumably under contact guidance, the typical polarised appearance would appear if enough Py cells were present in one area. This would also explain the greater incidence at 5 μg/ml than at 1 μg/ml, for the stimulatory effect increased in that order. The reduction in mitotic rate to approximately half in cells already polarised would then be secondary, and be due to the inhibition of growth ultimately seen in crowded Py cultures.

Polarisation could also have come about by a return, for some reason, of mutual contact inhibition between toluidine blue-treated Py cells, with or without the help of contact guidance. The phenomenon was never observed in dye-treated Py monolayers, suggesting that neither mutual interactions, nor increased growth rate were enough by themselves to produce polarisation.

Therefore, it seems most probable that polarisation was due to a degree of contact inhibition exerted upon the invading cells by surrounding heart cells, as described by Stoker in a monolayer situation with similar cells [15]. If the normal cells were arranged in a parallel fashion—and Fig. 9 shows that they can be—the invading cells, if under contact influence, would also line up. Invading and invaded cells have been seen to lie side-by-side and parallel after toluidine blue-treatment in the organ cultures, but it was not possible to determine whether the interaction was one of inhibition, or guidance or whether it was in fact fortuitous. The reduced mitotic rate of the polarised cells corresponds with this interpretation, since Stoker reported that Py cells aligned on non-dividing mouse fibroblasts were inhibited from dividing, and the fibroblasts in the present study were rarely seen to divide. The progressive increase in polarisation observed when cultures were grown in the presence of increasing amounts of the dye may thus be due to a reduction in surface charge following the adsorption of toluidine blue on the Py cell membrane, which had allowed the cells to approach more nearly and to align themselves more completely on the normal fibroblasts. The untransformed BHK21 cell usually invades in a polarised manner.

One other possible cause of polarisation should be mentioned briefly. Polylysine has been shown to link red blood cells, despite their electrostatic mutual repulsion [16]. However, since this effect was traced to the size of the molecule concerned as well as to its charge, it seems unlikely to have occurred with toluidine blue, which has a relatively small molecular weight. There is no evidence to suggest that toluidine blue was polymerised at the cell surface in the present study, thereby increasing its cationic effect. This limitation, and the failure to observe polarisation in homologous cultures of Py cells render the explanation untenable.

Invasion of BHK21 cells

The difference in invasion pattern between Py and BHK21 cells (p. 257) must be due to

some difference in the manner in which the two types of cells invade. From the experiments in the present study, it would seem that Py cells invade by means of free cell movements, cell division, and some capacity to nourish themselves on the normal tissue which they invade. There are suggestions that BHK21 cells are not capable of living off the explant to the same degree as Py cells are, but the possibility has yet to be fully explored.

If BHK21 cells were completely normal, it would be surprising that they invaded at all. In fact, this line is considered premalignant by several workers [17], in part because the cells are capable of forming multilayered monolayers. It seems unlikely, however, that the difference in invasive behaviour is due to a radical difference between mitosis control in Py and BHK21 cells, as this appears to be a matter rather of degree. The situation as regards growth-control in transformed and untransformed BHK cells is too unsettled at present to allow really categorical statements.

It seems more likely that the difference is due to a reduction of invasive movements in BHK21 cells. If intercellular control of movement operates in three dimensions, BHK21 cells, which are supposed to operate it in monolayer culture, might also do so in the invasive situation. As Abercrombie has pointed out [18], retention of mutual adhesions is not necessarily a barrier to invasion. In fact, Leighton suggested that aggregation into broad 'fronts' is necessary for some types of cells to invade, since their individual capacities to respond to metabolic and nutritional gradients are so small [19]. This would explain the 'en bloc' adherent nature of BHK21 invasion, in comparison with the infiltrating Py type.

The probability exists that BHK21 cells are subject to contact inhibition (of movement) from the normal cells of the explant, thereby dissemination is reduced. If this is so, and is also the cause of polarisation in invading Py cells (p. 260), the similarity between the two types of invasion would be explained satisfactorily. It is of interest to note that BHK21 cells, kept in monolayer culture long enough (approximately 6 weeks) for the inevitable 'spontaneous' transformation to a Py type of growth and appearance to occur, invaded under standard conditions in the typical Py manner (Fig. 10).

In conclusion, invasion in this *in vitro* system seems to be largely a matter of movement, variations were probably due to differences in intercellular controls, and toluidine blue reduced the dissemination of the invading cells. No evidence was found to support the proposal (p. 255) that the degree of contact inhibition is governed by the presence of charged molecules at the surface, and that reduction of this charge will prevent invasion *in vitro*.

RESUME

Une méthode expérimentale a été mise au point pour étudier in vitro *le rôle de la charge de surface sur l'invasion. Des nombres connus de cellules BHK*21 *Py furent ajoutés à des cultures organotypiques de coeur foetal de souris, et maintenus, pendant* 5 *jours, dans un milieu comportant de l'agar, le milieu de Eagle modifié, du sérum de cheval et de l'extrait embryonnaire. Les effets des* 2 *substances basiques, le bleu de toluidine et la poly-*L*-lysine, furent étudiés.*

Les concentrations du colorant, dépassant 1 µg/*ml du milieu de culture étaient léthales, tandis que la polylysine n'avait pas d'effet toxique, même à la plus forte concentration utilisée,* 150 µg/*ml.*

Les cellules prétraitées par des concentrations plus basses de polylysine, en l'absence de sérum, subissaient cependant un certain dommage; de plus, le pouvoir invasif de ces cellules était diminué. On suppose que l'adsorption de polylysine réduit le pouvoir invasif, en diminuant la motilité des cellules.

*La présence de bleu de toluidine, à des concentrations non léthales, provoquait, de façon typique, un phénomène que l'on a appelé la 'polarisation', c'est-à-dire une disposition des cellules envahissant le tissu normal semblable à celle des fibroblastes diploïdes normaux, poussant en couche monocellulaire sur le verre. Les raisons possibles de la polarisation sont discutées, en insistant particulièrement sur les modes d'invasion différents manifestés par les cellules BHK*21 *Py et par les fibroblastes non transformés BHK*21 *dont elles sont issues.*

SUMMARY

A culture and assay system was developed to investigate the role of surface charge on

invasion in vitro. *Known numbers of BHK21 Py cells were added to organ cultures of foetal mouse heart, and maintained for five days on a medium consisting of agar, modified Eagles medium, horse serum and embryo extract. The effects of treating with the basic substances toluidine blue and poly-L-lysine were then studied.*

Concentrations of the dye in the culture medium in excess of 1 μg/ml *were progressively lethal, though polylysine in the medium had no toxic effects even at the highest level tested* 150 μg/ml.

Cells pretreated with lower concentrations of polylysine in the absence of serum, however, showed a certain amount of damage; in addition, subsequent invasive powers were shown to be impaired. It is suggested that the reduced invasion was due to adsorption of the polylysine molecule, which decreased the capacity of the cells to move.

The presence of toluidine blue in the culture medium when not lethal, typically produced a phenomenon termed 'polarisation'—the arrangement of the invading transformed cells within the normal tissue in the manner of diploid normal fibroblasts, growing as a monolayer on glass. Possible reasons for polarisation are discussed, with particular reference to the differing modes of invasion displayed by BHK21 Py cells and by the BHK21 (untransformed) parent fibroblast.

ZUSAMMENFASSUNG

Es wurde ein System der Zellkultur entwickelt, um zu untersuchen, welche Rolle die Oberflächenspannung bei der Invasion in vitro *spielt. Eine bestimmte Anzahl von BHK21 Py-Zellen wurde Organkulturen von fötalem Mäuseherz beigegeben und für 5 Tage mit einem Medium, welches aus Agar, verändertem Eagles Medium, Pferdeserum und embryonalem Extrakt bestand, gehalten. Anschließend wurde die Wirkung der Behandlung mit den basischen Substanzen Toluidin-blau und Poly-L-lysin untersucht.*

Die Farbstoffkonzentrationen in Kulturmedien von mehr als 1 μg/ml *führten in zunehmendem Maße zum Tode der Zellen, während die stärkste Konzentration von* 150 μg/ml *Poly-L-lysin keine toxische Wirkung zeigte.*

Zellen, die bei Weglassen des Serums mit einer niedrigeren Poly-L-lysin-Konzentration vorbehandelt wurden, wiesen einen gewissen Schädigungsgrad auf; weiter zeigte sich, daß die Intensität der anschließenden Invasion beeinträchtigt war. Das läßt darauf schließen, daß die Beeinträchtigung der Invasion auf eine Adsorption des Poly-L-lysin-Moleküls zurückzuführen ist, wodurch die Bewegungsfähigkeit der Zellen herabgesetzt wird.

Die Anwesenheit von Toluidin-blau im Kulturmedium bei einer nicht tödlichen Konzentration rief ein typisches Phänomen hervor, die sogennante 'Polarisation'. Die veränderten Zellen, welche in das normale Gewebe eindringen, sind ähnlich diploiden Bindegewebszellen angeordnet, wie sie in einfacher Schicht auf Glas wachsen. Die Ursachen für die Polarisation werden diskutiert, bei besonderer Berücksichtigung der unterschiedlichen Arten der Invasion von BHK21 Py-Zellen und von BHK21 (unveränderten) Bindegewebezellen.

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Studies of Tumour Invasion in Organ Culture*

II. Effects of Enzyme Treatment

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INTRODUCTION

USING the assay method for invasion *in vitro* described earlier [1], the effect of the enzymatic removal of sialic acid from the surface of the invading cells was investigated. The rationale of this procedure has already been described. Neuraminidase removes the sialic acid and excess charge from the surface of BHK21 polyoma-transformed cells [2], and trypsin has been shown to reduce the electrophoretic mobility [3] and to release sialomucoprotein [4] from the surface of red blood cells.

METHODS

Organ cultures in which Py cells invaded foetal mouse heart muscle in a semi-reproducible manner were prepared as described previously, and results were interpreted in terms of deviations from 'norm' amounts of invasion and 'alpha' appearances of the invading cells, as before [1].

Substances tested

(a) *Neuraminidase.* (Crystalline, Beringwerke A. G., Marburg, W. Germany)

Py cells were pretreated in serum-free 'BHK' medium containing 10 i.u/ml neuraminidase, with $CaCl_2$ to make 0·04M [5], at 37° and pH 7·4 for 45 min. Treated cells were added to the explants without washing. Cultures were fixed in Bouin after 1, 2, 3, 4 and 5 days.

(b) *Trypsin.* (Difco 1:250)

This was added to the growth medium before gelation to give concentrations of 0·02%, 0·1%, 0·2% and 1%.

Py cells were also pretreated in serum-free 'BHK' medium containing 0·1% trypsin at 37° and pH 7·4 for 45 min. The reaction was stopped by adding 10% serum, and the cells were added to the explants without washing.

No mortality was associated with pretreatment with trypsin nor neuraminidase.

RESULTS

(a) *Lethal effects upon explants and invading cells*

Crystalline neuraminidase at the concentrations used had no adverse effects on either invading cells nor explants, although it had been found (unpublished) that Burroughs Wellcome 'Receptor Destroying Enzyme' (crude neuraminidase) was completely lethal at as low a concentration as 1 i.u./ml.

Complete digestion of explant and invading cells occurred when 1% trypsin was included in the growth medium for five days; 0·2% digested away all the explants and 2/3 of the invading cells (Fig. 1). Trypsin at a concentration of 0·1% had no ill-effects on the invading cells, but appeared to exert a slightly

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Table 1. *Effects of neuraminidase on the amount of invasion and the appearance of the invading cells*

		% Cultures with			
		reduced invasion	γ-cells	increased invasion	No. cultures
Py cells pretreated with 10 i.u./ml neuraminidase	after 1 day	100 (100)	36 (25)	0 (0)	7 (7)
	after 2 days	75 (89)	86 (44)	16 (0)	12 (9)
	after 3 days	50 (25)	80 (70)	12 (12)	8 (8)
	after 4 days	62 (0)	70 (25)	0 (14)	16 (7)
	after 5 days	19 (11)	19 (6)	10 (8)	21 (220)

Parentheses indicate control values.

digestive effect on the explant when present in the medium. The deposition of trypsin-treated Py cells was associated with the death of 18% of the explants.

(b) *Effects upon the amount of invasion*

Neither neuraminidase-treated nor untreated Py cells began to invade until 48 hr after deposition on the explant. After the second day in culture, less invasion was found where pretreated cells (Fig. 2) were present (Table 1) than where cells were untreated (Fig. 3). Pretreated cells remained less well able to invade until Day 4, and on Day 5, the amount of invasion was similar in both cases.

On the contrary, increased invasion (Table 2) followed trypsin treatment at all concentrations. Total digestion took place when 1% trypsin was present in the growth medium. When the concentration of trypsin in the medium was 0·1%, 74% of the cultures exhibited increased invasion.

Pretreatment with the same strength of trypsin caused increased invasion in only 15% of samples.

(c) *Effect upon the appearance and arrangement of Py cells*

Polarised Py cells appeared in neither neuraminidase nor trypsin-treated cultures. Gamma cells, however, were typical; they appeared in up to 45% of cultures during the second, third and fourth days after pretreatment (Fig. 4), and in 89% of cultures after 5 days in medium containing 0·1% trypsin (Fig. 5). Gamma cells were exhibited by 59% of the cultures following treatment of Py cells alone with trypsin.

(d) *Effect upon the type of invasion*

There was no evidence of any alteration in the type of invasion following neuraminidase- or trypsin-treatment.

(e) *Effect upon mitosis in Py cells*

In the invading cultures, the mitotic index of untreated Py cells fell from 2·2% after 24 hr to 1·1% after 5 days. That of neuraminidase-treated cells was also 2·2% after 24 hr, but fell less steeply to 1·1% i.e. the greatest drop in mitotic index among untreated cells occurred

Table 2. *Effects of trypsin on the amount of invasion and the appearance of invading cells*

	% Cultures with			
	reduced invasion	γ-cells	increased invasion	No. cultures
Control	11	6	8	220
0·1% trypsin in culture medium	0	95	82	15
0·1% trypsin used to pretreated Py cells	8	65	23	13
0·02% trypsin in culture medium	3	35	24	14

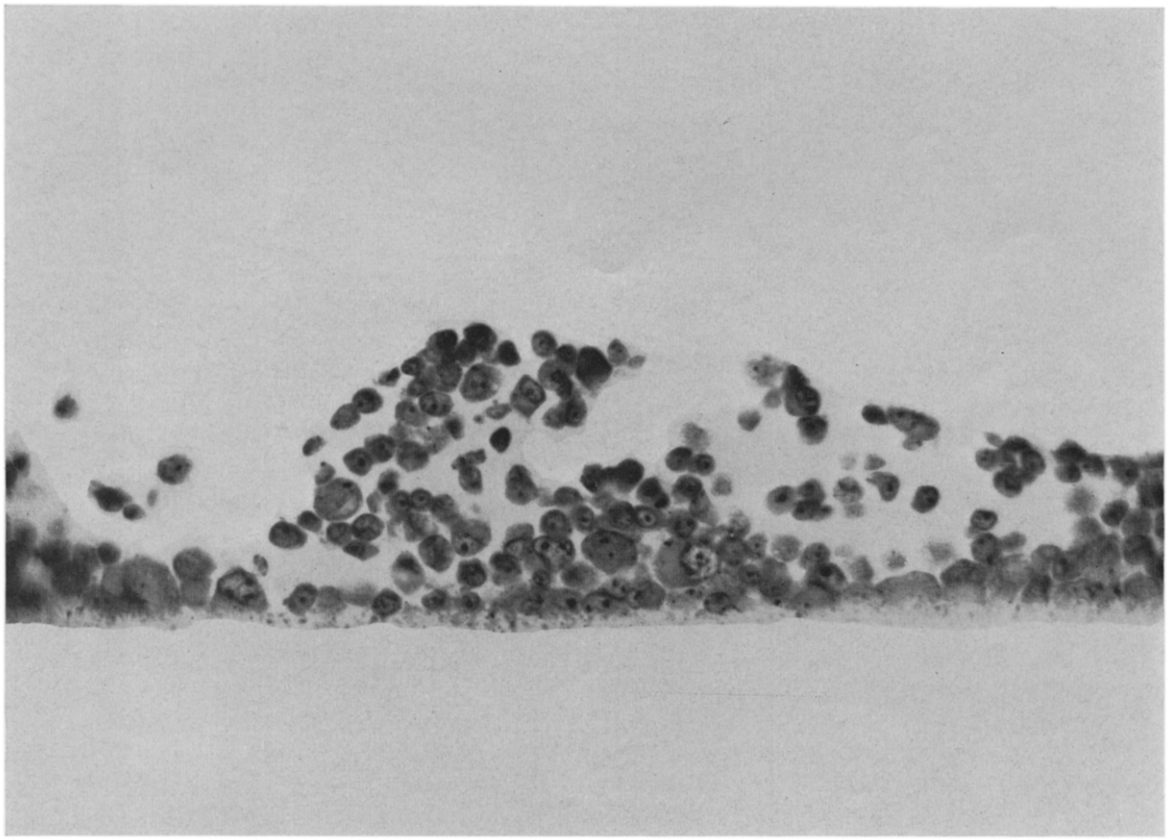

Fig. 1. *The effect of including* 0·2% *trypsin in the culture medium: digestion of the explant, survival of Py cells. H. and E.* × 300.

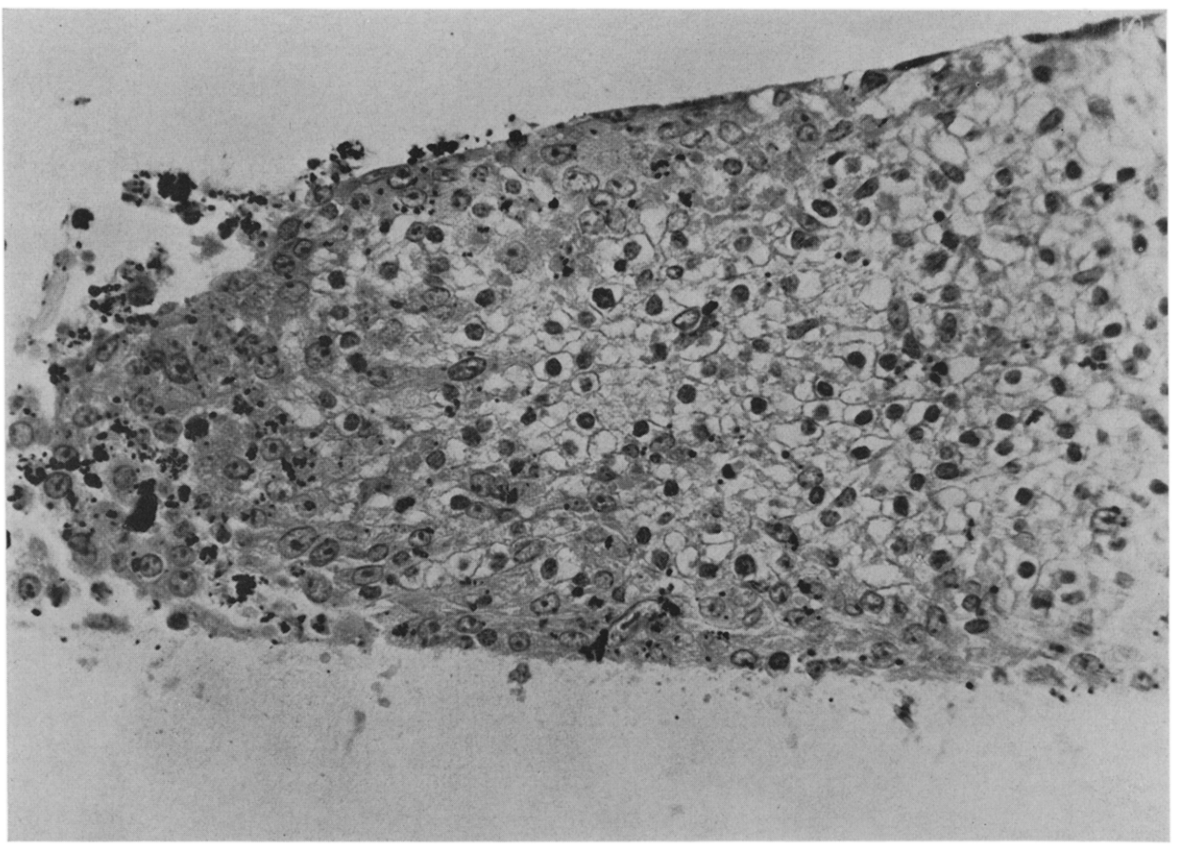

Fig. 2. *The effect, after* 3 *days' culture, of pretreating Py cells with* 10 *i.u./ml neuraminidase: reduced. invasion H. and E.* × 400.

(to face p. 266)

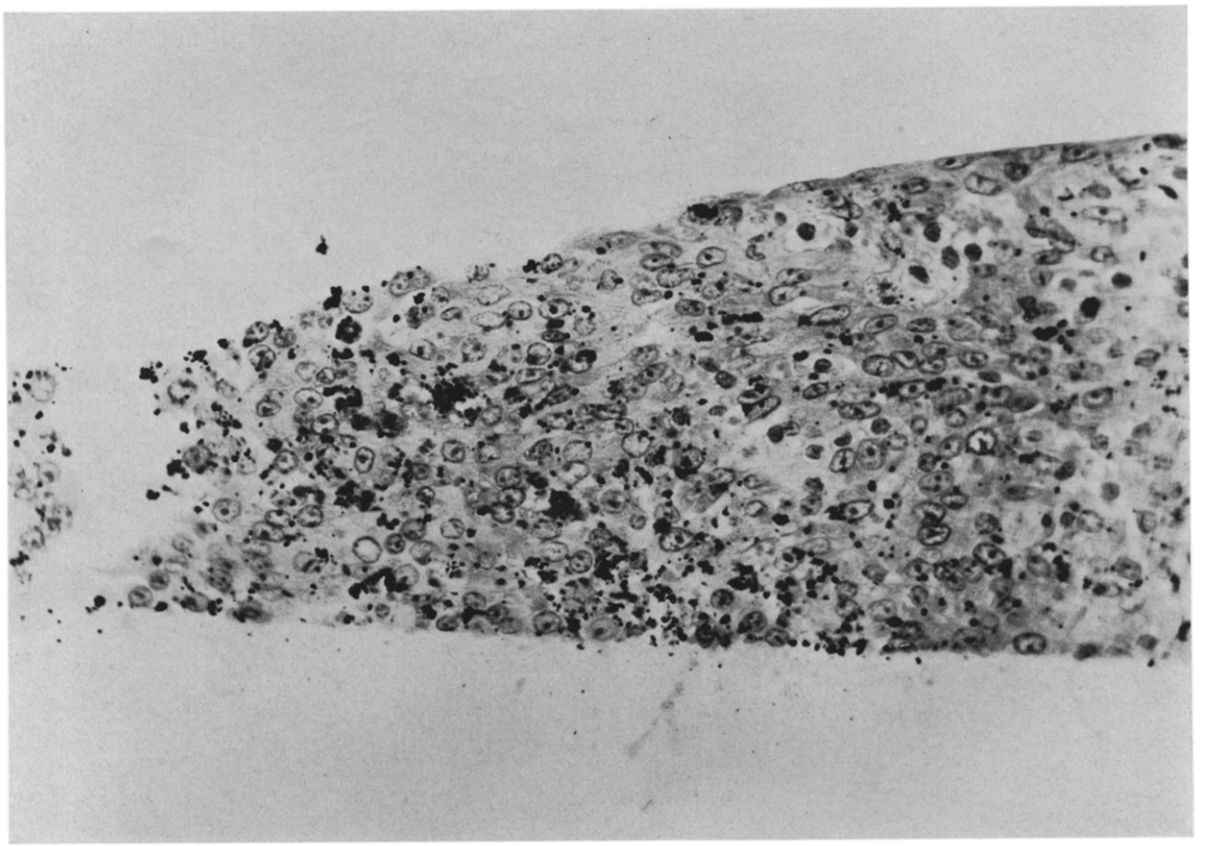

Fig. 3. *The normal amount of invasion* 3 *days after deposition of Py cells pretreated with medium only H. and E.* × 400.

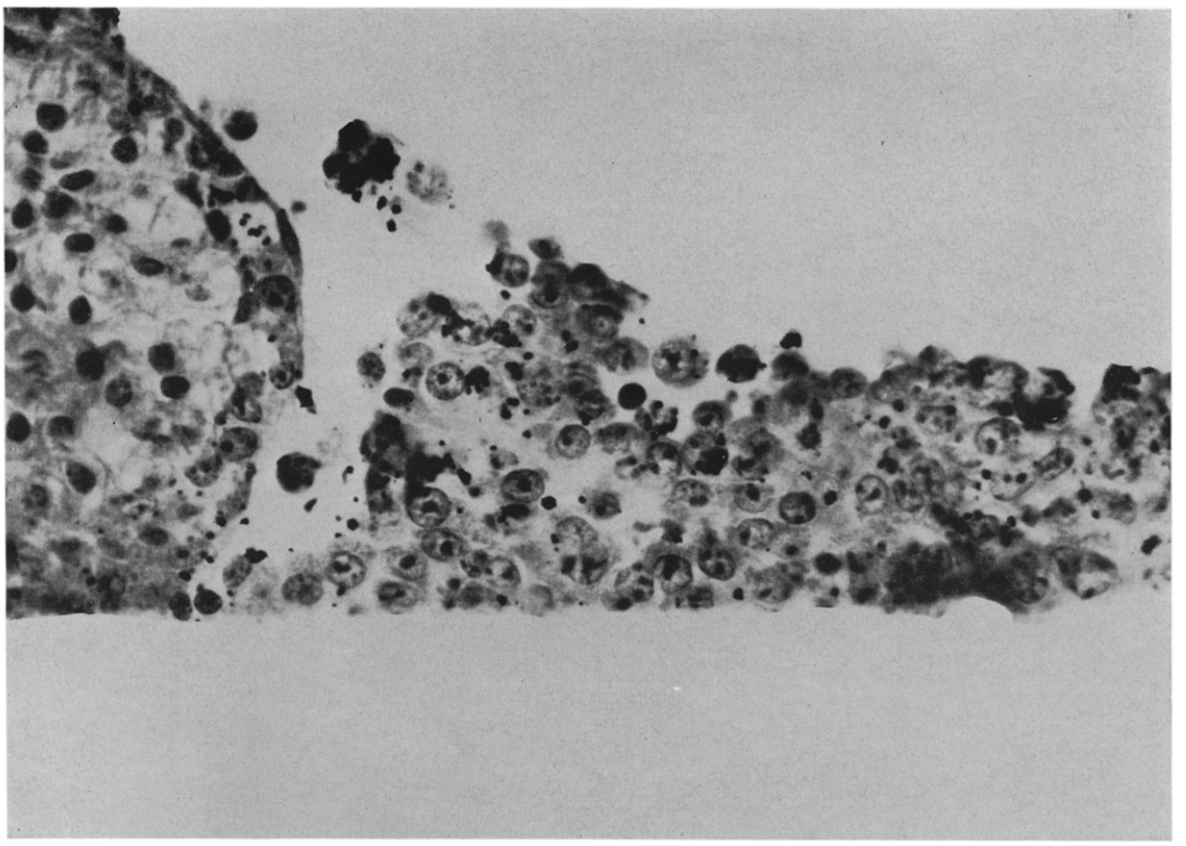

Fig. 4. *The effect of pretreating Py cells with* 10 *i.u./ml neuraminidase after* 2 *days: a high number of gamma cells. H. and E.* × 580.

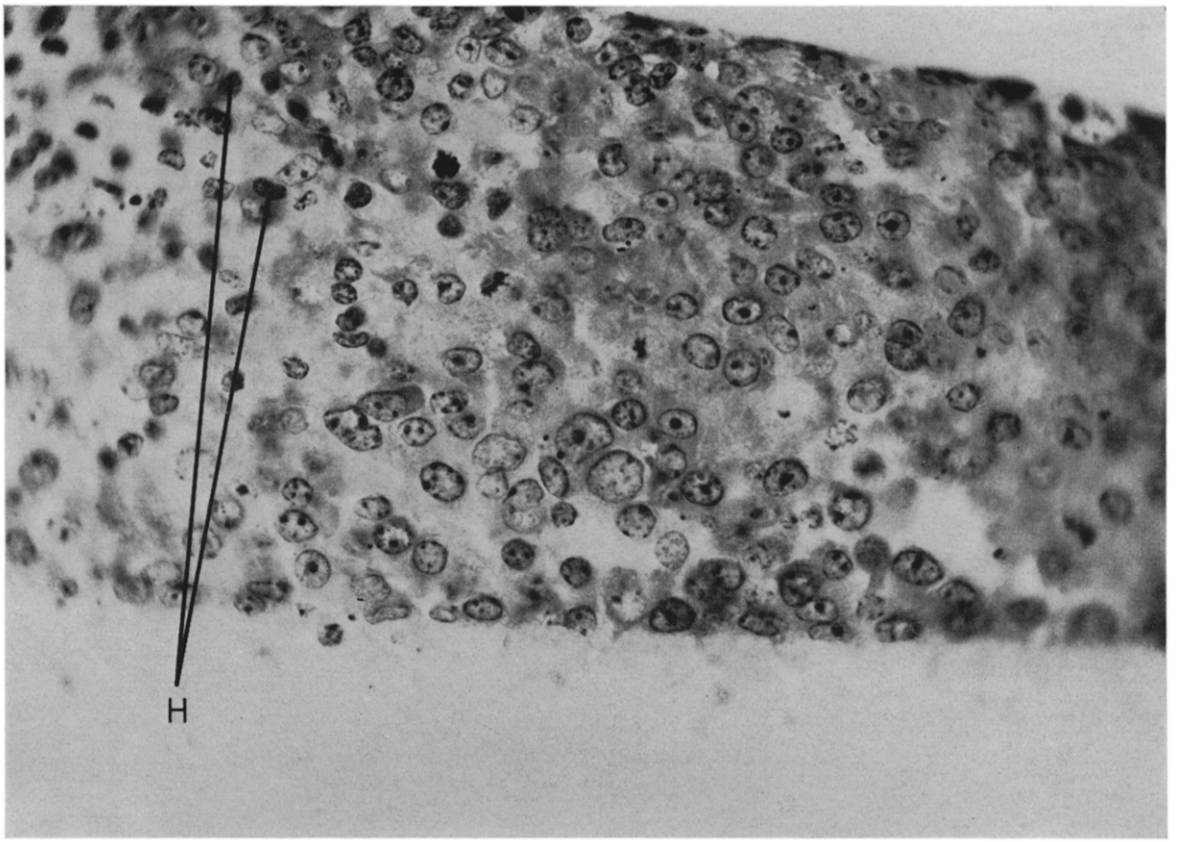

Fig. 5. *The effects of including* 0·1% *trypsin in the culture medium; some digestion of the explant* (*H*), *high numbers of gamma cells and mitoses. H. and E.* × 580.

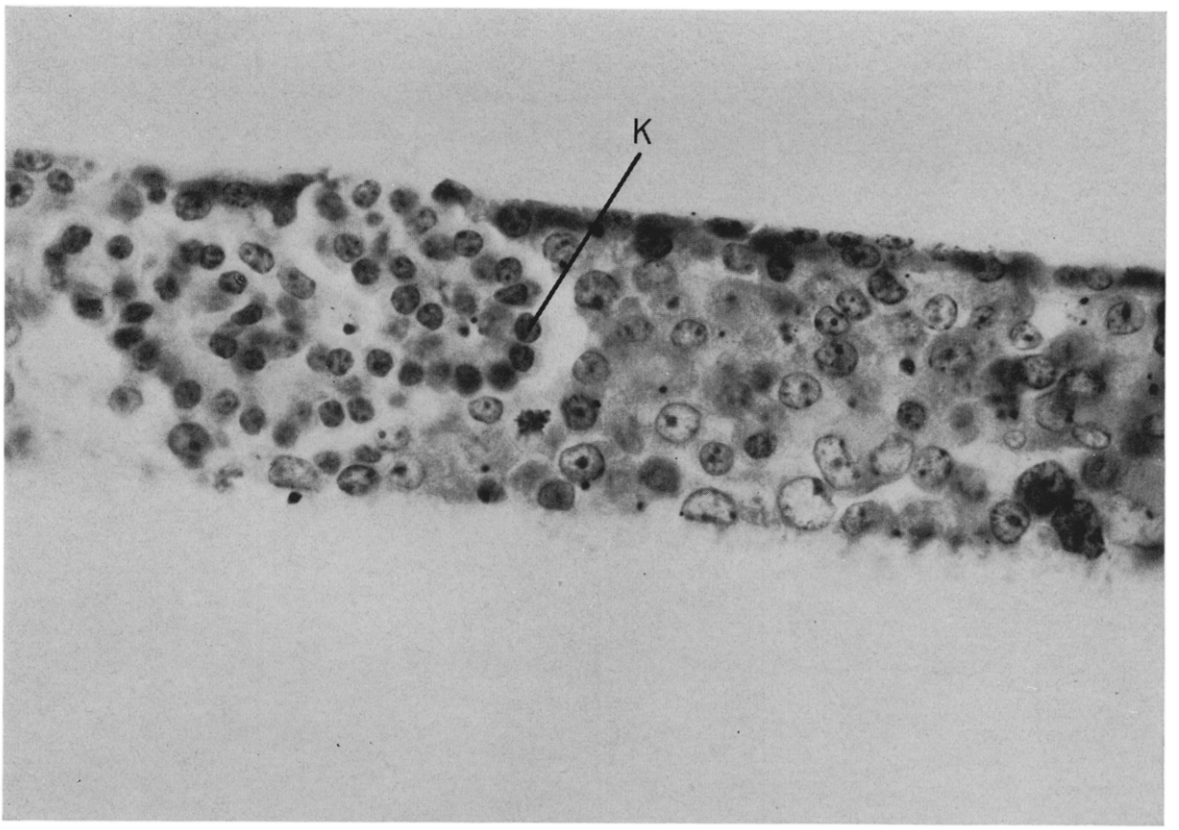

Fig. 6. *The effects of including* 0·1% *trypsin in the culture medium when the explant* (*K*) *was foetal mouse kidney: digestion of the explant, high numbers of gamma cells. H and E.* × 580.

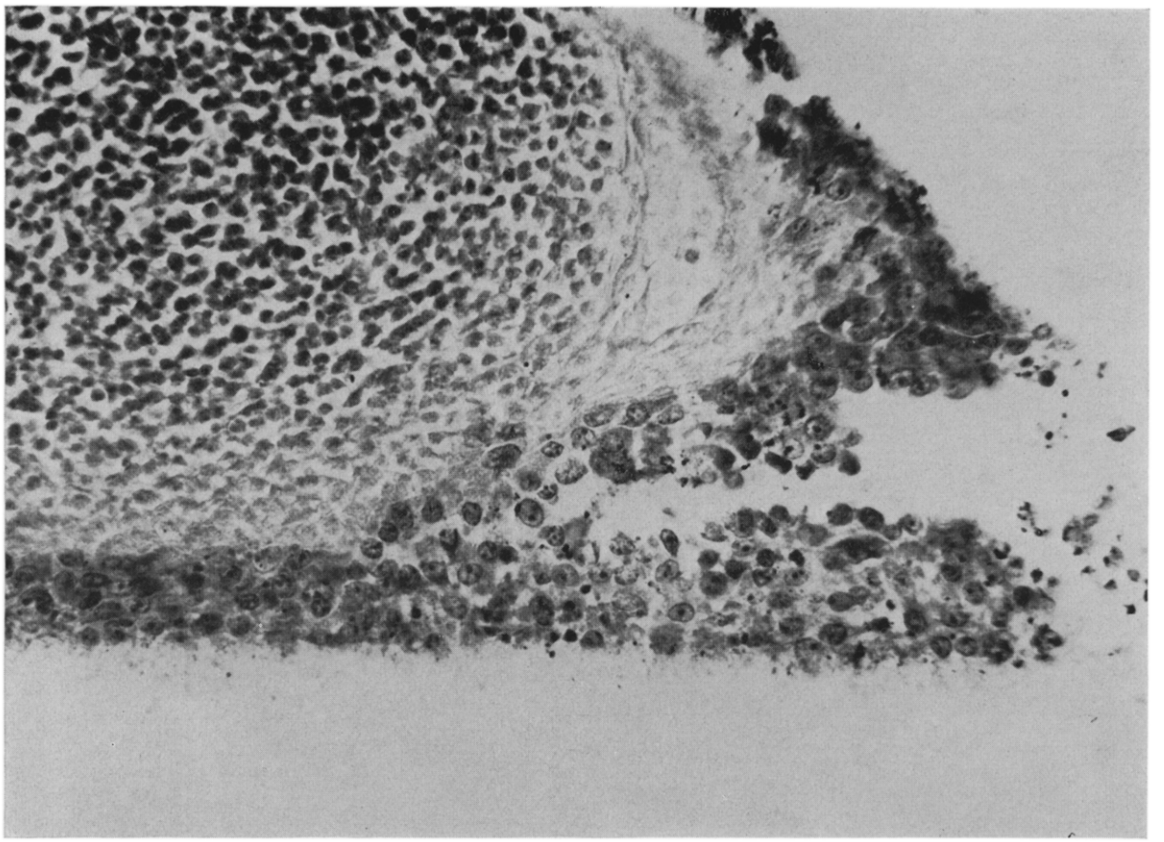

Fig. 7. The effect of killing the explant by autoclaving before depositing the Py cells: reduced invasion. H. and E. × 400.

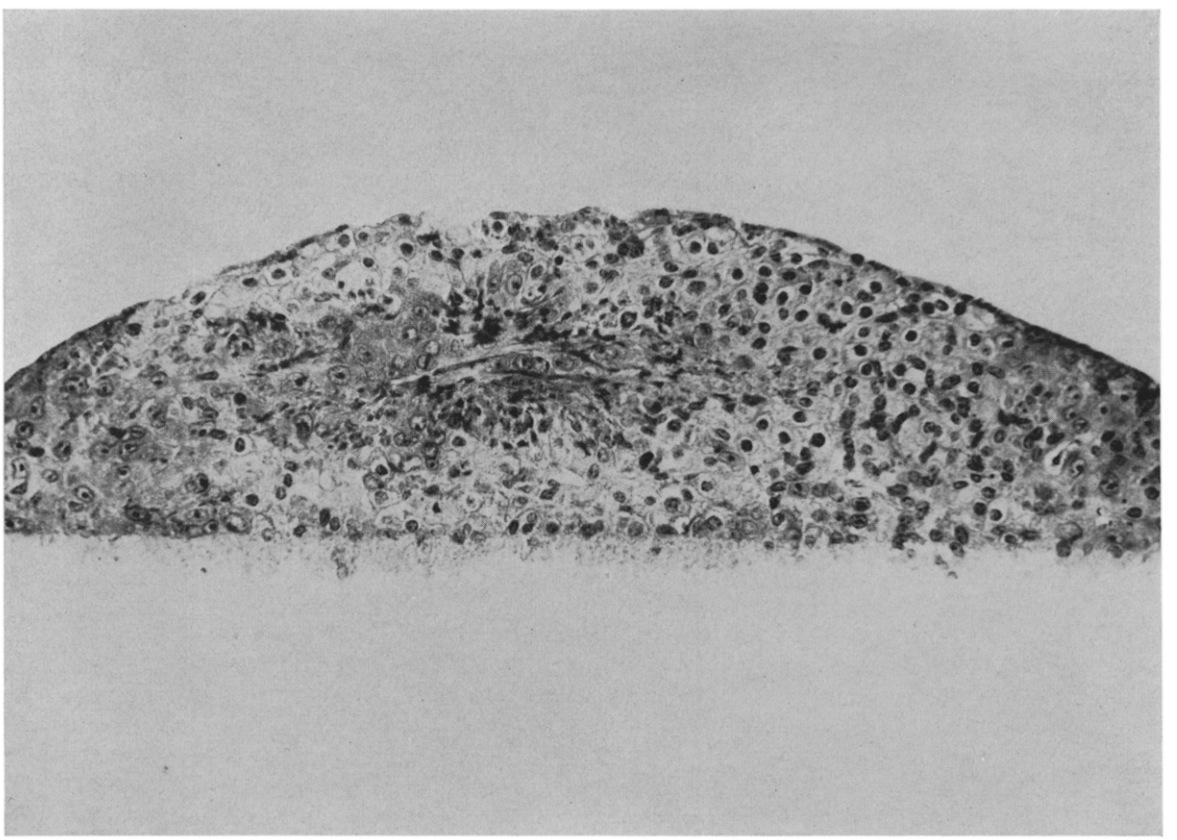

Fig. 8. Invasion of Py cells in a necrotic area of foetal mouse heart muscle. H. and E. × 300

between days 2 and 3, but in neuraminidase-treated cells it was between days 3 and 4 (more than 1000 cells counted in each case).

Pretreatment with 0·1% trypsin caused no substantial change from the control mitotic index of 1·1% (25,700 cells counted) but the presence of 0·1% trypsin in the growth medium was associated with a two-fold increase (7479 cells counted, Fig. 5).

DISCUSSION

Effect of neuraminidase on invasion

Since neuraminidase specifically removes neuraminic (sialic) acid, these results suggest that loss of sialic acid resulted in reduced invasion, and that it was replaced within four days of its removal from the cell surface.

The latter reaction, under present conditions, causes a reduction in the net negative surface charge of certain cells including transformed BHK21 [2, 6, 7]. There was no evidence that this was followed by greater mutual adhesion [8], nor was any return of mutual contact inhibition observed [9]. Neuraminidase-treatment was associated, however, with a slight change in the sequence of mitotic events, which may be connected with the fact that neuraminidase increases phagocytosis in monolayer cells [10]. Within the invasive context, this could lead to better nutrition and increased mitosis.

The other peculiarity of neuraminidase-treatment was the high proportion of gamma cells produced. In general (Table 1), these appeared lesss frequently in control cultures, and the incidence in treated cultures dropped sharply after the fourth day. This would suggest that the gamma appearance, in this case, was due to the removal of sialic acid. Since this has been implicated already as the cause of reduced invasion, it may be concluded tentatively that gamma cells are less well able to invade than alpha cells. Dumonde and Forrester (Personal communication) reported that neuraminidase-treated peritoneal macrophages were almost completely inhibited from moving on a glass survace. The reduction of invasion found in the present experiments following treatment with neuraminidase may have been due also to inhibition of movement, in addition to that which occurred in all cells during the first 48 hr. From results, it would appear that inhibition of mitosis plays no discernible part in reducing the invasive powers of treated cells.

Effect of trypsin on invasion

From these results it seems clear that the increase in invasion was due to the effect of trypsin on the explant, rather than on the invading cells and that is was partly caused by increased mitosis. Simms [11] showed that trypsin treatment stimulates the outgrowth of normal cells from explants, and Lefford [12] followed this up by finding that pretreatment with 0·1% crude trypsin stimulated outgrowth from adult explants. She drew the conclusion that emigration was encouraged by the breakdown of fibrous proteins by elastase and collagenase, both of which are probably present in crude trypsin. Lefford also attributed the increased emigration to 'mobilization'—a term introduced by Abercrombie [13]—which in turn was due to mitotic stimulation. Pace has shown that trypsin stimulates mitosis in 'L' and HeLa cells [14], not because of a direct effect on the cells, but because the serum in the culture medium is partially broken down by the trypsin and rendered more available to the cells.

In the same way in the present experiments, 0·1% trypsin may have caused just enough digestion of the explant to allow invading cells to move more freely, and the products of digestion allowed them then to divide at a faster rate than in control cultures, stimulating mobilisation. This interpretation is supported by the need of Py cells in monolayer culture for 10% 'tryptose phosphate broth' (Difco) to achieve maximum growth rates. Although the trypsin used was not identical with Lefford's. it was by no means crystalline. The same type of result was obtained when foetal kidney was used instead of foetal heart (Fig. 6), the explant was broken down and invasion and mitosis were stimulated. The lethal effects of trypsin were clearly digestive in origin.

The gamma cells commonly associated with trypsin-treatment were not associated with reduced invasion (perhaps because trypsin had other effects which caused, indirectly, a great increase in invasion), but were obviously produced, as in the case of neuraminidase-treatment, by removal of material from the cell surface.

Invasion of Py cells

Throughout this study it was noted that successful invasion of Py cells was dependent to some extent on the viability of the explant. To confirm, explants were killed by autoclaving, and Py cells deposited in the standard manner. Very little invasion was seen in these cultures (Fig. 7). It is possible, of course, that invasion was reduced because of denaturation of proteins and resulting greater toughness

of the explants. However, the fact remains that invasion is reduced in dead explants, which suggests that living tissues provide an environment which stimulates invasion.

In the context of invasion *in vitro*, this could be stimulation of movement, stimulation of mitosis, or a combination of both. A dead and disintegrating piece of tissue seems more likely to allow freer movement than a more resistant living explant, thus stimulation of movement by living tissues is mechanically less likely than stimulation of growth. Mitosis may be stimulated by something produced by normal embryonic cells, as proposed by Wolff and Wolff [15], or by a substance which becomes available only after the death of the tissue cell. The latter type of stimulation was described by Robin [16], and was observed when cultures were grown on medium containing trypsin (p. 267).

Fig. 8 shows the invasion of Py cells in an area of the explant which appears to be dead. Since invasion is discouraged by dead tissue, it seems that in this very commonly-observed situation, the invading cells had killed the normal tissue because only fresh products from newly-killed normal cells are capable of stimulating mitosis in invading cells. A similar situation was commented upon by Wolff in 1962 [17]. If this is so, it would explain the ability of Py cells to replace by invasion so reproducibly, half of the explant in a certain time. The invading cells would move, within a nutrition gradient, to areas still alive and healthy and invasion would be stimulated. By what means the invading Py cell could kill the living tissue is as yet undetermined, but it may be a combination of lysosomal enzymes and phagocytosis, as proposed by Sylven [18].

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RESUME

Utilisant la méthode déjà décrite pour étudier le pouvoir invasif des cellules BHK 21 Py dans des explants de coeur foetal de souris, la trypsine et la neuraminidase furent employées pour ôter le matériel adsorbé à la surface des cellules invasives.

Un traitement préalable à la neuraminidase réduisait le pouvoir invasif des cellules, apparamment jusqu'à ce que l'acide sialique de la surface cellulaire eût été reformé.

Un traitement préalable à la trypsine n'avait pas d'effet sur l'invasion, mais, lorsque la trypsine était présente à une concentration de 0,1% dans le milieu de culture, l'invasion était augmentée. Il s'agissait d'un effet nutritif, lié presque entièrement à la lyse enzymatique de l'explant.

La nature du pouvoir invasif des cellules BHK 21 Py est discutée, et certaines conclusions sont présentées.

SUMMARY

Using the technique already described for studying the invasion of BHK21 Py cells into foetal mouse heart explants, trypsin and neuraminidase were used to remove surface material from the invading cells.

Pretreatment with neuraminidase reduced the capacity of the cells to invade, apparently until sialic acid on the cell surface had been reformed.

Pretreatment with trypsin had no effect on invasion, but when 0·1% trypsin was present in the culture medium, invasion was stimulated. This was shown to be a nutrition effect, and to be due almost entirely to the enzymic breakdown of the explant.

The nature of the capacity of the BHK21 Py cell to invade is discussed and some tentative conclusions are reached.

ZUSAMMENFASSUNG

Mit der zuvor beschriebenen Methode der Untersuchung der Invasion von BHK 21 Py-Zellen in Organkulturen von fötalem Mäuseherz wurde geprüft, welcehn Ekefft die Entfernung von Oberffächenbestandteilen der eindringenden Zellen hat.

Die Invasionsfähigkeit der Zelle wurde nach Vorbehandlung mit Neuraminidase herabgesetzt, und zwar solange, bis sich die Sialsäure wieder gebildet hatte.

Die Vorbehandlung mit Trypsin hatte auf die Invasion keine Einwirkung; die Invasion wurde aber durch die Anwesenheit von 0,1% Trypsin im Kulturmedium angeregt. Dies

erwies sich als ein Ernährungseffekt und die Erscheinung ist fast ausschließlich auf den Enzym-Zerfall in der Organkultur zurückzuführen.

Das Wesen der Invasionsfähigkeit der BHK 21 Py-Zelle wird diskutiert und mögliche Schlußfolgerungen werden gezogen.

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Combination of Arabinosyl Cytosine, Methylglyoxal bis (Guanylhydrazone), 6-Mercaptopurine and Prednisone in the Treatment of Acute Myelocytic Leukemia

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INTRODUCTION

THE EFFECTIVENESS of two drugs has been recently demonstrated for the treatment of acute myelocytic leukemia (AML): methylglyoxal bis (guanylhydrazone (methyl-GAG)* and arabinosyl cytosine (ara-C)†.

Although the toxicity elicited by methyl-GAG was severe, its activity in AML was unquestionable when it was given either alone [1–4] or in combination with 6-mercaptopurine [5].

The antitumor activity of ara-C, a synthetic pyrimidine nucleoside differing in the sugar moiety from the normal metabolites cytidine and deoxycytidine was reported by several investigators in patients with malignant diseases [6–9] and prompted the Acute Leukemia Group B to initiate a study for the treatment of acute leukemia in adults with ara-C. Preliminary results showed that out of 59 patients with (AML), 16 complete remissions were obtained [10]. The value of ara-C was confirmed by overall results of protocols ALGB 6503 and ALGB 6506 [11].

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*Guanidine, 1,1'—methylelethane diylidene dinitrilo di—dihydrochloride, hydrate; methyl-GAG. NSC 32946.

†B-D Arabinofuranosyl Cytosine; ara-C. NSC 63878.

It appeared logical from these previous data to combine in the same study ara-C, methyl-GAG and 6-mercaptopurine (6-MP) which had proved useful in the treatment of acute myelocytic leukemia, for which, in contrast with acute lymphocytic leukemia, little progress in therapy has occurred.

The addition of moderate doses of prednisone to this program was inspired by the feeling that prednisone may bring some comfort to these severely ill patients.

MATERIAL AND METHODS

Thirty-seven patients (30 adults, 7 children), 33 previously untreated, who had acute myelocytic leukemia were treated in the Hematologic department of the Saint-Louis Hospital, Paris, from February 1 to October 31 1966.

Prednisone, 40 mg/m2, and 6-MP, 90 mg/m2, were given daily orally (p.o.).Methyl-GAG, 350 mg/m2/3 times a week was injected in the tubing of a running isotonic glucose perfusion. The doses of ara-C were adjusted during the study: 16 patients were given ara-C,30 mg/m2/3 times a week intravenously (I.v.); 21 were given ara-C, 30 mg/m2/daily i.v. Ara-C was dissolved in 300 ml isotonic glucose and given over a 3–4 hr period.

The criteria for complete remission were those outlined by the Clinical Studies Panel,

Table 1. *Results of combination therapy (methyl-GAG+ara-C+6-MP+prednisone) in* 37 *patients with acute myelocytic leukemia*

Doses of ara-C		Responses of adults				Responses of children			
		Total adults treated	Complete remission	Partial remission	Failure	Total children treated	Complete remission	Partial remission	Failure
3 times a week	First attack	15	2	2	11	4	3	1	
Daily	First attack	13	5	2	6	1		1	
	Relapse	2	1		1	2	2		
		30	8	4	18	7	5	2	

Cancer Chemotherapy National Service Center [12].

However, in most cases the hemoglobin level was slightly depressed (about 12g/100 ml) during the first weeks of the remissions. If this abnormality was unique, we considered those cases (M1, P1, S1, and H2 because of this anemia) as complete remissions provided that the blood picture finally returned to normal. This rating was confirmed by the fact that the duration of remissions was equivalent in anemic and non anemic patients.

RESULTS

The results are summarized in Table 1. Thirteen complete remissions were obtained (33%).

The results in children were very good since we noted 5 complete remissions and 2 partial remissions out of 7 cases.

Owing to the small number of patients in each series, it appears difficult to compare the results obtained when ara-C was given daily or when it was given 3 times a week.

We noted however that in 15 adults who were given ara-C 30 mg/m2/daily, 6 complete remissions and 2 partial remissions were obtained, whereas out of 15 patients treated by ara-C 30 mg/m2/3 times a week, 2 complete remissions and 2 partial remissions were noted only.

It is thus possible that better results are obtained when ara-C is given daily. A more extensive study should be made in order to confirm this point.

Complete remissions (13 *patients*)

The decrease of blastic cells was progressive and the disappearance of bone marrow hemoblasts generally occurred during the 2nd or the 3rd week of treatment. In our experience, the complete remission was obtained at the end of the 3rd week (median 27 days, ranges 11–37 days).

In seven cases, the M1 status occurred after a period of tolerable bone marrow depression, with lymphocytosis and decreased cellularity; in no case this hypoplasia was life-threatening and white blood cells transfusions were not required but in one case.

Maintenance therapy was begun after the completion of remission. The patients received 6-MP 90 mg/m2 daily p.o. and ara-C 30mg/m2/weekly subcutaneously; if weekly blood tests showed a leukopenia, 6-MP was reduced temporarily or definitively if leukopenia was recurrent; thus in two patients the maximum tolerable dose of 6-MP was 60 mg/m2/daily.

Two patients (1 child, 1 adult) have had a complete remission for more than 24 months at the moment (December 1968). (24+, 30+). Eleven patients relapsed after a median remission of 5 months (1·5; 2·5; 5; 5; 7·5; 8·5; 20 for first attacks; 1·5; 2; 8·5 for relapses)

Partial remission (6 *cases*)

In 2 children the disappearance of blastic cells from bone marrow was obtained and there were symptoms confirming a regenerative bone marrow (increase of platelets and of neutrophils) but death occurred before M1 was attained: in one case the death was due to hepatic abcesses and in the other case to an acute tubulo-nephritis; in this later case the renal lesions histologically assessed seemed to be secondary to a Gram-negative septicemia and not to a specific drug toxicity.

In 3 adults, the combination therapy resulted into a marked but not total reduction of bone marrow hemoblasts (persistence of 20% blastic cells whereas blood examinations returned to normal. M2; H1; P1; S1). These partial remissions lasted respectively 5, 5, 4 months with the maintenance treatment.

Table 2. Toxic effects of combination therapy methyl-GAG, ara-C, 6-MP and prednisone in 37 patients with acute myelocytic leukemia

Patients	Clinical toxicity			Hematological toxicity	
	Hepatic lesions	Gastro-intestinal lesions	Bucopha-ryngeal lesions	Bone marrow aplasia reversible	irreversible
Children				4	
Adults	2	2	4	7	3

In one adult the total disappearance of hemoblasts was obtained but in consideration of the persistent thrombocytopenia and of the early relapse 1 month later, this result was rated partial rather than complete remission. (M1; H2; P1; S1).

Failures (18 *cases*)

One adult, treated for his first relapse, died from a sepsis on day 4 after the beginning of the treatment. One has been completely resistant with an increase of blast cells on treatment. In the other patients a decrease (10 cases) or a disappearance (3 cases) of blasts were noted but those patients died from sepsis (10 cases) or bleeding (2 cases) during the hypoplastic period (median day of death 19, range 4–46).

Toxicity

The toxic effects of combination therapy are summarized in Table 2. Hepatic lesions were questionable: an icterus was noted at the preterminal stage in a patient suffering also from a Hodgkin's disease since 1956; in another patient, toxic histologic lesions were observed at the post-mortem examination. In two patients, digestive bleeding might have been related to drug-induced mucous ulcerations. In 4 patients buccal ulcerations possibly due to methyl-GAG were noted. In 14 patients, a bone marrow depression was observed and was fatal in 3 cases: the aplasias occurring within the 2 first weeks of treatment were more easily reversible than those occurring later.

DISCUSSION

Although the number of cases in this study is too small for a precise evaluation, the rate of remission induced by the combination of ara-C, methyl-GAG, 6-MP and prednisone seems greater than the rate of remission observed with ara-C alone.

In a preliminary study [10], we noted 27% complete remissions in acute myelocytic leukemia trated with ara-C. In the more complete study conducted by R. R. Ellison [11], there are 14% remissions complete in all aspects, 3% complete except for hemoglobin level, and 7% partial remissions among 178 adults with acute myelocytic leukemia trated with ara-C.

Another complete and more precise study would be required to compare the rate of remissions obtained by the combination of ara-C, methyl-GAG, 6-MP and prednisone on the one hand, of methyl-GAG and 6-MP on the other hand, which in a previous investigation gave us 33% complete remissions out of 42 patients with AML [5].

Concerning the doses of ara-C included in this combination therapy, the better results generally obtained in this study with the daily administration of ara-C seem to demonstrate that initial high doses of ara-C are valuable. Further studies remain necessary to determine the possibility for using in the combination daily doses of ara-C larger than 30 mg/m2.

A new antibiotic, daunomycin or Rubidomycin [13] has recently been used in the treatment of acute myelocytic leukemia; it is very active but toxic for bone marrow and therefore its management is especially difficult. It would certainly be of interest to find out the good way to combine it to the four drugs mentioned in this work.

RESUME

Trente-sept malades (30 *adultes*, 7 *enfants*), *atteints de leucémie aiguë myéloblastique, ont été traités par une association de prednisone* (40 *mg/m2, par jour, per os*), 6-*mercaptopurine* (90 *mg/m2, par jour, per os*), *méthyglyoxal bis* (*guanylhydrazone*) (350 *mg/m2, 3 fois par semaine, par voie intraveineuse*) *et arabinosyl cytosine* (30 *mg/m2, 3 fois par semaine, ou chaque jour, par voie intraveineuse*).

Huit adultes et 5 enfants ont eu une rémission complète. Onze de ces malades ont eu une récidive de la maladie.

Deux malades, 1 adulte et 1 enfant, sont encore en rémission après plus de 24 mois.

Les manifestations toxiques gastro-intestinales et cutanéo-muqueuses ont été relativement modérées, mais 14 malades ont eu une aplasie médullaire qui a été fatale dans 3 cas.

SUMMARY

Thirty-seven patients (30 adults, 7 children) with acute myelocytic leukemia were treated with a combination of prednisone (40 mg/m2/daily, p.o.), 6-mercaptopurine (90 mg/m2/daily, p.o.), methylglyoxal bis (guanylhydrazone) (350 mg/m2, 3 times a week, i.v.) and arabinosyl cytosine (30 mg/m2, i.v., 3 times a week or daily). Eight adults and 5 children attained complete remission. Eleven of these patients relapsed after a median time of 5 months. Two patients: 1 adult and 1 child are still in remission after more than 24 months.

Gastrointestinal and mucocutaneous toxic effects were relatively moderate, but 14 patients had bone marrow aplasia which was fatal to 3.

ZUSAMMENFASSUNG

Siebenunddreizig patienten (30 Erwachsene, 7 Kinder) mit akuter myeloischer Leukämie wurden mit einer Kombination, bestehend aus Prednison (40 mg/m2/täglich p.o.), Methylglyoxal-bis-guanylhydrazon 350 mg/m2/dreimal wöchentlich i.v.) und Arabinosyl-Cytosin (30 mg/m2 i.v./dreimal wöchentlich oder täglich) behandelt. Bei 8 Erwachsenen und 5 Kindern trat eine vollständinge Remission ein. 11 dieser Patienten bekamen nach einem Zeitraum von 5 Monaten einen Rückfall. Bei 2 Patienten, einem Erwachsenen und einem Kind, trat eine Remission von mehr als 24 Monaten ein.

Toxische Effekte im Magen-Darmbereich, sowie der Haut- und Schleimhaut traten relativ selten auf, aber 14 Patienten wiesen Knochenmarksaplasien auf, welche bie 3 Patienten zum Tode führten.

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Carcinoma of the Bladder treated by Suprapubic Radium implants

The Value of Additional External Irradiation

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BETWEEN 1950 and 1967, 1343 patients with neoplasms of the urinary bladder were treated at the Rotterdam Radiotherapeutic Institute. All cases were examined jointly by a urologist and a radiotherapist, and in all a confirmatory biopsy was performed. The proportional distribution of tumour stages according to the international T.N.M. system is presented in Fig. 1. The various curative and palliative modalities of treatment applied in patients who were followed for at least 3 years, are shown in Table 1.

After treatment, all patients were submitted to regular follow-up examinations including cystoscopy (unless this was prevented by poor condition). With the exception of one patient who emigrated, none was lost to follow-up.

The purpose of this paper is to analyse the results of radium implant therapy, with special regard to additional pre- or post-operative external irradiation.

MATERIAL AND METHODS

Radium implantation was performed in case of bladder cancer belonging to stages T_1M_0, $T_{II}M_0$ and $T_{III}M_0$, provided the diameter did not exceed 5 cm and the general condition of the patient permitted suprapubic intervention. Details of this technique have been published in a previous paper [1].

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In the cases treated up to 1956, no additional external irradiation was given. Between 1956 and 1962, in an attempt to prevent scar implants, post-operative irradiation was applied to the healed operation wound: a skin dose of about 1500 rad. was given in 4 daily fractions (250 keV, 1 Th. Filter, 40 cm FSD). After 1962, in an attempt to reduce other possible iatrogenic metastases, the entire small pelvis was irradiated by a telecobalt source on 3 consecutive days prior to radium implantation: a total midline dose of 1050 rad. was delivered. These patients received no post-operative irradiation. In most cases in this latter group, a biopsy was taken during operation from apparently healthy bladder tissue at 2-cm distance from the tumour in order to study the possible implications of the microscopical findings on the incidence of local or marginal recurrences.

RESULTS

(1) *General*

The average age of the patients treated with external supervoltage irradiation was 68·5 years (95% confidence limit of the average: 10 months), as compared with 62·5 years (95% confidence limit of the average: 12 months) of the patients treated with radium needles. In the latter group fluctuations of the average in the various stages and sub-groups were of no significance.

The sex ratio in the groups analysed varied between 3 and 6 males to 1 female.

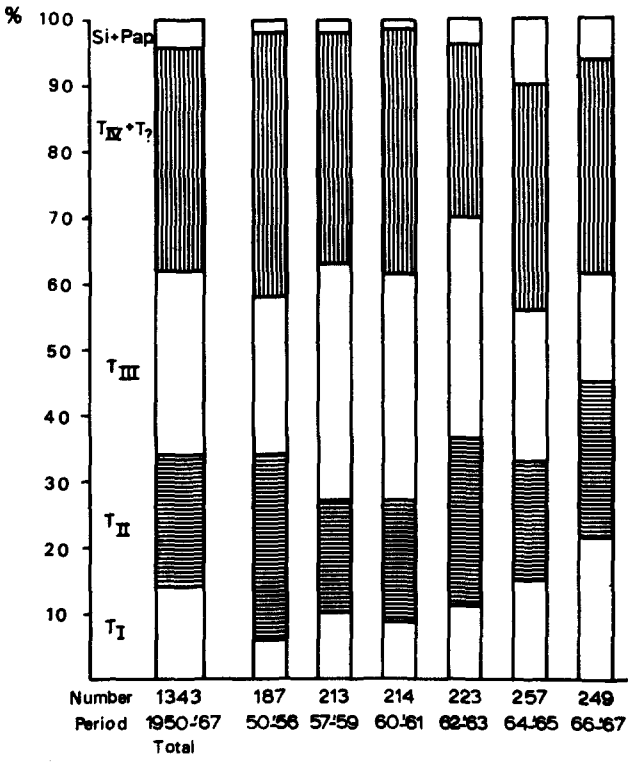

Fig. 1. *All bladder tumours treated at the R.R.T.I. between* 1950 *and* 1967. *Proportional distribution of tumour stages in the various periods.*

(2) *Survival rates and course after radium implantation*

For purpose of comparison, 3- and 5-year survival rates were calculated by stages for all patients who received curative radiotherapy (Figs. 2 and 3). Out of 353 patients who received palliative treatment only 17 (5%) were alive and apparently free of disease after 3 years. Tables 2 and 3 reflect 3- and 5-year survival rates according to the stage after interstitial radium therapy, in regard to additional pre- or post-operative external irradiation. They also show the cause of death in each group.

In stage T_I, neither additional pre- nor post-operative external irradiation appeared to alter the course of the disease.

In stage T_{II}, in contrast to radium only, post-operative irradiation slightly reduces the mortality from metastases (without local recurrence), while pre-operative irradiation lowers the mortality somewhat from local recurrence as well as from metastases. These latter two effects, neither of statistical significance, jointly improve prognosis considerably.

In stage T_{III}, an increase in 3- and 5-year survival rates after additional pre- and post-operative irradiation is demonstrable at a

Table 1. *Carcinoma of the bladder, treated at the R.R.T.I. between* 1950 *and* 1965. (*Ca.* in situ *and papilloma excluded*)

	With curative intent	With pall. intent
Radium implantation	336	5 (T_{IV} prostatic)
External supervoltage irradiation	300	226
External 250 kV irradiation (before 1957)	—	98
Intravaginal radium	—	12
Surgery only (T.U.R.: 4, segmental resection: 2 cystectomy: 5)	11	—
No treatment	—	12
Total	647	353

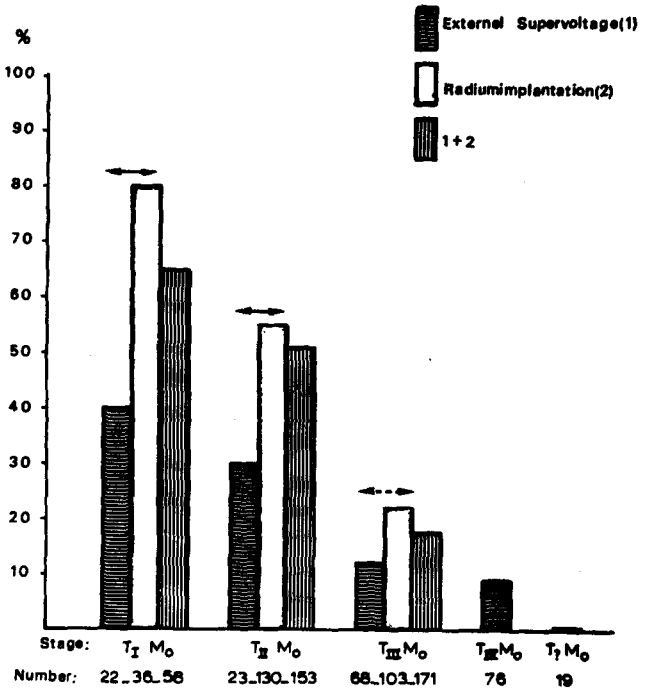

Fig. 2. *Carcinoma of the bladder treated with curative intent by radium implantation or by full course of supervoltage irradiation. R.R.T.I.* 1950–1965. 3-*year survival.*

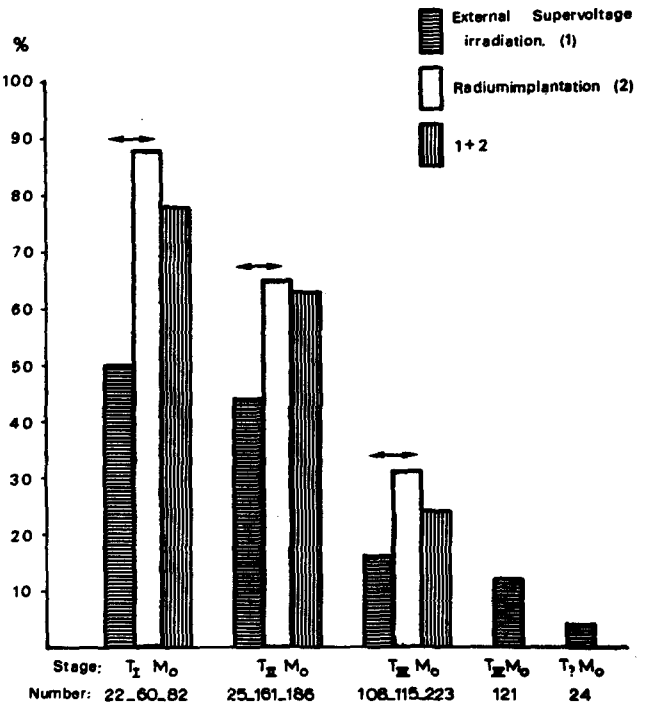

Fig. 3. *Carcinoma of the bladder treated with curative intent by radium implantation or by full course of supervoltage irradiation. R.R.T.I.* 1950–1963. 5-*year survival.*

$\longleftrightarrow$ Connect percentages that differ significantly at $P \leqslant 0 \cdot 05$ level.
$\leftarrow - - \rightarrow$ Connect percentages that differ significantly at $0 \cdot 05 > P < 0 \cdot 1$ level.

Table 2. Three-year course after radium implantation with respect to additional external irradiation

	Stage T_I			Stage T_{II}			Stage T_{III}		
	A	B	C	A	B	C	A	B	C
Number treated	9	23	28	32	81	48	32	56	27
3-yr Survival	7 78%	21 91%	25 89%	18 56%	51 63%	36 75%	5 16·5%	18 32%	13 48%
† Loc. rec. (with or without metast.)	—	1 4%	1 3·5%	4 12·5%	12 15%	1 2%	8 25%	16 28%	6 22%
†Metastasis (incl. scar impl.) (without loc. rec.)	1 11%	—	—	7 22%	8 10%	7 14·5%	16 50%	20 36%	3 11·5%
† Operation or late complications	1 11%	—	—	3 9%	—	1 2%	3 9%	—	—
†Intercurrent	— —	1 4%	2 7%	— —	10 12%	3 6%	— —	2 4%	5 18·5%

A No additional external irradiation.
B Post-operative scar irradiation.
C Pre-operative irradiation.

Table 3. Five-year course after radium implantation with respect to additional external irradiation

	Stage T_I			Stage T_{II}			Stage T_{III}		
	A	B	C	A	B	C	A	B	C
Number treated	8	23	5	32	79	19	32	56	15
5-yr survival	4 50%	21 91%	4 80%	16 50%	42 53%	13 67%	4 12·5%	12 21%	7 47%
† Loc. rec. (with or without metast.)	1 12·5%	1 4%	—	5 15·5%	15 19%	2 11%	8 25%	17 32%	3 22%
†Metastasis (incl. scar impl.) (without loc. rec.)	1 12·5%	—	—	7 22%	12 15%	2 11%	16 50%	23 41%	2 14%
† Operation or late complications	1 12·5%	—	—	3 9%	—	—	3 9%	—	—
† Intercurrent	1 12·5%	1 4%	1 20%	1 3%	10 12·5%	2 11%	1 3%	4 7%	3 21%

A No additional external irradiation.
B Post-operative scar irradiation.
C Pre-operative irradiation

Table 4. Incidence of local and marginal recurrence after radium implantation of bladder cancer, with regard to additional external irradiation

	No ext. irrad.	Post-op. irrad.	Pre-op. irrad.
Stage T_IM_0			
Total number	9	23	51
? Loc. rec.	1	—	6
Loc. recurr.	1/8	4/23	4/45
	12·5%	17·5%	9%
Stage $T_{II}M_0$			
Total number	32	81	62
? Loc. rec.	7	13	7
Loc. rec.	5/25	16/68	8/55
	20%	24%	14·5%
Stage $T_{III}M_0$			
Total number	32	56	37
? Loc. rec.	9	12	2
Loc. rec.	8/23	20/44	8/35
	35%	45%	23%

statistically reliable level. This is the result mainly of a significant decrease of the death rate due to metastases (metastasis without local recurrence).

In each stage and in each sub-group, the prognosis was found to be unrelated to the histological grade of the tumour.

(3) *Local (and marginal) recurrence and metastasis*

An analysis of local recurrences and metastases appeared necessary, because the mortality from either is not the correct indicator for the incidence of each.

In stages T_I and T_{II}, 95% of the local recurrences (with and without metastases) and of the metastases (with or without local recurrence) became evident within two years. In stage T_{III} growths, this period was shorter: 1 year. Consequently, all radium implant cases with at least a 2 year (or 1-year, in stage T_{III} cases) follow-up were included in an additional analysis of local recurrences and metastases. Patients dying before the end of the relevant period of observation without local recurrence were classified as indeterminate ("? local recurrence") and were omitted from the local recurrence study. In an analogous way, the other indeterminate group ("? metastasis") was omitted from the metastasis study.

Table 4 depicts the incidence of local recurrences in each stage with regard to additional external irradiation.

In stages T_I and T_{II} with overall local recurrence rates of 12% and 20% respectively, no statistically significant differences were demonstrated in connection with any type of additional external irradiation. In stage T_{III} the overall local recurrence rate of 35% was reduced by more than 10% following pre-operative irradiation.

The occurrence of metastasis in stage T_I (7%) was unaffected by any type of additional external irradiation. In stage T_{II} with an overall metastasis rate of 27% this incidence is markedly reduced (from 39% after radium only to 18% after pre-operative irradiation). In stage T_{III} with a 44% incidence of metastases the influence of additional external irradiation is even more pronounced: 65% metastasis after radium only, 49% after additional post-operative irradiation and 18% after pre-operative irradiation (Table 5).

The effect of various types of additional irradiation on the incidence of scar implants is shown in Table 6.

The histological grade of the tumour considered by stage was found to have no bearing on the incidence of either local recurrences or metastasis in either category.

Bladder-wall biopsy at a distance of 2 cm from the tumour in 133 patients who received

pre-radium irradiation revealed carcinoma in 31 (32%). A positive 2 cm biopsy indicated a markedly higher likelihood of the patient developing a local recurrence (32% in 31 cases) than a negative biopsy (10% in 102 cases).

Table 7 reflects a slight increase of positive biopsies with increasing stage. It also shows that a substantial fraction (67% of 31 cases) failed to develop local or marginal recurrences in spite of malignancy being demonstrated beyond or at the border of the adequately implanted area.

Table 5. Incidence of metastasis, including scar implants, after radium implantation of bladder cancer with regard to additional external irradiation

	No ext. irrad.	Post-op. irrad.	Pre-op. irrad.
Stage T_IM_0			
Total number	9	23	51
? Metast.	1	1	7
Metastasis	1/8 12·5%	2/22 9%	2/44 5%
Metastasis without Loc. rec.	1/8 12·5%	1/22 4·5%	2/44 4·5%
Stage $T_{II}M_0$			
Total number	32	81	62
? Metast.	4	7	2
Metastasis	11/28 39%	21/74 26%	11/60 18%
Metastasis without Loc. rec.	8/25 29%	14/81 17%	6/80 13%
Stage $T_{III}M_0$			
Total number	32	56	37
? Metast.	3	3	3
Metastasis	19/29 65%	26/53 49%	6/34 18%
Metastasis without Loc. rec.	16/29 55%	23/53 43·5%	4/34 12%

Table 6. Incidence of scar implants after radium implantation according to stage and additional external irradiation

	Scarimplants		
Stage	No ext. irr.	Post-op. irr.	Pre-op. irr.
T_I	0/9 0%	0/23 0%	0/51 0%
T_{II}	2/28 7%	1/74 1·5%	0/60 0%
T_{III}	7/29 24%	3/53 5·5%	0/34 0%
Total	9/66 14%	4/150 2·5%	0/145 0%

Table 7. Carcinoma of the bladder, treated by radium implant and pre-operative external irradiation
Relation between positive "2-cm biopsy" and local recurrence

Stage	Pos. "2-cm biopsy"	Loc. recurrence
T_I	8/43 18·5%	0/8
T_{II}	11/55 20%	4/11
T_{III}	12/35 34%	6/12
Total	31/133 23%	10/31 33%

A second bladder cancer, arising beyond the site of the treated primary growth, was diagnosed in 5% of all cases. These second growths became evident 2–13 years after treatment of the first growth. In view of the variations in survival rate and the length of the follow-up periods, conclusive information concerning the rate of incidence cannot be calculated in the various groups.

(4) *Complications*

Complications following radium implantation were observed in 7·5% of 431 cases (Table 8). No relationship could be demonstrated between these complications and the stage or histological grade of the tumour or the type of additional external irradiation.

DISCUSSION AND CONCLUSIONS

In cases of carcinoma of the bladder, the prognosis is distinctly better after radium implantation than after external irradiation (Figs. 2 and 3). In part this is due to case selection, as indicated above. However, the main reason is the higher dose of ionizing irradiation delivered by radium to the tumour (which is usually only moderately radiosensitive), without damaging the surrounding healthy tissues [1–4].

Mortality following interstitial radium therapy is caused—apart from intercurrent death—by complications, local or marginal recurrence and by metastases (Tables 2 and 3). By reducing these causes, the prognosis can be improved. The incidence of fatal complications has probably already reached the lowest level possible, in the present state of knowledge (Tables 2, 3 and 8).

Local and marginal recurrence—an accurate distinction is impossible—will originate from residual viable tumour cells in the main growth or from the tumour foci in the adjacent bladder wall. The likelihood of the latter event occurring is supported by microscopic evidence of malignancy in the bladder wall at a 2-cm distance from the tumour. The true prevalence of such malignant foci is likely to be much higher because this biopsy merely represents a random sample. Baker [5], in cystectomy and autopsy specimens, demonstrated malig-

Table 8. Complications after radium implantation of bladder cancer. R.R.T.I. 1950–1967.

Total number treated with radium	431	
† Operation	7	
† Pyonephrosis	1	
† Tumour perforation+sepsis	1	
Nephrectomy because of pyonephrosis	1	
Ureter deviation because of necrosis	7	
Cystectomy because of small bladder-capacity+necrosis	3	
Complaints due to necrosis, cured with 1 year.	7	
All complications	32/431	7·5%
Death due to operation or complications	9/431	2%

nant infiltration beyond the clinically involved area, its extension increasing with advancing stage. It is not unlikely that radium implants eradicate tumour foci adjacent to the clinically affected area, especially in cases of less deeply infiltrating growth in which the bladder wall retains its suppleness and collapses around the implant. This might explain the "local cure" obtained in the stage T_I and T_{II} cases that gave positive 2-cm biopsies (Table 7). This "cure" cannot be attributed to pre-operative irradiation, because in these stages the local recurrence rate did not decline after its application. However, in stage T_{III} it probably contributed to the eradication of adjacent malignant cell nests and to reducing the main tumour mass, thus accounting for a significantly lower incidence of local recurrence (Table 4).

A simple increase in radium dosis (above the equivalent of 6–7000 rad/168 hr) and enlargement of the implanted area might forestall local recurrence but these measures carry the risk of producing irreversible necrosis [1]. This complication necessitates ureteral transplantation thus negating one of the major advantages of radium implantation as compared with cystectomy, viz the preservation o normal bladder function.

Additional pre-operative external irradiation neither delayed wound healing nor affected the nature or incidence of serious complications.

Metastases which are present though undetected at the time of the first treatment are at present not amenable to curative treatment. Their incidence constitutes the potential curability of the patients [1, 6].

Metastases originating from local recurrence or residual growth may be forestalled by eliminating their resources. In the stage T_{III} cases, pre-operative irradiation diminished the occurrence of local recurrences and thus the persistence of metastatic sources. Part of the reduced metastasis rate in stage T_{III} might be attributable to this mode of action. In stage T_{II} this is not demonstrable.

Iatrogenic scar implants may be prevented to a certain extent by careful handling of the tumour and by bathing the operation site with cytoxic agents. In this series post-operative irradiation to the scar without any other precaution appeared capable of destroying "spilled" cells, thus reducing the occurrence of scar implants (Table 6).

Iatrogenic lymphatic or bloodborne metastases are easily conceivable, because the bladder tumour is inevitably compressed during implantation of the radium needles. The deeper the infiltration of the growth, the more extensive its involvement in vascular structures [1]. Consequently, the risk of iatrogenic spread might be expected to increase with each advancing stage.

Animal experiments [8–10] have demonstrated that irradiation with moderate non-tumour lethal doses can reduce the viability of tumour cells and hence their ability to establish metastases. It is not unlikely that in this series pre-operative irradiation affected the incidence of metastasis in the same way; a complete absence of scar implants (Table 6) and a significant decrease of all types of metastases in stages T_{II} and T_{III}. In stage T_I, where metastases are rare anyhow, no such influence could be elicited (Table 5). Apart from a devitalizing effect on the cells of the primary growth, pre-operative irradiation could conceivably also sterilize small tumour foci in the pelvic lymphatics and thus remove potential sources of metastasis.

It is a fact that the three modalities of treatment viz radium implantation only, additional external pre-operative irradiation and external post-operative irradiation, have not been applied in randomized series. This flaw can be mitigated by considering that neither implantation techniques nor surgical methods have been altered; staging carried out by the same clinical team has not shifted in such a way as to include "better" cases in more advanced stages. On the contrary, a proportional increase of stage T_I cases may be noted after 1962, probably due to a growing alertness of both general practitioners and urologists to the problem (Fig. 1). It might therefore be concluded that in the case of bladder cancer treated by radium implant, pre-operative irradiation significantly reduces the occurrence of metastases in stages T_{II} and T_{III} and of local recurrences in stage T_{III}, thus considerably improving prognosis.

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RESUME

Les résultats de la radiumthérapie interstitielle, pour des cas sélectionnés de cancer de la vessie, sont supérieurs à ceux de l'irradiation externe par supervoltage.

Une dose de 1050 *rad, appliqués durant une période de* 3 *jours avant l'insertion des aiguilles de radium, améliore considérablement le pronostic pour les stades* T_{II} *et* T_{III}.

Cette amélioration peut être expliquée par une diminution du pourcentage des récidives locales au stade T_{III}, *par la suppression complète des implantations cancéreuses cicatricielles, et par la réduction de la dissémination métastatique pour les stades* T_{II} *et* T_{III}.

SUMMARY

Radium implantation in selected cases of cancer of the urinary bladder offers a high cure rate with preservation of normal bladder function. A 3*-day course of supervoltage irradiation to the small pelvis on three consecutive days immediately prior to operation was shown considerably to increase the survival rates in stage* T_{II} *and* T_{III} *cases. This was effected by reducing the incidence of local recurrence in stage* T_{III}, *by completely forestalling scar implants and by decreasing the rate of metastasis in stages* T_{II} *and* T_{III}.

ZUSAMMENFASSUNG

Die Prognose nach interstitieller Radiumbehandlung von ausgewählten Blasenkrebspatienten ist bedeutend besser als nach perkutaner Supervoltbestrahlung.

Die Heilungsrate im Stadium T_{II} *und* T_{III} *wird durch zusätzliche präoperative Supervoltbestrahlung des kleinen Beckens* (1050 *rad. "midline" Dosis in* 3 *Bestrahlungen*) *erhöht. Dies wird durch Minderung der lokalen Rezidivfrequenz im Stadium* T_{III} *bewirkt, durch vollständinge Unterdrückung der Narbenmetastasten und durch Minderung der lympho- und hämatogenen Metastasierungsfrequenz in den Stadien* T_{II} *und* T_{III}.

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The Growth of Heterologous Tumour Cells in Chick Embryos

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INTRODUCTION

THE TECHNIQUES available for the maintenance of human tumours in foreign hosts are relatively few [1–3]. The embryonated hen's egg would appear to offer a number of advantages as a host system. The transplantation techniques involved are simple and the embryos are reputed to be incapable of producing an effective immune response before hatching [4]. Leighton [5] has reported that a variety of human tumours can be maintained as grafts on the chorioallantoic membrane, but even his results, the best reported so far with this system, demonstrate that only half of the specimens can be grafted successfully and most attempts to passage the tumours from egg to egg have failed.

The factors responsible for the success or failure of this host system are largely unknown, and it seemed desirable to investigate these factors systematically using a variety of experimental animal tumours. Tumour cell suspensions were inoculated intravenously into the embryo in order to bring a known number of tumour cells into immediate contact with the embryonic tissues and fluids in a reproducible manner, and the extent of tumour growth in the various organs assessed histologically. The effects on tumour growth of varying the age of the embryo, the number and type of tumour cells injected, and also various immunosuppressive procedures have been investigated.

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MATERIAL AND METHODS

Eggs

Fertilized White Leghorn eggs were kept in a humidified incubator at 37–38°C. Intravenous injections were made via the chorioallantoic vein as described by Beveridge and Burnett [6]. The embryos and extraembryonic membranes were removed at varying times and the tissues fixed and stained for histological examination. A minimum total of twenty embryos were injected for every tumour examined. The eggs were examined twenty-four hours after injection and those in which the embryos had died were rejected. The most frequent causes of embryonic death appeared to be excessive haemorrhage at the injection site, and the use of cell suspensions containing large aggregates of cells or debris.

Cell suspensions

The majority of tumours studied were in the ascitic form. After withdrawal from the peritoneal cavity they were washed twice with cold Hank's solution and resuspended in Hank's solution or serum-free culture medium or occasionally in cell-free ascitic fluid. The concentration of cells was adjusted to give the appropriate number of cells per embryo in an inoculum of 0·05–0·10 ml. In general, cell suspensions were not used if they contained more than 5% of cells stained by Trypan Blue. Suspensions of cells were also prepared from the solid mouse tumours, 6C3HED, multiple myeloma and the Friend leukaemia, and also from normal spleen and lymph nodes of mice

by chopping the tissues with crossed-scalpels and teasing with needles. With benzpyrene-induced rat tumours which were more resistant to dispersion, trypsinization and subsequent washing yielded satifactory cell suspensions. Suspensions of the cultured cells, normal hamster kidney fibroblasts BHK 21, clone 13, and polyoma-virus transformed cells (Py), obtained initially from Professor M. Stoker, were prepared by mild treatment of the cells growing on glass with trypsin–versene solution followed by thorough washing with Hank's solution.

Assessment of tumour growth in tissues

Three different methods were used. Firstly, gross macroscopic observations of the organs on dissection was of considerable value, any abnormalities in organ size or appearance being noted. For example, extensive growth of tumour in the liver of mesonephros resulted in an increase in their size, frequently accompanied by a 'pocked' and haemorrhagic appearance of the surface of the organs.

The second method of assessing tumour growth was by microscopic examination of sections of the tissues, which consisted of the liver, mesonephros, metanephros, brain, heart, stomach, lungs, spleen, eyes, skeletal muscle, yolk sac and chorioallantoic membranes. Complete organs were dissected from the chick embryo, fixed and stained with H. and E. and 8–10 sections of each specimen examined. It was relatively easy to distinguish most individual mouse or rat tumour cells from chick embryo cells by the larger size and different staining properties of the nuclei of the tumour cells. When more than a few tumour cells formed a group they could be identified and counted with speed and accuracy. The amount of tumour growth was assessed from examination of the sections.

Various qualitative criteria were also taken into account in the scoring of the extent of growth. For example, tumour growth in the mesonephros was almost always confined initially to the glomeruli, but if growth was sufficiently extensive, invasion and replacement of the tubules was observed. In the heart, tumour cells were initially confined to the periphery, but as tumour growth proceeded columns of tumour cells could be seen invading between the muscle fibres. Growth was always scored independently by at least two observers and the results compared.

The third method used was that of Hally [7], a quantitative method of measuring the area occupied by the tumour cells in the total area of the section, using a squared graticule eyepiece. This method gave highly reproducible results in the hands of different observers but was too laborious to be used for routine scoring.

Immunosuppressive procedures

(1) *Cortisone.* Preliminary experiments established that a single intravenous injection of 0·5 mg hydrocortisone sodium succinate into 10-day chick embryos produced retardation of growth, extensive liver necrosis and death of embryos by day 17. Injection of 0·1 mg hydrocortisone produced some liver necrosis by day 14, which disappeared by day 17, and this quantity was administered to 10-day embryos.

(2) *X-irradiation.* 10-day eggs were irradiated with 460R using 220kV, at a rate of 18R per minute with 2mm Cu and 1mm Al filters.

(3) *Chemical bursectomy.* Chemical bursectomy was consistently achieved by injecting 0·08 ml of a 5% solution of testosterone propionate in ethyl oleate into the allantoic cavity of 6-day chick embryos [8]. No bursal remnants were detectable in treated embryos on day 18.

(4) *Attempted absorption of maternal antibody.* Large numbers ($100–200 \times 10^6$) of viable Landschütz or L5178 cells were injected into the yolk sacs of 10- and 11-day embryos in attempts to absorb any anti-tumour factors present in the yolk sac.

The embryos subjected to treatment with cortisone, X-rays, testosterone propionate and yolk sac injection of tumour cells were injected intravenously on day 11 with the mimimum quantity of Landschütz ascites tumour cells (5×10^5) which gave reproducible growth in untreated embryos, or with 10×10^6 L5178 cells which grew only in the embryo brain. The extent of tumour growth in many organs of the treated embryos was compared with that in control embryos.

The effect of L-*asparagine on the growth of L*5178 *cells*

Embryos were injected intra-yolk sac on day 10 and each subsequent day until day 17 with 1 ml of a solution containing 20 mg L-asparagine. On day 11 the embryos were injected intravenously with 20×10^6 L5178 cells and the growth of tumour in these embryos compared with that in the saline injected control 3 and 6 days later.

Comparison of the growth of tumour in chick and duck embryos

Landschütz ascites cells (4×10^6) were in-

jected into 11 day duck and chick embryos. Embryos were sacrificed at 14, 17, 19, and in the case of duck embryos at 24 days of incubation, and the organs examined for tumour growth.

RESULTS

General observations

Examination of sections of organs fixed at intervals from 5 min to 48 hr after the injection of tumour cells suspensions into 11-day embryos revealed the presence of tumour cells in the blood vessels in decreasing quantities. By 18 hr the number of identifiable tumour cells present within any organ had decreased, and most of the remaining cells had become extravascular, invading the tissues of the organs. With some tumours the number of tumour cells continued to decrease until by the third day few, if any, viable cells could be detected. Other tumours grew so vigorously that they could result in death of the embryo within 3–5 days. A few tumours, notably the Du, Compton and S-180 ascites of mice resulted in the death of most of the inoculated embryos within 2–3 days, although tumour growth was not extensive at this time. Bacteriological tests failed to reveal any bacteria contaminating these suspensions. Embryos from 9 days onwards would tolerate inoculation with up to 40×10^6 cells of other tumours quite well.

Confirmation that the cells identified in the chick organs were indeed viable tumour cells was obtained by taking a portion of the liver of an 18-day chick embryo inoculated at 11 days with 4×10^5 Landschütz ascites tumour cells. A section of the remainder of the liver revealed masses of tumour cells occupying more than half of the tissue area. The rest of the liver was teased apart in Hank's solution and a suspension of large, apparently viable cells obtained. Three mice were each inoculated with 2×10^5 cells, and thirteen days later all three had developed a large volume of ascitic fluid averaging 15 ml, in which the number of cells was greater than 2000×10^6, i.e. 1000 times the inoculum injected 13 days previously.

TUMOUR GROWTH

Most of the tumours studied fell into two well-defined groups with respect to their capacity to grow within the organs following injection of tumour cells into 11-day embryos.

1. *Tumours which did not grow well in chick embryos*

This group consisted of tumours of the rat and mouse which were strain specific, i.e. they would only grow successfully from a small inoculum of cells if they were injected into the strain of rat or mouse from which the tumour originated. Of the fourteen strain specific tumours tested, eleven did not yield any growth in any of the chick embryo organs examined, except sometimes in the brain, even with inocula up to 40×10^6 tumour cells per embryo (see Table 1). Occasionally a few isolated tumour cells appeared to survive in the heart but mitotic figures were rarely seen. The other three strain specific tumours did not show the characteristic lack of growth. Two of them, the Moloney-virus induced lymphoma YAC, and the EL4 leukaemia, were very erratic in their behaviour. Eight out of sixty-seven embryos inoculated with $7–25\times10^6$ EL4 tumour cells on different occasions showed vigorous growth in the lung, mesonephros, and heart, but relatively little growth in the liver. Eighteen out of forty-six embryos inoculated with $5–30\times10^6$ YAC tumour cells showed similar growth in these organs. In neither case was any correlation found between the sex of the embryo and the growth of the tumour. With both these tumours the other embryos injected with the cell suspensions showed no sign of tumour growth except in the brain. The third atypical strain specific tumour, the Hepatoma 129, behaved reproducibly in that it always grew vigorously in many of the embryonic organs, provided a minimum inoculum of 7×10^6 cells was injected. Repeated attempts to grow these three tumours in strains of mice of different genetic constitution proved unsuccessful. At best, using large inocula, the tumour would grow for a few days, often producing large volumes of ascitic fluid containing few tumour cells, but after 7–10 days most of the ascitic fluid disappeared and no animals died as a result of tumour growth.

2. *Tumours which grew extensively in chick embryos*

The second group consisted of nine tumours of the rat and mouse which had lost their strain specificity in the original host (see Table 1). These tumours grew in many chick embryonic organs following intravenous injection into 11-day embryos particularly in the liver, mesonephros, brain and heart, and also in the chorioallantoic and yolk sac membranes. Moderate growth of tumours in this group was obtained with inocula (200,000–500,000), more than one hundred times smaller than those which were used without success for the strain specific group. Three of these non-strain specific tumours, the S-180, Du and Compton ascites of the mouse proved to be extremely

Table 1

Tumour strain specific	Host	Sites of tumour grown	Tumour strain specific	Host	Sites of tumour grown
L5178	DBA2 mice	Brain (Heart, eye)	Ehrlich	BALB/C⁻ and CBA/CB mice	Liver, brain, cam, meso., heart, eye, yolk sac, stomach
L1210	DBA2 mice	None	Landschütz	BALB/c mice	Heart, eye, yolk sac, stomach
C.B. ascites hepatoma	DBA2 mice	Brain	Krebs	BALB/c mice	Heart, eye, yolk sac, stomach
TL1 lymphoma	DBA2 mice	None	NK lymphoma sens. and res.	BALB/c mice	Heart, eye, yolk sac, stomach
6C3HED lymphona	CBA/CB mice	None	Walker 256	C.B. stock rats	Liver, brain, heart, cam, meso., wing joints
Mult. myelomas, PC5, PC6	BALB/c mice	Brain			
L1512	CBA/CB mice	Brain	Yoshida sarcoma	C.B. stock rats	Liver, brain, meso., cam, heart, spleen
CL6	BALB/c mice	None	S-180	BALB/c mice	Some growth, very toxic
Friend virus leukaemia	BALB/c mice	None	D-180	BALB/c mice	Some growth, very toxic
C+leukaemia	BALB/c mice	None	Compton 22	BALB/c mice	Some growth, very toxic
Benzpyrene sarcomas	Wistar rats	None	Cultured 'normal' hamster kidney fibroblasts, BHK 21, C13		Very little growth
EL4	C57BL mice	Brain 12% with extensive growth in lung, heart, meso., cam, liver	Cultured polyoma virus transformed hamster fibroblasts		Liver, meso., cam, brain, heart, (muscle)
YAC (Moloney) (Virus)	A mice	Brain 40% with extensive growth in lung, liver, meso, spleen, cam	Py		
HEP 129	CBA/CB mice	Brain, lung, liver, heart, meso., cam (muscle)			

toxic to chick embryos, most of which died within 1–3 days of receiving an inoculum of 1×10^6 tumour cells. In the few surviving embryos tumour growth in the liver, mesonephros lung and brain was moderate but not as extensive as with the same inocula of the other non-strain specific tumours. There were, however, many indications of damage and death of chick embryo cells in many organs. Repeated bacteriological tests on these three ascites tumours failed to yield any evidence of bacterial contamination.

The remaining six tumours of this group (see Table 1) grew progressively and vigorously resulting in death of the embryo when large inocula ($>5 \times 10^6$ cells) were used. In such cases many of the organs, particularly the liver and mesonephros were considerably enlarged weighing more than twice the weight of control organs, and tumour occupied from $\frac{2}{3}$ to $\frac{3}{4}$ of the tissue. Growth of tumour in the spleen was rarely observed even when growth in many other organs was extensive. Two of these tumours grew in organs which were not usually affected by the other tumours. The Yoshida rat sarcoma grew very vigorously in the lung and spleen, and the Walker 256 grew so well in the wing joints that large nodules were formed. Other tumours did not grow significantly in these sites.

Other types of cells

Cell suspensions obtained from cultures of so-called normal hamster fibroblasts (C13 clone of BHK21) appeared to grow initially following injection into 11 day embryos. Small foci consisting of three or four cells could be detected three days after the injection of $5–10 \times 10^6$ cells, but subsequently no further growth was observed and the cells appeared to become very swollen and vacuolated, with empty or disintegrating nuclei. The corresponding polyoma-virus transformed cells (Py) grew vigorously with inocula of $1–5 \times 10^6$ cells, chiefly in the liver, yolk sac membrane, CAM, mesonephros lung and brain. A feature of some interest was observed with these cells. If suspensions containing a moderate number of cell aggregrates were injected, tumour growth was much reduced in the liver and became very extensive in the pectoral muscle. When suspensions contained very few cell aggregates, growth was much more extensive in the liver than in the pectoral muscle. Similar effects had been noted with the HEP 129 tumour.

The injection of 16×10^6 lymphoid cells obtained from the spleen and lymph nodes of adult male Balb/c mice did not result in any splenomegaly, nor was splenomegaly detected following injection of any of the tumour cell suspensions, except in the rare case where extensive tumour growth in the spleen was observed.

The effect of varying the tumour inoculum using embryos of the same age

This was investigated mainly with 11 day embryos, but similar results were obtained with embryos from 7 to 14 days of development. Injecting embryos of the same age with 5-fold serial dilutions of tumour cells, always revealed an increase of tumour growth and an increase of the number of organs affected when larger inocula were used. When the dilution factor was smaller than 5-fold it became increasingly difficult to distinguish between the amounts of tumour growth obtained, and larger numbers of eggs had to be used.

Much more clear-cut differences were obtained when the lower concentration of cells was close to the minimum which gave tumour growth; for example, 2×10^6 HEP 129 cells gave negligible growth of tumour in any organ except the brain at 3, 6 and 7 days after injection, whereas 10×10^6 cells resulted in massive tumour growth in pectoral muscles, liver, heart, spleen, mesonephros, stomach and brain after 6 days in all embryos injected.

The effect of varying the age of the embryo

The injection of the same number of tumour cells into embryos aged from 7 to 14 days always resulted in greater growth in the earlier embryos after the same period of time. Thus, injection of 3×10^4 Ehrlich ascites tumour cells into 12-day embryos resulted in no detectable growth at any time up to hatching, whereas embryos injected with the same number of cells at day 7 had appreciable amounts of tumour in the liver, mesonephros and brain by day 14. When strain-specific tumour cells were injected into younger embryos a similar increase in tumour growth was observed. The extent of increased growth varied with the stain-specific tumour injected. Some, such as the L5178, showed increased growth in younger embryos in the brain only, whilst others, such as the ADJ/PC5 gave considerable growth in many organs following injection into 7–9-day embryos whereas growth occurred only in the brain following injection of 11-day embryos.

This increase in tumour growth associated with injection of younger embryos was not due solely to the increase in the ratio of tumour cells to normal tissue weight in the younger embryos, since similar increases in growth

were observed when the number of tumour cells injected into the younger embryos was reduced in direct proportion to the embryonic weight.

The effect of immunosuppressive treatment, yolk sac injections of tumour cells, and L-*asparagine*

Treatment of embryos with cortisone, X-irradiation or testosterone propionate did not result in any detectable increase in the growth of intravenously injected Landschütz or L5178 tumour cells relative to that in untreated control embryos.

No enhancement of growth of L5178 cells was observed in embryos receiving prior yolk sac injection of these cells. The results obtained with the Landschütz tumour were erratic in that enhancement of tumour growth was observed in two out of five separate experiments. In none of these experiments were viable tumour cells detected within the yolk sac 5 days after injection of 200×10^6 cells into the yolk sac.

The daily injection of 20 mg of L-asparagine into the yolk sacs did not have any detectable effect on the growth of the L5178 tumour in any organ examined.

Comparison of chick and duck embryos

The growth of the Landschütz tumour in all the organs examined was much less extensive in duck embryos than in chick embryos at 3, 6 and 8 days after injection. Eight days after injection many of the chick embryos had died with massive tumour growth, whilst tumour cells had almost completely disappeared from all duck embryonic organs except the brain. Thirteen days after injection tumour could not be detected even in the brain of duck embryos.

DISCUSSION

One of the most striking observations was the difference in behaviour of non-strain specific and strain specific tumours following intravenous injection into the chick embryo. It was quite unexpected that the embryo should be capable of distinguishing between these two categories of tumour cell derived from the same foreign species.

Many, though not all, of the non-growing strain-specific tumours tested were lymphomas and leukaemias, some of which are known to be inhibited by treatment with guinea pig serum which contains the enzyme asparaginase. It was possible that these cells which require L-asparagine for growth might have been inhibited by asparaginase present in the embryo, since Ohnuma *et al.* [9] have shown that adult chicken liver contains asparaginase. No asparaginase activity could be detected, however, in the blood of 14- and 17-day chick embryos, and repeated daily injections of 20 mg L-asparagine into the yolk sac did not result in any detectable increase in the growth of the asparagine dependent tumour L5178. Moreover, one of the tumours reported by Broome [10] to be sensitive to asparaginase was the Walker tumour which grows well in the chick embryo, as previously reported by Leighton [11].

The proliferative capacity of many strain specific tumours is comparable with that of many non-strain specific tumours. The strain specific L5178 tumour, for example, has a doubling time of 11 hr in DBA2 mice, and the tumour will take in these mice from an inoculum of 50 cells [12]. It is unlikely, therefore, that the failure of strain specific tumours to grow well in chick embryos is due to a slower rate of proliferation of these tumours. Moreover, the growth of many strain specific tumours in the embryo brain demonstrates their capacity for proliferation.

As the use of the chick embryo limits the time for tumour growth to 8–9 days, a few experiments were carried out using duck eggs, which have a longer incubation period (27 days), as hosts for tumour growth. Unfortunately, the growth of non-strain specific tumours such as the Landschütz was much less vigorous and more rapidly inhibited in the duck embryo than in the chick embryo.

The only procedure found so far which consistently and markedly favours the growth of intravenously injected tumour cells in chick embryos is the use of younger embryos. Resistance to tumour growth appears to increase progressively with embryonic developments. Two of the most relevant changes taking place in the embryo during the period studied are the appearance of maternal antibodies in the embryonic circulation and the possible onset of specific immune responses.

The growth of many of the strain-specific tumours exclusively in the embryonic brain, commonly considered to be an immunologically protected site, suggests that an immunological mechanism, either active or passive, could be involved in the resistance of the embryo to the growth of these tumours. Normal adult fowl serum contains antibodies against erythrocytes of all species tested so far, and their transmission to chick embryos via the yolk has been confirmed [13]. Kaminski and Durieux [14] using gel diffusion tests found that serum γ-globulins could be detected in

the embryonic circulation by day 14, and sufficient quantities might be present at 11 days to account for the inability of many tumours to grow. The enhanced growth of most tumours following injection into 7–8 day embryos would be in agreement with this, since once the tumour had penetrated the tissues and proliferated the subsequent growth might well outstrip the inhibition resulting from the progressive release of γ-globulins into the circulation.

The failure to detact any viable tumour cells in the yolk sac or embryo a few days after the injection of up to 200×10^6 Landschütz tumour cells also indicates that there may be factors present in the yolk which are incompatible with tumour cells survival. The observation that such intra-yolk sac injection can sometimes enhance subsequent growth of tumour cells injected intravenously may be due to the partial absorption or inactivation of the anti-tumour factors. Previous work by Green and Lorincz [15] also support the conclusion that circulating antibody, probably of maternal origin, may be responsible for the resistance of the embryo to tumour growth. They found that growth of Kreb's mouse ascites tumour was inhibited if non-immune adult fowl serum was injected following intravenous injection of the tumour cells.

The possibility of an active specific cell mediated immune response by the embryo cannot be completely excluded since Seto and Albright [16] on the basis of partial inhibition of splenomegaly following inoculation of adult hen lymphoid cells concluded that the embryo is capable of initiating an immune cellular response on day 14–15. The failure of X-irradiation and cortisone treatment to produce any increase in tumour growth indicates, however, that the resistance of the embryo was probably not associated with a specific immune cellular response. In addition, chemical bursectomy, which has been shown to inhibit completely the synthesis of specific antibody by the chick embryo [8], also failed to produce any enhancement of tumour growth.

Two further observations which indicate that an active specific immune response is not responsible for the resistance to tumour growth are based on the speed with which the embryo eliminates many tumours. Firstly, electron microscopic examination (unpublished) of the livers of embryos injected at 11 days with non-growing strain specific tumours such as L5178 and ADJ/PC5 revealed that a high proportion of the tumour cells were being phagoctyosed by host cells as early as 3 hr after injection. Secondly, as shown in the following paper, as much as 25% of the total radioactivity derived soely from tumour cells, labelled with tritiated thymidine before injection, was associated with chick embryo cells present in the blood within 24 hours after injection. Such a rapid reutilization of the labelled DNA derived from the tumour cells, combined with the extensive phagocytosis observed within a few hours of tumour cell injection cannot readily be accounted for by an active induced specific immune response by the host.

However, resistance of the growth of tumours associated with natural maternal antibodies could account for the different growth capacities of non-strain specific and strain specific tumours following injection into the embryos. Möller [17] has suggested that the main change responsible for non-specific tumour growth is the decreased concentration of isoantigenic receptors on the tumour cell surface, which results in an increased resistance to the homograft reaction and to the cytotoxic effects of antibodies [18]. Mayer [19], for example, was unable to demonstrate strong histocompatibility antigens on the Ehrlich ascites tumour cells, which were lacking the D, A and K antigens of the H-2 type. The ability of the chick embryo to support the vigorous growth of non-strain specific tumours and to resist the growth of strain-specific tumours may be connected with the presence of natural maternal antibodies derived from the yolk which can react with antigens present on the surface of the strain specific cells but not with the non-strain specific cells, many of whose surface antigens may be absent or masked.

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RESUME

Afin de déterminer les facteurs responsables des variations de vitesse de croissance de différentes tumeurs au contact des tissus de l'oeuf embryonné de poule, on a étudié la croissance de cellules tumorales provenant de rats, de souris et de hamsters, après les avoir injectées dans la veine chorio-allantoïdienne.

Les tumeurs peuvent être réparties en deux groupes distincts, sur la base de leur capacité de croissance, après injection à des embryons de 11 jours. Les tumeurs qui avaient perdu leur spécificité de lignée dans leur espèce d'origine croissaient vigoureusement dans plusieurs organes de l'embryon de poulet et sur la membrane chorio-allantoïdienne, ainsi que dans le sac vitellin. La plupart des tumeurs qui avaient conservé leur spécificité de lignée ne croissaient dans aucune organe, excepté dans le cerveau, même avec le maximum d'inoculum toléré.

Les moyens immuno-suppresseurs, y compris l'irradiation des embryons, le traitement à la cortisone, et la bursectomie, chimique n'augmentaient ni n'inhibaient, de façon significative, la croissance des tumeurs représentatives desdeux groupes, indiquant ainsi que la résistance de l'hôte n'était pas due à une immunité spécifique. L'emploi d'embryons plus jeunes (7–8 jours) favorisait la croissance de toutes les tumeurs, quelquefois de façon spectaculaire. On suppose que la résistance des embryons à la croissance de certaines tumeurs est due au passage d'anticorps naturels d'origine maternelle, passant du sac vitellin dans la circulation embryonnaire, au cours du développement.

SUMMARY

In order to investigate the factors responsible for the varying capacities of different tumours to grow in contact with the tissues of embryonated hen's eggs the growth of tumour cells derived from rats, mice and hamsters following intravenous injection via the chorio-allantoic vein has been examined.

Tumours can be divided into two fairly distinct groups on the basis of their capactiy to grow following injection into 11 day embryos. Those tumours which had lost their strain specificity in the species of origin grew vigorously in many organs of the chick embryo and also in the chorioallantoic and yolk sac membranes. Most of the tumours which had retained their strain specificity did not grow in any organs except the brain, even with maximum tolerated inoculum.

Immunosuppressive procedures including X-irradiation of the embryos, treatment with cortison and chemical bursectomy did not significantly enhance or inhibit the growth of representative tumours from both groups, indicating that host resistance was not associated with an active specific immune response. The use of younger embryos (7–8 days) enhanced the growth of all tumours, sometimes dramatically. It is suggested that the resistance of embryos to the growth of certain tumours is associated with the release of natural meternal antibodies from the yolk into the embryonic circulation during development.

ZUSAMMENFASSUNG

Um die Faktoren für die Ursachen des Wachstumsfähigkeit verschiedener Tumoren im Kontakt mit embryonalem Gewebe der Hühnereier zu untersuchen, wurde das Wachstum der Tumorzellen von Ratten, Mäusen und Hamstern nach intravenöser Injektion in die Chorional antoisgefäße geprüft.

Die Tumoren können in 2 deutlich verschiedene Gruppen unterteilt werden, je nach ihrer Wachstumskapazität nach Verimpfung auf 11 Tage alte Embryonen. Solche Tumoren, welche ihre Artspezifität verloren hatten, wuchsen kräftig in vielen Organen des Hühnchenembryos sowie auf den Membranen der Chorionallantois und des Dottersackes. Die Tumoren, welche ihre Spezifität behalten hatten, wuchsen in keinem Organ, mit Ausnahme des Gehirnes, selbst wenn sie mit der maximal verträglichen Menge geimpft waren.

Immunsuppression, etwa durch Röntgen-Bestrahlung von Embryonen oder Behandlung mit Cortison oder chemische Bursektomie, zeigte keine signifikante Wirkung auf das Wachstum typischer Tumoren beider Gruppen. Dies läßt darauf schließen, daß die Abwehr des Wirts nicht eine spezifische Immunleistung darstellt. Bei der Verwendung von jüngeren Embryonen (7 bis 8 Tage alt) zeigte sich bei allen Tumoren eine, manchmal dramatische, Wachstum steigerung. Dies läßt darauf schließen, daß die Resistenz von Embryonen gegen das Wachstum einiger Tumoren mit der Abgabe von natürlichen mütterlichen Antikörpern vom Dotter zum embryonalen Kreislauf während der Entwicklung verbunden ist.

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The Distribution of Heterologous Tumour Cells in Chick Embryos following Intravenous Injection

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IN THE preceding paper [1], concerned with factors influencing the growth of heterologous tumour cells in chick embryos, results were obtained which made it desirable to investigate the distribution of these cells in the embryonic organs following injection. The following questions arose from this previous work.

1. Could the relatively poor growth of most strain specific tumours compared with the vigorous growth of non-strain specific tumours be accounted for by differences in organ distribution?

2. For any single tumour is the extent of subsequent tumour growth in the organs directly related to the relative ability of the organs to trap tumour cells, or do certain organs appear to provide a particularly favourable or unfavourable environment for tumour growth?

3. Is there any indication of 'specificity' of localization of different tumours in organs, and if so, how is this related to the size of the injected cells?

In this work tumour cells were labelled with tritiated thymidine and the distribution in various embryonic organs measured. In addition, the effect of injecting varying numbers and proportions of labelled cells on the organ distribution was investigated. Variation in the distribution of radioactivity with the time following injection was investigated using several tumours, and the influence of the age of the embryo on organ distribution of one tumour was also examined.

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MATERIAL AND METHODS

The procedures for incubating and injecting the eggs have been described in the preceding publication [1].

Labelling of cells

Ascites tumour cells were labelled *in vivo* by injecting tritiated thymidine intraperitoneally three times at intervals of three hours. Each injection consisted of 0·2 ml ^{3}H TDR (Specific activity: 5–12 c/mole) containing 20 μc. Twenty-four hours after the first injection the mouse was sacrificed and the ascitic suspension withdrawn, washed five times with 10 ml quantities of Hank's solution, and finally suspended in Hank's solution or serum-free tissue culture medium to give the desired concentration of cells to be injected. The cell suspensions usually contained less than 3% cells stained by Trypan Blue. To determine the amount of radioactivity contained in the inoculum, 0·1 ml of the cell suspension was added to 0·9 ml saline, the cells separated by centrifugation and the radio-activity in the cells and supernatant measured. The supernatant always contained less than 1% of the activity present in the cells. A smear of the labelled cells was was made for autoradiography in each case in order to estimate the percentage of labelled cells (generally 70–80% were found to be labelled).

Table 1. *The initial distribution of* ^{3}*H-labelled cells in* 11*-day chick embryos*

(Expressed as percentages of the total injected)

	Landschütz (16)* (%M.D.)	L5178 (10) (%M.D.)	HEP129 (6) (%M.D.)	YAC (12) (%M.D.)	EL4 (6) (%M.D.)
Cam+amnion	65·7±4·1	63·3±6·9	53·7±3·9		
Yolk sac	6·4±2·3	11·0±3·5	3·2±0·5		
Liver	15·6±2·9	7·3±1·4	13·6±1·9	5·2±1·7	3·4±0·76
Mesonephros	0·97±0·19	0·47±0·10	0·75±0·28	0·61±0·15	1·0±0·21
Brain	0·56±0·10	0·68±0·19	0·60±0·17		
Heart	0·35±0·07	0·30±0·05	0·44±0·11		
Stomach	0·30±0·07	0·30±0·08	0·29±0·06		
Lungs	0·26±0·04	0·24±0·05	0·60±0·10	2·1±0·07	2·2±0·32
Spleen	0·22±0·06	0·09±0·02	0·26±0·10	0·83±0·06	
Eyes	0·15±0·05	0·43±0·13	0·21±0·07		
Metanephros	0·10±0·04	0·10±0·06	0·12±0·05		
Rest of embryo	3·6±1·7	3·7±1·0	5·8±0·60		
Blood cells†	0·05±0·02	0·48±0·20	0·07±0·03		
Plasma†	0·12±0·05	0·37±0·13	0·31±0·07		

The values were derived from specimens taken 1–6 hr after injection.
*No. of embryos used.
†Values for 0·2 ml embryo blood.

Measurement of radioactivty present in the organs

The embryonic organs were dissected, weighed immediately, 0·5–1·0 ml 10% TCA added and heated at 80°C for ½ hr. A sample (0·5 ml) of the supernatant of each organ was used for the measurement of ^{3}H activity in a liquid scintillation counter, Packard 3003 series. The extraembryonic membranes comprising the chorioallantoic membrane+amnion, and the yolk sac membrane, the remainder of the embryo, and the extra-embryonic fluids were each homogenized in a Potter homogenizer and aliquots were extracted with TCA. The ^{3}H activity of each sample was corrected to dpm and the total activity within the organ calculated. The results were expressed either as

$$\frac{\text{dpm/0·1g tissue}}{\text{total dpm injected}}$$

which was a measure of the relative efficiency of the tissue in trapping radioactivty, or as the percentage of the total radioactivity injected,

$$\frac{\text{dpm in the tissue}}{\text{total dpm injected}}$$

Autoradiographs of organs, blood smears and cells suspensions were obtained using the dipping-film technique.

Cell size distribution

Measurements of the cell size distribution of various tumour cell populations were carried out using a Coulter counter, model B.

Changes in the distribution of ^{3}*H with time*

The organ distribution of ^{3}H was measured at 5 min and 1, 2, 3, 4, 6, 8, 24, 48, and occasionally 72 hr after injection of the labelled cells. The organs examined are listed in Table 1, and three tumours, the Landschütz, L5178, and HEP 129 were compared in detail and two others, YAC and EL4, less extensively. In addition, samples of blood (usually 0·2 ml) were removed whenever possible, diluted with 0·8 ml saline, centrifuged, and the radioactivity in the sedimented blood cells and supernatant plasma solution measured.

The effect of varying the number of cells injected on the organ distribution

The ^{3}H-labelled Landschütz cells were suspended in Hank's solution to give two cell suspensions containing $2·9\times10^6$ and 1×10^5 cells/0·1 ml (the volume injected), respectively. These suspensions were injected into 11-day embryos and at 3 and 20 hr the livers were removed, weighed and the radioactive content determined.

The effect of varying the proportion of labelled cells injected

As the cells in any given suspension would not be uniformly labelled with ^{3}H, and the degree of labelling would vary with different suspensions, it was necessary to ascertain that the distribution of the labelled cells was reasonably representative of that of the whole population. This was investigated by injecting one group of 11 day embryos with $3·5\times10^6$ ^{3}H-labelled YAC cells, and another group

with the same number of YAC cells of which the number of labelled cells had been diluted to 1:11 with unlabelled YAC cells. Two hours after injection the livers and lungs were removed and the counts/min/mg weight of tissue determined.

The distribution of Landschütz ascites tumour cells in 8-day embryos

Four 8-day embryos were injected with $2{\cdot}5 \times 16^6$ ^{3}H-labelled Landschütz tumour cells, the livers and lungs removed at $\frac{1}{2}$, 3, 8 and 18 hr after injection, and the counts/min/mg determined and compared with that of 11-day embryos.

RESULTS

Recovery of radioactivity

The recovery of tritium in experiments where all the contents of the egg were estimated was between 70 and 90%. The only portion of the egg whose tritium content varied erratically was the combined extraembryonic fluids, which usually contained negligible quantities of tritium, but occasionally contained as much as 10% of the total injected. Some of this tritium was probably derived from contamination of the fluids with small fragments of the high-activity membranes, but another source of error was the very large dilution factor ($\times 400$) involved in the estimation of activity in these fluids. This factor would magnify considerably the errors involved in counting the very low levels of radioactivity present in the sample.

Changes in distribution with time

Histological examination of sections of the organs revealed that most of the viable tumour cells had become extravascular 24 hr after injection. Before this time it was impossible from the measurements of radioactivity present in the organs to be certain that some tumour cells were not escaping from the organs. From 1–8 hr after injection, the estimated percentage of the radioactivity present in the total volume of blood at the times of removal was about 1% for non-strain specific tumours and from 1 to 5% for the strain specific tumours. From autoradiographs of blood smears, it was evident that most of this radioactivity was due to the presence of debris from dead cells, but it cannot be excluded that there may have been a slow and continuous release of tumour cells from the organs.

Dissections of the organs could be completed within 5 min of injection, but the quantities of radioactivity found in any organ at this time were more variable than those found in organs removed 1 hr after injection, or later. The organs whose radioactive content was measured are listed in Table 1. Figures 1–5 illustrate some of the results obtained in one of many experiments in which ^{3}H-labelled tumour cells were injected into 11-day embryos.

For all the tumours examined the radioactivity in various organs listed in Table 1 was essentially constant for the first 8 hr, but began to change by 24 hr. The liver and the CAM+amnion were the only tissues in which the amount of radioactivity began to fall at 24 hr, whilst it began to rise in all other tissues at this time. For reasons which will be considered in detail in the discussion, the initial distribution of ^{3}H from 1 to 6 hr was considered to reflect the distribution of the tumour cells, whilst later values at 24 and 48 hr were complicated by loss of ^{3}H from tumour cells and its reutilization by chick embryo cells.

Table 2. The relative tumour trapping capacity of 11-day chick embryo tissues

(Expressed as percentages per 100 mg wet wt. of tissue)

	Landschütz	L5178	HEP 129	YAC	EL4
Liver	45	22	28	8·0	5·3
Spleen	22	9·1	25	80	
Cam+amnion	6·6	6·3	5·4		
Mesonephros	5·7	2·9	3·1	5·8	7·2
Metanephros	2·5	1·8	1·5		
Heart	1·3	1·1	0·9		
Lungs	0·7	0·7	1·2	3·3	5·0
Stomach	0·5	0·9	0·5		
Muscle, thigh	0·3	0·5	0·3		
Muscle, pectoral	0·2	0·4	0·4		
Brain	0·3	0·4	0·3		
Yolk sac	0·3	0·5	0·2		
Rest of embryo	0·2	0·3	0·2		
Liver/lung	64	31	23	2·4	1·1
Mean cell dia. μ	13·8	12·6	15·6	12·4	11·6

The initial organ distribution of labelled tumour cells

The organs which trapped the most tumour cells were the CAM, the liver and the yolk sac (Table 1). If the distribution was considered on the basis of the percentage/100 mg tissue, the liver and spleen emerged as the most efficient tumour trapping organs, followed by the CAM and mesonephros (Table 2).

Comparison of the organ distribution of different tumours in 11-day embryos revealed significant differences. For example (Table 2), the liver trapped 64 times as many tumour cells as the lung per unit weight of tissues when Landschütz ascites tumour was injected, whereas using the YAC and EL4 tumours the liver trapped only 1–2 times as many tumour cells as the lung. Other differences in organ distribution and their possible significance will be considered in the discussion.

The effect of varying the numbers of cells injected

The results obtained were:

	counts/min/mg liver (using $2{\cdot}9\times10^6$ cells)	counts/min/mg liver (using 1×10^5 cells)	ratio
3 hr	150	3·7	40
20 hr	71	1·0	71
		Theoretical ratio=29	

The ratio of counts/min/mg liver for the two inocula at 3 hr was reasonably close (40) to that which would be expected if the distribution of the cells was independent of the number of cells injected (29), but by 20 hr, radioactivity, and presumably viable cells, were being lost from the livers of embryos injected with 1×10^5 cells at a greater rate than from those injected with $2{\cdot}9\times10^6$ cells. It should be mentioned that 1×10^5 Landschütz cells is about the minimum which would grow following injection into 11-day embryos, whilst 3×10^6 gives consistent growth.

The effect of varying the proportion of labelled cells injected

If the organ distribution of tumour cells was independent of the proportion of cells labelled with ^{3}H, then the radioactivity/mg wt. of tissue should be directly proportional to the radioactivity of the original cell suspensions. In this experiment comparison was made between the results obtained by injecting two cell suspensions with identical numbers of cells but with different proportions of labelled cells. The ratio of radioactivity present in the original cell suspensions was 9·3. After injection and extraction of ^{3}H from the tissues the ratio was 11·4 for the livers and 11·1 for the lungs. It appeared, therefore, that the distribution of ^{3}H-labelled cells in the organs was reasonably representative of the distribution of the whole population of cells injected.

Comparison of distribution of Landschütz cells in 8 and 11-day embryos

The mean percentage of the total radioactivity injected which was found in the livers of 8-day embryos was $2{\cdot}4\pm0{\cdot}65\%$, and in the lungs $0{\cdot}06\pm0{\cdot}02\%$, both of which were about 1/5th of the percentage found in 11-day embryo livers and lungs for this tumour. The counts/min/mg wt. were, however, very similar to those obtained using 11-day embryos:

	% of total ^{3}H injected/ 0·1 g. tissue for 8-day embryos	% of total ^{3}H injected/ 0·1 g tissue for 11-day embryos
Liver	36	45
Lung	0·42	0·54

These results indicate that the numbers of tumour cells trapped by a *unit weight* of liver and lung were similar for 8- and 11-day chick embryos.

DISCUSSION

Survival of tumour cells in the embryos

The rapid death of injected strain specific tumour cells was confirmed both by the histological observation that viable tumour cells were rarely observed in any tissue except the brain one to two days after injection, and by the very high levels of radioactivity (up to an estimated 25% of the total injected, see Fig. 5) found in the circulating blood 24–48 hr after injecting viable ^{3}H-labelled L5178 tumour cells. Most of the radioactivity was present in the sedimented blood cells, and autoradiographs of blood smears revealed that all the ^{3}H-labelled cells were of chick origin. Even the non-strain specific tumour cells underwent cytolysis to a certain extent, as there was a ten-fold increase in radioactivity, representing 1–2% of the total radioactivity injected, in the blood during the first 48 hr, which was almost exclusively associated with chick cells.

Initial distribution of tumour cells

Before measurements of the quantities of tritium localized in the embryonic organs and extraembryonic structures could be interpreted it was necessary to ascertain the conditions under which the distribution of tritium reflects the distribution of the cells. This was particu-

larly important when strain specific tumour cells, most of which die following injection, were used, as reutilization of ^{3}H-labelled material by proliferating and phagocytic chick cells present in most organs could confuse the interpretation. Fortunately, all the tumours examined gave reasonably constant levels of radioactivity in the organs for the first six hours following injection, as illustrated by Figs. 1–4. This stability over this period was also found in the constant levels of radioactivity present in the blood (Fig. 5). It, therefore, seemed reasonable to assume that the average values for the organ distribution over the first six hours reflected the initial organ distribution.

By 24 to 48 hr after injection, this assumption was certainly no longer valid in the case of the strain specific tumours. At 48 hr, examination of sections of organs revealed that most of the remaining tumour cells were degenerating, and this was reflected in the very pronounced rise in the amount of radioactivity associated

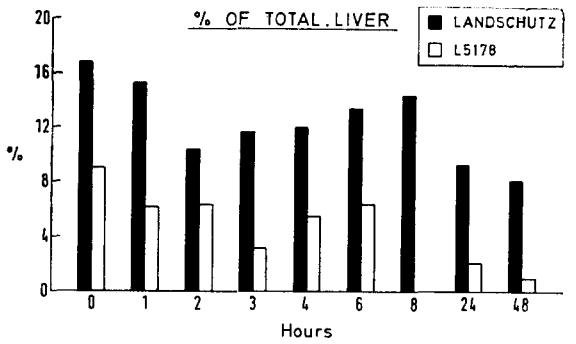


Fig. 1.

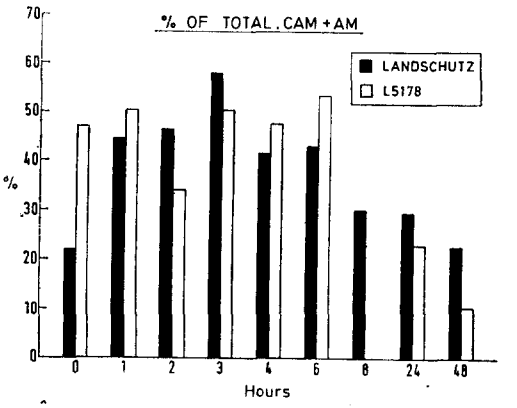
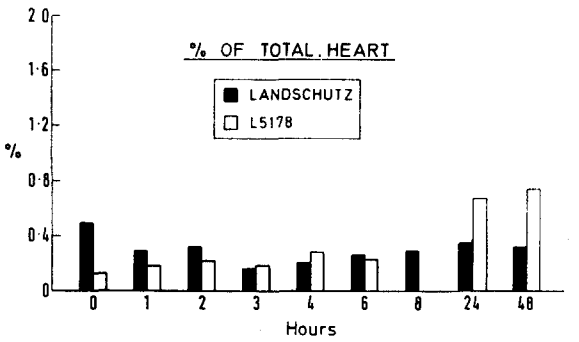


Fig. 2.



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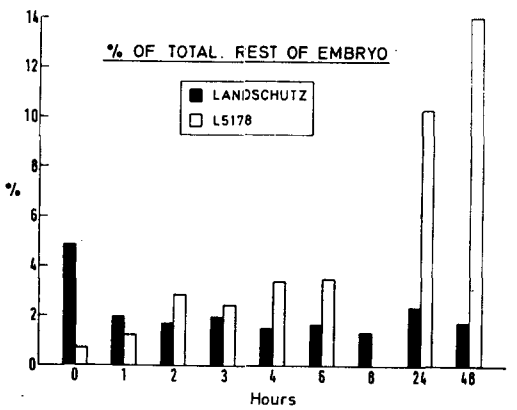

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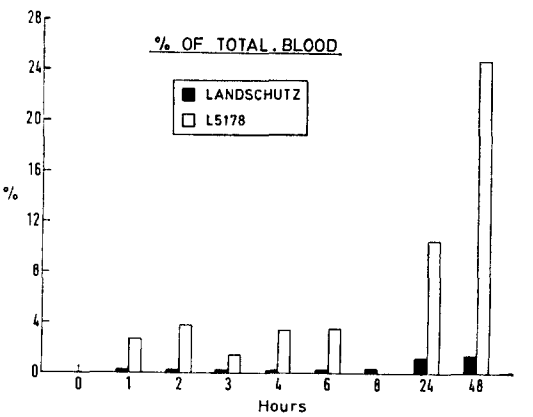

Fig. 5.

Figs. 1–5. *The effect of time on the distribution of ^{3}H in various chick embryo tissues following injection of ^{3}H-labelled cells. Figures* 1–5 *illustrate typical results obtained in individual experiments using Landschütz and* L5178 *tumour cells. The ordinates represent the percentage of the total ^{3}H injected and the abscissae the time following injection.*

with the chick embryo blood cells, and also with a rise in the percentage of radioactivity present in most tissues except the liver and CAM, which suffered a decline in the percentage of radioactivity. The liver and CAM were two of the most efficient tumour-trapping tissues, and their loss of radioactivity due to tumour cell death was probably greater than that acquired by proliferating embryonic cells within these organs. The level of radioactivity in all other organs increased, probably as a result of reutilization or phagocytosis of the ^3H compounds released following the death of a high proportion of the large number of tumour cells initially trapped in the liver and CAM.

Since ascites tumours do not contain an homogeneous population of cells and as the cells were labelled to varying degrees it was necessary to establish that the initial distribution of the labelled cells was representative of the total population. This was investigated by injecting two groups of embryos with suspension of tumour cells containing equal concentrations of cells but with different proportions labelled with ^3H. The ratio of radioactivity present in the two cell suspensions was found to be 9·3 and the ratios of radioactivity/mg tissue for the two groups of embryos actually found were 11·1 for the lungs and 11·4 for the livers. This indicates that the distribution of the labelled cells reflects with reasonable accuracy the distribution of the whole population. It cannot be excluded, however, that some organs may preferentially trap a certain proportion of the cell population differing significantly from the rest in some property such as cell size, surface charge density, etc.

Another factor which might have influenced the organ distribution, and hence the sites of tumour growth, was variations in the number of cells injected. Distribution studies were carried out with five ascites tumours (Table 1) using inocula varying from 2×10^6 to 10×10^6 for any individual tumour without obtaining any significant effect on the organ distribution. It is of interest, however, to compare the distribution of Landschütz tumour cells in two groups of 11-day embryos injected with inocula differing by a much larger factor ($\times29$). The results obtained revealed that 3 hr after injection the ratio of radioactivity/mg wt. of the livers was 40:1 instead of the theoretical 29:1 but by 20 hr the ratio had risen to 71:1. This increase in the ratio at 20 hr was due to a greater loss of radioactivity from the livers of embryos receiving the smaller inoculum. Since this lower inoculum, 1×10^5 cells, was about the mimimum required to obtain tumour growth it is probable that a much higher proportion of tumour cells were dying following injection, than with the larger inoculum, $2\cdot9\times10^6$ cells, which gave vigorous and consistent growth. It would appear that the size of the inoculum had little effect on the distribution, but that it was desirable to avoid using inocula close to the mimimum necessary for tumour growth.

Variations in distribution with age of the embryo

The enhanced tumour growth observed in younger embryos when equal numbers of tumour cells were injected into 7- and 11-day embryos could have been accounted for by the trapping of a proportionately larger number of cells in the smaller embryonic organs of the younger embryos. As shown in the results, however, the mean percentages of the total radioactivity trapped in the livers and lungs of the younger embryos was about 1/5th of that found for the older embryos, and since the weights of the younger embryonic organs were about 1/5th of those of the 11-day embryos the number of tumour cells/mg of tissue was almost identical for 8- and 11-day embryos. There is a smaller difference in the weights of the chorioallantoic membranes of 8- and 11-day embryos [2], and probably proportionately more tumour cells were trapped in the membranes of the younger embryos. The enhanced growth of the younger embryos cannot, therefore, be due to the trapping of proportionately more cells in the livers and lungs of the younger embryos. It may be significant, as discussed in the preceding paper, that serum γ-globulins do not appear in the embryonic circulation until day 14 [3], and the enhanced growth of tumour could be due to the absence of maternal antibody in the early embryo.

Strain-specific vs. non-strain-specific tumours

The comparison of the *initial* organ distribution of the strain specific L5178 tumour cells with that of the non-strain specific Landschütz cells revealed no striking differences (Table 1). The failure of the strain specific tumours to grow in the embryonic organs was not due, therefore, to failure of the organs to trap tumour cells. The growth of L5178 cells only in the brain could not be due to localization of a high proportion of these cells in that organ since it was one of the least effective organs for trapping tumour cells (Table 2).

Organ distribution of tumour cells in relation to subsequent growth

The organ distribution of Landschütz tumour

cells revealed that those organs which were the most efficient in trapping tumour cells, i.e. the liver, mesonephros and CAM, were those in which tumour growth was most extensive. This relationship between cell-trapping and subsequent tumour growth did not, however, hold for all organs. The spleen, which was second only to the liver in its efficiency in trapping tumour cells (Table 2), rarely contained detectable tumour cells three to four days after injection, even when the tumour was growing vigorously in other organs. There is some evidence which indicates that it may be possible to induce an immunological response in chick embryos by day 15 [4], and it is possible that a weak immune reponse may be capable of destroying tumour cells localized in the spleen but not on other organs. In addition, the chick embryo spleen possesses considerable RES activity as demonstrated by Kent [5] using carbon particles.

The vigorous growth of tumours in the brain, in spite of its low tumour-trapping efficiency, may be due to it being an immunologically protected site. The brain has been used successfully for the growth of tumours in immunologically competent, adult heterologous animals [6], and similar factors may operate in the chick embryo brain.

Differences in the organ distribution of different tumours

Surprisingly large differences were observed in the relative distributions of different tumour cells in some embryonic organs. When the quantities of tumour cells trapped/100 mg tissue in the livers and lungs of embryos injected with different ^{3}H-labelled tumours were compared (Table 2), the ratio liver/lung was found to vary from 64 for the Landschütz tumour down to 1 for the EL4 tumour. This means that when the Landschütz tumour was injected 64 tumour cells were trapped in a unit weight of liver for every single tumour cell trapped in the same weight of lung, whereas when the EL4 tumour was injected almost as many tumour cells were trapped in a unit wt. of lung as in the liver. These differences in the liver/lung ratios were paralleled by the relative amounts of tumour growth in these two organs. Thus, the Landschütz tumour grew very vigorously in the liver, but only small areas of tumour growth were occasionally seen in the lungs, even with large inocula. The EL4 tumour grew better in the lung than the liver, and extensive growth was frequently observed in the lungs of embryos whose livers contained no detectable viable tumour cells. Very similar results were obtained using YAC tumour which gave a liver/lung ratio of 2·4. The HEP 129 tumour with a ratio of 23 was intermediate between the Landschütz and EL4, and tumour growth was almost equal in the liver and lungs. In general, it would appear that in this system if the liver and lungs are seeded with equal numbers of tumour cells per unit weight of tissue the lung provides an environment more suited for tumour growth than the liver. The factors responsible for such large differences in the organ distribution of different tumours are probably very complex. There does not appear to be any simple correlation between the size of the tumour cells and the proportions trapped in the liver and lung (Table 2). Other cell properties, not yet investigated in this system, which may influence the number of cells trapped in various organs include the deformability of the tumour cells, their adhesiveness, and capacity to penetrate or injure the vascular endothelium.

The observation of quite large differences in organ distribution of different tumours in the chick embryo is perhaps surprising in view of recent work by Burdick [7], in which he studied the distribution of suspensions of cells of the chick embryo mesonephros, limb and heart ventricle following intravenous injection into 5-day chick embryos and found no tendency of the cells to home or migrate to the organ source. It is clearly impossible in the case where heterologous tumour cells are injected to consider specificity of lodgement in the sense used by Burdick. If differences in the tumour cell-organ adhesions are responsible for the observed differences in organ distribution, they must be considered fortuitous and their origin, at present, unknown.

Comparison of the amount of tumour growth in different organs of the chick embryo indicated that, in general, it was directly related to the density of seeding of the organs with tumour cells, which would support the mechanical circulatory theory of the sites of metastasis formation [8] which has received considerable support from the work of Coman and his colleagues [9, 10]. On the other hand, the relationship between the liver/lung trapping ratios of all the tumours examined and the relative amounts of tumour growth in these two organs, indicated that, in general, the chick embryo lung provides a much better environment for tumour growth than the liver. This would appear to support the 'soil' theory of Paget [11] which has received impressive supports from experiments such as those of Kinsey [12]. He observed that metastatic

lesions would form only in grafts of lung tissue, and not in grafts of other normal tissues, following the intravascular inoculation of S-91 mouse melanoma which forms metastases in the lungs in normal animals. In the chick embryo system, however, it would appear that the 'soil' factor can be dominated by the trapping factor when tumours such as the Landschütz ascites are injected. Thus, although certain organs of the chick embryo such as the lung, would appear to provide a better environment for tumour growth than other organs, such as the liver, the trapping factor may be so high that it determines the site of metastasis formation, e.g. the liver: lung trapping ratio of 64:1 in the case of the Landschütz tumour results in much greater tumour growth in the liver than in the lung.

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RESUME

On a étudié la distribution quantitative de cellules marquées au tritium, provenant de 5 *tumeurs différentes de la souris, après injection intraveineuse à des embryons de poulet.*

L'implantation de ces cellules dans les organes fut très rapide. La destruction des tumeurs non évolutives fut constatée par une réutilisation importante du radioisotope par les cellules de l'embryon de poulet. La croissance relativement faible des tumeurs spécifiques de lignée n'était due à aucune différence caractéristique de leur distribution dans les organes, ni à la vitesse à laquelle elles étaient fixées.

Pour chaque tumeur, l'importance de la croissance ultérieure, dans les différents organes, correspondait à la capacité de fixation de ces différents organes, à deux exceptions près. Le cerveau retenait peu de cellules tumorales, mais permettait cependant une croissance vigoureuse de la tumeur, vraisemblablement parce qu'il est situé à l'abri des mécanismes immunitaires. La rate retenait beaucoup de cellules, mais constituait un mauvais terrain pour les tumeurs, vraisemblablement à cause de l'abondance du tissu réticulo-endothélial. Pour une même densité d'ensemencement par les cellules tumorales, les poumons se montraient plus favorables à la croissance des tumeurs que le foie.

Plusieurs organes avaient des capacités de fixation très différentes pour les diverses tumeurs, et ces différences étaient en correlation avec la croissance ultérieure des tumeurs. Les différences de distribution ne pouvaient pas s'expliquer uniquement par des différences de taille des cellules. Des facteurs mécaniques et des facteurs spécifiques de la tumeur et de l'organe semblent être impliqués dans la formation et la localisation des métastases.

SUMMARY

The quantitative distribution of ^{3}H-labelled cells from 5 *different mouse tumours following intravenous injection into chick embryos has been investigated.*

The lodgement of tumour cells in the organs was very rapid. Death of non-growing tumours was reflected in the extensive reutilization of radioisotope by chick embryo cells. The relatively poor growth of strain specific tumours was not due to any characteristic differences in their organ distribution or the speed with which they were trapped.

For any single tumour the extent of subsequent growth within different organs corresponded well with the tumour trapping efficiency of the organs, with two exceptions. The brain trapped few tumour cells but supported vigorous tumour growth, possibly because it is an immunologically protected site. The spleen trapped many cells but was a poor site for tumour growth, possibly because of its high R.E.S activity. There were indications that with equal density of seeding oftumour cells the lung provided a much better site for tumour growth than the liver.

With different tumours there were large differences in the tumour trapping efficiencies of several organs, and these differences corresponded well with the extent of subsequent tumour growth. These differences in distribution could not be solely accounted for by differences in cell size. Both mechanical and organ- and tumour-specific factors appear to be involved in the site of formation of metastases in this system.

ZUSAMMENFASSUNG

Es wurde die Verteilung von ^3H-markierten Zellen von 5 verschiedenen Mäusetumoren nach intravenöser Injektion bei Hühnchenembryonen quantitativ untersucht.

Die Ansiedlung der Tumorzellen in den Organen erfolgte rasch. Der Tod nicht wachsender Tumoren spiegelte sich in dem Einbau von Radioisotopen in den Zellen des Hühnchenembryos wieder. Das verhältnismäßig schwache Wachstum von artspezifischen Tumoren war nicht ausschlaggebend für irgendeinen charakteristischen Unterschied in der Organverteilung oder der Geschwindigkeit, mit welcher sie zertört wurden.

Das Ausmaß des späteren Wachstums jedes einzelnen Tumors innerhalb der verschiedenen Organe entsprach deren Leistung beim Tumoreinfangen, mit 2 Ausnahmen: das Gehirn enthielt weniger Tumorzellen, unterstützte aber kräftig das Tumorwachstum, weil es möglicherweise eine immunologisch indifferente Region darstellt. Viele Zellen wurden in der Milz angesammelt, sie war jedoch eine schwache Basis für das Tumorwachstum; möglicherweise beruht dies auf ihrer hohen RES-Aktivität. Es waren Anzeichen vorhanden daß bei gleicher Dichte der Ansiedlung der Tumorzellen die Lunge einen viel besseren Nährboden für das Tumorwachstum als die Leber darstellt.

Bei verschiedenen Tumoren wurden große Unterschiede in der Sammlungsfähigkeit durch verschiedene Organe festgestellt, und diese Unterschiede entsprachen dem Ausmaß des späteren Tumorwachstums. Die Differenzen der Verteilung konnten nicht allein durch die Unterschiede der Zellgrößen bedingt sein. Sowohl mechanische als organ- und tumorspezifische Faktoren erscheinen bei der Metastasenbildung in diesem System beteiligt.

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Rous Sarcomas in Mice: The Chromosomal Progression in Primary Tumours

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INTRODUCTION

MOST primary tumours, animal as well as human, have been studied chromosomally at a more or less arbitrary time of their growth period. The chain of chromosomal events before and after the karyological analysis remains either completely obscure or in some cases only theoretically deducible [1–11].

The present work is focused on the chromosomal progression in Rous sarcomas in mice. In a previous report [5], a detailed description of the chromosomal spectrum was given on the basis of the findings in 91 different Rous mouse sarcomas; about 40% of the tumours had a normal diploid karyotype, the main heteroploid groups being hyperdiploid and tetraploid. This pattern is compatible with the conclusion that all the sarcomas were initially diploid, and that this state persisted for a variable period of time but ultimately changed into heteroploidy by the overgrowth of more viable variant cells. The aim of the present investigation is to test this hypothesis and to elucidate as far as possible the evolutionary steps and pathways in the *in vivo* heteroploid transformation. A related question, also studied, is the chromosomal progression in primary sarcomas with an already established heteroploid stemline.

MATERIAL AND METHODS

All animals belonged to the inbred strain CBA. As described in detail elsewhere [5, 10] newborn mice were inoculated with a finely minced suspension of Rous chicken sarcoma (strain RSV-SR). Eleven sarcomas located in one of the hindlegs or subcutaneously in the back, were partly excised, only leaving a small peripheral brim of tumour tissue. To avoid a too rapid recurrency, about 90% of the sarcoma was as a rule extirpated each time. The excised specimen was used for several purposes; direct fixation (D), explantation into primary culture for later fixation (Pc), histopathological examination; often also for *in vivo* transplantation and sometimes for storage by freezing. The methods used for D and Pc were described earlier [5, 10].

Successful fixations were obtained from 11 of 19 tumours operated upon and Table 1 is a survey of data other than chromosomal from the 11 tumours. They are numbered as in the previous report [5]. Roman numerals indicate the operation sample, the highest numeral referring to the final recurrency. Histopathologically, the tumours fall into 5 groups [6]. These are with decreasing degree of maturity: well and less differentiated fibrosarcomas (F_1 and F_2, respectively), well and less differentiated spindle sarcomas (Sp_1 and Sp_2, respectively), anaplastic sarcomas (A).

TERMINOLOGY

As in previous reports [5–6, 10–11], *S* is a symbol for the stemline, *i.e.* the most frequent karyotype of the tumour cell population, and *s* (s_1, s_2, etc) a symbol for the sideline(s), *i.e.* other karyotypes of less frequent occurrence than the stemline but present in a frequency of 10% or more. Different morphological types of markers are indicated by the following abbreviations:

- T_1 Telocentric marker longer than the longest normal pair (No. 1).
- T_{min} Telocentric marker similar in size to or shorter than the shortest normal chromosomes (pair No 20 and the Y chromosome=No. 21).
- *M* Biarmed marker with arms of equal size.
- *m-sm* Biarmed marker with an arm index between 1 and 3.

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Table 1. *Non-chromosomal data for the* 11 *operated primary tumours*

Tumour No.	Sample I–V	Sex	Latent period (days)	Growth period before fixation (days)	Longest tumour dia. (mm)	Histological type
3	I			128	15	Sp1
	II	M	335	153	21	Sp1
	III			171	27	Sp1
5	I	F	567	53	14	Sp2
	II			73	20	Sp2
6	I	M	723	45	25	Sp2
	II			81	21	A
16	I			41	22	F2
	II			62	16	F2
	III	F	315	78	19	Sp1
	IV			95	22	Sp1
	V			108	31	Sp1
17	I			31	10	Sp1
	II	M	281	74	16	Sp2
	III			123	25	Sp2
33	I	M	633	19	12	Sp2
	II			44	20	Sp2
36	I	F	429	16	18	F2
	II			38	21	A
38	I	F	545	68	17	Sp1
	II			108	28	Sp1
57	I			22	12	F2
	II	M	523	42	16	F2
	III			62	20	Sp1
75	I	M	66	42	20	A
	II			67	22	A
79	I	M	720	45	18	Sp1
	II			99	20	Sp1

OBSERVATIONS

1. *Hypodiploid sarcomas*

Three tumours, Nos. 3, 5, and 6, belonged to this group (Table 2). They will be treated individually.

Sarcoma No. 3. All three samples of this male sarcoma showed a hypodiploid stemline with $S=38$ (Fig. 1a) and with no sidelines. Despite the loss of 2 chromosomes and no discernible structural deviation, the S showed considerable stability. This tumour is unique in the present material by its lack of a progressional trend. There were only single cells in the hypotetraploid region, which never developed into a sideline. Such an evolution would have furnished material for genic variation compensating the chromosomal losses in the stemline.

Sarcoma No. 5. In sample I of this female sarcoma there was a hypodiploid stemline with $S=39$ (Fig. 1b) and a minor hypotetraploid sideline with $s=76$ (Fig. 1c). In the hypodiploid region there was a considerable fraction of cells with only 38 chromosomes from which the s was most probably derived. In sample II, the stemline number changed from 39 to 38 (Fig. 1d). The asymmetry of the distribution was reversed in comparison with sample I, with the persistence of a large fraction of cells with 39 chromosomes. The original hypotetraploid s was still present but with too few cells to be termed an s.

A further analysis of the readjustments in the hypodiploid region was prevented by the absence in both samples of markers or involvements of identifiable chromosomes.

Sarcoma No. 6. The chromosomal findings in both samples of this male tumour were discussed in detail in a previous report [10]. The main interest at that time was directed to the occurrence of a rare chromosomal aberration, termed double-minutes. It will suffice to mention here that in sample II the s of sample I had taken over the stemline corresponding to a numerical change of S from 39 to 39–40, and a change in the marker set from one T_1, one T_{min}, and one M to just one T_1, and one T_{min}. Doubling-products of the new S (sample II) formed the most prominent sideline,

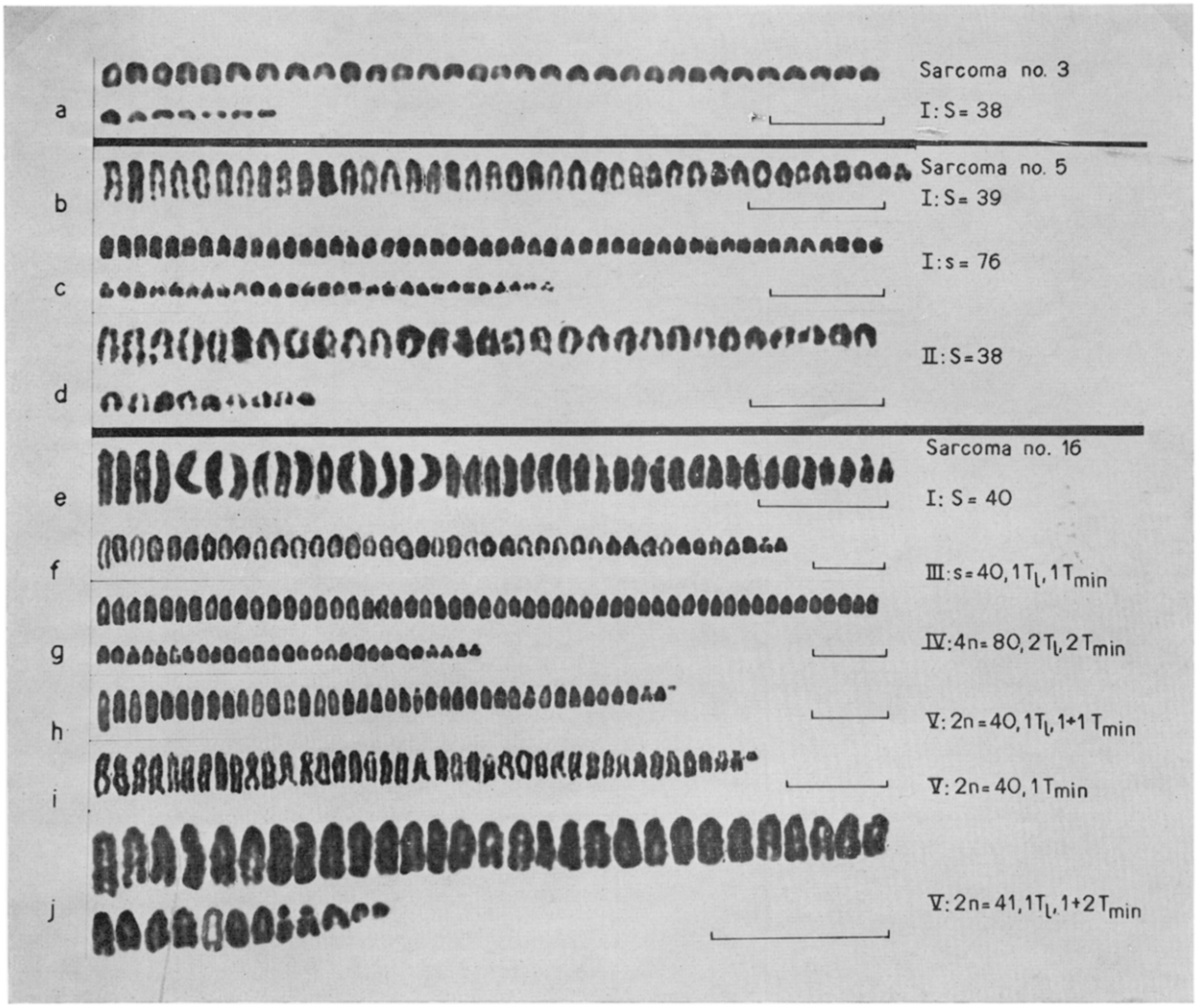

Fig. 1. *Karyotypes illustrating the chromosomal progression in the primary sarcomas Nos.* 3, 5 *and* 16. *The line below each karyotype* = 10μ.

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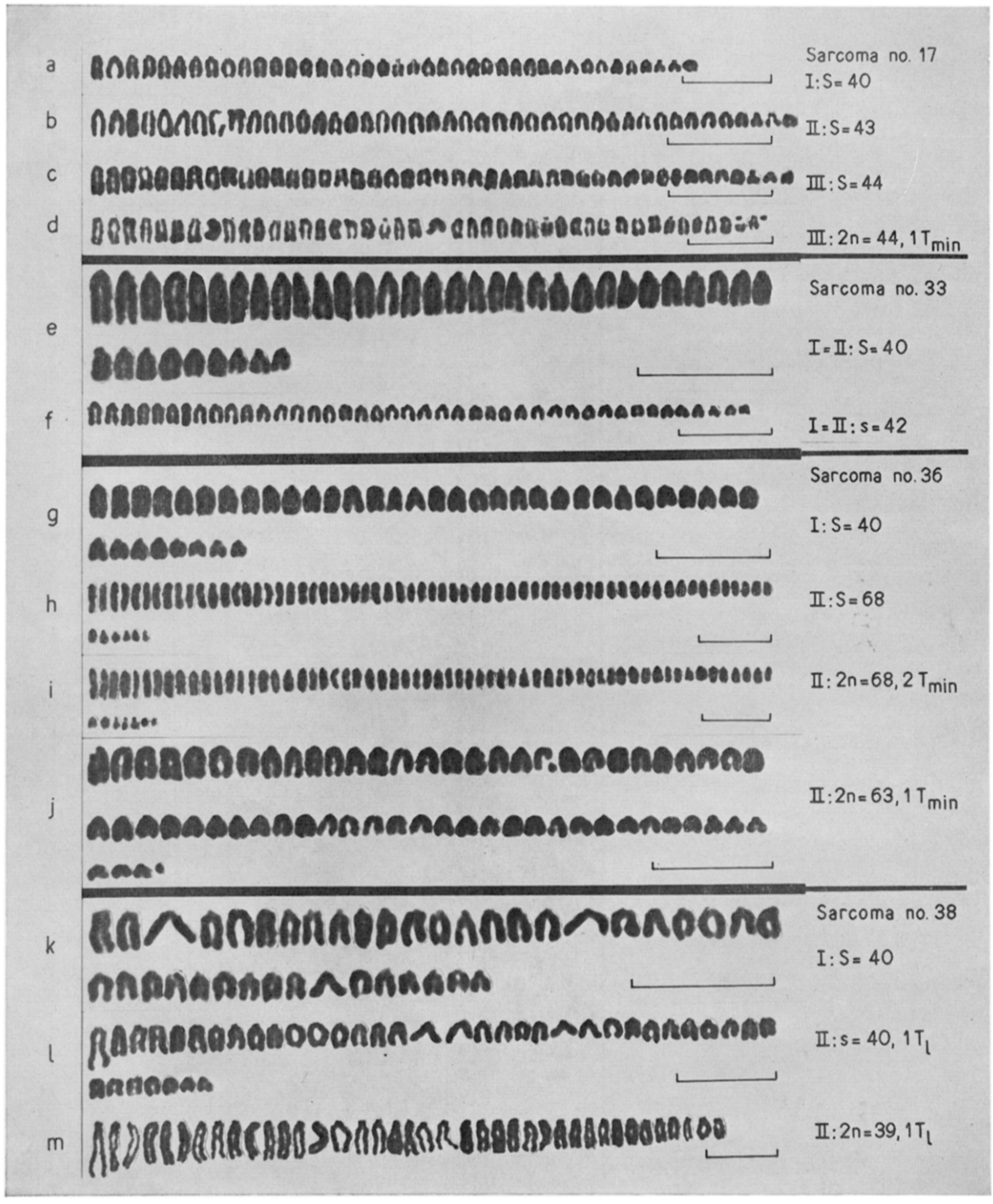

Fig. 2. Karytopyes illustrating the progression in the primary sarcomas Nos. 17, 33, 36 *and* 38.

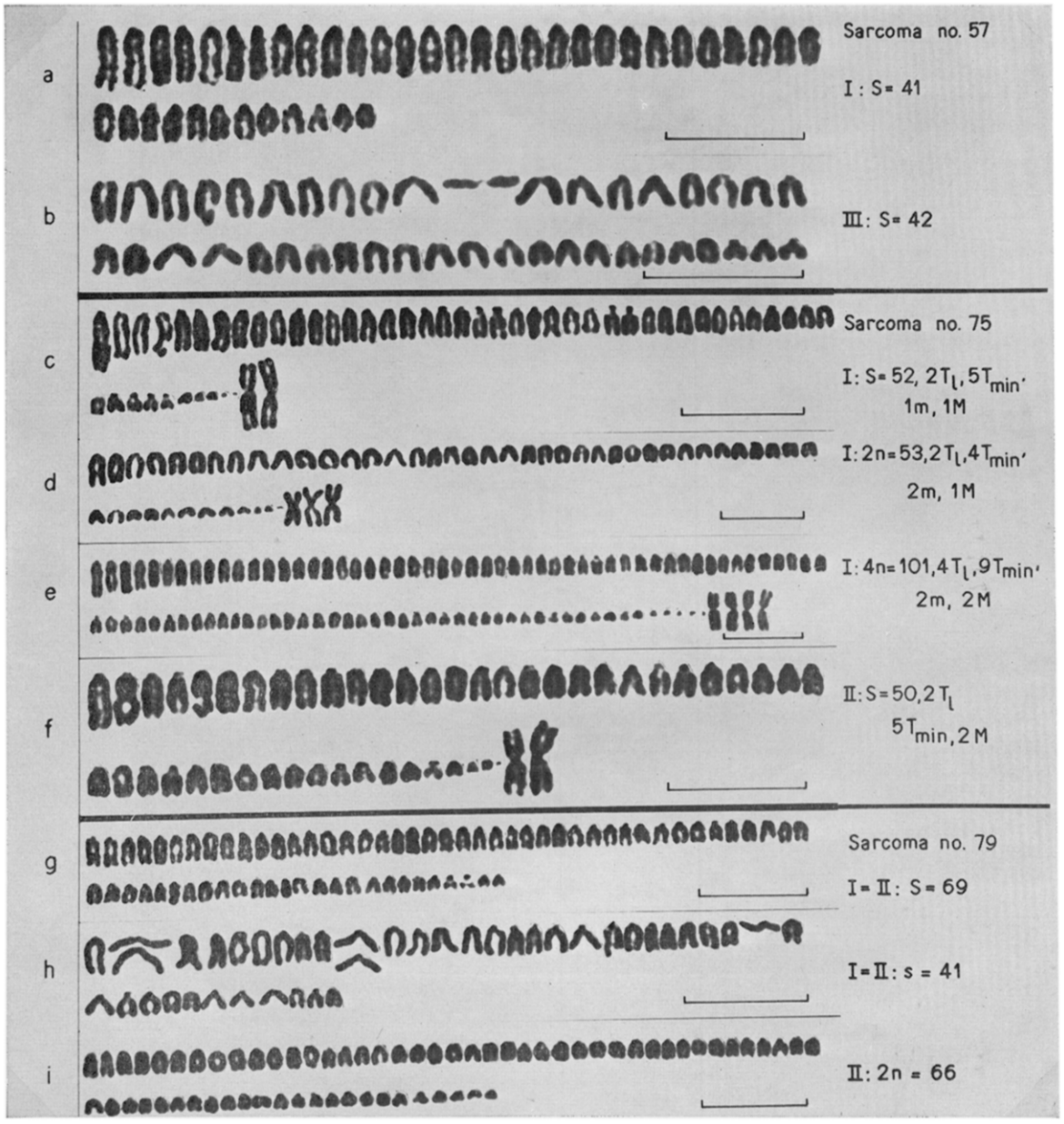

Fig. 3. Karyotypes illustrating the progression in the primary sarcomas Nos. 57, 75 and 79.

s_1=80 with two T_1s and two T_{min}s, but the original hypodiploid *S* (sample I) was still present in sample II as a minor second sideline, s_2.

The progression is thought to have proceeded as follows: The markers T_1 and the T_{min}, and perhaps also the double-minutes, may be signs of a period of breakage prior to sample I. During the subsequent adaptation, a stemline with an added *M* developed and became temporarily superior. This stemline was again out-grown by derivatives of the population readjusted with just the two telocentric markers (sample II). Experiences from *in vivo* transplantations of primary Rous sarcomas in mice (unpublished) have repeatedly indicated the occurrence of similar processes, *i.e.* an appearance of a temporary new near-diploid or polyploid stemline followed sooner or later by the return of the original stemline or derivatives of it.

2. *Diploid sarcomas*

Five tumours, Nos. 16, 17, 33, 36, and 38 (Table 2) belonged to this group.

Sarcoma No. 16. In sample I, this female tumour showed a normal diploid stemline (Fig. 1e) without any sidelines. The fixation of sample II was unsuccessful, but the progression could be studied in specimens from 3 further recurrencies. Thus, in samples III and IV, the normal diploid S persisted unchanged but its dominance was successively reduced due to the appearance of a pseudodiploid *s*. The karyotype of this sideline, similar in both samples is shown in Fig. 1f. About 12% of the tumour cells in sample III belonged to this s against 33% in sample IV. Finally, in sample V, the former pseudodiploid *s* had taken over the stemline and the original normal diploid *S* now constituted an *s* corresponding to about 25% of the cells in mitosis. Since no structurally changed variant cells had been observed in sample I, it may be concluded that the pseudodiploid *s* and *S* were later products of the progression.

The pseudodiploid karyotype was characterized by the presence of one T_1 and one T_{min} and the absence of one member of the smallest pair, No. 20 (Fig. 1f). As a rule the T_1 was easily distinguishable, but its size was not constant, and it was sometimes only a little longer than the longest normal pair, No. 1 (Fig. 1g, h). The T_{min} had a size similar to pair 20 but could be distinguished due to the lack of the prominent proximal secondary constriction characteristic of this pair in CBA mice (Fig. 1e, f). The considerable variability of the karyotype of this tumour was reflected by the occurrence in several cells with only one T_1, one T_{min} (Fig. 1i), one or two additional and smaller T_{min}s (Fig. 1h–j). Several cells had two chromosomes No. 20 (Fig. 1i, j) in addition to the marker set and, in some cases, with the probable disappearance of one chromosome No. 19 (Fig. 1i). The frequency of the variant cells derived from the pseudodiploid *s* or *S* was 5, 12 and 18%, respectively, in samples III, IV and V. The rising frequency of these variant cells may be due to a marked instability of the developed pseudodiploid side-stemline. The frequent variability of chromosome No. 20 also points to the common incidence of mitotic disturbances of the non-disjunctional type.

Sarcoma No. 17. This male tumour also started out with a normal diploid stemline (Fig. 2a) without any sidelines. In sample II a profound change in the chromosomal picture became apparent, the mode now being in the hyperdiploid region with *S*=43 (Fig. 2b) and with the original normal diploid *S* not even discernible as an *s*. The next sample, No. III, had essentially the same picture, with the mode in the hyperdiploid region, however, now moved to the higher level of *S*=44 (Fig. 2c). The stemline of sample III was apparently derived from that of sample II; the close numerical relation between them and the persistence in sample III of a considerable fraction of cells with 43 chromosomes are compatible with this assumption. The presence of single hyperdiploid cells in sample I, probably the result of non-disjunctional variation, foreshadowed the later appearance of the 43–44 chromosome stemline. It seems that a superior competitive capacity has been reached at the *S*=43–44 level without any intervening 41–42-stem- or sidelines, thus an instance of discontinuous stemline shift during progression.

A T_{min} (Fig. 2d) was found in only a few cells of sample II and III. As a rule, pair No. 20 and the Y chromosome were present in normal numbers in the hyperdiploid cells (Fig. 2a–d) and the extra chromosomes were thus among the unidentifiable chromosomes.

Sarcoma No. 33. The two samples of this male tumour showed a similar chromosomal picture, a normal diploid stemline (Fig. 2e) and a hyperdiploid sideline (Fig. 2f). In sample II, however, the prominence of the *s* was increased, a small but definite change pointing towards a stemline shift, approaching slowly. The progression is of the same kind as that of sample II of sarcoma No. 17, although proceeding more slowly. Even among tumours

with apparently normal diploid stemline, it should be expected that the stemline shift may take place with different rapidity. This is an indication that the individual tumours are dissimilar at the submicroscopical level due to mutations or cryptostructural deviations. Pseudodiploidy could also play a significant part without being detected in karyotypes such as the present.

Sarcoma No. 36. In sample I of this female tumour a normal diploid stemline was found (Fig. 2g) and, in addition, a hypertriploid sideline with $s=68$ (Fig. 2h). No markers were noticed in the *s*. In sample II, the original normal *S* had disappeared completely, and the hypertriploid *s* had taken over the stemline. The abruptness of this stemline shift is quite different from the slow and gradual change in the previous tumour, sarcoma No. 33, and also to some measure in sarcoma No. 17.

Although the *S*-number of sample II agreed with the *s*-number of sample I, the numerical distribution was different with many counts below 68 (Fig. 2j). The asymmetrical distribution probably indicates an early phase of an 'Ising' effect, the well-known adaptive reaction in advanced polyploid mouse tumours by which a gradual shift towards lower chromosome numbers takes place [12, 13]. Another difference, in relation to sample I, was the occurrence of one or two $T_{min}s$ in about 20% of the cells in sample II (Fig. 2i, j). Since no mode could be found, no *s* could be defined on this basis. Two cells with one T_{min} were recorded among the hypertriploid metaphases of sample I, a frequency roughly corresponding to 8% cells with markers in the triploid population of sample I. Also, the increased structural variability on the polyploid level of the present primary sarcoma is in accordance with experiences from advanced polyploid mouse tumours [12, 13].

Sarcoma No. 38. In sample I of this female tumour, a normal diploid stemline was found (Fig. 2k) and, in addition, a minor hypotetraploid sideline with $s=76$ without markers. In sample II, the stemline was unchanged but the hypotetraploid sideline was replaced by a pseudodiploid *s*. The latter sideline and some variant cells, adding up to about 24% of the population of sample II, showed a T_1 with a vague secondary constriction in the middle part (Fig. 2l, m). Two of the diploid cells of sample I had a similar T_1. Despite its scarcity, the characteristic marker indicates that the pseudodiploid *s* of sample II was derived from these variant cells of sample I. The disappearance of the hypotetraploid *s* of sample I is an unusual development and the reasons remain obscure.

3. *Hyperdiploid sarcoma*

Sarcoma No. 57. In sample I this male tumour showed a hyperdiploid stemline with $S=41$ (Fig. 3a). In the next successful sample, No. III, the *S* was 42 (Fig. 3b); as in sample I, there were no sidelines and the stemline showed no markers or identifiable extra chromosomes. In both samples, the distribution was asymmetrical with a considerable fraction of cells in the *S*-1 class. In the case of sample I, this is thought to illustrate the persistence of a fraction of the original normal diploid stemline, in the case of sample III the persistence of the stemline of sample I. In contrast to sarcomas Nos. 17 and 33 the hyperdiploid progression here seems to be a continuous process.

4. *Near-triploid sarcomas*

Sarcoma No. 75. This exceptional male tumour showed a hypotriploid stemline in sample I with $S=52$ (Fig. 3c) with no sidelines. In sample II, the *S*-number was reduced to 50 (Fig. 3f), thus being on the border line between hyperdiploidy and hypotriploidy. As in sample I, no sidelines were found. In comparison with the numerical distribution in sample I, there was in sample II a slight decrease in the spread around the *S*-number and a pronounced decrease of the doubling-products and their derivatives (Fig. 3e). The latter trend is reminiscent of that found in sample II of sarcoma No. 38.

The *S* of sample I had on an average 8–9 markers (Fig. 3c, d) covering a wide spectrum of diverse telocentric and biarmed markers. In sample II, there were minor changes but the basic composition of this marker spectrum was retained (Fig. 3f). The loss or gain of the different marker types in sample II indicates a liability of newformed chromosomes, observable as markers, to be involved in mitotic irregularities and also to be involved in further structural remodelling. According to this view, the mosaicism recognizable as a variation in the marker set may depend on the continuous generation of variant cells, rather than the evolution and persistence of many clones. The evolution proposed is supported by the high degree of resemblance, structurally, between the individual cells in both samples. Thus, the great spread in both samples, numerically, is not unexpected, and is probably accentuated by the stemline being located in the triploid region. The negative scewness in

the distribution of sample I is probably not artificially produced by losses during squashing. It seems more plausible that it represents early stages of the "Ising" effect. This interpretation corresponds with the lowering of the stemline number in sample II.

Sarcoma No. 79. Both samples of this male tumour showed a hypertriploid stemline with $S=69$ (Fig. 3g), and a hyperdiploid sideline with $s=41$ (Fig. 3h). Neither the stemlines nor the sidelines had markers. The only noticeable change from sample I to sample II was a slight but definite increase in the counts below $S=69$ (Fig. 3i). As suggested in the case of sample I of the previous sarcoma, No. 75, this asymmetry of the numerical distribution could be an early indication of an 'Ising' effect.

Whether the stemline of sarcoma No. 79, as well as the only other hypertriploid distribution in this material, *viz.* the sarcoma No. 36, are originally triploids or have been derived from the tetraploid level of variation could not be determined. The 'Ising' effect is the progressional trend to be expected in near-tetraploid tumours, a category not represented in the present study.

5. *Progression and frequency of normal diploid cells*

The frequency of normal diploid cells during aging of the sarcomas is illustrated in Fig. 4. With few exceptions the trend is a fall in this frequency. The tumours with a normal diploid stemline in sample I showed great differences in the rate of decrease. This finding corresponds well with the diversity in progressional pathways and demonstrate the competitive value of the different heteroploid stem-sidelines in comparison with their original, apparently normal, diploid *S*. The slight increase in the normal fraction, found in sarcoma No. 38, could be an incidental finding, due to an unrevealed pseudodiploidy or reflect the disappearance of less competitive variant cells and sidelines.

The picture of the heteroploid transformation is that of a non-linear process. Sarcoma No. 16 figures a slow initiation, but also indicates a slow end. The last trend seems to be a common feature according to the findings in the sarcomas with a heteroploid stemline, already established. Counts of normal, stromal or inflammatory cells might sometimes blur the picture, however, the general trend is hardly influenced by this. The persistence, late in the growth phase, of a small fraction of the normal, diploid stemline is interesting as a potential source for the origin of new heteroploid stemlines. In heteroploid tumours the persistence of remnants of an early stemline after further reshuffling is a parallel phenomenon.

6. *Progression in relation to growth rate and histopathological type*

The cumulative curves of Fig. 5 show the changes in the growth rate in the 11 sarcomas. As expected, the general trend is a growth rate increasing with age. Consideration of the chromosomal data presented in previous sections shows that there is no clear and constant relation between chromosomal progression and changes in growth rate. In sarcoma No. 3, for example, there is an obvious increase in the growth rate despite a complete lack of discernible progressional steps, and in sarcoma No. 36 the growth rate seemed unchanged despite a change in stemline from a normal diploid one to a triploid *S*. It can not be denied that, as a rule, stemline shifts as well as minor fluctuations in the composition of the population must be due to differences in the competitive capacity of different genotypes of the *S* and *s*. The visible differences are small or

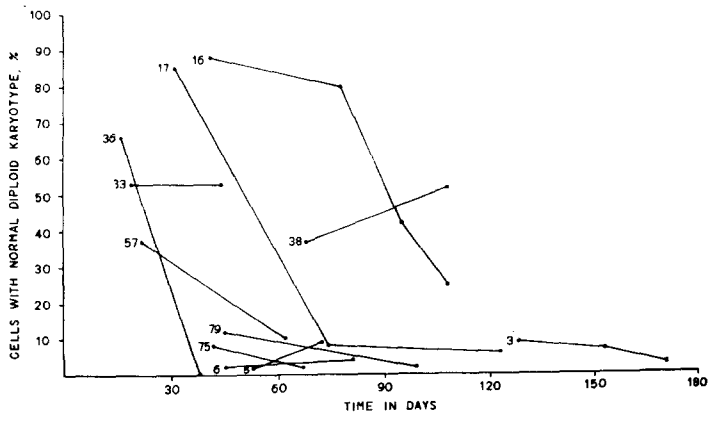

Fig. 4. *The relation between the frequency of cells with a normal, diploid karyotype and the age of the* 11 *primary sarcomas.*

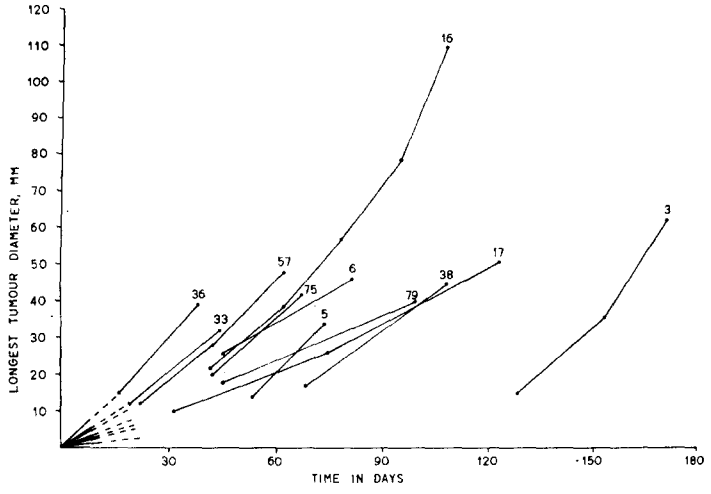

Fig. 5. *The growth rate in the* 11 *primary sarcomas during aging. The longest diameter* (*Table* 1) *of the original tumour and the recurrency*(*ies*) *is used as an index of the size at the time of the operation. A cumulative curve is formed for each sarcoma by adding the diameter of sample II to that of sample I, etc.*

lacking in many cases, due perhaps to the need of further adaptation of the genic material before optimal viability is achieved. Sarcoma No. 3, with its lack of visible progressional trends could be such an example. The operations and the age of the animal, as well as other host factors, are further causes, which also might have influenced on the results, though the extent could not be determined in the present experiments.

In all cases with a shift of the *S* from one karyotypic category to another, there was a change in the histological picture (Tables 1 and 2: sarcomas Nos. 16, 17 and 36). In sarcoma No. 36 with its shift from a normal diploid stemline to a triploid, the change was discontinuous (F2–A); in the other tumours the histological picture was changed to the nearest, less differentiated type, *i.e.* F2 to Sp1, Sp2 to A, etc. Modifications of heteroploid stemlines without a change of the S-category, as well as the appearance of new sidelines, were accompanied only occasionally by a continuous change in the histological picture (sarcomas Nos. 6 and 57). Although the correlation between chromosomal progression and histopathological picture is by no means an absolute one and is sometimes inappreciable in tumours undergoing only minor chromosomal changes, it is felt that the histological picture is a parameter more sensitive and more closely related to chromosomal progression than the growth rate.

CONCLUSIONS AND DISCUSSION

The results presented above, demonstrate that, as a rule, chromosomal evolutionary forces are operating very actively in primary Rous sarcomas in mice. The findings in the present material do not indicate initial or very early numerical and/or structural chromosomal changes [14]. The normal diploid stemlines seem to possess the latent capacity of heteroploid transformation along a diversity of pathways at different times, and often comparatively late, in tumour development. The results, illustrating the steps in this transformation, can be summarized as follows:

1. The formation of a few variant cell types: deviating numerically and/or structurally from the normal, diploid *S* (sarcomas Nos. 17 and 38).

2. The development of a heteroploid *s*: sometimes traceable to a certain fraction of variant cells (sarcomas Nos. 16, 17 and 38).

3. The heteroploid sideline takes over the stemline (sarcomas Nos. 16, 17 and 38).

4. Modifications of the heteroploid *S* (but without a departure from the originally established stemline category, *i.e.* the progressional pathway chosen by a particular tumour). The four possible pathways are considered separately (a–d).

a. Hypodiploid stemlines. They showed no similarities in the progression. Thus, in one case (sarcoma No. 3) there were no discernible changes, in another case the hypodiploid character was accentuated by a lowering of the *S*-number (sarcoma No. 5), and in a third case a sideline, numerically and structurally related to the *S* took over the stemline (sarcoma No. 6).

b. Pseudodiploid stemlines. The progressively increasing numerical and structural pleomorphism in sarcoma No. 16 could be an indication of a probable further reshuffling of the genic material before departure from diploidy.

c. Hyperdiploid stemlines. In both cases analyzed (sarcomas Nos. 17 and 57) the hyper-

diploid character was accentuated by a further elevation of the *S*-number.

d. Polyploid stemlines. A more or less marked 'Ising' effect was seen in both the hypo- and hypertriploid region (sarcomas Nos. 36, 75 and 79). No tumour with tetraploid *S* was included in the material, but the trend in the triploid region is a phenomenon often seen in transplanted tetraploid mouse tumours [12, 13].

Step 1–3 was initiated or completed in all sarcomas studied. Step 4, covering the whole spectrum of possible heteroploid pathways, is probably not completed in most tumours. In some of the sarcomas this step might proceed indefinitely by a continuous reshufflling of the genic material without a change of the karyotypic stemline category, whereas in others it might be possible to define a fifth step due to the occurrence of such a shift in the *S*-group.

The diversity in progressional pathways, as revealed by the present investigation, is in line with the broad variation found within the chromosomal spectrum of primary Rous sarcomas in mice. The variation and the progressional pathways chosen are phenomena related to, and to a great extent probably dependent on, the nature of the carcinogen. Other mouse tumours such as mammary carcinomas, the lymphomas-leukemias, and probably some of the hepatomas (for review see reference 11), differ from the present tumours by their tendency to retain diploid-near-diploid stemlines even after a comparatively long time of transplantation.

The observations of the chromosomal spectrum of primary Rous sarcomas in mice and of their progression indicate a specific evolution for each individual sarcoma; in this respect the picture is similar to that found in sarcomas induced by polymer implants in mice [14]. If so, the chromosomal changes during early stages of the neoplastic conversion would be unspecific; either due to extensive and widely distributed mutational or cryptostructural changes; or due to the involvement of general functions, such as disturbances of the normal mitotic function. In an investigation of primary Rous sarcomas in the Chinese hamster, however, Kato [3] found a more frequent involvement of certain chromosome pairs. However, in mouse tumours, the uniform complement prevents this kind of analysis.

In the present context it is of interest to mention that a specific and fairly constant chromosomal change was noted in thyroid cells in rats maintained on an iodine-deficient diet [15, 16]. The frequency of the aneuploid cells with 41 or 43 chromosomes ($2n=42$) increased with time; 90–100% of the metaphases with only 41 chromosomes had lost one member of pair 15. This specific loss persisted in well established tumours developed after transplantation of the precancerous cells but in many cases there were, in addition, further numerical and structural changes, different for each tumour.

In human primary tumours normal diploid stemlines are rare, except in leukemias; the heteroploid *S*-numbers, though often clustered in the near-triploid region, cover a broad range. The information about the extent of superimposed chromosomal changes, influencing this picture are scanty. On the basis of the distribution of markers in the tumour cell population a clonal origin has been suggested in human tumours, and the possibilities of superimposed coincidental changes, hiding early, specific rearrangements, has been discussed [3]. However, systematic studies of the chromosomal progression in primary human neoplasms are still scanty [2, 4, 7–9, 17]. In comparison between non-invasive and invasive tumours of the bladder it was found that the former group had chromosome numbers predominantly in the diploid region while the latter group had chromosome numbers ranging from 40 to more than 100 without any preferred level of ploidy (Lamb cited by Hughes in [2]). Other studies with a bearing on this problem are those dealing with cervical neoplasia, dysplasia, carcinoma *in situ* and invasive carcinoma [7–9, 17]. The frequent occurrence of heteroploid stemlines in early stages of this tumour type makes it probable that extensive changes have often taken place before the appearance of the invasive carcinoma. A less pronounced but similar picture has been found in the chromosomal studies of chorionic lesions [4].

The results already obtained are of essential importance for the evaluation of chromosomal findings in other classes of primary human tumours. They also underline the need for studies of the chromosomal progression in different categories of neoplasms, especially regarding their early progression. In comparison to Rous mouse sarcomas the human tumours, referred to above, seem to show an earlier heteroploid transformation, often already discernible in premalignant, non-invasive lesions.

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RESUME

La progression chromosomique a été étudiée dans 11 *sarcomes de Rous primitifs de la souris, analysés à des stades successifs de leur développement. Cinq sarcomes, qui avaient à l'origine une lignée diploïde normale, ont constitué le matériel qui a permis l'étude des étapes vers la transplantation hétéroploïde. La nouvelle lignée pouvait souvent être rattachée à une lignée collatérale hétéroploïde, et, dans certains cas, il était possible de retrouver l'origine de cette lignée collatérale dans une petite fraction de cellules différant de la lignée principale.*

Les sarcomes qui avaient, dès le premier examen, une lignée hétéroploïde, présentaient souvent des réarrangements chromosomiques supplémentaires, mais sans qu'il y eût modification de la catégorie de la lignée principale originelle. Ils conservaient donc la direction de la progression choisie par la tumeur. Alors que les étapes de la progression avaient un caractère général, la route suivie et la vitesse de la progression étaient propres à chaque tumeur.

Il n'y avait pas de corrélation nette entre les changements de la vitesse de croissance et la progression chromosomique. L'aspect histopathologique, en revanche, montrait une corrélation avec les principales modifications de la progression.

Le tableau chromosomique plein de dynamisme qui caractérise le sarcome de Rous est comparé aux observations faites dans d'autres tumeurs expérimentales chez l'animal, et dans les cancers chez l'homme.

SUMMARY

The chromosomal progression was studied in 11 *primary Rous sarcomas in mice, analyzed at successive stages of their development. Five sarcomas, originally having a normal diploid stemline, furnished material for studies of the steps in heteroploid transformation. The new stemline could often be traced to a preceding heteroploid sideline, and it was possible in some cases to trace the origin of this sideline to a small fraction of variant cells.*

Sarcomas with a heteroploid stemline, established at the time of the first analysis, often underwent further chromosomal rearrangements but without changing the original stemline category, i.e. *the progressional pathway chosen by the tumour. Whereas the progressional steps were of general character, the route and rate of progression were individual for each tumour.*

No clear-cut correlation was found between changes in growth rate and chromosomal progression; the histopathological picture, however, was a parameter showing correlation to the major progressional changes.

The highly dynamic chromosomal picture found in Rous sarcomas is discussed in relation to tumours in other animal model systems and to human neoplasms.

ZUSAMMENFASSUNG

Bei 11 *primären Rous-Sarkomen in Mäusen wurde die chromosomale Progression verfolgt und in den folgenden Entwicklungsstadien analysiert. Fünf Sarkome mit ursprünglich normaler diploider Stammlinie lieferten Material zum Studium der einzelnen Schritte, die zur heteroploiden Transformation führten. Die neue Stammlinie konte oft auf eine vorhergehende heteroploide Seitenlinie zurückgeführt werden, und in einigen Fällen war es möglich, den Ursprung dieser Seitenlinie in einer kleinen Fraktion verschiedener Zellen zu finden. Sarkome mit einer, zum Zeitpunkt der ersten Analyse, heteroploiden Stammlinie durchliefen oft chromosomale Umordnungen jedoch ohne die ursprüngliche Stammlinienkategorie zu ändern, i.e. der Tumor behielt den einmal eingeschlagenen Entwicklungsweg bei. Während die einzelnen Stufen der Progression generellen Charakters waren, blieben Weg und Rate individuell für jeden Tumor.*

Kein klaren Zusammenhang zwischen Wachstumsrate und chromosomaler Progression wurde gefunden. Der Parameter des histopathologischen Bildes blieb jedoch in Zusammenhang mit den fortschreitenden Veränderungen.

Das sehr dynamische chromosomale Bild, das in Rous-Sarkomen gefunden wurde, wird in Verbindung mit Tumoren in anderen Tiermodellsystemen und in menschlichen Neoplasmen diskutiert.

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Rous Sarcomas in Mice: The Chromosomal Progression in Primary Tumours

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INTRODUCTION

MOST primary tumours, animal as well as human, have been studied chromosomally at a more or less arbitrary time of their growth period. The chain of chromosomal events before and after the karyological analysis remains either completely obscure or in some cases only theoretically deducible [1–11].

The present work is focused on the chromosomal progression in Rous sarcomas in mice. In a previous report [5], a detailed description of the chromosomal spectrum was given on the basis of the findings in 91 different Rous mouse sarcomas; about 40% of the tumours had a normal diploid karyotype, the main heteroploid groups being hyperdiploid and tetraploid. This pattern is compatible with the conclusion that all the sarcomas were initially diploid, and that this state persisted for a variable period of time but ultimately changed into heteroploidy by the overgrowth of more viable variant cells. The aim of the present investigation is to test this hypothesis and to elucidate as far as possible the evolutionary steps and pathways in the *in vivo* heteroploid transformation. A related question, also studied, is the chromosomal progression in primary sarcomas with an already established heteroploid stemline.

MATERIAL AND METHODS

All animals belonged to the inbred strain CBA. As described in detail elsewhere [5, 10] newborn mice were inoculated with a finely minced suspension of Rous chicken sarcoma (strain RSV-SR). Eleven sarcomas located in one of the hindlegs or subcutaneously in the back, were partly excised, only leaving a small peripheral brim of tumour tissue. To avoid a too rapid recurrency, about 90% of the sarcoma was as a rule extirpated each time. The excised specimen was used for several purposes; direct fixation (D), explantation into primary culture for later fixation (Pc), histopathological examination; often also for *in vivo* transplantation and sometimes for storage by freezing. The methods used for D and Pc were described earlier [5, 10].

Successful fixations were obtained from 11 of 19 tumours operated upon and Table 1 is a survey of data other than chromosomal from the 11 tumours. They are numbered as in the previous report [5]. Roman numerals indicate the operation sample, the highest numeral referring to the final recurrency. Histopathologically, the tumours fall into 5 groups [6]. These are with decreasing degree of maturity: well and less differentiated fibrosarcomas (F_1 and F_2, respectively), well and less differentiated spindle sarcomas (Sp_1 and Sp_2, respectively), anaplastic sarcomas (A).

TERMINOLOGY

As in previous reports [5–6, 10–11], *S* is a symbol for the stemline, *i.e.* the most frequent karyotype of the tumour cell population, and *s* (s_1, s_2, etc) a symbol for the sideline(s), *i.e.* other karyotypes of less frequent occurrence than the stemline but present in a frequency of 10% or more. Different morphological types of markers are indicated by the following abbreviations:

- T_1 Telocentric marker longer than the longest normal pair (No. 1).
- T_{min} Telocentric marker similar in size to or shorter than the shortest normal chromosomes (pair No 20 and the Y chromosome=No. 21).
- *M* Biarmed marker with arms of equal size.
- *m-sm* Biarmed marker with an arm index between 1 and 3.

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Table 1. *Non-chromosomal data for the* 11 *operated primary tumours*

Tumour No.	Sample I–V	Sex	Latent period (days)	Growth period before fixation (days)	Longest tumour dia. (mm)	Histological type
3	I			128	15	Sp1
	II	M	335	153	21	Sp1
	III			171	27	Sp1
5	I	F	567	53	14	Sp2
	II			73	20	Sp2
6	I	M	723	45	25	Sp2
	II			81	21	A
16	I			41	22	F2
	II			62	16	F2
	III	F	315	78	19	Sp1
	IV			95	22	Sp1
	V			108	31	Sp1
17	I			31	10	Sp1
	II	M	281	74	16	Sp2
	III			123	25	Sp2
33	I	M	633	19	12	Sp2
	II			44	20	Sp2
36	I	F	429	16	18	F2
	II			38	21	A
38	I	F	545	68	17	Sp1
	II			108	28	Sp1
57	I			22	12	F2
	II	M	523	42	16	F2
	III			62	20	Sp1
75	I	M	66	42	20	A
	II			67	22	A
79	I	M	720	45	18	Sp1
	II			99	20	Sp1

OBSERVATIONS

1. *Hypodiploid sarcomas*

Three tumours, Nos. 3, 5, and 6, belonged to this group (Table 2). They will be treated individually.

Sarcoma No. 3. All three samples of this male sarcoma showed a hypodiploid stemline with $S=38$ (Fig. 1a) and with no sidelines. Despite the loss of 2 chromosomes and no discernible structural deviation, the S showed considerable stability. This tumour is unique in the present material by its lack of a progressional trend. There were only single cells in the hypotetraploid region, which never developed into a sideline. Such an evolution would have furnished material for genic variation compensating the chromosomal losses in the stemline.

Sarcoma No. 5. In sample I of this female sarcoma there was a hypodiploid stemline with $S=39$ (Fig. 1b) and a minor hypotetraploid sideline with $s=76$ (Fig. 1c). In the hypodiploid region there was a considerable fraction of cells with only 38 chromosomes from which the s was most probably derived. In sample II, the stemline number changed from 39 to 38 (Fig. 1d). The asymmetry of the distribution was reversed in comparison with sample I, with the persistence of a large fraction of cells with 39 chromosomes. The original hypotetraploid s was still present but with too few cells to be termed an s.

A further analysis of the readjustments in the hypodiploid region was prevented by the absence in both samples of markers or involvements of identifiable chromosomes.

Sarcoma No. 6. The chromosomal findings in both samples of this male tumour were discussed in detail in a previous report [10]. The main interest at that time was directed to the occurrence of a rare chromosomal aberration, termed double-minutes. It will suffice to mention here that in sample II the s of sample I had taken over the stemline corresponding to a numerical change of S from 39 to 39–40, and a change in the marker set from one T_1, one T_{min}, and one M to just one T_1, and one T_{min}. Doubling-products of the new S (sample II) formed the most prominent sideline,

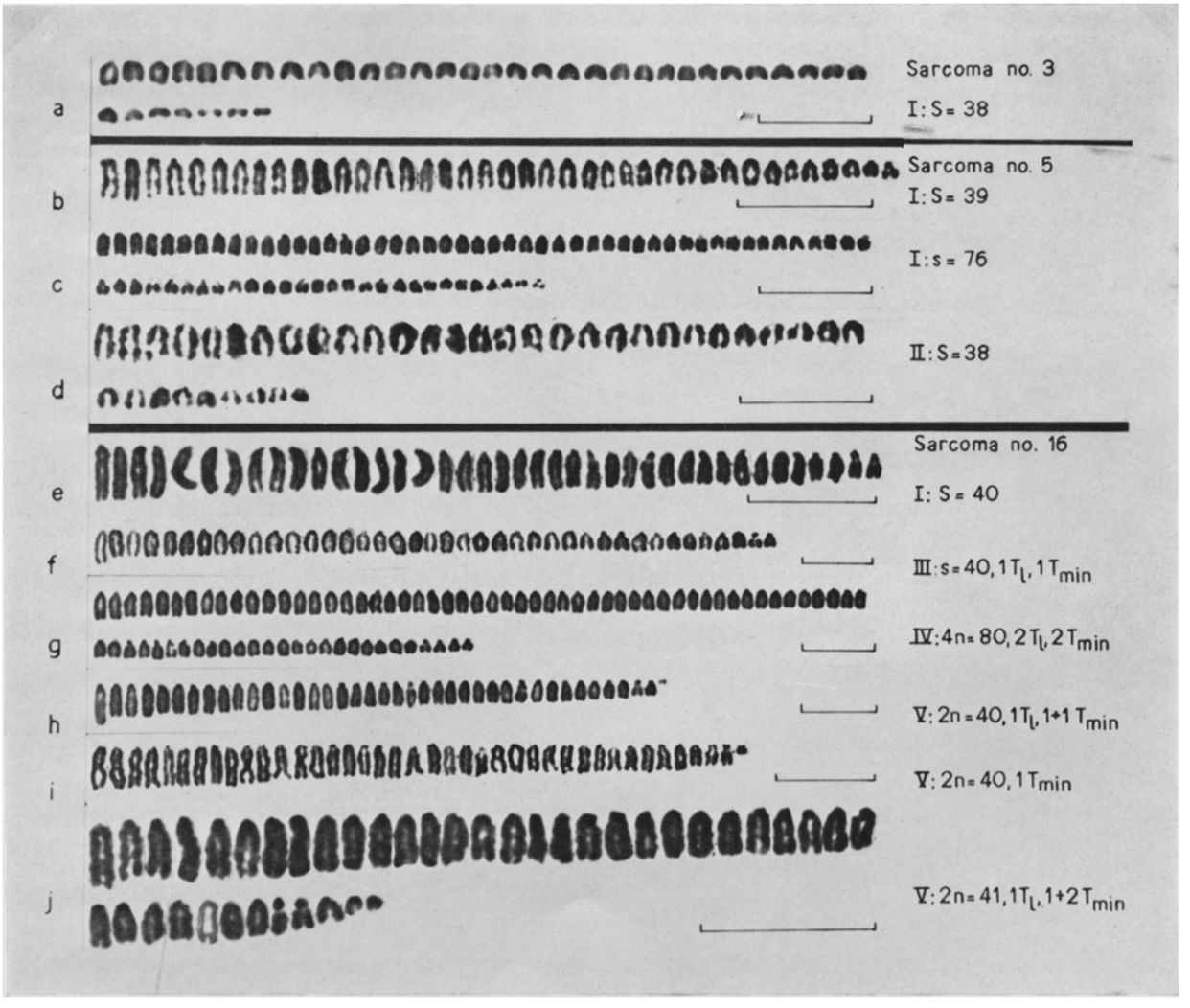

Fig. 1. Karyotypes illustrating the chromosomal progression in the primary sarcomas Nos. 3, 5 and 16. The line below each karyotype = 10μ.

(to face p. 308)

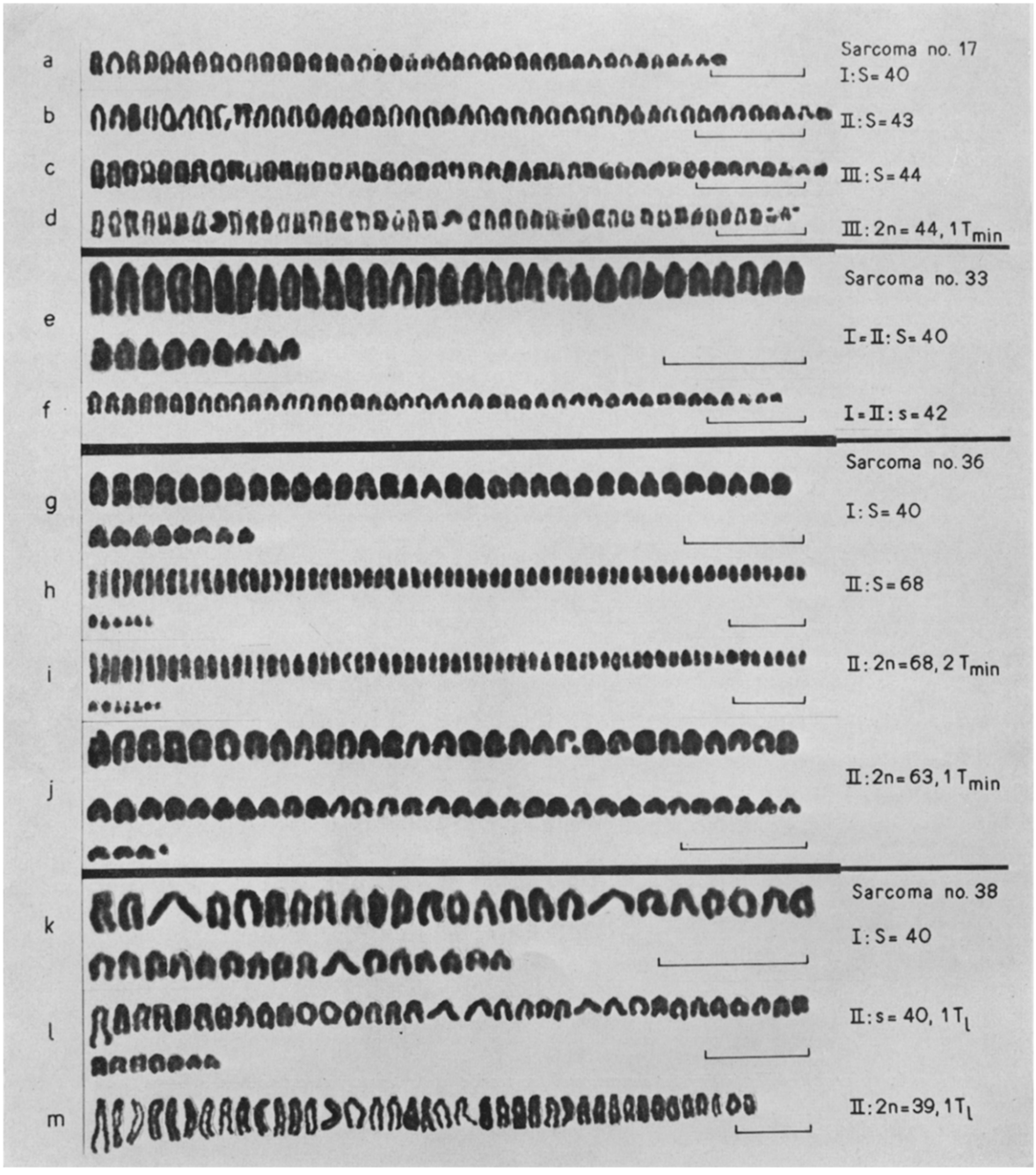

Fig. 2. Karyotypes illustrating the progression in the primary sarcomas Nos. 17, 33, 36 *and* 38.

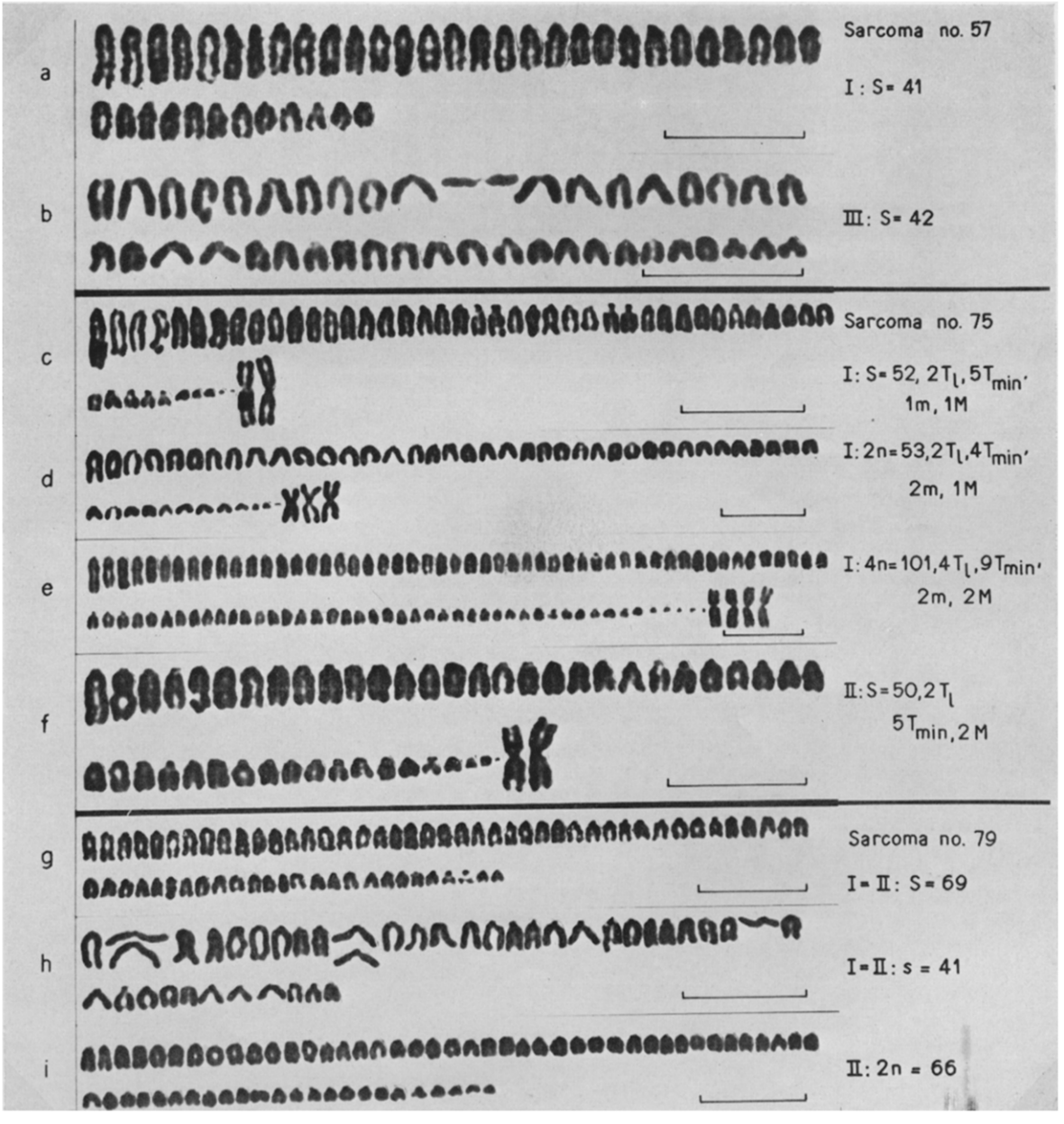

Fig. 3. Karyotypes illustrating the progression in the primary sarcomas Nos. 57, 75 and 79.

Tumour No.	Sample I–V	D or Pc	2x 34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57
3	I	Pcs	1	2	4	24	158	20	20	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—
	II	D	—	—	1	2	16	3	2	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	III	D	—	—	2	4	19	2	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
5	I	D	—	—	—	2	7	20	1	2	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	II	D	—	—	—	6	44	22	8	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
6	I	D	—	—	—	1	4	19	11	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	II	D	—	—	—	1	2	22	15	2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
16	I	D	—	—	—	—	—	2	22	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	III	D	—	—	—	—	—	1	48	3	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	IV	D	—	—	—	—	—	1	32	4	2	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	V	D	—	—	—	—	—	2	86	5	1	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—
17	I	D	—	—	—	—	—	1	17	—	1	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	II	D	—	—	—	—	—	—	2	1	4	14	1	—	—	—	—	—	—	—	—	—	—	—	—	—
	III	D	—	—	—	—	—	3	4	2	3	8	36	4	—	1	—	—	—	—	—	—	—	—	—	—
33	I	D	—	—	—	1	1	2	24	5	9	2	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	II	D	—	—	—	—	—	1	26	4	15	1	—	1	—	—	—	—	—	—	—	—	—	—	—	—
36	I	D	—	—	—	—	—	2	71	—	1	1	—	1	—	—	—	—	—	—	—	1	—	—	—	—
	II	D	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1
38	I	D	—	—	—	—	—	2	18	4	—	3	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	II	D	—	—	—	—	1	3	76	9	3	2	—	—	—	—	—	—	—	—	—	—	—	—	—	—
57	I	D	—	—	—	—	—	—	13	18	3	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	III	D	—	—	—	—	—	1	5	12	27	3	—	—	—	—	—	—	—	—	—	—	—	—	—	—
75	I	D	—	—	—	—	1	—	9	—	—	—	1	2	3	3	1	5	7	8	10	4	3	3	2	2
	II	D	—	—	—	—	—	—	1	—	—	—	1	2	2	1	2	5	12	6	6	4	2	—	1	—
79	I	D	—	—	—	—	—	—	6	10	1	—	1	—	—	—	1	—	—	—	—	—	—	—	—	—
	II	D	—	—	—	—	—	—	1	8	2	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—

*Double-minutes excluded [8].

Table 2. Chromosomal findings in operation samples from 11 primary sarcomas

Chromosome Numbers																														
		3x																				4x								
8	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86
–	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	3	—	1	—	1	—	—	—	—	—	—
–	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	2	—	—	—	—	—	—	—	—	—	—
–	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	—	—	—	2	—	—	—	—	—	—
–	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	2	1	5	1	1	—	—	—	—	—	—	—	—
–	—	—	—	—	—	—	—	—	—	—	—	—	—	1	—	1	—	6	—	—	—	—	—	—	—	—	—	—
–	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	—	3	—	2	—	—	—	—	—	—
–	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	—	5	—	1	—	—	—	—
–	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
–	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
–	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	—	2	—	—	—	—	—	—
–	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	4	1	—	—	—	—	—
–	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
–	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	—	—	—	—	—	2
–	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	—	—	—	1	—	—
–	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	—	—	—	—
–	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	—	—	—	—	—	—
–	—	—	1	1	1	—	2	1	3	9	6	1	—	—	—	—	—	—	—	1	—	2	—	—	—	—	—	—
1	—	—	1	3	1	4	3	12	12	14	7	6	5	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—
–	—	—	—	—	—	—	—	—	—	1	—	1	—	—	—	1	2	7	2	—	2	—	—	—	—	—	—	—
–	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	4	—	2	—	—	—	—
–	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
–	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	—	—	—
–	—	1	—	—	—	—	—	—	—	—	—	—	—	—	1	—	1	2	1	—	1	2	2	—	—	5	3	3
–	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	—	—	—	—	—	—	1
–	—	—	—	—	—	—	—	2	2	2	21	1	1	2	—	—	—	—	—	—	—	—	—	—	—	—	—	—
–	—	—	—	—	—	—	1	2	6	8	15	2	1	—	—	—	—	—	—	1	—	—	—	2	—	—	—	—

No.	87	88	89	90 110	6–8x –111– 160	Total cells	Stemline S	Stemline T_1	Stemline T_{min}	Stemline M	Stemline m-sm	Sidelines s_1–s_2	Sidelines T_1	Sidelines T_{min}	Sidelines M
3	—	—	—	—	—	235	38	—	—	—	—	—	—	—	—
	—	—	—	—	—	27	38	—	—	—	—	—	—	—	—
	—	—	—	—	—	31	38	—	—	—	—	—	—	—	—
5	—	—	—	—	—	44	39	—	—	—	—	s=76	—	—	—
	—	—	—	—	—	88	38	—	—	—	—	—	—	—	—
6	—	—	—	—	—	42	39*	1	1	1	—	s=39–40*	1	1	—
												s_1=80	2	2	—
	—	—	—	—	—	49	39–40*	1	1	—	—	s_2=39*	1	1	1
16	—	—	—	—	—	25	40	—	—	—	—	—	—	—	—
	—	—	—	—	—	52	40	—	—	—	—	s=40	1	1	—
	—	—	—	—	—	43	40	—	—	—	—	s=40	1	1	—
	—	—	—	—	—	100	40	1	1	—	—	s=40	—	—	—
17	—	—	—	—	—	20	40	—	—	—	—	—	—	—	—
	—	—	—	—	—	25	43	—	—	—	—	—	—	—	—
	—	3	—	—	—	66	44	—	—	—	—	—	—	—	—
33	—	—	—	—	—	45	40	—	—	—	—	s=42	—	—	—
	—	—	—	—	—	49	40	—	—	—	—	s=42	—	—	—
36	—	—	—	—	2	107	40	—	—	—	—	s=68	—	—	—
	—	—	—	—	1	72	68	—	—	—	—	—	—	—	—
38	—	—	—	—	—	43	40	—	—	—	—	s=76	—	—	—
	—	—	—	—	—	100	40	—	—	—	—	s=40	1	—	—
57	—	—	—	—	—	35	41	—	—	—	—	—	—	—	—
	—	1	—	—	—	50	42	—	—	—	—	—	—	—	—
75	2	5	2	12	1	108	52	2	4–5	1	1	—	—	—	—
	—	—	—	3	—	50	50	1–2	4–5	2	0–1	—	—	—	—
79	—	—	—	—	—	50	69	—	—	—	—	s=41	—	—	—
	—	—	—	—	—	50	69	—	—	—	—	s=41	—	—	—

*Doub

s_1=80 with two T_1s and two T_{min}s, but the original hypodiploid *S* (sample I) was still present in sample II as a minor second sideline, s_2.

The progression is thought to have proceeded as follows: The markers T_1 and the T_{min}, and perhaps also the double-minutes, may be signs of a period of breakage prior to sample I. During the subsequent adaptation, a stemline with an added *M* developed and became temporarily superior. This stemline was again out-grown by derivatives of the population readjusted with just the two telocentric markers (sample II). Experiences from *in vivo* transplantations of primary Rous sarcomas in mice (unpublished) have repeatedly indicated the occurrence of similar processes, *i.e.* an appearance of a temporary new near-diploid or polyploid stemline followed sooner or later by the return of the original stemline or derivatives of it.

2. *Diploid sarcomas*

Five tumours, Nos. 16, 17, 33, 36, and 38 (Table 2) belonged to this group.

Sarcoma No. 16. In sample I, this female tumour showed a normal diploid stemline (Fig. 1e) without any sidelines. The fixation of sample II was unsuccessful, but the progression could be studied in specimens from 3 further recurrencies. Thus, in samples III and IV, the normal diploid S persisted unchanged but its dominance was successively reduced due to the appearance of a pseudodiploid *s*. The karyotype of this sideline, similar in both samples is shown in Fig. 1f. About 12% of the tumour cells in sample III belonged to this s against 33% in sample IV. Finally, in sample V, the former pseudodiploid *s* had taken over the stemline and the original normal diploid *S* now constituted an *s* corresponding to about 25% of the cells in mitosis. Since no structurally changed variant cells had been observed in sample I, it may be concluded that the pseudodiploid *s* and *S* were later products of the progression.

The pseudodiploid karyotype was characterized by the presence of one T_1 and one T_{min} and the absence of one member of the smallest pair, No. 20 (Fig. 1f). As a rule the T_1 was easily distinguishable, but its size was not constant, and it was sometimes only a little longer than the longest normal pair, No. 1 (Fig. 1g, h). The T_{min} had a size similar to pair 20 but could be distinguished due to the lack of the prominent proximal secondary constriction characteristic of this pair in CBA mice (Fig. 1e, f). The considerable variability of the karyotype of this tumour was reflected by the occurrence in several cells with only one T_1, one T_{min} (Fig. 1i), one or two additional and smaller T_{min}s (Fig. 1h–j). Several cells had two chromosomes No. 20 (Fig. 1i, j) in addition to the marker set and, in some cases, with the probable disappearance of one chromosome No. 19 (Fig. 1i). The frequency of the variant cells derived from the pseudodiploid *s* or *S* was 5, 12 and 18%, respectively, in samples III, IV and V. The rising frequency of these variant cells may be due to a marked instability of the developed pseudodiploid side-stemline. The frequent variability of chromosome No. 20 also points to the common incidence of mitotic disturbances of the non-disjunctional type.

Sarcoma No. 17. This male tumour also started out with a normal diploid stemline (Fig. 2a) without any sidelines. In sample II a profound change in the chromosomal picture became apparent, the mode now being in the hyperdiploid region with *S*=43 (Fig. 2b) and with the original normal diploid *S* not even discernible as an *s*. The next sample, No. III, had essentially the same picture, with the mode in the hyperdiploid region, however, now moved to the higher level of *S*=44 (Fig. 2c). The stemline of sample III was apparently derived from that of sample II; the close numerical relation between them and the persistence in sample III of a considerable fraction of cells with 43 chromosomes are compatible with this assumption. The presence of single hyperdiploid cells in sample I, probably the result of non-disjunctional variation, foreshadowed the later appearance of the 43–44 chromosome stemline. It seems that a superior competitive capacity has been reached at the *S*=43–44 level without any intervening 41–42-stem- or sidelines, thus an instance of discontinuous stemline shift during progression.

A T_{min} (Fig. 2d) was found in only a few cells of sample II and III. As a rule, pair No. 20 and the Y chromosome were present in normal numbers in the hyperdiploid cells (Fig. 2a–d) and the extra chromosomes were thus among the unidentifiable chromosomes.

Sarcoma No. 33. The two samples of this male tumour showed a similar chromosomal picture, a normal diploid stemline (Fig. 2e) and a hyperdiploid sideline (Fig. 2f). In sample II, however, the prominence of the *s* was increased, a small but definite change pointing towards a stemline shift, approaching slowly. The progression is of the same kind as that of sample II of sarcoma No. 17, although proceeding more slowly. Even among tumours

with apparently normal diploid stemline, it should be expected that the stemline shift may take place with different rapidity. This is an indication that the individual tumours are dissimilar at the submicroscopical level due to mutations or cryptostructural deviations. Pseudodiploidy could also play a significant part without being detected in karyotypes such as the present.

Sarcoma No. 36. In sample I of this female tumour a normal diploid stemline was found (Fig. 2g) and, in addition, a hypertriploid sideline with $s=68$ (Fig. 2h). No markers were noticed in the *s*. In sample II, the original normal *S* had disappeared completely, and the hypertriploid *s* had taken over the stemline. The abruptness of this stemline shift is quite different from the slow and gradual change in the previous tumour, sarcoma No. 33, and also to some measure in sarcoma No. 17.

Although the *S*-number of sample II agreed with the *s*-number of sample I, the numerical distribution was different with many counts below 68 (Fig. 2j). The asymmetrical distribution probably indicates an early phase of an 'Ising' effect, the well-known adaptive reaction in advanced polyploid mouse tumours by which a gradual shift towards lower chromosome numbers takes place [12, 13]. Another difference, in relation to sample I, was the occurrence of one or two $T_{min}s$ in about 20% of the cells in sample II (Fig. 2i, j). Since no mode could be found, no *s* could be defined on this basis. Two cells with one T_{min} were recorded among the hypertriploid metaphases of sample I, a frequency roughly corresponding to 8% cells with markers in the triploid population of sample I. Also, the increased structural variability on the polyploid level of the present primary sarcoma is in accordance with experiences from advanced polyploid mouse tumours [12, 13].

Sarcoma No. 38. In sample I of this female tumour, a normal diploid stemline was found (Fig. 2k) and, in addition, a minor hypotetraploid sideline with $s=76$ without markers. In sample II, the stemline was unchanged but the hypotetraploid sideline was replaced by a pseudodiploid *s*. The latter sideline and some variant cells, adding up to about 24% of the population of sample II, showed a T_1 with a vague secondary constriction in the middle part (Fig. 2l, m). Two of the diploid cells of sample I had a similar T_1. Despite its scarcity, the characteristic marker indicates that the pseudodiploid *s* of sample II was derived from these variant cells of sample I. The disappearance of the hypotetraploid *s* of sample I is an unusual development and the reasons remain obscure.

3. *Hyperdiploid sarcoma*

Sarcoma No. 57. In sample I this male tumour showed a hyperdiploid stemline with $S=41$ (Fig. 3a). In the next successful sample, No. III, the *S* was 42 (Fig. 3b); as in sample I, there were no sidelines and the stemline showed no markers or identifiable extra chromosomes. In both samples, the distribution was asymmetrical with a considerable fraction of cells in the *S*-1 class. In the case of sample I, this is thought to illustrate the persistence of a fraction of the original normal diploid stemline, in the case of sample III the persistence of the stemline of sample I. In contrast to sarcomas Nos. 17 and 33 the hyperdiploid progression here seems to be a continuous process.

4. *Near-triploid sarcomas*

Sarcoma No. 75. This exceptional male tumour showed a hypotriploid stemline in sample I with $S=52$ (Fig. 3c) with no sidelines. In sample II, the *S*-number was reduced to 50 (Fig. 3f), thus being on the border line between hyperdiploidy and hypotriploidy. As in sample I, no sidelines were found. In comparison with the numerical distribution in sample I, there was in sample II a slight decrease in the spread around the *S*-number and a pronounced decrease of the doubling-products and their derivatives (Fig. 3e). The latter trend is reminiscent of that found in sample II of sarcoma No. 38.

The *S* of sample I had on an average 8–9 markers (Fig. 3c, d) covering a wide spectrum of diverse telocentric and biarmed markers. In sample II, there were minor changes but the basic composition of this marker spectrum was retained (Fig. 3f). The loss or gain of the different marker types in sample II indicates a liability of newformed chromosomes, observable as markers, to be involved in mitotic irregularities and also to be involved in further structural remodelling. According to this view, the mosaicism recognizable as a variation in the marker set may depend on the continuous generation of variant cells, rather than the evolution and persistence of many clones. The evolution proposed is supported by the high degree of resemblance, structurally, between the individual cells in both samples. Thus, the great spread in both samples, numerically, is not unexpected, and is probably accentuated by the stemline being located in the triploid region. The negative scewness in

the distribution of sample I is probably not artificially produced by losses during squashing. It seems more plausible that it represents early stages of the "Ising" effect. This interpretation corresponds with the lowering of the stemline number in sample II.

Sarcoma No. 79. Both samples of this male tumour showed a hypertriploid stemline with $S=69$ (Fig. 3g), and a hyperdiploid sideline with $s=41$ (Fig. 3h). Neither the stemlines nor the sidelines had markers. The only noticeable change from sample I to sample II was a slight but definite increase in the counts below $S=69$ (Fig. 3i). As suggested in the case of sample I of the previous sarcoma, No. 75, this asymmetry of the numerical distribution could be an early indication of an 'Ising' effect.

Whether the stemline of sarcoma No. 79, as well as the only other hypertriploid distribution in this material, *viz.* the sarcoma No. 36, are originally triploids or have been derived from the tetraploid level of variation could not be determined. The 'Ising' effect is the progressional trend to be expected in near-tetraploid tumours, a category not represented in the present study.

5. *Progression and frequency of normal diploid cells*

The frequency of normal diploid cells during aging of the sarcomas is illustrated in Fig. 4. With few exceptions the trend is a fall in this frequency. The tumours with a normal diploid stemline in sample I showed great differences in the rate of decrease. This finding corresponds well with the diversity in progressional pathways and demonstrate the competitive value of the different heteroploid stem-sidelines in comparison with their original, apparently normal, diploid *S*. The slight increase in the normal fraction, found in sarcoma No. 38, could be an incidental finding, due to an unrevealed pseudodiploidy or reflect the disappearance of less competitive variant cells and sidelines.

The picture of the heteroploid transformation is that of a non-linear process. Sarcoma No. 16 figures a slow initiation, but also indicates a slow end. The last trend seems to be a common feature according to the findings in the sarcomas with a heteroploid stemline, already established. Counts of normal, stromal or inflammatory cells might sometimes blur the picture, however, the general trend is hardly influenced by this. The persistence, late in the growth phase, of a small fraction of the normal, diploid stemline is interesting as a potential source for the origin of new heteroploid stemlines. In heteroploid tumours the persistence of remnants of an early stemline after further reshuffling is a parallel phenomenon.

6. *Progression in relation to growth rate and histopathological type*

The cumulative curves of Fig. 5 show the changes in the growth rate in the 11 sarcomas. As expected, the general trend is a growth rate increasing with age. Consideration of the chromosomal data presented in previous sections shows that there is no clear and constant relation between chromosomal progression and changes in growth rate. In sarcoma No. 3, for example, there is an obvious increase in the growth rate despite a complete lack of discernible progressional steps, and in sarcoma No. 36 the growth rate seemed unchanged despite a change in stemline from a normal diploid one to a triploid *S*. It can not be denied that, as a rule, stemline shifts as well as minor fluctuations in the composition of the population must be due to differences in the competitive capacity of different genotypes of the *S* and *s*. The visible differences are small or

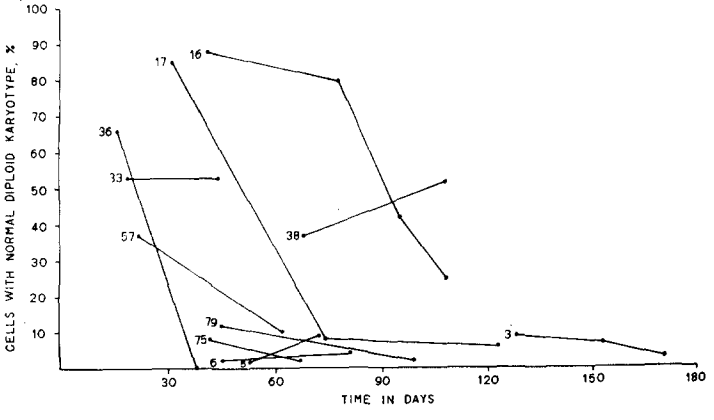

Fig. 4. The relation between the frequency of cells with a normal, diploid karyotype and the age of the 11 primary sarcomas.

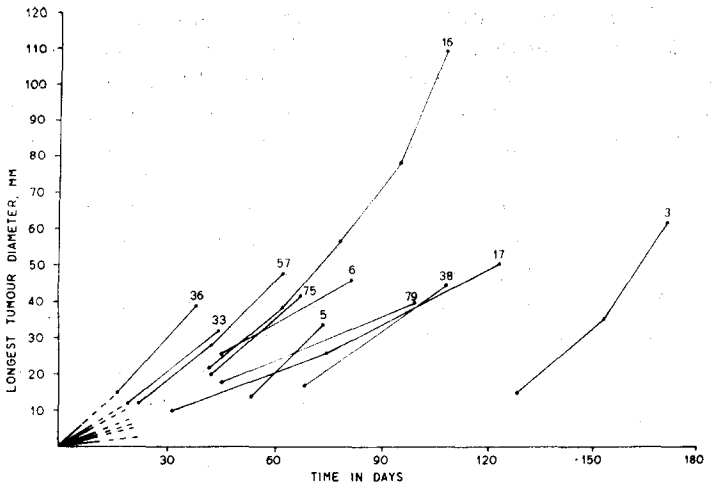

Fig. 5. *The growth rate in the* 11 *primary sarcomas during aging. The longest diameter* (*Table* 1) *of the original tumour and the recurrency(ies) is used as an index of the size at the time of the operation. A cumulative curve is formed for each sarcoma by adding the diameter of sample II to that of sample I, etc.*

lacking in many cases, due perhaps to the need of further adaptation of the genic material before optimal viability is achieved. Sarcoma No. 3, with its lack of visible progressional trends could be such an example. The operations and the age of the animal, as well as other host factors, are further causes, which also might have influenced on the results, though the extent could not be determined in the present experiments.

In all cases with a shift of the *S* from one karyotypic category to another, there was a change in the histological picture (Tables 1 and 2: sarcomas Nos. 16, 17 and 36). In sarcoma No. 36 with its shift from a normal diploid stemline to a triploid, the change was discontinuous (F2–A); in the other tumours the histological picture was changed to the nearest, less differentiated type, *i.e.* F2 to Sp1, Sp2 to A, etc. Modifications of heteroploid stemlines without a change of the S-category, as well as the appearance of new sidelines, were accompanied only occasionally by a continuous change in the histological picture (sarcomas Nos. 6 and 57). Although the correlation between chromosomal progression and histopathological picture is by no means an absolute one and is sometimes inappreciable in tumours undergoing only minor chromosomal changes, it is felt that the histological picture is a parameter more sensitive and more closely related to chromosomal progression than the growth rate.

CONCLUSIONS AND DISCUSSION

The results presented above, demonstrate that, as a rule, chromosomal evolutionary forces are operating very actively in primary Rous sarcomas in mice. The findings in the present material do not indicate initial or very early numerical and/or structural chromosomal changes [14]. The normal diploid stemlines seem to possess the latent capacity of heteroploid transformation along a diversity of pathways at different times, and often comparatively late, in tumour development. The results, illustrating the steps in this transformation, can be summarized as follows:

1. The formation of a few variant cell types: deviating numerically and/or structurally from the normal, diploid *S* (sarcomas Nos. 17 and 38).

2. The development of a heteroploid *s*: sometimes traceable to a certain fraction of variant cells (sarcomas Nos. 16, 17 and 38).

3. The heteroploid sideline takes over the stemline (sarcomas Nos. 16, 17 and 38).

4. Modifications of the heteroploid *S* (but without a departure from the originally established stemline category, *i.e.* the progressional pathway chosen by a particular tumour). The four possible pathways are considered separately (a–d).

a. Hypodiploid stemlines. They showed no similarities in the progression. Thus, in one case (sarcoma No. 3) there were no discernible changes, in another case the hypodiploid character was accentuated by a lowering of the *S*-number (sarcoma No. 5), and in a third case a sideline, numerically and structurally related to the *S* took over the stemline (sarcoma No. 6).

b. Pseudodiploid stemlines. The progressively increasing numerical and structural pleomorphism in sarcoma No. 16 could be an indication of a probable further reshuffling of the genic material before departure from diploidy.

c. Hyperdiploid stemlines. In both cases analyzed (sarcomas Nos. 17 and 57) the hyper-

diploid character was accentuated by a further elevation of the *S*-number.

d. Polyploid stemlines. A more or less marked 'Ising' effect was seen in both the hypo- and hypertriploid region (sarcomas Nos. 36, 75 and 79). No tumour with tetraploid *S* was included in the material, but the trend in the triploid region is a phenomenon often seen in transplanted tetraploid mouse tumours [12, 13].

Step 1–3 was initiated or completed in all sarcomas studied. Step 4, covering the whole spectrum of possible heteroploid pathways, is probably not completed in most tumours. In some of the sarcomas this step might proceed indefinitely by a continuous reshufflling of the genic material without a change of the karyotypic stemline category, whereas in others it might be possible to define a fifth step due to the occurrence of such a shift in the *S*-group.

The diversity in progressional pathways, as revealed by the present investigation, is in line with the broad variation found within the chromosomal spectrum of primary Rous sarcomas in mice. The variation and the progressional pathways chosen are phenomena related to, and to a great extent probably dependent on, the nature of the carcinogen. Other mouse tumours such as mammary carcinomas, the lymphomas-leukemias, and probably some of the hepatomas (for review see reference 11), differ from the present tumours by their tendency to retain diploid-near-diploid stemlines even after a comparatively long time of transplantation.

The observations of the chromosomal spectrum of primary Rous sarcomas in mice and of their progression indicate a specific evolution for each individual sarcoma; in this respect the picture is similar to that found in sarcomas induced by polymer implants in mice [14]. If so, the chromosomal changes during early stages of the neoplastic conversion would be unspecific; either due to extensive and widely distributed mutational or cryptostructural changes; or due to the involvement of general functions, such as disturbances of the normal mitotic function. In an investigation of primary Rous sarcomas in the Chinese hamster, however, Kato [3] found a more frequent involvement of certain chromosome pairs. However, in mouse tumours, the uniform complement prevents this kind of analysis.

In the present context it is of interest to mention that a specific and fairly constant chromosomal change was noted in thyroid cells in rats maintained on an iodine-deficient diet [15, 16]. The frequency of the aneuploid cells with 41 or 43 chromosomes ($2n=42$) increased with time; 90–100% of the metaphases with only 41 chromosomes had lost one member of pair 15. This specific loss persisted in well established tumours developed after transplantation of the precancerous cells but in many cases there were, in addition, further numerical and structural changes, different for each tumour.

In human primary tumours normal diploid stemlines are rare, except in leukemias; the heteroploid *S*-numbers, though often clustered in the near-triploid region, cover a broad range. The information about the extent of superimposed chromosomal changes, influencing this picture are scanty. On the basis of the distribution of markers in the tumour cell population a clonal origin has been suggested in human tumours, and the possibilities of superimposed coincidental changes, hiding early, specific rearrangements, has been discussed [3]. However, systematic studies of the chromosomal progression in primary human neoplasms are still scanty [2, 4, 7–9, 17]. In comparison between non-invasive and invasive tumours of the bladder it was found that the former group had chromosome numbers predominantly in the diploid region while the latter group had chromosome numbers ranging from 40 to more than 100 without any preferred level of ploidy (Lamb cited by Hughes in [2]). Other studies with a bearing on this problem are those dealing with cervical neoplasia, dysplasia, carcinoma *in situ* and invasive carcinoma [7–9, 17]. The frequent occurrence of heteroploid stemlines in early stages of this tumour type makes it probable that extensive changes have often taken place before the appearance of the invasive carcinoma. A less pronounced but similar picture has been found in the chromosomal studies of chorionic lesions [4].

The results already obtained are of essential importance for the evaluation of chromosomal findings in other classes of primary human tumours. They also underline the need for studies of the chromosomal progression in different categories of neoplasms, especially regarding their early progression. In comparison to Rous mouse sarcomas the human tumours, referred to above, seem to show an earlier heteroploid transformation, often already discernible in premalignant, non-invasive lesions.

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RESUME

La progression chromosomique a été étudiée dans 11 *sarcomes de Rous primitifs de la souris, analysés à des stades successifs de leur développement. Cinq sarcomes, qui avaient à l'origine une lignée diploïde normale, ont constitué le matériel qui a permis l'étude des étapes vers la transplantation hétéroploïde. La nouvelle lignée pouvait souvent être rattachée à une lignée collatérale hétéroploïde, et, dans certains cas, il était possible de retrouver l'origine de cette lignée collatérale dans une petite fraction de cellules différant de la lignée principale.*

Les sarcomes qui avaient, dès le premier examen, une lignée hétéroploïde, présentaient souvent des réarrangements chromosomiques supplémentaires, mais sans qu'il y eût modification de la catégorie de la lignée principale originelle. Ils conservaient donc la direction de la progression choisie par la tumeur. Alors que les étapes de la progression avaient un caractère général, la route suivie et la vitesse de la progression étaient propres à chaque tumeur.

Il n'y avait pas de corrélation nette entre les changements de la vitesse de croissance et la progression chromosomique. L'aspect histopathologique, en revanche, montrait une corrélation avec les principales modifications de la progression.

Le tableau chromosomique plein de dynamisme qui caractérise le sarcome de Rous est comparé aux observations faites dans d'autres tumeurs expérimentales chez l'animal, et dans les cancers chez l'homme.

SUMMARY

The chromosomal progression was studied in 11 *primary Rous sarcomas in mice, analyzed at successive stages of their development. Five sarcomas, originally having a normal diploid stemline, furnished material for studies of the steps in heteroploid transformation. The new stemline could often be traced to a preceding heteroploid sideline, and it was possible in some cases to trace the origin of this sideline to a small fraction of variant cells.*

Sarcomas with a heteroploid stemline, established at the time of the first analysis, often underwent further chromosomal rearrangements but without changing the original stemline category, i.e. *the progressional pathway chosen by the tumour. Whereas the progressional steps were of general character, the route and rate of progression were individual for each tumour.*

No clear-cut correlation was found between changes in growth rate and chromosomal progression; the histopathological picture, however, was a parameter showing correlation to the major progressional changes.

The highly dynamic chromosomal picture found in Rous sarcomas is discussed in relation to tumours in other animal model systems and to human neoplasms.

ZUSAMMENFASSUNG

Bei 11 *primären Rous-Sarkomen in Mäusen wurde die chromosomale Progression verfolgt und in den folgenden Entwicklungsstadien analysiert. Fünf Sarkome mit ursprünglich normaler diploider Stammlinie lieferten Material zum Studium der einzelnen Schritte, die zur heteroploiden Transformation führten. Die neue Stammlinie konte oft auf eine vorhergehende heteroploide Seitenlinie zurückgeführt werden, und in einigen Fällen war es möglich, den Ursprung dieser Seitenlinie in einer kleinen Fraktion verschiedener Zellen zu finden. Sarkome mit einer, zum Zeitpunkt der ersten Analyse, heteroploiden Stammlinie durchliefen oft chromosomale Umordnungen jedoch ohne die ursprüngliche Stammlinienkategorie zu ändern, i.e. der Tumor behielt den einmal eingeschlagenen Entwicklungsweg bei. Während die einzelnen Stufen der Progression generellen Charakters waren, blieben Weg und Rate individuell für jeden Tumor.*

Kein klaren Zusammenhang zwischen Wachstumsrate und chromosomaler Progression wurde gefunden. Der Parameter des histopathologischen Bildes blieb jedoch in Zusammenhang mit den fortschreitenden Veränderungen.

Das sehr dynamische chromosomale Bild, das in Rous-Sarkomen gefunden wurde, wird in Verbindung mit Tumoren in anderen Tiermodellsystemen und in menschlichen Neoplasmen diskutiert.

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Some Effects of Daunomycin on the Nucleic Acid Synthesis in Synchronized *L*-Cells

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INTRODUCTION

DAUNOMYCIN is an antibiotic with marked antitumor activity. During recent years it was introduced into the therapy of acute leukemias with considerable success, causing regression in between 35 and 55% of patients [1].

Daunomycin and the structural identical antibiotic rubidomycin were in the early sixties isolated for cultures of streptomyces by Italian [2, 3] and French workers [4]. It consists of an aglycon chromophore of the polyoxyanthraquinone group (daunomycinone) linked to an aminosugar (daunosamine).

The effect of the antibiotic itself has been studied on a variety of transplantable tumors of solid and ascites forms, *in vivo* and *in vitro*, and has been found to inhibit cell growth, to decrease the mitotic index and to produce chromosomal aberrations. However, just as is the case with most other cytostatics, the effects on nucleic acid synthesis has not been fully clarified.

The enzymatic synthesis of DNA-dependent RNA was found to be inhibited in a cell-free system at the same time as the DNA-dependent-DNA synthesis was inhibited to a much smaller degree than the RNA synthesis [6].

Other authors [7] showed that daunomycin binds to DNA both *in vivo* and *in vitro*, forming a complex involving hydrogen bonds and electrostatic forces, and suggested that this complex, rather than the inhibition of macromolecular synthesis *per se* may be responsible for the biological activities of daunomycin. Thus, by combining with DNA, daunomycin prevents the separation of the two strands, thereby secondarily hampering the function of the DNA-dependent-RNA polymerase system.

This would imply that daunomycin acts in the same way as does actinomycin D. However, daunomycin does not require any particular base in order to interact with DNA [8], in contrast to actinomycin D, which preferently binds to deoxyguanosine residues [9] like mitomycin C [10] and a number of other cytostatics. It was lately reported, [11] that daunomycin is a more effective inhibitor of DNA synthesis than of RNA synthesis, and hence suggested that its biological mechanism differs from that of actinomycin D.

The effects of actinomycin D in the G1 and S phase of mechanically synchronized *L*-cells have been investigated by others [12, 13], and we have therefore found it of interest to test daunomycin in a parallel system.

MATERIALS AND METHODS

Cell lines

Four sublines of *L*-929 mouse fibroblasts, all growing as monolayers, were cultivated. One subline (c) was kindly placed at our disposal by Christain Mittermayer, MD, Freiburg

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Abbreviations:
DNA, deoxyribonucleic acid; RNA, ribonucleic acid; DNA-polymerase, deoxynucleosidetriphosphate: DNA deoxynucleotidyltransferase (EC 2.7.7.7); SIRC, rabbit corneal cells; PCA, perchloric acid; *m*-RNA, messenger-ribonucleic acid; TTP thymidine-5′-triphosphate; FUDR, 5-fluoru-2′-deoxyuridine.

University, Germany, one (*s*) by J. Leerhoey, MD, Statens Seruminstitut, Copenhagen and two (b_1 and b_2) by J. Genner, MD, Department of Microbiology, University of Copenhagen.

SIRC cells were kindly supplied by Statens Seruminstitut through the courtesy of J. Leerhoey.

Burkitt lymphoma cells (sublines Annah, Esther and Silfere) established as suspension cultures, was a gift from docent Eva Klein, Tumorbiologiska Institutet, Karolinska Institutet, Stockholm, Sweden.

Media

L-cells were grown in modified Eagle's medium [14], supplemented with 5% heat inactivated and filtered calf serum. SIRC cells [15] were also grown in this medium but containing 10% heat inactivated calf serum, sterilized by filtration through Millipore filters 0·22 μ pore size, and supplemented with Lactalbumin hydrolysate (g/l) 1·7 g and yeast extract 'Difco' 0·57 g.

Burkitt lymphoma cells were kept in stock suspension cultures in Parker medium 199, supplemented with 10% filtered fetal calf serum. When subjected to experiments designed for the establishment of monolayers, Burkitt cells were transferred to different modifications of the above mentioned modified Eagle's medium and kept under observation for at least four weeks.

Except for the Parker medium, which contained 100 i.e. penicillin and 50 μg streptomycin sulphate per ml, none of the medias contained antibiotics.

Treatment of glassware

Since the adhesion and growth behaviour of mammalian cells are dependent on quality and treatment of the culture vessels [16] our routine washing procedure will be briefly reviewed.

In the adhesion and growth behaviour experiments the same kind of glassware (Pyrex Brand, Bellco) was used throughout. In the modified Prior tissue culture chamber for microcinematographic registration, soft-glass cover-slides were employed. These slides were used once only.

Prewashing. All glassware was treated overnight in 10% RBS 25° followed by thorough rinsing in distilled water. This treatment was followed by another prewash in 70°C hot water for 10 min, wash in 5% Trinon NJ° for 40 min, rinsing in four changes of cold tap water and four changes of ion-exchanged pyogen-free water. All glassware, except cover-slides, underwent this treatment and was therefore dried in an oven at 110°C for 1 hr followed by sterilisation by hot air at 180°C for 2 hr. Cover-slides were immersed in 10% RBS 25° for 2 hr, rinsed in distilled water, and dried by passing into absolute ethyl alcohol twice and then into diethylether. Sterilisation was affected by autoclaving at 120°C for 30 min.

Mechanical syncrhonization

Cell cultures (*L*-929) were propagated and synchronized in 1000 ml Pyrex glass Blake bottles (Bellco Glass, Vineland N.Y.) according to a method previously described [17].

By this method mitotic cells are gently detached from the glass surface by a manual shaking procedure, whereby 10 ml medium is push-pulled 10 times over the cell layer within 20 sec. The medium is then transferred to the next bottle, while the first bottle is refed with its original conditioned medium. The process is repeated with 10 bottles, giving finally 10 ml medium containing $2·5–3 \times 10^4$ cell/ml. These cells are then transferred into a 125 ml Erlenmeyer bottle (Bellco Glass, Vineland) and grown until they reach the required age.

Total cell concentration

In random cultures cell counts were obtained after wash with Hank's solution twice, followed by trypsin digestion (0·5% trypsin, Difco 1:250, in Sorensen phosphate buffer, pH 7·4) [18] for 5 min at 34°C. The treatment was stopped by adding icecold Hank's solution and aliquots counted in a coulter counter model F (Coutler Electronic, Beds, Dunstable, England).

From synchronous cultures aliquots of 1 ml were diluted with 9 ml isotonic saline in samples later used for incorporation studies, while 5 ml aliquots were diluted with 5 ml isotonic saline in experiments designed to control the yield of cells in successive synchronizations. In this way counts of $1·2–1·5 \times 10^4$ were obtained giving a statistical error of less than 1%.

Mitotic index determination

Aliquots of 5 ml from synchronized cell cultures were centrifuged at 800 rev/min for 5 min at 2°C in a Christ KII refrigerated centrifuge, and the pellet resuspended by dropwise addition of 5 ml, 4°C, 0·1 M sucroseunder continuous agitation. After 3 min in the centrifuge chamber, the suspension was centrifuged once more as above. Acetic-acid methanol (1:3 v/v) at 4°C was added dropwise with continuous stirring to a final volume of 5 ml. The sample was then stored in an icebath for 10 min. Following another centrifugation as

above, the cell pellet was resuspended in 0·1 ml of acetic acid–methanol fixative and applied to a slide, air dried, and stained with a modified Jenner–Giemsa technique. From each sample a total of 500 cells were scored for mitotic figures.

Preparation of slides for microscopical examination after daunomycin treatment was done as previously described [19]. Slides were stained with Feulgen–Fast Green for DNA and RNA respectively [20] or with haematoxylin-eosine for morphological studies. Further details are given in the legends to individual figures.

Microcinematography

For microcinematographic experiments cells in standard medium were inoculated into a slightly modified Prior tissue culture chamber (Bie & Berntsen, Copenhagen) which holds approximately 1 ml cell suspension when space is left for an airbubble of 3 mm dia. Equipment for time-lapse was constructed from a Carl Zeiss photomicroscope II equipped for phase-contrast, by adding a hot chamber to the stage and connecting the photomultiplier to a self constructed phototimer. The phototimer automatically turns on the light-source and exposes the film in preselected intervals. Between two exposures cells were grown in the dark. Slides were evaluated by projecting on a screen at approximately 50× magnification.

Isolation of nuclei and mitochondrions

Cells were incubated at 37°C for 60 min in medium containing ^{3}H-thymidine 1 μc/ml and, for inhibition studies, various amounts of daunomycin. Incubation was terminated by washing twice with icecold Eagle's medium containing 100 μg thymidine/ml. Isolation was done in the following way: cells in exponential growth were trypsinized as above. After 20 sec the trypsin was discharged and the cultures left at 34°C until detachment of the cells was finished (roughly 5–10 min). Cells were then washed twice in serum-free Eagle's medium and collected by centrifugation at 800 rev/min for 10 min at 2°C. The pellet was resuspended in 5 ml cold medium and homogenized in a Dounce all glass homogenizer (849S Braun, Melsungen) 80 strokes followed by 2 min pipetting in a 100 μl constriction pipette (Bie & Berntsen, Copenhagen). The nuclear pellet was collected by centrifugation at 2°C, 500 rev/min for 5 min. Resuspension, pipetting and centrifugation was repeated twice. The final pellet containing the nuclei was hydrolysed in 0·5 M NaOH overnight at room temperature, and aliquots taken for scintillation counting and protein determination. The supernatants from all centrifugations following the homogenization were pooled and centrifuged at 2500 g for 15 min. The pellet was then layered on ×0·25 M sucrose and centrifuged 15 min at 2650 g, 2°C. This treatment was repeated twice and the resulting pellet containing the mitochondrial fraction was hydrolysed as above.

Determination of DNA and RNA synthesis

DNA and RNA synthesis were determined by measuring the incorporation of radioactive precursors into PCA precipitable material as described previously [21]. Measurements were done in a Pachard Tri-Carb liquid scintillations counter. Bray's scintillation fluid was used throughout the experiments. Quinching was corrected by internal and external standards.

Protein determination

Proteins were determined in a Beckman DU or a Carl Zeiss PMQII spectrophotometer according to [22].

Chemicals

^{3}H-thymidine (3 c/mM), thymidine-2-^{14}C (60·5 mc/mM) and uridine 5-^{3}H (5 c/mM) were purchased from the Radiochemical Centre, Amersham, U.K. Daunomycin was kindly supplied in substance from Medical department E, Finsen Institute. DNA (thymonucleic acid, sodium salt, high molecular) was obtained from Fluka, Schweiz. Salmin sulphate was purchased from British Drug Houses Ltd., U.K. The rest of the chemicals used in these investigations were analytical grade.

RESULTS

The mechanical synchronization of mammalian cells has been a valuable tool in the investigation of the cell cycle, since the method was introduced in 1963 [23]. However, only few cell lines have been found suitable for this procedure, and among these, *L*-cells is one of the more promising [12, 17].

For the establishment of synchronous cell cultures, we chose *L*-929, mouse fibroblasts. Since this line has been grown in culture for more than 20 years [24] it was supposed that certain genetical and morphological differences might exist between various sublines influencing their ability to detach from glass when subjected to sheering forces.

To investigate this we grew four sublines cultured for years in Danish or German laboratories. Prior to experiments all cell lines were grown in our standard medium [14]

Table 1. *The mitotic indices of different L-929 sublines upon mechanical synchronization. From each synchronous cell sample* 500 *cells were scored for mitotic figures.*

Synchrony	Mitotic index Cell lines			
No.	S	C	B_1	B_2
1	47·0	52·8	63·4	64·6
2	61·2	59·6	65·4	68·0
3	61·8	59·2	34·8	64·6
4	66·8	72·6	75·8	73·4
5	66·8	71·0	79·8	76·0

for several passages. On a given day five well-grown cultures were selected by microscopical examination from each of the four sublines and subjected to mechanical synchronization with intervals of 2 hr. As will be seen from Table 1, the four sublines differ in their reaction to mechanical forces which made us select the subline B1 for further experiments. All efforts to syndronize Burkitt lymphoma cells and SIRC cells were unsuccessful.

Characterisation of the synchronous cell system

Ideally, a synchronous cell system should fullfil a number of demands. The section of the cell cycle cut out for analysis should be well-defined and repeated syndronizations should yield about the same number of viable cells. Furthermore cell cycles should be synchronized to reach the second cycle simultaneously. In short, the method should not interfere with the cells and their mileu, but yield large quantities of biological material.

Variations found in mitotic index and in the number of detached cells upon repeated synchronizations are presented in Fig. 1. Usually desynchronization factors, resulting in the detachment of interphase cells, will decrease linearly during the first four shakings, and reach a 'steady-state' at about synchrony number five, yielding a mitotic index better than 80% from then on. As a consequence the first four synchronisations have been dis-

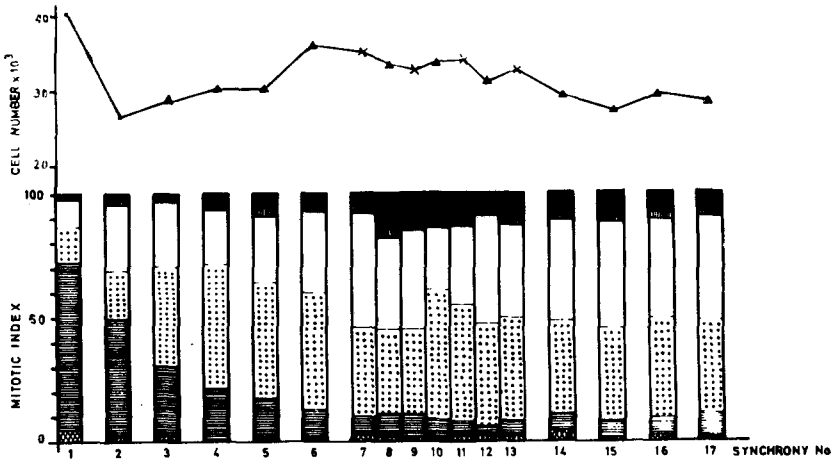

Fig. 1. *Variations of the mitotic index and cell number as a result of repeated synchronizations.* 2×10 *Blake bottles containing* 24 *hr old cultures* ($3 \cdot 5$–4×10^6 *cells/bottle*), *were synchronized by shaking with* 10 *ml medium. Ten bottles* (*group A*) *were synchronized as described in the text, while* 10 *others,* (*group B*) *were started after* 4 *synchronizations of group A. Of group B the first* 2 *samples were discharged while the following synchronisations were done alternating with group A. In this way synchrony number* 1–6 *and* 14–17 *became separated by* 2 *hr intervals, while synchrony number* 7–13 *coinciding with the suspected maximal DNA and RNA synthesis, had intervals of* 1 *hr.*

The 10 *ml from each sample was divided in two equal parts, one of which was used for mitotic index determination* (*lower part of the figure*). *The other half was used for cell counting* (*upper part*) *as described in* Methods.

Coulter settings: Attenuation: $0 \cdot 707$; *Aperture:* 16, *Treshold:* 25.
▲—▲: *group a*
×—×: *group b*
●—●: *discharged samples from group a*
hatched: pyknosis, horizontal lines: interphasis, dotted: prophasis,
vertical lines: anaphasis, blank: metaphasis, closed: telophasis

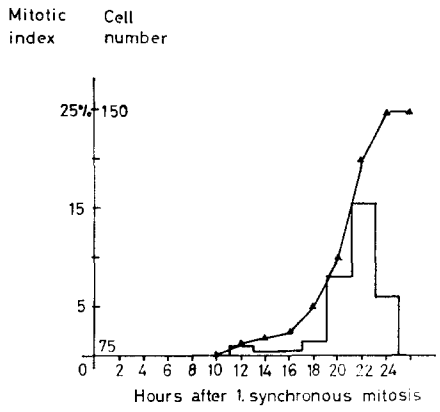

Fig. 2. The kinetic of the second synchronous mitosis. Histogram: mitotic index, solid line: number of living cells. For further details see Methods.

charged in all subsequent experiments. By scoring of individual mitotic figures, prophases constitute 40–55%, metaphases 25–45%, anaphases 1–5%, telophases 5–12%, interphases 4–18% and pyknosis 2–4% of the population.

The absolute number of cells detached are graphed in the upper part of Fig. 1. Since the mitotic index of randomly dividing *L*-cells in log-phase is about 4·5% it can be calculated, that these cells represent only 25% of the cells in mitosis at any given moment.

Time-lapse cinematography was used to investigate the viability and plating efficiency of mechanical selected mitotic cells (Figure 2). Time-lapse was done on cells growing in a modified Prior tissue culture chamber, at 37°C, in the dark. It was found that cells begin to adhere after about 20 min and that adhesion is completed after about 2 hr. However, this requires optimal conditions of growth, and temperature changes of 37°C±2°C protracts the time for plating to a maximal of 5 hr.

Under optimal conditions the first cells will adhere within minutes. Multiplication will begin after 18·5 hr, so that the cells double within 26 hr, with a plating efficiency and viability of 90–96%. Generation time in time-lapse studies were estimated to 22 hr± 10·5%. This means that the cells reach the second synchronous mitosis under a considerable loss in synchrony. Whether this be due to differences in generation time or to the different time used by individual cells for plating, or both, one can only speculate.

Biochemical characterisation of the cell system

The sequence of DNA and RNA synthesis in mechanically synchronized *L*-cells are graphed in Fig. 3. From this we estimated the duration of G1, S and G2 phase to an equal 8 hr duration. In our cells the main DNA synthesis (S-phase DNA) is preceded by a

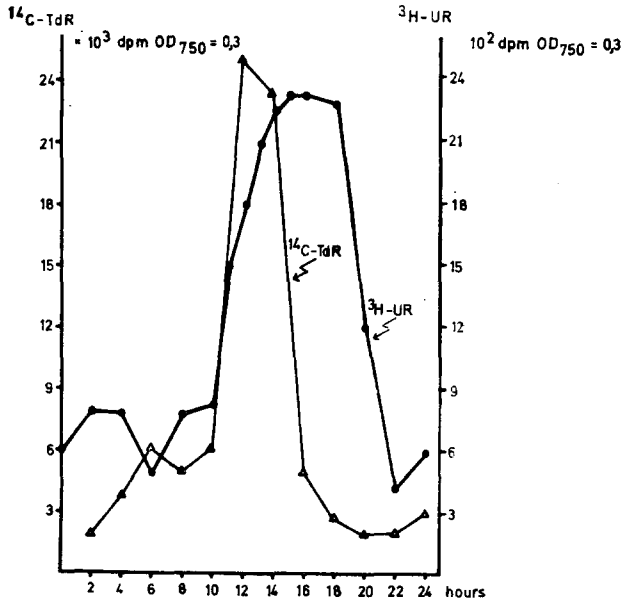

Fig. 3. Determination of the DNA and RNA synthesis in synchronized cells.
Twelve Blake bottles were synchronized in 2 hr intervals. From each sample 2 ml aliquots were taken for mitotic index determination.
After 24 hr when the first synchronized culture had passed its second synchronous mitosis, the cells were pulse labelled for 15 min at 37°C, in 10 ml slightly used (conditioned) medium comtaining the radioactive precursors ^{14}C-thymidine ($4{\cdot}44\times10^4$ cpm/ml medium) and ^{4}H-uridine ($6{\cdot}6\times10^5$ cpm/ml medium). The incorporation was stopped by washing three times with 5% PCA, 0°C, followed by hydrolysis in 4 ml 0·5 NaOH overnight at room temperature. Of the hydrolysates 1 ml aliquots were taken for radioactivity determinations, and 1 ml for protein measurements.

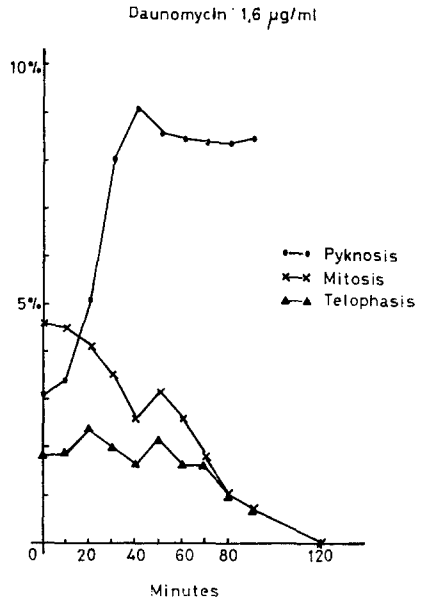

Fig. 4. Determination of the mitotic index of populations grown for various periods under the influence of 1·6 µg *daunomycin/ml medium.*

Cells were grown on object slides in Petri-dishes for 24 *hr. Then transformed to medium containing the cytostatic and grown for the indicated periods of time. Fixation was performed in Lillie's AAF fixative at* 0°C *for* 40 *min and the slides were stained Feulgen–Fast-green FCF. From each slide* 1000 *cells were scored for mitotic figures.*

burst of RNA synthesis in the G1 phase, beginning immediately after the first synchronous mitosis. At 6 hr, a drop in RNA synthesis is experienced, followed by another raise after 8 hr, just preceding the S-phase DNA synthesis by about 2 hr. In the S-phase reaching into the G2-phase the main RNA synthesis takes place headed by a five times increase in DNA synthesis from the 10th to the 14th hour. An interesting feature is the minor peak of DNA synthesis found in the G1-phase. This peak seems to be intimately connected with the second raise in G1 RNA synthesis and may possibley reflect a two-step kinetic of the DNA synthesis. Connected with the second synchronous mitosis a sharp fall in RNA synthesis is seen, delayed by about 4 hr from a parallel drop in DNA synthesis.

Effects of daunomycin on cell division, morphology and viability

To ensure that the experimental set-up was adequate for the investigation of daunomycin, a number of control experiments were carried out.

The most conspicuous change in randomly dividing *L*-cells treated with daunomycin, was a rapid decrease in mitotic index and a corresponding increase in pyknotic cells (Fig. 4). Generally pyknotic cells were found to be in interphase, but also direct action on mitotic cells was seen. Moreover an accumulation of mitosis in telophase and disappearance of all other figures suggest that daunomycin blocks some substances needed for cells to enter cytokinesis. Morphological alterations in the nucleus of cells treated for prolonged periods of time (24 hr, 1·6 µg daunomycin/ml) were quite obvious, leading to condensation of the nucleoli and an increase in heterochromatic material, followed by slightly impaired stainability of the cytoplasm (Fast–Green

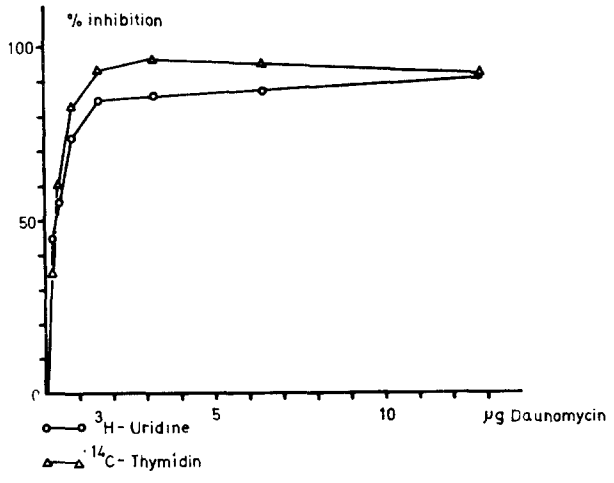

Fig. 5. Effects of different concentrations of daunomycin on the incorporation of ^{14}*C-thymidine and* ^{3}*H-uridine into PCA precipitable material of randomly dividing cultures.*

Nine Blake bottles, each containing approximately 2×10^{6} *cells/*80 *ml medium, were supplemented with* 1 *ml medium containg* 81 *times the indicated concentrations of daunomycin. After* 2 *hr the cells were washed once with* 20 *ml Hank's solution and then incubated for* 15 *min at* 37° *in* 20 *ml conditioned medium containing the radioactive precursors in concentrations as explained in the legend to Fig.* 3. *Termination of the reaction, hydrolysis, radioactivity measurements and protein determination were carried out as described in Fig.* 3.

FCF). The few mitotic figures seen in cultures treated for more than 20 min showed swollen and occasionally broken chromosomes, while the cells were generally slightly enlarged with granulated cytoplasms, containing a few vacuoles. The cells surviving treatment with daunomycin continued to grow and gain in volume for about 72 hr, then ceased, and stayed in culture as giant cells of more than double the normal volume. Apparently the treatment had caused them to enter a state of unbalanced growth [25, 26] characterized by cellular and nuclear enlargement. However, nucleoli remained hypoplastic even in these cells. Lately, studies in unbalanced growth caused by blockage of DNA synthesis by thymidine [27] have shown the stage to be reversible even after prolonged treatment. Similarly, we found the changes induced by daunomycin reversible even after 24 hours' inhibition of nucleic acid synthesis, cells entering mitosis shortly after removal of the inhibitor.

Effects of daunomycin on DNA and RNA synthesis in random cultures

In our cell system DNA synthesis is inhibited to about 50% of control values by concentrations of daunomycin about 0·2 μg/ml (Fig. 5). Maximal inhibition (95%) is reached at concentrations of 3·2 μg/ml. The inhibition is independent of further increases in the concentration of daunomycin within the range investigated. The inhibition of RNA follows an almost identical curve. From the observation that daunomycin binds to both native and denatured DNA *in vivo* and *in vitro* [7] and from a number of reports [28, 29, 30] on the uptake of heterologous high polymer DNA in mammalian cells by pinocytosis [31], we found it advisable to add high polymer DNA followed by daunomycin to randomly dividing log-phase *L*-cells. As a result we found an enhancement for the cytostatic in concentrations up to 6 μg/ml medium concerning the DNA synthesis, while for higher concentrations of daunomycin, the effect of added DNA was reversed (Fig. 6). That the increased inhibition of DNA synthesis is only partially dependent of added heterologous DNA is in accordance with the earlier observations [32]. These authors postulated that the number of binding sites for external DNA are limited and that these are saturated by doses of DNA slightly above 1 μg/ml. Addition of DNA alone caused no inhibition of cellular DNA synthesis.

To investigate the efficiency of added daunomycin as inhibitor of DNA and RNA synthesis

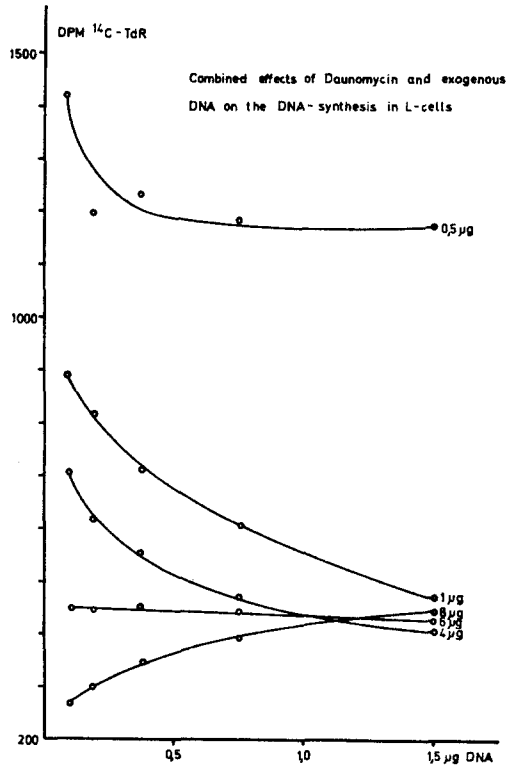

Fig. 6. Cells growing in Blake bottles received the indicated concentrations of DNA. The cultures were divided into five sublines each receiving the indicated concentrations of daunomycin after 20 min. After another 2 hr, the medium was changed to fresh medium containing the radioactive precursors ^{14}C-thymidine (4·44 × 10^6 cpm/ml medium). Each point represent one bottle.

in long term experiments, we chose from Fig. 5 a concentration of 1·6 μg/ml medium, attaining about 90% inhibition of DNA and RNA synthesis after 2 hr. This concentration was chosen since the oscillations caused by minor decreases in inhibitor efficiency would in this way be maximized.

Figure 7 indicates the time course of DNA and RNA synthesis when cells were subjected to daunomycin for 24 hr. After an initial drop to about 5% of normal DNA synthesis, a slow linear increase is noted leading to about 15% of control values after 20 hr. The RNA synthesis drops off parallel to the DNA synthesis reflecting the data shown in Fig. 5. After 6 hr the RNA synthesis reach a minimum of 20% of control values. Beginning at 12 hr an exponential increase in RNA synthesis is seen, leading to about 60% of normal values after 21 hr.

In an effort to elucidate the nature of this secondary increase for which more explanations might be forwarded, a number of control experiments were carried out.

Daunomycin enriched medium was transferred from cells treated for 12 hr to fresh cultures, while the treated cells had fresh medium without cytostatics. In the fresh

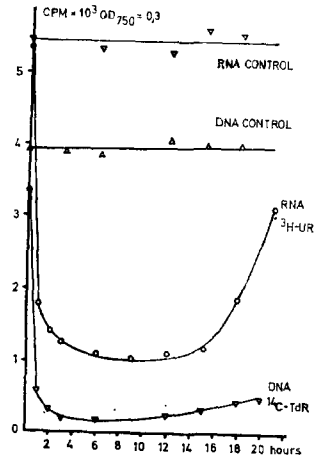

FIG. 7.

DAUNOMYCIN 1·6 µg/ml

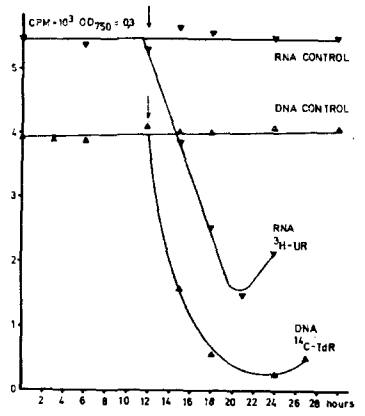

FIG. 8.

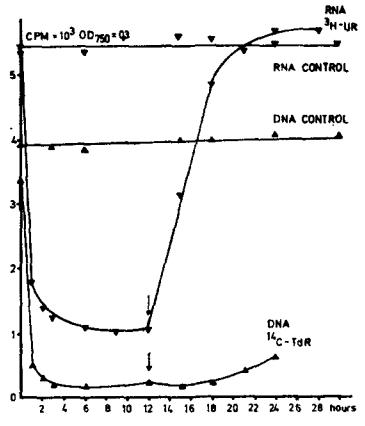

FIG. 9.

DAUNOMYCIN
1·6 µg/ml

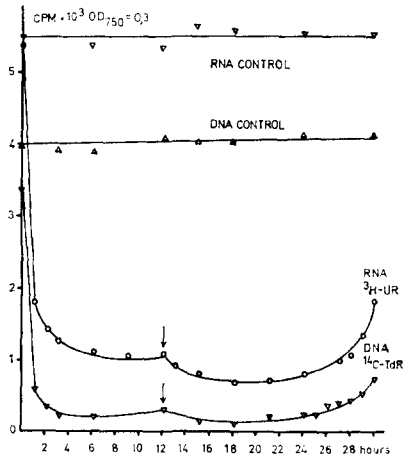

FIG. 10.

Figs. 7, 8, 9 *and* 10. *Determinations on the relationships between the duration of daunomycin treatment and macromolecular synthesis in randomly dividing cultures.*

Cells were grown in Blake bottles under continuous influence of daunomycin 1·6 µg/ml *medium. At the indicated times, cultures were washed once in Hank's solution, and then pulselabeled, terminated and further treated as indicated in the legend to Fig.* 3. *The arrows indicated (in Figs.* 8 *and* 10) *the addition of daunomycin as explained under results. The arrow in Fig.* 9 *indicated the removal of daunomycin from the cultures.*

cultures once more parallel drops in DNA and RNA synthesis (Fig. 8) were encountered both reaching a minimum at 10–11 hr, followed by a slow but still parallel increase. In the released cultures (Fig. 9) it was demonstrated that the inhibition of DNA synthesis is almost irreversible, in contrast to RNA synthesis, since the DNA synthesis remained on low values while RNA synthesis rapidly recovered to control values within 9 hr.

Possible decreases in cellular permeability in respect to daunomycin or acquired tolerance to the drug were investigated by adding another dose of the cytostatic in fresh medium to cells inhibited for 12 hr. This treatment postponed the secondary increase in macromolecular synthesis by about 12 hr and caused the RNA synthesis to remain on much lower values than after a single dose of inhibitor (Fig. 10).

Investigations on cellular fractions

Since even prolonged treatment with daunomycin or repeated high doses of the inhibitor could not eliminate the DNA synthesis completely, we took interest in the question whether nuclear and cytoplasmatic DNA synthesis would be inhibited to the same extent. As a consequence we isolated nuclei and mitochondrions from cells grown in the presence of daunomycin prior to pulse-labeling. While under these conditions the nuclear DNA synthesis can be reduced almost to zero values, no effect on mitochondrial DNA synthesis could be found. On the other hand, nuclear DNA seems to be able to serve as template

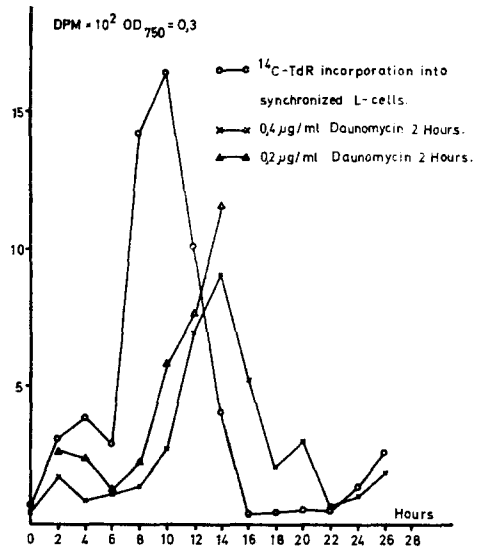

Fig. 11. *Determination of the influence on the DNA synthesis of the application of daunomycin to different points of the division cycle.*

Daunomycin were suspended in conditioned, refiltered medium and added to the synchronous cultures by changing 1 *ml growth medium with* 1 *ml daunomycin suspension, giving the indicated final concentrations of* 0·2 *or* 0·4 µg/*ml medium. After* 2 *hr cells were washed once with medium and then labeled for* 20 *min at* 37°*C with* ^{14}*C-thymidine (radioactive concentration:* 4·44× 10^6 *cpm/ml medium). The reaction was terminated by washing three times with ice-cold PCA, followed by hydrolysis in* 2 *ml* 0·5 M *NaOH at room temperature overnight.* 1 *ml hydrolysate was used for radioactivity determination while the other was used for protein measurement.*

Control cultures were treated in exactly the same way, except that they received 1 *ml conditioned medium without cytostatic* 2 *hr before pulse-labeling.*

Counts are referred to protein concentrations (OD_{750}±0·3) *and corrected for quinching by internal and external standards.*

for RNA synthesis even after conjugation with daunomycin, while the replication of DNA itself is arrested or strongly inhibited in spite of normal DNA-polymerase activities.

Effects of daunomycin on DNA synthesis in synchronous cultures

The addition of daunomycin (0·2 or 0·4 µg/ml) for 2 hr prior to pulse-labeling caused a delay in maximal DNA synthesis by about four hr (Fig. 11). It seems from the Figure that no special part of the DNA synthesis is hit by daunomycin, and that only quantitative differences exist between the inhibition caused by the high and the low concentration investigated. Whether the peak found in G2 phase represents fluctuations in mitochondrial DNA synthesis, as found in Physarum polycephalum [33], has not been established, but remains a tempting possibility.

Fig. 12. *Determination of the influence on the RNA synthesis of the application of daunomycin* (2 µg/*ml medium*) *to different points of the division cycle.*

The experiment was carried out as explained in the legend to Fig. 11, *except that the cells were pulselabeled with* ^{3}*H-uridine (radioactive concentration:* 6·6×10^5 *cpm/ml).*

Effects of daunomycin on RNA synthesis in synchronous cultures

While daunomycin does not show any preference for special steps in the DNA replication, RNA production (at least at this low drug concentration) (Fig. 12), is mainly inhibited in the S and G2 phase. Early RNA synthesis, which in *L*-cells is blocked by actinomycin D [12, 13] remains on control values as does the second pre-S phase RNA burste. This indicates that the effects of daunomycin on nucleic acid synthesis are not identical with those of actinomycin D. The results are in agreement with previous observations made in morphological and autoradiographic experiments [34] and also with the finding [13] that RNA's synthesized in late S-phase and G2 phase mainly constitute ribosomal precursors.

DISCUSSION

In the division cycle of normal and neoplastic cells, DNA synthesis takes place during interphase within a distinct time interval, separated from mitosis by two periods termed G1 and G2 respectively [35] during which no nuclear DNA synthesis is observed. A number of methods have been applied in studies of the division cycle in relation to the duration of the

different phases and the macromolecular synthesis. Of these autoradiography after incorporation of labeled DNA precursors, eventually in combination with quantitative cytophotometry, has been used as well *in vivo* as *in vitro* [35–40]. In addition, cell cultures may be partially synchronized by reversible inhibition of a process which is specific for one part of the cell cycle, an approach which permits analysis of relationships between different phases of the cell cycle. Such chemical synchronisation attempts have largely been based on inhibitors of the TTP-biosynthesis, (FUDR, amethoptherine)[41] which is known to regulate DNA synthesis [42] (for discussions see references 43, 17). Furthermore, in cell culture, the individuals in one part of the cell cycle may be separated from the remainder of the population by physical means. In our studies, a modification of the original method [23, 17] was used for the selective detachment of tenuously attached cells grown in monolayer cell cultures, resulting in production of cell populations with high mitotic indices, under near physiological conditions. Such cultures were found suitable for investigations on macromolecular synthesis and drug response in relation to cell cycle. In addition, such populations are excellent for basic investigations on mitosis and differentiation [21], in contrast to chemically synchronized cultures whose growth becomes quickly disturbed [42, 48, 49].

In our hands, the segment cut out of the cell cycle represents less than 5% of the generation time (22 hr) and since the percentage of mitosis exceeds 80% the synchrony was considered satisfactory for our purposes. However, the system has one major disadvantage since the cells reach the second synchronous mitoses somewhat out of step, mainly due to differences in generation time. So far a number of studies have been published in which various cytostatics and antimetabolites have been applied as more or less selective inhibitors to cells in different phases of the cell cycle in order to gain information on the interrelation between DNA synthesis and processes which might regulate or depend upon DNA synthesis. Among these actinomycin D was intensively studied [50], as an inhibitor of *m*-RNA synthesis. Thus, RNA synthesis in Physarum polycephalum was reduced by one half, following the addition of actinomycin D, in concentrations without effect on DNA synthesis, but delaying the mitosis after the interphase in which the inhibitor was added. Similar results have been obtained in mammalian cells [12, 13]. Like actinomycin D, daunomycin has been supposed to inhibit preferably the RNA synthesis while the DNA synthesis was inhibited only at higher concentrations, and therefore presumably possesses a biologic mechanism similar to that of actinomycin D. In studies on daunomycin and its effects on ^{32}P incorporation into DNA and RNA of 6C3HD ascites tumor *in vivo* and *in vitro* and of the regenerating liver after partial hepatectomy, it was found [11] that daunomycin is a more effective inhibitor of DNA than of RNA synthesis. These authors therefore suggested the two antibiotics to differ in respect to biological mechanism of action. Our observations lend support to this concept. The divergencies between [11] and earlier publications, on the primary effects on nucleic acid synthesis may be due to differences in observation time. This view would be in accordance with our finding, that inhibitions of DNA and RNA synthesis run parallel for a period up to 12 hr, at which time RNA synthesis is gradually released, suggesting that DNA synthesis is selectively inhibited. Contrarily, actinomycin D in small concentrations inhibits selectively the DNA directed RNA synthesis [51–53] which is preferently situated in the G1 phase of *L*-cells [13]. Daunomycin does not inhibit this synthesis at all. In contrast the synthesis of ribosomal precursors, mainly situated in the S and G2 phases [13] is suppressed to about one half, as is the DNA synthesis, suggesting a direct transcription of newly synthesized DNA in the S and G2 phases. Furthermore, the nucleolar changes found by us and others [34] seem to support the concept that ribosomal precursors are preferably inhibited by daunomycin.

Daunomycin, however, seems to be a multifocal depressor, since it reduces the mitotic index of treated cultures so drastically that virtually no mitoses are seen after 2 hr at a moment when at least some G2 phase cells should be expected to occur, having replicated their DNA before the addition of daunomycin. Furthermore, the cytostatic causes chromosomes swelling in cells when added after the beginning of mitosis.

Uptake and retention of daunomycin have lately been introduced in the discussion [54], as factors in drug response. However, these authors and others [7] found the uptake process to be unsaturable. In contrast, we found barriers to uptake since both DNA and RNA synthesis were inhibited only to a certain degree. Since inhibition has a maximum it seems reasonable to postulate a transport mechanism involving a limited number of

receptors situated at the cellular membrane. Further support of this hypothesis may be found in the observation that the addition of external DNA followed by daunomycin will cause a proportional increase in the inhibition of DNA synthesis. Since daunomycin is bound to DNA *in vitro* [7] which is pinocytosed by mammalian cells [31] to a limited degree depending on a saturable mechanism [32] this means that the supposed barriers to daunomycin uptake may be overcome by the addition of a secondary transport system. The data further suggest that the binding sites for daunomycin and those for daunomycin plus DNA are not identical as might be expected from the differences in molecular size.

RESUME

Les auteurs décrivent brièvement un système de cellules synchronisées, basé sur la sélection mécanique des cellules en mitose. L'index mitotique, lors de la première division synchrome, dépasse 80%. *Les cellules sont réparties de la façon suivante: prophases* 40 *à* 55%, *métaphases* 25 *à* 45%, *anaphases* 1 *à* 5%, *télophases* 5 *à* 12%, *interphases* 4 *à* 18%, *cellules en pycnose* 2 *à* 4%. *La seconde division synchrone est atteinte, par* 96% *des cellules, après un délai de* 22 *h.*$\pm$10,5%. *La durée des phases G*1, *S et G*2, *mesurée par l'incorporation de précurseurs marqués de l'acide désoxyribonucléique est, pour chacune d'elles, d'environ un tiers du cycle cellulaire.*

La daunomycine réduit l'index mitotique par une action au moment de l'interphase et par une action directe sur la mitose. Les modifications morphologiques observées sont discutées en rapport avec le phénomène de la croissance déséquilibrée. La nature de l'inhibition de la synthèse des acides nucléiques, causée par la daunomycine, est disuttée en fonction du temps d'observation de la perméabilité de la membrane et des fractions cellulaires. Enfin, les auteurs décrivent les résultats de leurs recherches sur l'inhibition séquentielle des fractions de l'acide nucléique.

SUMMARY

A synchronous cell system, based on the mechanical selection of mitotic cells, is briefly characterized. The mitotic index at the first synchronous division is better than 80%. *Prophases constitute* 40–55%, *metaphases* 25–45%, *anaphases* 1–5%, *telophases* 5–12%, *interphases* 4–18% *and pyknoses* 2–4%. *Second synchronous mitotis is reached within* 22 *hr*$\pm$10·5% *by* 96% *of the cells. The G*1, *S and G*2 *phases measured from the incorporation of radioactive-labelled precursors into DNA constitute each* 1/3 *of the cell cycle.*

Daunomycin is found to decrease the mitotic index both by action in the interphase and by direct action on mitosis. Also morphological changes are reported and discussed in relation to the phenomenon of unbalanced growth. Some obersvations on the nature of the inhibition of nucleic acid synthesis brought about by daunomycin is discussed in relation to observation time, membrane permeability and cellular fractions. Finally the results of investigations on the sequential inhibition of nucleic acid fractions are reported.

ZUSAMMENFASSUNG

Ein synchronisiertes Zellsystem, welches durch mechanische Selektion von Mitosezellen erhalten wurde, wird kurz charakterisiert. Nach der ersten synchronen Teilung betrug der Mitoseindex mehr als 80%. *Die erste Phase der Zellteilung macht* 40 *bis* 55% *aus, Metaphasen* 25 *bis* 45%, *Anaphasen* 1 *bis* 5%, *Telophasen* 5 *bis* 12%, *Interphasen* 4 *bis* 18% *und Pyknosen* 2 *bis* 4%. *Innerhalb von* 22 *Stunden trat bei* 96% *der Zellen* $\pm$10,5% *die zweite synchrone Mitose ein. Die* G_1, *S und* G_2 *Phasen, die nach der Inkorporation von radioaktivmarkierten Vorstufen des DNS gemessen wurden, machen je ein Drittel des Zellzyklus aus.*

Daunomycin reduziert den Mitoseindex sowohl durch Wirkung auf die Interphase als durch direkte Einwirkung auf die Mitose. Auch morphologische Veränderungen werden im Verhältnis zum gestärten Wachstum beschrieben und diskutiert. Einige Beobachtungen über das Wesen der Hemmung der Nukleinsäuresynthese, die Daunomycin bewirkt, werden im Verhältnis zur Beobachtungszeit, Permeabilität der Membranen und zu verschiedenen Zellfraktionen diskutiert. Schließlich folgt ein Bericht von Untersuchungen über die aufeinanderfolgende Hemmung der Bildung von Nukleinsäurefraktionen.

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La Sensibilité Différentielle d'une Tumeur Humaine et des Tissus Somatiques et Germinaux des Gonades Embryonnaires en Culture Organotypique *in vitro*

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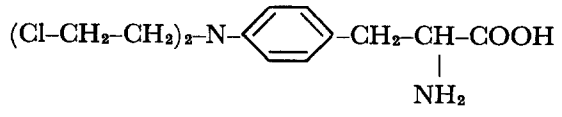
INTRODUCTION

LE MELPHALAN appartient à un groupe de substances, les agents alkylants [1], utilisés en chimiothérapie du cancer. L'Alkeran, ou melphalan (substance CB 3025), est un dérivé de l'ypérite azotée et a été fabriqué pour la première fois en 1953 par Bergel. La formule chimique est *p*–di (2 chloroéthyl) amino L phénylalanine:



La melphalan est le L isomère de la moutarde phénylalanine.

La phénylalanine sert de molécule porteuse d'un groupe cytotoxique. Sans cette molécule, la substance pénètre indifféremment dans les cellules normales ou dans les cellules cancéreuses [2]. L'activité antitumorale du melphalan dépend des propriétés optiques [3].

Lawley et Brookes [4] pensent que les moutardes doivent leur effet cytotoxique à une interaction avec l'azote de l'unité guanine de l'ADN, qui mène à une destruction de la chaîne nucléique.

Le melphalan a un effet inhibiteur sur un grand nombre de tumeurs animales, notamment la tumeur 256 de Walker, la leucémie 1 RC1 741, l'épithélioma T8 de Guérin, le carcinome de Flexner-Gobling, le carcinome d'Ehrlich [5], et le mélanome S91 [6]. Stubblefield et Hsu [7] ont essayé la substance sur les cultures de tissu *in vitro*.

Ils ont trouvé que les tissus normaux sont plus résistants que les tissus cancéreux. Le melphalan est utilisé dans le traitement des cancers lymphatiques et ovariens, et également dans les myélomatoses et les mélanomes.

Dans ce travail, nous avons étudié les effets comparés du melphalan sur les gonades de l'embryon de Poulet et sur les cancers humains transplantés en culture organotypique. L'intérêt des gonades est qu'elles comprennent un tissu somatique et des cellules germinales; celles-ci étant généralement très sensibles aux agents destructeurs, il était intéressant de comparer leur sensibilité au melphalan à celle des cellules cancéreuses.

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MATERIEL ET METHODES

La souche du cancer qui a été utilisée pour ces expériences a été maintenue en culture organotypique pendant six ans par Wolff et Wolff [8]. Cette souche appelée Z200 est une tumeur maligne humaine provenant d'une métastase hépatique d'origine gastrique [9]. La souche fut entretenue *in vitro* selon les techniques de culture organotypique mises au point par Wolff et Wolff [10, 11]. Les frag-

ments de tumeurs, prélevés directement sur le patient, étaient associés à des explants du mésonéphros d'embryon de Poulet de 8½ jours. Le mésonéphros fut séparé du cancer par un fragment de membrane vitelline d'oeuf de Poule. Plus tard, des explants de mésonéphros furent remplacés par un dialysat d'extraits de foie embryonnaire de Poulet de 8 à 10 jours [13].

Les gonades ont été prélevées sur l'embryon de Poulet, de la race Leghorn blanche, après 8 ou 10 jours d'incubation. Nous avons prélevé les deux testicules de chaque animal et les ovaires gauches des femelles.

Les gonades et le cancer étaient cultivés sur le même milieu. Ce milieu contenait de la gélose, du sérum, de l'extrait d'embryon de 8 jours, du dilaysat d'extraits de foie embryonnaire de 8 à 10 jours, et de la pénicilline. Les cultures furent maintenues à 37°C.

Le melphalan a été fabriqué par le Chester Beatty Research Institute, London. La substance fut dissoute dans de l'eau distillée stérilisée, et les solutions furent fraîchement préparées pour toutes les expériences. Les concentrations utilisées furent les suivantes: solution C 0,4 g pour 100 ml; solution D 0,67 g pour 100 ml; solution E 0,2 g pour 100 ml; et solution F 0,133 g pour 100 ml.

Le melphalan fut introduit, en faible quantité (0,04 ml par salière), dans le milieu de culture. Les concentration finales des solutions dans le milieu furent; solution C—0,66 mM, solution D—1,1 mM, solution E—0,33 mM, et solution F—0,22 mM.

Une partie des explants fut fixée après 7 jours, et l'autre fut repiquée sur un milieu frais et maintenue en culture pendant une deuxième semaine. Dans ce dernier cas, la substance fut ajoutée à certains milieux de culture, d'autres n'en contenaient pas.

La plupart des résultats sont d'ordre histologique. Les cultures furent fixées dans le liquide de Bouin et les coupes furent colorées à l'hématoxyline-éosine. Nous avons utilisé, comme critère de toxicité, l'intensité de la nécrose observée dans les cultures. Nous avons compté les gonocytes présents dans les gonades après la culture. Une numération était faite toutes les dix coupes.

Nous avons employé d'autres critères pour les expériences faites avec la solution C de melphalan. Nous avons dessiné les explants à la chambre claire, et nous en avons mesuré les surfaces avec un planimètre. Les possibilités d'erreur, dans ces dernières mesures, sont de 2,36 unités arbitraires (1 unité est égale à 9,046 mm^2).

Pour affiirmer qu'il y a une différence entre les résultats obtenus dans les cultures expérimentales et les cultures témoins, nous avons fait la comparaison entre les deux moyennes à l'aide du test de Student (calcul de t).

A. Action de différentes concentrations de la substance alkylante sur les tumeurs et les gonades

Les résultats que nous avons obtenus avec diverses concentrations de melphalan, introduites dans le milieu, sont rapportés dans le Tableau 1.

Aspect morphologique. Les gonades cultivées sur milieu contenant du melphalan avaient un aspect macroscopique différent des témoins. Elles devenaient plus petites et plus rondes. Les témoins et expérimentées s'aplatissaient en seulement après deux semaines de culture. On a remarqué également que les gonades témoins et expérimentées s'aplatissaient en leur milieu. Dans les cultures de cancer, nous avons observé des auréoles importantes de cellules nécrosées autour d'un noyau central sain.

Aspect histologique

1. *Forte concentration.* Pour la plus forte concentration (solution D 1,1 mM) le melphalan avait un effet très nocif sur les cellules cancéreuses. Sur 51 cultures, 10 moururent après une semaine. Des 41 restantes, 24 ont été repiquées. Les coupes histologiques de ces dernières montrèrent d'énormes masses de cellules nécrosées (Fig. 2, comparée avec le témoin Fig. 1). Parmi celles qui ont été repiquées une deuxième fois, soit sur un milieu avec melphalan, soit sur un milieu standard sans cette substance, toutes, sauf une, étaient en dégénérescence après 7 jours (Fig. 4).

Nous n'avons pas observé de nécroses dans les témoins. Ils grandissaient, étaient en bon état, et présentaient à l'histologie de nombreuses mitoses (Fig. 3).

Après une semaine de culture sur le milieu qui contenait la substance, les gonades d'embryons de 8 jours montrèrent une inhibition de croissance. Elles étaient beaucoup plus petites que les témoins. Histologiquement, elles ne montraient pas de nécrose, mais étaient complètement privées de gonocytes. La croissance des témoins n'était pas inhibée. Ils présentaient en moyenne plus de 1.000 spermatocytes par testicule et 2.000 oocytes par ovaire (Fig. 5 et 7). Les cellules somatiques des mâles et femelles avaient un aspect semblable à celles des témoins et elles ne semblaient pas être affectées par cette substance.

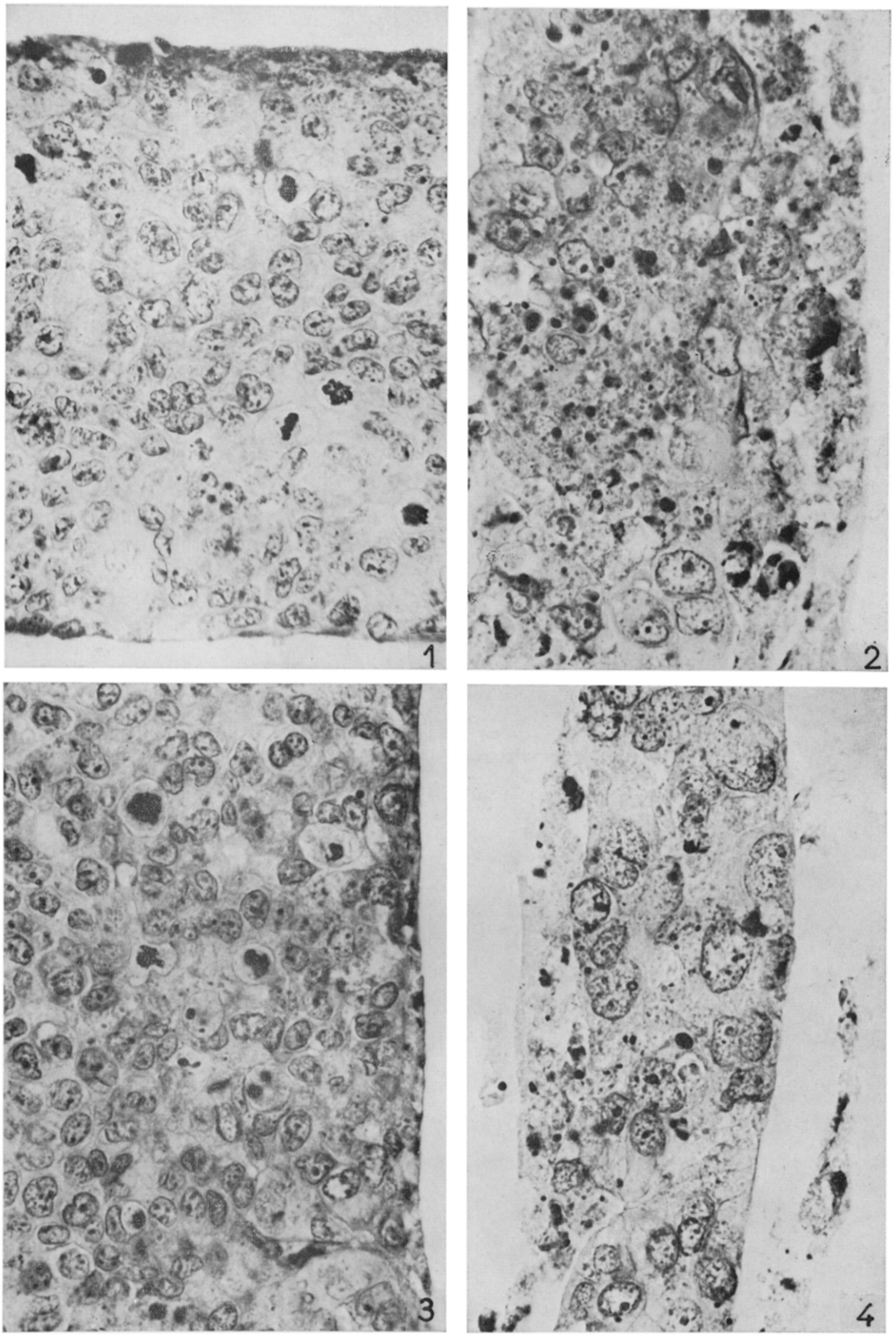

Fig. 1. *Cancer témoin, cultivé pendant* 7 *jours sur milieu standard.*

Fig. 2. *Cancer traité, cultivé pendant* 7 *jours sur milieu contenant du melphalan, à la concentration de* 0,4 *g*/100 *ml.*

Fig. 3. *Cancer témoin, repiqué pendant deux semaines sur milieu standard.*

Fig. 4. *Cancer traité, repiqué pendant deux semaines sur milieu contenant du melphalan, à la concentration de* 0,4 *g*/100 *ml. Noter l'absence de mitoses et l'hypertrophie pathologique des noyaux des cellules tumorales.*

(to face p. 332)

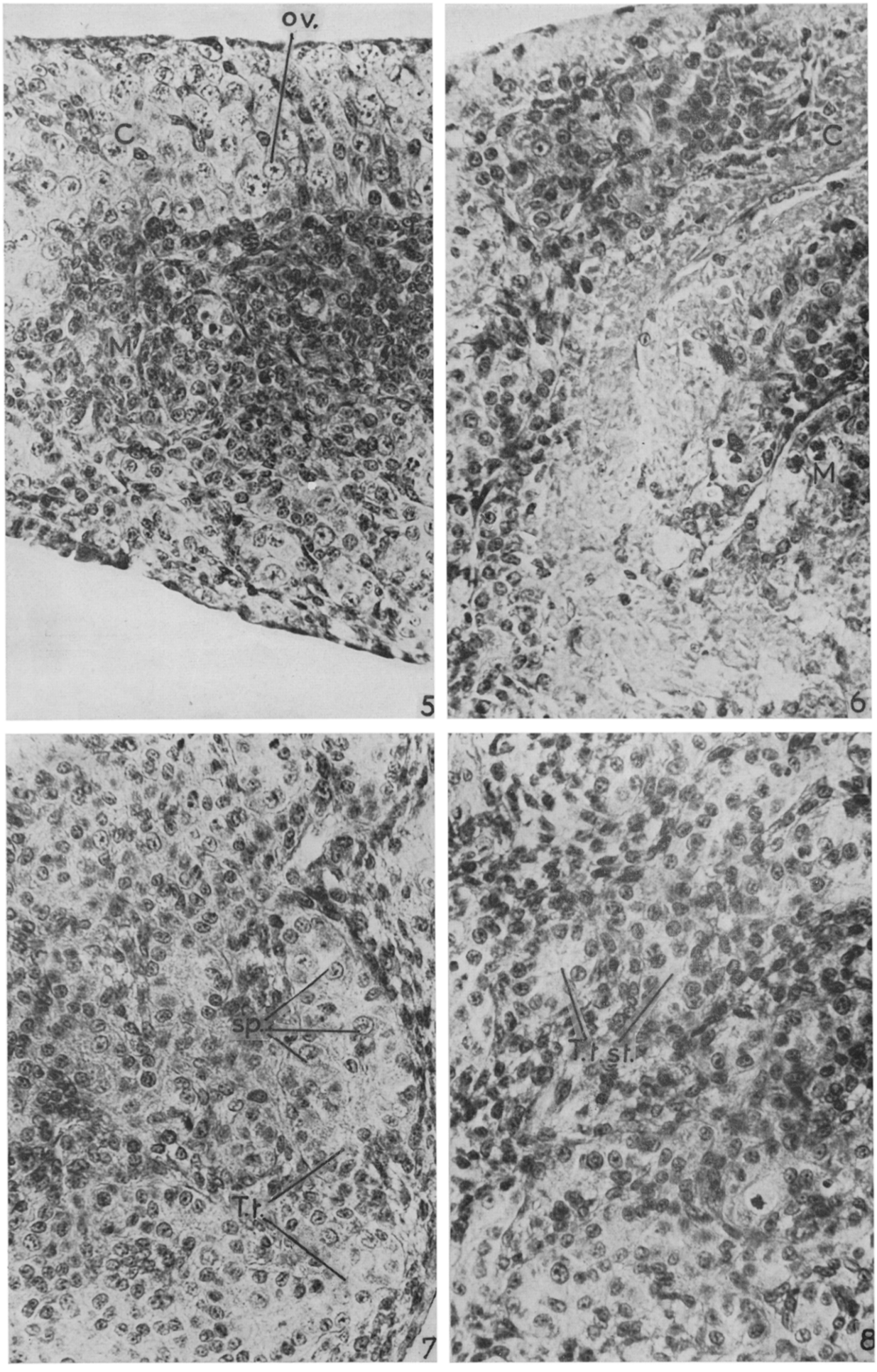

Fig. 5. *Ovaire témoin, cultivé pendant une semaine sur milieu standard.* $G \times 600$. *ov.—ovocytes; C—cortex; M—médulaire.*

Fig. 6. *Ovaire traité, cultivé pendant une semaine sur milieu contenant du melphalan à la concentration de* 0,4 *g*/100 *ml et ne montrant pas de gonocytes.* $G \times 100$. *C—cortex; M—medullaire.*

Fig. 7. *Testicule témoin, cultivé pendant une semaine sur milieu standard.* $G \times 600$. *sp.—spermatogonies T.t.—tube testiculaire.*

Fig. 8. *Testicule traité, cultivé pendant une semaine sur milieu contenant du melphalan, à la concentration de* 0,4 *g*/100 *ml; et montrant peu de gonocytes.* $G \times 600$. *T.t.st.—tube testiculaire stérile.*

Tableau 1. *Action du melphalan sur les cultures á diverses concentrations.*

	Concentrations	Cancer	Testicules	Ovaires
Après 7 jours de culture sur milieu contenant du melphalan	*D* 0,67 g/100 ml	Nécroses abondantes Pas de mitoses	Pas de nécrose Inhibition Pas de gonocytes	Pas de nécrose Inhibition Pas de gonocytes
	C 0,4 g/100 ml	Nécroses abondantes Pas de mitoses	Pas de nécrose Inhibition Peu de gonocytes	Pas de nécrose Inhibition Peu de gonocytes
	E 0,2 g/100 ml	Nécrose localisée	Pas de nécrose Peu de gonocytes	Pas de nécrose Peu de gonocytes
	F 0,133 g/100 ml	Pas de nécrose Peu de mitoses	Peu de nécrose Gonocytes présents	Pas de nécrose Gonocytes présents
Repiquées une deuxième semaine sur milieu sans melphalan	*D*	Mort de 100% des cultures	Peu de nécrose Pas de gonocytes	Peu de nécrose Pas de gonocytes
	C	Nécrose Mort de 99% des cultures	Pas de nécrose Inhibition Peu de gonocytes	Pas de nécrose Inhibition Très peu de gonocytes
	E	Nécrose localisée Quelques rares mitoses	Inhibition Quelques gonocytes	Inhibition Quelques gonocytes
	F	Pas de nécrose Mitoses fréquentes	Pas d'inhibition Gonocytes fréquents	Pas d'inhibition Gonocytes fréquents
Repiquées une deuxième semaine sur milieu contenant du melphalan	*D* 0,67 g/100 ml	Mort de 100% des cultures	Nécrose localisée Inhibition Pas de gonocytes	Nécrose localisée Pas de gonocytes
	C 0,4 g/100 ml	Mort de 99% des cultures	Nécrose localisée Peu de gonocytes	Nécrose localisée Pas de gonocytes
	E 0,2 g/100 ml	Nécrose localisée	Pas de nécrose Peu de gonocytes	Peu de nécrose Peu de gonocytes
	F 0,133 g/100 ml	Nécrose très localisée Mitoses rares	Pas de nécrose Peu de gonocytes	Pas de nécrose Peu de gonocytes

Après deux semaines de culture en présence de melphalan, les cellules somatiques furent atteintes par la nécrose. Chez les témoins des nécroses n'apparaissaient que dans quelques cas. Les témoins présentaient cependant une diminution de taille.

2. *Concentration moyenne.* Les cancers cultivés sur le milieu contenant du melphalan en solution C, concentration finale: 0,66 mM, montrèrent des résultats semblables à ceux obtenus avec la solution D. Les gonades montrèrent très peu de gonocytes, mais nous n'observâmes pas d'absence totale (Fig. 6 et 8). Pour cette concentration, les expériences seront décrites plus complètement aux paragraphes B 1 et 2.

3. *Faible concentration.* Avec de faibles concentrations des solutions E et F, 0,33 et 0,22 mM, l'effet sur les cultures fut moins prononcé. Les cellules cancéreuses étaient très nécrosées, mais elles reprenaient un aspect normal quand elles étaient repiquées sur un milieu sans melphalan. Les gonades présentaient peu de gonocytes, mais il n'y avait pas de nécrose.

B. Résultats quantitatifs obtenus avec une concentration constante de melphalan

Après incorporation du melphalan dans le milieu. La solution C avec melphalan, à la concentration de 0,4 g pour 100 ml, a été considérée comme la concentration la plus faible ayant un effet léthal sur les cellules cancéreuses. La concentration finale de cette solution dans le milieu est de 0,66 mM environ. Nous avons mesuré l'effet de la substance, à cette concentration, sur les cancers et les témoins, en évaluant la surface des explants et en dénombrant les gonocytes. Les mesures de la surface plus la variation standard sont présentées au Tableau 2.

Tumeurs. Sur 46 cultures cultivées sous l'influence de la substance, 7 étaient entièrement nécrosées. Des 39 restantes, 20 ont été repiquées. Après 7 jours, toutes étaient nécrosées sauf une (Tableau 3). En revanche, les 48 témoins cultivés pendant 7 jours étaient sains; 20 de ces témoins, repiqués durant la deuxième semaine, l'étaient encore. La croissance des cancers témoins (Fig. 9), après trois jours, est hautement significative. Mais,

Tableau 2. *Mersures de la surface des explants cultivés sur milieu contenant du melphalan (solution C) pendant 7 jours (unités arbitraires, 1 unité étant égale à 9046 mm²)*

	0 jour	Nombre de cas	3 jours	Nombre de cas	7 jours	Nombre de cas
Cancers avec melphalan	71,13±21	46	73±21	46	32,25±15	46
témoins	56,78±11	48	92,44±19	48	78,56±30	48
Ovaires avec melphalan	189±11	17	177±10	16	127±18	16
témoins	229±16	16	235±15	16	203±12	16
Testicules avec melphalan	84±25	32	111±18	30	101±23	30
témoins	77±14	32	103±20	32	117±35	32
					10 jours	n
			Cancer témoins		95,94±20	20

après 7 jours, il y eut une faible diminution de la surface. Cette diminution n'est pas significative; elle est probablement due à des conditions nutritives. En effet, si les cultures sont repiquées sur un milieu frais, la croissance reprend. La diminution de la surface observée pour les cancers traités est hautement significative.

L'examen histologique des cancers traités montra des nécroses abondantes, et aucune mitose n'était présente. Les témoins, en revanche, montrèrent beaucoup de mitoses, et il n'y avait pas de nécrose.

Testicules. Les gonades ont été prélevées à 10 jours. La mesure de la surface des testicules ne montra aucune différence statistiquement significative entre les gonades traitées et le témoins. Après 7 jours de culture, l'augmentation de la surface des témoins ne fut pas significative. Il en est de même pour les expérimentés (Fig. 10). On y observe beaucoup moins de cellules germinales mâles que dans les témoins: 601 contre 1.173 (Tableau 4).

Ovaires. Les ovaires cultivés sur les milieux contenant la substance se sont révélés très sensibles. Malheureusement la différence entre les témoins et les expérimentés était déjà significative dès le départ (0 jour). Ceci était dû au hasard. Donc les différences après 3 et 7 jours n'ont pu être comparées. Cependant, les surfaces mesurées des témoins pour 0, 3, et 7 jours de culture ne montrèrent aucune différence significative entre elles, alors que la diminution de surface des ovaires expérimentés entre 3 et 7 jours fut hautement significative (Fig. 11).

Aucun ovaire ne montrait de nécroses après 7 jours. Les cellules somatiques avaient un aspect semblable dans les expérimentés et les témoins. Les cellules germinales femelles étaient présentes en très faible quantité dans les expérimentés: 115 contre 4.345 dans les témoins (Tableau 4).

DISCUSSION

Le melphalan, utilisé à la concentration la plus forte, a un effet léthal sur le tissu cancéreux, et produit des nécroses importantes dans les gonades après deux semaines. Aux concentrations un peu plus faibles (solution C), l'effet est plus nuancé. La croissance des gonades est inhibée, mais il n'y a pas d'action sur les cellules somatiques. Les gonocytes cependant montrent une sensibilité aussi grande

Tableau 3. *Mortalité des cultures cultivées sur milieu contenant du melphalan*

	Nombre de cultures					
		Après 7 jours			Après 14 jours	
	Au début	Mortes	Vivantes	Repiquées	Mortes	Vivantes
Cancers avec melphalan	46	7	39	20	19	1
témoins	48	0	48	20	0	20
Ovaires avec melphalan	16	0	16	8	0	8
témoins	16	0	16	8	0	8
Testicules avec melphalan	30	0	30	15	0	15
témoins	32	0	32	15	0	15

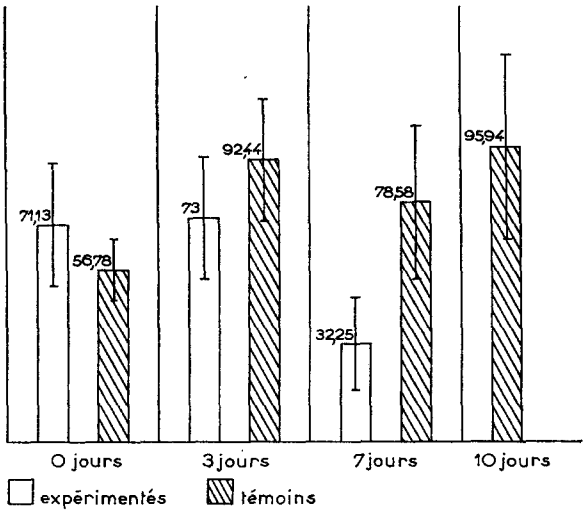

Fig. 9. *Variations de la surface des explants cancéreux cultivés sur milieu contenant le melphalan pendant* 7 *jours (unités arbitraires;* 1 *unité est égale à* 9,046 mm^2).

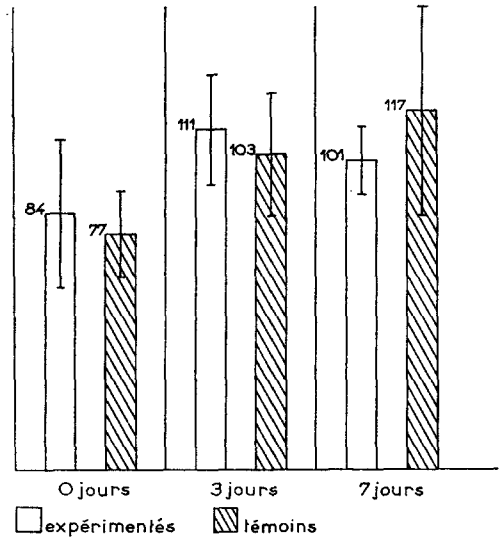

Fig. 10. *Variations de la surface des testicules cultivés sur milieu contenant le melphalan pendant* 7 *jours (unités arbitraires;* 1 *unité est égale a* 9,046 mm^2).

que les cellules cancéreuses. Les cellules germinales mâles sont également plus résistantes que les cellules germinales femelles. Johnson et Bergel [1] ont trouvé que le melphalan n'a aucune action sur la spermatogénèse des Rats adultes après injection *in vivo*.

Stubblefield et Hsu [7] ont essayé l'effet du melphalan sur les tissus en culture. Leurs expériences ont porté sur les tissus néoplasiques

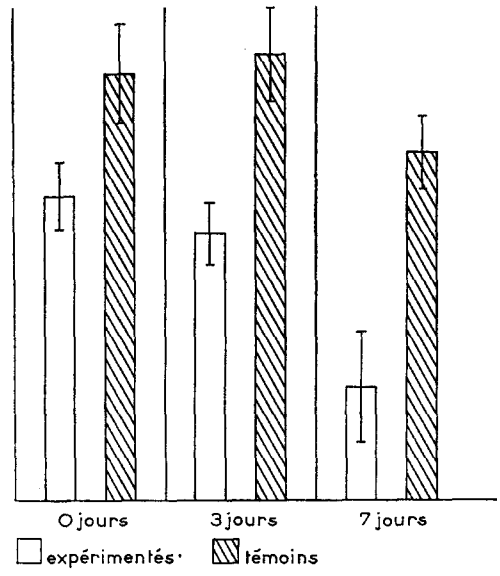

Fig. 11. *Variations de surface des ovaires cultivés sur milieu contenant le melphalan pendant* 7 *jours (unités arbitraires;* 1 *unité est égale à* 9,046 mm^2).

suivants: les cellules HeLa, le mélanome S-91 de Cloudman et le mélanome de Harding Passey, ainsi que sur les tissus normaux suivants: le coeur, les poumons et la peau de Souris de 16 jours et les papilles rénales de Souris adulte. A la concentration de 1 mM pendant 24 heures, tous les tissus sont morts. Mais aux concentrations d'environ 0,1 m-mole, l'effet était nuancé. Les mélanomes étaient plus résistants que les cellules HeLa, mais les tissus normaux étaient les plus résistants. Leurs résultats avaient porté sur les pycnoses de noyau. Ces auteurs avaient remarqué que, dans tous les tissus traités avec le melphalan, il y avait une absence de mitoses. Les résultats présentés ici confirment ceux de Stubblefield et Hsu.

Remerciements—L'auteur tient à exprimer sa reconnaissance au Professeur Etienne Wolff pour l'hospitalité qu'il lui a accordée dans son laboratoire, et pour les conseils qu'il lui a prodigués durant ses recherches. Il remercie vivement le Professeur F. Bergel et le Docteur J. L. Everett du Chester Beatty Research Institute, de Londres, qui lui ont fourni gracieusement les substances alkylantes.

Ce travail a été fait grâce à l'aide d'une bourse du Gouvernement français.

Tableau 4. *Nombre de gonocytes comptés après* 7 *jours (dénombrement portant sur* 1 *coupe toutes les* 10 *coupes)*

	Nombre de cas	Gonocytes
Testicules avec melphalan	11	601
témoins	11	1173
Ovaires avec melphalan	6	115
témoins	6	4345

RESUME

1. *Les cellules cancéreuses humaines et les gonocytes d'embryon de Poulet montrèrent une très grande sensibilité à la* p–*di* (2-*chloroéthyl*) *amino* L *phénylalanine. Leur traitement avec une concentration forte de* 0,67*g/ml, pendant une semaine, produisit des nécroses abondantes dans les tumeurs et une absence de gonocytes dans les gonades.*

2. *Les cellules germinales femelles traitées entre le* 10 *et le* 17*e, jour d'incubation furent plus sensibles que les cellules germinales mâles. Les testicules traités avec la* p–*di* (2-*chloroéthyl*) *amino* L *phénylalanine ne montrent que* 50% *de la quantité de gonocytes comptée dans les témoins. Les ovaires traités ne montrent que* 2,5% *de la quantité de gonocytes comptée dans les témoins.*

3. *Les tissus somatiques des gonades de l'embryon de Poulet sont plus résistant aux mêmes stades de traitement. Ils montrent une inhibition de croissance et un manque de mitoses, mais il n'y a pas de nécrose.*

SUMMARY

1. *A great sensitivity towards* p–*di* (2-*chloroethyl*) *amino* L *phenylalanine is shown by the cells of a human tumour and the gonocytes of the chick embryo. Treatment throughout one week with a strong concentration of* 0,67 *g/*100 *ml produces abundant necrosis in the tumour and a complete absence of gonocytes in the gonads.*

2. *Female germinal cells treated between* 10 *and* 17 *days of incubation show a greater sensitivity towards the* p–*di* (2-*chloroethyl*) *amino* L *phenylalanine than the male germinal cells. Testicles treated with the substance show only* 50% *of the number of gonocytes that can be counted in the controls. Ovaries treated show only* 2,5% *of the number of gonocytes counted in the controls.*

3. *The somatic tissue of chick embryo gonads shows the greatest resistance. Growth is inhibited and there is a distinctive lack of mitosis but there is no necrosis.*

ZUSAMMENFASSUNG

1. *Bei den menschlichen Tumorzellen und bei den Keimzellen des Hühnchenembryos wird eine große Überempfindlichkeit gegen* p–*Di*–(2-*chloraethyl*)-*amino*-L-*phenylalanin festgestellt. Nach einwöchiger Behandlung mit einer starken Konzentration von* 0,67 *g/*100 *ml wurden in dem Tumor reichlich Nekrosen beobachtet, sowie ein vollständiges Fehlen der Keimzellen in den Gonaden.*

2. *Weibliche Keimzellen, die zwischen* 10 *und* 17 *Tage inkubiert wurden, zeigten eine größere Überempfindlichkeit gegen* p–*Di*–(2-*chloraethyl*)-*amino*-L-*phenylalanin als die männlichen Keimzellen. Hoden, die mit der Substanz behandelt wurden, zeigten im Gegensatz zu den Kontrollen nur* 50% *der Keimzellen. Ovarien, die ebenfalls behandelt wurden, zeigten im Gegensatz zu den Kontrollen nur* 2,5% *der Keimzellen.*

3. *Das somatische Gewebe der Gonaden im Hühnchenembryo zeigt die größte Resistenz. Das Wachstum ist gehemmt; ein deutlicher Mangel an Mitosen ist erkennbar und es sind keine Nekrosen festzustellen.*

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Essai de Traitement des Leucémies Aiguës Granulocytaires par la Daunomycine*

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(O.E.R.T.C.)

LES RÉSULTATS publiés sur la capacité de la daunomycine (ou rubidomycine) d'induire des rémissions chez les patients atteints de leucémies aiguës granulocytaires sont très variables: Boiron *et coll.* [1] ont observé 51% de rémissions complètes, tandis que, pour d'autres [2, 3], la fréquence des rémissions complètes est de l'ordre de 20%. On sait que, en toute rigueur statistique, il n'est pas possible de comparer ces pourcentages, d'autant que plusieurs variables peuvent expliquer ces différences: âge des malades, stade de la maladie (première phase ou rechutes), modalités d'administration de la chimiothérapie, définition de la 'rémission complète'. En fait, pour avoir plus de précision sur le pourcentage réel d'induction de rémissions complètes dans les leucémies aiguës granulocytaires, il fallait faire un essai sur un plus grand nombre de malades. Aussi nous a-t-il paru utile que notre groupe soumette un nombre suffisant de patients atteints de leucémie aiguë granulocytaire à un schema thérapeutique homogène, semblable à celui qui avait donné les meilleurs résultats.

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Les demandes de tirés à part sont à adresser au secrétaire, le Docteur M. Hayat, Hôpital Paul-Brousse, Institut de Cancérologie et d'Immunogénétique, 14, Avenue Paul-Vaillant-Couturier, 94-Villejuif.

(1) *Les malades*

Soixante et onze sujets ayant une leucémie aiguë granulocytaire en phase détectable ont été inclus dans cet essai. Le diagnostic a été établi par un examen cytologique du sang et de la moelle osseuse.

Les malades étaient âgés de 9 ans à 75 ans; on comptait 37 sujets du sexe masculin et 34 sujets du sexe féminin. Tous les malades étaient en phase détectable de la maladie, mais celle-ci varie de la première à la troisième.

(2) *Modalités d'administration du produit*

La substance utilisée a été la daunomycine, présentée en ampoules de 20 mg. Elle a été

administrée par voie intraveineuse, selon des doses répondant au schéma suivant: lors d'une première injection, on administra 2 mg par kg de poids ou 70 mg par m^2 de surface corporelle. Quatre jours après, la dose de la deuxième injection dépendait du taux des polynucléaires. Si le taux des polynucléaires était égal ou supérieur à 2.000, on pouvait réadministrer la même dose si le madade n'était pas encore en rémission complète. Au contraire, si le taux de polynucléaires était compris entre 1.500 et 2.000, on ne donnait qu'une demi-dose lors de cette deuxième injection. Enfin, si le taux était inférieur à 1.500, on faisait un myélogramme pour voir si la moelle était aplasique ou non, et c'est selon ces résultats que l'on décidait de refaire ou non la deuxième injection de daunomycine. Les doses totales ont varié de 100 mg à 720 mg. La dose totale n'a pas été fixée à l'avance; elle fut soit la dose maximale tolérée, soit la dose suffisante pour obtenir un effet optimal.

(3) *Surveillance*

Les malades étaient hospitalisés et soumis à des examens cliniques bi-quotidiens et hématologiques tri-hebdomadaires. D'autre part, la surveillance de l'état cardiovasculaire fut très minutieuse, avec dosage de la créatine phosphokinase et électrocardiogramme systématiques.

(4) *Appréciation des résultats*

Les résultats furent appréciés sur deux sortes de symptômes: (a) cliniques, (b) hématocytologiques. L'essai ne comporta pas de sujets témoins, notre but, dans ce premier temps, n'étant pas de comparer l'efficacité de la daunomycine à celle d'un autre produit, mais seulement de rechercher si elle était efficace, et d'avoir une idée du degré d'efficacité. En revanche, chaque période de traitement par le produit à l'étude fut comparée à une période précédente, de durée variable, et pendant laquelle le patient pouvait avoir, ou non, reçu une autre substance. Nous considérons que l'on peut attribuer au produit à l'étude un effet favorable observé, si les symptômes de la maladie s'aggravaient ou demeuraient stationnaires pendant la période témoin.

OBSERVATIONS ET ANALYSE DES RESULTATS

(1) *Tolérance*

Comme cela a été déjà observé avec la daunomycine, les signes de toxicité sont de deux ordres: aplasie médullaire d'une part, et manifestations cardiovasculaire d'intolérance d'autre part. Sur les 71 malades inclus dans l'essai, on a observé 23 aplasies médullaires sévères avec taux de polynucléaires inférieur à 400 par mm^3. Dans de nombreux cas, il y a eu des complications infectieuses sévères, et l'on a observé notamment 4 septicémies, dont une à Pseudomonas et 3 à autres germes gram négatifs. Les complications cardiovasculaires ont été soit relativement bénignes, soit graves. Parmi les complications d'évolution favorable, il faut signaler les accès de tachycardie se manifestant aussitôt après la première injection de daunomycine, et qui ont été observés dans 5 cas, des modifications de l'électrocardiogramme avec, dans 1 cas, une sous-dénivellation du segment ST et, dans un autre cas, des extrasystoles supra-ventriculaires. Les complications cardiovasculaires graves ont été observées dans 3 cas, avec évolution fatale le lendemain de la première injection de daunomycine pour 2 d'entre eux. Il s'agissait, pour l'un des cas, d'une femme âgée de 69 ans, qui avait reçu 70 mg et qui, le lendemain, est décédée dans un état de collapsus irréversible. L'autre malade, âgé de 50 ans, avait reçu également une seule injection, et est décédée le lendemain de la première administration du produit. Il faut signaler encore les manifestations digestives qui ont été observées dans 5 cas, et consistaient essentiellement en nausées et vomissements.

(2) *Résultats*

Pour les apprécier, on distingue, au terme de la cure thérapeutique, quatre éventualités possibles. Il y a une rémission complète lorsque tout les signes cliniques ont régressé et que l'examen hématocytologique est normal, le médullogramme comportant moins de 5% de myéloblastes. La rémission est incomplète lorsqu'il y a régression de tous les signes, mais de façon incomplète, avec, dans le médullogramme, un taux de myéloblastes certes abaissé, mais restant pathologique. On dit qu'il y a échec non total lorsque peu de signes de la maladie ont régressé, et partiellement. La quatrième éventualité est l'échec total lorsque l'état reste stationnaire ou s'est aggravé.

(a) *Résultats globaux.* Les résultats d'ensemble sont résumés dans le Tableau 1: sur 71 cures thérapeutiques, on compte 18 rémissions complètes, 11 rémission incomplètes, 11 échecs partiels et 31 échecs totaux. Lorsqu'une rémission complète a été obtenue, la durée du traitement a varié d'une semaine à 5 semaines, et c'est en moyenne après la quatrième

Tableau 1. *Effets de la daunomycine dans les leucémies aiguës granulocytaires*

Phase détectable	Nbre de cas	Rémissions complètes	Rémissions incomplètes	Echecs non totaux	Echecs totaux
1	43	13	7	6	17
2	27	5	4	5	13
3	1				1
Total	71	18	11	11	31

injection que l'effet maximum du produit a été obtenu.

Dans ce type d'essai, il n'est pas possible d'apprécier la durée des rémissions puisque les malades sont soumis à une chimiothérapie d'entretien après la cure de daunomycine ayant permis d'obtenir une rémission apparemment complète.

(b) *Etude analytique des résultats.* L'étude analytique des résultats, comme on peut le voir dans le Tableau 1, montre que les rémissions complètes, ainsi que les rémissions incomplètes, paraissent plus fréquentes sur les premières phases détectables de la maladie. Il n'y a pas de différence selon le sexe, ni selon l'âge.

DISCUSSION

Le présent essai confirme donc la valeur de la daunomycine comme traitement des leucémies aiguës granulocytaires, mais il ne retrouve pas le haut pourcentage de rémissions antérieurement publié par certains [1]; il retrouve le pourcentage modeste indiqué par la plupart des autres auteurs [2, 3]. Il confirme la haute toxicité de la drogue, notamment cardio-vasculaire.

Ces résultats sont donc du même ordre que ceux obtenus par la méthylglyoxal-bis (guanylhydrazone) (26% de rémissions complètes selon Mathé *et coll.* [4]) et la cytosine-arabinoside (26% de rémissions complètes selon Mathé *et coll.* [5]).

Les résultats obtenus avec chacune des trois substances utilisées isolément sont particulièrement médiocres et incitent à étudier l'effet d'associations comportant une de ces drogues efficaces et d'autres composés qui ne le sont qu'exceptionnellement. Notre prochain essai consistera à étudier, sur un grand nombre de malades, l'association de prednisone, vincristine et daunomycine, cette dernière, donnée à moindre dose pour éviter les complications toxiques, notamment cardio-vasculaires.

Tableau 2. *Toxicité de la daunomycine dans* 71 *cas de leucémies aiguës granulocytaires*

		Nombre de cas
Toxicité hématologique	Sévère	23
	Modérée	14
Toxicité cardiovasculaire	Sévère	3 (dont 2 décès)
	Modérée	5
Toxicité digestive		5

RESUME

Les résultats obtenus par la daunomycine dans les leucémies aiguës granulocytaires sont très variables selon les auteurs, certains ayant publié plus de 50% *de rémissoins complètes. Le présent essai coopératif porte sur* 71 *malades;* 18 *rémissions apparemment complètes seulement ont été obtenues, soit* 25%.

SUMMARY

The results obtained with daunomoycin in the treatment of acute leukemias appear to be most variable according to the different authors some having found more than 50% *complete remissions. The present cooperative work included* 71 *patients:* 18 *remissions only (apparently complete) were obtained, i.e.* 25%.

ZUSAMMENFASSUNG

Die Ergebnisse von einer Daunomycin Behandlung in den akuten granulocytären Leukämien sind sehr verschieden den Autoren nach. Diese kooperative Arbeit betrifft 71 *Kranken: nur* 18 *anscheinend volle Remissionen wurden erhalten, d.h.* 25%.

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Unusual Blast Proliferation and Kinetics in Acute Lymphoblastic Leukaemia*

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INTRODUCTION

IN ACUTE leukaemia only a small quota of the blast population is in DNA-synthesis stage at any one time. It is however recognized that low ^{3}H-thymidine labelling indices (L.I.) in this disease [1–4] are not the result of long generation times but an expression of the fact that the blast population is composed of both dividing and non-dividing leukaemic cells [5–10]. Comparison of marrow and peripheral blood L.I.'s has regularly shown that marrow indices are significantly higher in acute leukaemia [4, 11–13] and this has been attributed to: (a) loss of the capacity to divide on the part of aging blasts [6] and (b) the elective release of such forms into the peripheral blood [7, 9, 12, 14, 15]. This has led to the statement that a non-proliferating compartment predominates in the peripheral blast population. Recent personal investigations suggest, however, that this statement is not of universal application in acute leukaemia. In some acute lymphoblastic cases, we observed clearly higher circulating blast L.I. and the present paper reports the results of *in vitro* and *in vivo* investigations of these patients.

MATERIALS AND METHODS

In vitro studies were carried out on 5 untreated acute lymphoblastic leukaemia (A.L.L.) cases in the 'steady state' (clinical and blood pictures stationary for 10–15 days before and during the experiment): blasts predominated in both the marrow and the peripheral cell populations in all patients (Table 1). The 5 cases under study were culled from 27 A.L.L. patients observed in this laboratory. Diagnosis was founded on the usual clinical and blood signs. In particular, the sudden onset of both the clinical and the blood pictures, unheralded by adenomegalia, excluded the possibility of lymphoma even in the 2 patients (cases 1 and 4) with frank leucocytosis. Spleen enlargement was patent in all cases and accompanied by hepatomegaly in cases 1, 2 and 5.

In vitro labelling with ^{3}H-thymidine was carried out by means of already reported techniques [1, 16, 17]. Previous studies have subdivided acute leukaemia blasts on the basis of diameter [6], with higher L.I. in larger cells and nil values in blasts of smaller diameter (Fig. 2). Our blast populations were therefore divided into a proliferating and a non-proliferating compartment on the basis of the thymidine uptake of cell classes of different diameters (in μ) [18]. This division was itself made subject to a correction factor of $10\% \pm 1{\cdot}5$, representing the mean increase in cell diameter during the S phase. This factor was obtained by comparing the mean diameter values of a ^{3}H-thymidine pulse-labelled cohort: (a) 1 hr after labelling; (b) immediately after division and at different times after the first

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Table 1. Clinical data, labelling index (L.I.) and mitotic index (M.I.) values, and proportion of proliferating blasts in 5 cases of acute lymphoblastic leukaemia

No.	Case	Age	Disease stage	Peripheral blood					Bone marrow			
				No. cells/ mm³	Percent. of blasts	M.I. (‰)	L.I. (%)	Evaluated proportion of prolif. blasts (% ± S.D.)	Percent. of blasts	M.I. (‰)	L.I. (%)	Evaluated proportion of prolif. blasts (% ± S.D.)
1	M.C. ♂	8	Steady state	390·000	99	1·8	6·1	67±2·0	99	2·1	5·9	56±2·6
2	D.P. ♂	2	Steady state	31·000	85	0·9	11·9	74±4·2	89	1·6	7·4	58±4·4
3	M.A. ♀	9	Steady state	18·100	96	1·8	19·9	64±3·7	99	2·2	5·9	51±4·5
4	R.B. ♀	2	Steady state	105·000	98	2·1	3·9	73±1·7	97	1·5	1·9	38±1·3
5	M.E. ♀	5	Steady state	2900	66	0·7	6·4	47±2·9	99	1·4	1·7	33±2·2

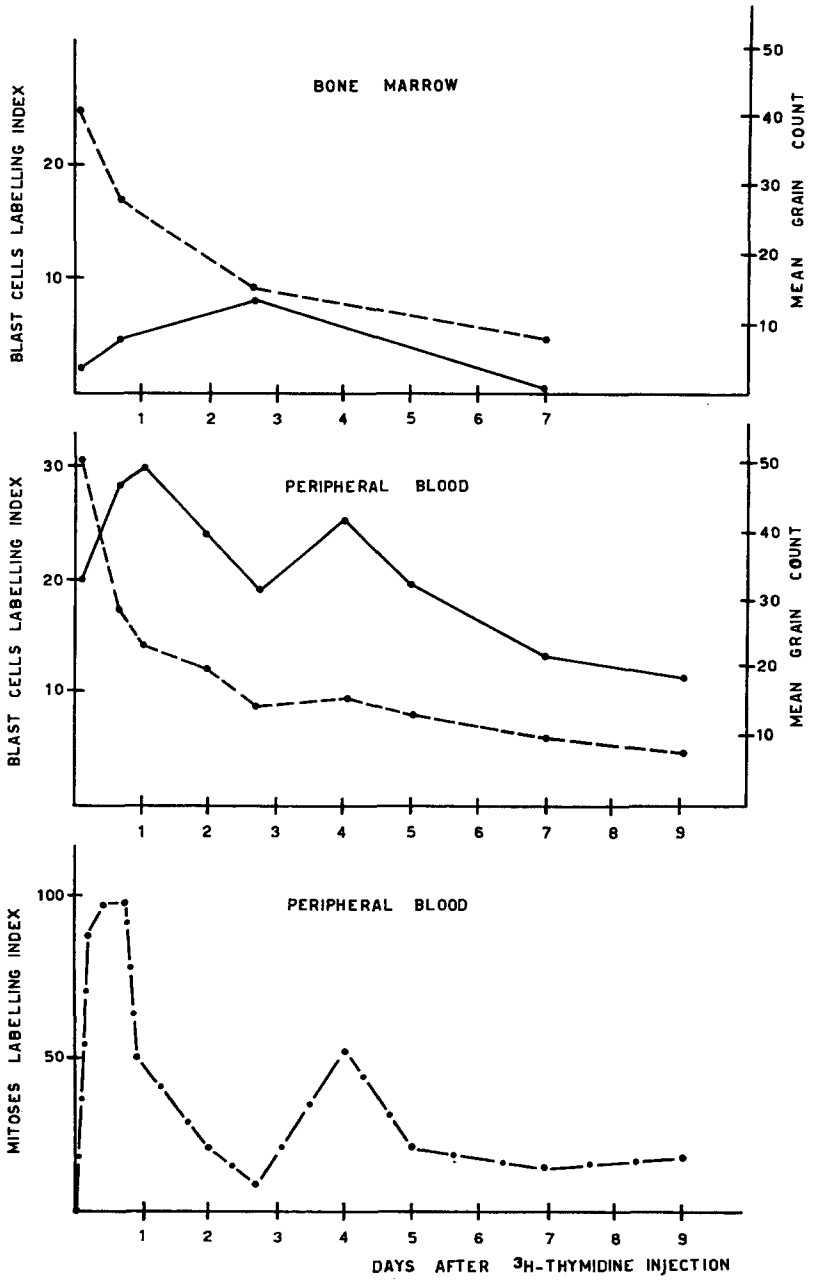

Fig. 1. Labelling index of blast cells ——, mean grain count - - - - - and mitosis labelling index —·—·— after in vivo *injection of thymidine-³H.*

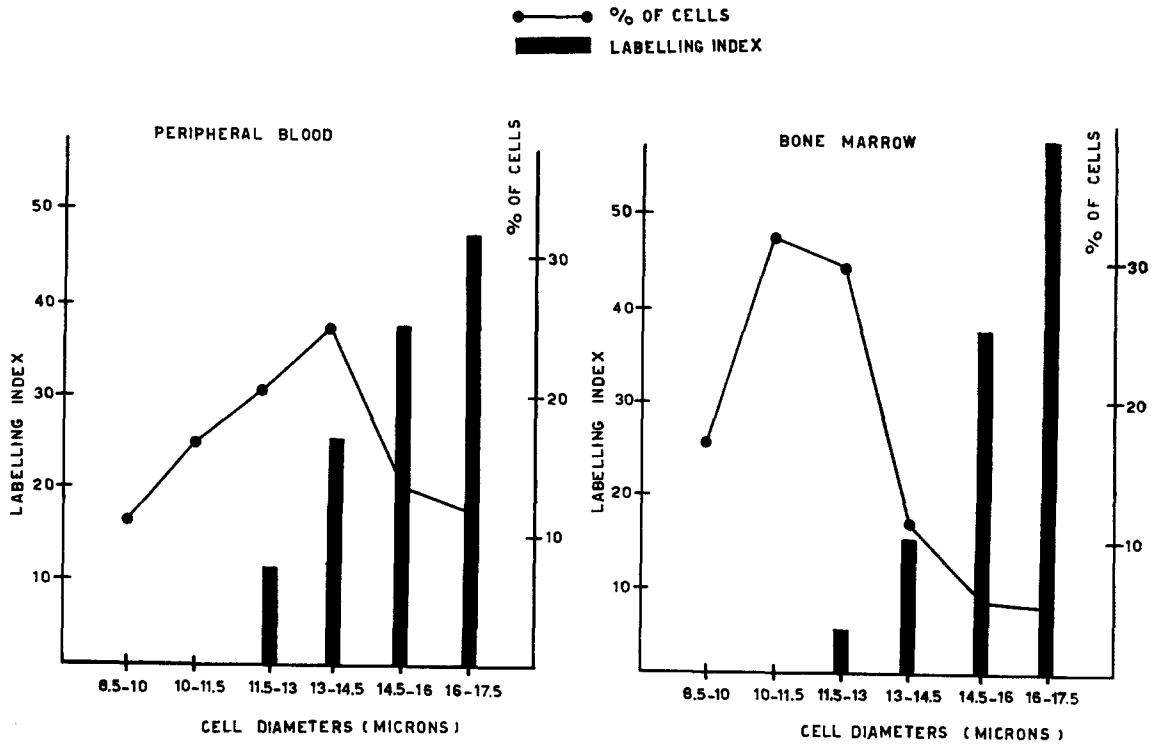

Fig. 2. Percentage of blast distribution and labelling indices in 6 classes with different diameters 1 hr after in vivo *thymidine-^3H injection.*

grain halving. Possibly significant differences in cell size, attributable to slide characteristics, were also taken into account. At least 500 cells were measured in each slide area and χ^2 analysis showed that no meaningful differences existed between different slide areas or between different slides with substantially similar cell densities per unit of surface area.

Evaluation *in vivo* was also carried out in one A.L.L. case (M.A.). Serial bone marrow samples (1, 18 and 66 hr, and 7 days) were obtained after a single intravenous injection of ^{3}H-thymidine (0·2 μC/g of body weight; specific activity 2 C/mM). Blood smears were taken at frequent intervals till the 9th post-injection day.

Standard techniques were used in the preparation of autoradiographic marrow and peripheral blood smears [8], which were exposed for 150 days at 4°C. L.I. values were determined on at least 1000 cells, mean grain counts (M.G.C.) on at least 200 labelled blasts, and labelled and non-labelled mitosis figures were counted on at least 50 figures at each interval. Blast division into proliferating and non-proliferating compartments (including the application of the correction factor) was determined on 1 hr post-^{3}H-thymidine labelling marrow and blood samples as already described (Fig. 2).

In all cases, the mitotic index (M.I.) was determined from the number of mitotic figures per 10000 cells observed in May-Grünwald–Giemsa stained bone marrow and peripheral blood smears.

RESULTS

Table 1 shows clinical data, L.I. and M.I. values, and proportion of proliferating blasts in 5 cases of A.L.L. In all cases a higher L.I. and a higher proportion of large proliferating blasts in peripheral blood as opposed to marrow are present; except for case 4, however, M.I. values were lower in the peripheral blood.

Figure 1 shows the peripheral blood and marrow L.I., M.G.C. and peripheral blood labelled mitosis curves after *in vivo* injection of thymidine. By comparison with the monophasic pattern of the bone marrow L.I. curve, that of the circulating blasts presents two clear peaks reaching highly significant levels, at the 24th hour and the 4th day.

The blast population was subdivided on the basis of cell diameter so as to identify the proliferating compartment (Fig. 2). This method gave proliferating compartment percents of 64% (peripheral blood) and 51% (marrow) respectively. These values are close to those given by the ratio between cell and mitosis L.I. values after desynchronisation of the labelled blast cohort [19]. Values of 0·61 (peripheral blood) and of 0·26 (marrow) were obtained on the 9th and on the 7th post-injection day respectively. This similarity is

particularly striking with respect to the peripheral blood whereas a wide range of difference is observed in marrow levels. Here, however, it may be noted that Mendelsohn's formula is only of limited value since it cannot be applied to cases of intercompartmental migration.

Since the number of samples available was sufficiently large, the duration of the S phase was determined on peripheral blood in terms of the time interval between the upward and downward halfway points of the labelled mitoses percentage curve. A value of 19 hr was obtained.

Three generation times were determined: (a) 86 hr, representing the interval between 2 successive peaks of the labelled mitoses curve; (b) 61 hr, being the value given by the formula: $Ns/Np = Ts/Tg$, where Ns=number of labelled cells, Np=number of proliferating cells, Ts=duration of DNA synthesis, Tg=generation time [20]; (c) 99 hr, being the value given by the formula: $Ms/M = Ts/Tg$, where Ms=number of labelled mitoses after desynchronisation, M=total number of mitoses [21].

DISCUSSION

Various points of interest emerge from these data. In the first place, L.I.'s were higher and the proliferating blast compartment was of greater size in the peripheral blood in all 5 cases; a considerable number of peripheral mitotic figures were also present in all patients. These findings are unusual since the normal acute leukaemia picture is one of greater proliferating activity in marrow and the elective release into the peripheral blood of aged, non-dividing blasts which have matured or mature to sterile forms [14]. It should also be noted that relatively few mitotic figures were observed in the peripheral blood in spite of the high L.I. values, especially in cases 2 and 4. As a result, the S period would be extraordinarily long — about 100-fold that of mitosis — in these 2 cases. It seems likely that cells in mitosis are withdrawn from the circulating blood. That this occurs at random — i.e. bears no relation to the labelling or non-labelling of the figures — is shown by the presence of a 100% labelled first wave in Fig. 1. This random sequestration of mitotic figures justifies the employment of mitosis L.I. values in our kinetics evaluation. Taken as a whole, our data suggest the hypothesis that the preferential site for the production of new leukaemic cells lies outside the marrow, with a resulting marrow-wards movement of blasts. Some of the *in vivo* findings can be interpreted in this light. Figure 1 shows that in peripheral blood — i.e. the area of more intense proliferative activity — there is a 50% increase in L.I. after the first grain halving. This is followed by a return to the start value and then by a second significant increase on the 4th day. In marrow, on the other hand, the L.I. made a distinct increase to 3 times the start value by the 66th hour. Apart from unusual proliferate activity, three other points of interest emerge from the peripheral blood data: (1) the 50% L.I. increase is less than would be expected if all cells remained there, and is in sharp contrast with the outstanding increase observed in marrow; (2) persistence of high peripheral L.I. values on the 7th and 9th post-thymidine days as compared with virtually nil marrow levels: this finding suggests a primarily extra-medullary origin for circulating blasts; (3) the two-peak pattern of the L.I. curve, with the second peak occurring at the 4th day: this could be the result of a fresh surge of labelled blasts into the peripheral blood from another side. These features would seem to be more in keeping with a kinetic view of the peripheral blood as a transit area, fed by the arrival of blasts and capable, in turn, of supplying other areas, rather than as a terminus area, as is usually suggested. This view will require further study, with special attention to lymphnode, spleen and thoracic duct lymphoblast kinetics, not forgetting the recirculation of lymphocytes. For the moment, it can safely be said that blast circulation in A.L.L. may be substantially different to that in other (particularly myeloblastic) leukaemias.

RESUME

Dans cinq cas de leucémie lymphoblastique aiguë (tirés d'un matériel plus vaste de leucémies lymphoblastiques aiguës), l'indice de marquage par thymidine-^3H des éléments blastiques circulants s'est révélé supérieur à celui des éléments blastiques médullaires et, au niveau périphérique, une plus large dimension du compartiment proliférant a été observée.

Les recherches cinétiques, effectuées in vivo *dans l'un de ces cinq cas, ont permis de préciser la dimension du compartiment proliférant et non proliférant de la moelle et du*

sang périphérique et, en outre, un certain nombre de particularités du comportement cinétique de la population blastique ont été relevées par rapport à ce qui peut être communément observé dans les cas de leucémie myéloblastique aiguë.

Dans ces cas particuliers de leucémie lymphoblastique aiguë, les auteurs suggèrent donc une origine surtout extramédullaire des éléments leucémiques, ce qui fait que les éléments circulants, au lieu de représenter, comme dans les cas de leucémie myéloblastique aiguë, un territoire terminal, présenteraient un territoire de passage avec possibilité de recirculation.

SUMMARY

In five cases of acute lymphoblastic leukaemia (taken from a larger series of acute lymphoblastic leukaemias) the thymidine-^3H labelling index of circulating blast cells proved to be higher than that of bone marrow blasts. It was also found that the proliferating compartment was larger at peripheral level.

In vivo *kinetic studies of one of the five cases made it possible to quantify the sizes of bone marrow and peripheral proliferating and non-proliferating compartments. Some features of blast population kinetic behaviour differing from what is normally observed in cases of acute myeloblastic leukaemia were also brought to light.*

A prevalently extra-medullary origin of leukaemic cells is proposed for these special cases of acute lymphoblastic leukaemia. Thus, the circulating cells may not represent a terminal zone as in acute myeloblastic leukaemia but a transit zone with recirculation possibilities.

ZUSAMMENFASSUNG

In fünf Fällen von akuter lymphoblastischer Leukämie (einem umfangreicheren Krankengut von akuten lymphoblastischen Leukämien entnommen) erwies sich der Markierungsindex mit ^3H-Thymidin der zirkulierenden blastischen Elemente höher als jener der blastischen Markelemente und in der Peripherie wurde ein größerer proliferierender Anteil beobachtet.

Die kinetischen Untersuchungen, welche in vivo *in einem dieser fünf Fälle durchgeführt wurden, ermöglichten es, das Ausmaß des proliferierenden und des nicht proliferierenden Anteils des Markes und des peripheren Blutes näher zu bestimmen. Ferner wurden einige Eigentümlichkeiten des kinetischen Verhaltens der blastischen Zellpopulation im Vergleich mit den üblichen Beobachtungen in Fällen von akuter myeloblastischer Leukämie festgestellt.*

In diesen besonderen Fällen von akuter lymphoblastischer Leukämie wird auf die zum größten Teil extramedulläre Herkunft der Leukämieelemente gedeutet, so daß die zirkulierenden Elemente, anstatt wie bei akuter myeloblastischer Leukämie einen Endbezirk, hier einen Durchgangsbezirk darstellen mit der Möglichkeit, wieder in den Kreislauf einzugehen.

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Ultrastructural Studies on Normal and Leukaemic Human Haematopoietic Cells

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INTRODUCTION

THE MOST important single step in the demonstration that a virus is involved in the aetiology of a leukaemia is the transmission of the disease by means of a cell free filtrate of material from a known case. Useful supporting evidence can, however, be obtained by the demonstration of virus particles in the leukaemic cells or other cells of the diseased animals by electron microscopy. In man transmission experiments pose obvious difficulties and therefore numerous attempts have been made to support the idea of a virus aetiology of human leukaemia by examining material from leukaemic patients in the electron microscope. Much of the work done in this field has been on negatively stained or thin sectioned plasma pellets. The difficulty in interpreting the findings in such material is considerable and frequently authors come to quite contradictory conclusions [1, 2].

Two types of particle would be of special interest. A herpes-like virus has been found to be associated with cultured cells from lymphomas and leukaemias in man [3] and a similar particle [4] appears in chickens with Marek's disease, one of whose features is the occurrence of lymphomas. Secondly, type-C particles occur in the murine, avian and feline leukaemias. It is now recognised that it is especially important to look for budding type-C particles. Amongst the great many reports of the finding of 'virus' in human leukaemic material there are only rare reports of budding [5]. Newell *et al.* [2] stress the need for more work on thin sectioned cellular material.

The present report deals with the examination of thin sectioned material from leukaemic patients and controls for virus-like particles. At the same time any unusual features were noted in an effort to detect any cellular features which may be specific for a particular kind of leukaemia.

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MATERIAL AND METHODS

Blood and bone marrow samples

Blood samples were examined from 38 patients with leukaemia and 12 subjects with a normal haematological picture. Bone marrow samples were examined from 12 patients with leukaemia and from 12 individuals with a normal haematological picture. Plasma pellets were examined from two leukaemia patients and one haematologically normal control, while one lymph node biopsy was examined from a case of chronic lymphatic leukaemia.

The different types of leukaemia included in the study were chronic myeloid leukaemia (13 bloods, 9 bone marrows, 1 plasma pellet), acute transformation of chronic myeloid leukaemia (7 bloods, 2 bone marrows), acute myeloid leukaemia (5 bloods, 1 bone marrow, 1 plasma pellet), acute monocytic leukaemia (4 bloods), acute lymphatic leukaemia (5 bloods) and chronic lymphatic leukaemia (4 bloods and 1 lymph node biopsy).

Full clinical and haematological studies

were made on all 38 leukaemic patients. Table 1 gives details of the age, sex, treatment and state of health at the time the samples were taken. The latter is included since Levine *et al.* [1] have suggested that this might influence the frequency with which virus-like particles are observed.

The blood samples were all obtained by

Table 1. *Details of patients included in the study*

No.	Age	Sex	Diagnosis	State of health	Treatment prior to sample	Specimen			
						PB	BM	LN	PP
1	56	F	CML	Well	Nil	+			
2	65	F	CML	Well	Busulphan	+	+		
3	72	M	CML	Well (Rem)	Busulphan	+			
4	64	M	CML	Ill	Nil	+			
5	40	M	CML	Well (Rem)	Busulphan	+			
6	69	M	CML	Well	Nil	+	+		
7	40	M	CML	Well	Nil	+	+		
8	37	F	CML	Ill	Nil	+	+		
9	52	F	CML	Well (Rem)	Busulphan	+			
10	44	F	CML	Well (Rem)	Busulphan	+	+		
11	70	M	CML	Ill	Splen. Irr.	+	+		
12	51	M	CML	Well (Rem)	Busulphan	+			
13	65	M	CML	Well	Nil	+			+
14	44	M	CML	Well	Nil		+		
15	58	F	CML	Well	Nil		+		
16	50	M	CML	Ill	Nil		+		
17	61	F	CML–A	Well	6M-P		+		
18	25	M	CML–A	Very ill (Rel)	Busulphan	+			
19	43	F	CML–A	Very Ill (Rel)	Splen. Irr.	+			
20	36	F	CML–A	Very Ill (Rel)	Splen. Irr. 6 M.P. Prednisolone	+			
21	49	F	CML–A	Ill (Rel)	Splen. Irr.	+			
22	54	F	CML–A	Very Ill (Rel)	Busulphan Splen. Irr.	+			
23	60	F	CML–A	Ill	Nil	+			
24	20	M	CML–A	Very Ill (Rel)	Busulphan	+	+		
25	71	F	AML	Very Ill	Nil	+	+		
26	29	M	AML	Ill	Prednisolone	+			
27	62	M	AML	Ill	Prednisolone 6 M.P.	+			+
28	66	F	AML	Very Ill	Nil	+			
29	56	M	AML	Ill	Prednisolone	+			
30	53	F	A. Mono L.	Very ill	Nil	+			
31	53	M	A. Mono L.	Ill (Rel)	Cyclophosphamide Prednisolone	+			
32	52	M	A. Mono L.	Very Ill	Nil	+			
33	52	M	A. Mono L.	Ill (Rel)	Prednisolone Cyclophosphamide	+			
34	6	M	ALL	Very Ill	Prednisolone	+			
35	13	M	ALL	Well (Rem)	Vincoblastine Prednisolone	+			
36	2	F	ALL	Ill	Nil	+			
37	6	M	ALL	Ill	Methotrexate 6 M.P. Prednisolone	+			
38	96	M	ALL	Ill	Nil	+			
39	66	M	CLL	Well	Nil	+		+	
40	67	M	CLL	Well	Nil	+			
41	62	F	CLL	Ill	Nil	+			
42	79	M	CLL	Well (Rem)	Chlorambucil	+			

CML=chronic myeloid leukaemia; CML–A=chronic myeloid leukaemia in the acute phase; AML=acute myeloid leukaemia; A. Mono. L.=acute monocytic leukaemia; ALL=acute lymphatic leukaemia; CLL=chronic lymphatic leukaemia; Rem=remission; Rel=relapse; PB=peripheral blood; BM=bone marrow; LN=lymph node; PP=plasma pellet.

venipuncture and the bone marrow samples from the leukaemic patients by aspiration from the sternum or iliac crest. The normal bone marrow samples were obtained either by aspiration from volunteers or from ribs which had been removed during the course of thoracic surgery. Most of these latter patients had a carcinoma of the lung but the bone marrow was considered to be haematologically normal. In some of the latter cases delays of up to 30 min occurred between the removal of the rib and the marrow being put into fixative.

Preparation of peripheral blood samples

The procedure used to prepare the specimens of peripheral blood leucocytes is the method of Anderson [6] with minor modifications. Briefly, 5–20 ml heparinised venous blood is spun at 3000 rev/min for 10 min in a siliconised centrifuge tube and plasma removed without disturbing the buffy layer till only about 1/16 in. of plasma is left. Gluteraldehyde (5% in 0·1 M phosphate or 0·1 M cacodylate buffer at pH 7·2) is then added slowly so as not to disturb the buffy layer. After a minimum of 1 hr in fixative the disc is removed gently and thin slices not more than 1 mm thick are cut with a razor blade. These can be divided into approximately four thin broad blocks which, when embedded in araldite, will orientate themselves correctly by falling broad-face down to the bottom, so that the cutting face is at right angles to the buffy layer. The blocks are then removed, washed in cacodylate or phosphate buffered 10% sucrose, post fixed in 2% unbuffered osmium tetroxide for 1 hr, dehydrated, cleared in epoxy propane and finally embedded in araldite polymerised at 60°C for 48–72 hr.

Before thin sections are cut it is necessary to examine a thick section of the block under the light microscope, to ensure that the final trimmed block face includes the full thickness of the buffy layer. If the buffy layer is too thick to be included in a single section, one block is trimmed to include the cells at the top of the buffy layer and another block trimmed to include cells at the bottom of the layer. Thick sections for light microscopy are adequately stained after 5 min in a mixture of 10% Giemsa in distilled water pre-heated to 60°C. After final trimming of the block ultra thin sections are cut using the LKB ultratome and double stained with uranyl acetate and lead citrate using the following technique. A drop of stain is deposited on to a paraffin wax coated petri dish which is flamed just before use to clean the surface. The grid is immersed in the drop of stain and allowed to rest on the bottom of the petri dish with the sections uppermost, and the lid is then placed on the petri dish. It is found that this procedure always gives sections free of stain precipitate even when times of up to 15 min are used for either stain. The sections were examined and photographed using an AEI EM6B Electron Microscope.

Other tissues

Specimens of bone marrow and lymph node are fixed in 5% glutaraldehyde in phosphate or cacodylate buffer, washed and post-fixed in 1% osmium tetroxide and processed as above.

Ultracentrifuged plasma pellets are prepared according to the method of Porter *et al.* [7], fixed in 1% osmium tetroxide or Dalton's fixative, and processed as above.

Scanning procedure

Up to 10 blocks were prepared from each buffy coat, the blocks always being taken from different parts of the disc. Approximately 5 thin sections (400–500Å) were cut from each of five complete buffy coats, and systematically examined. Virtually all cells on each section were examined. In addition, for the bone marrow samples, areas containing several megakaryocytes were located in thick sections in the light microscope and several serial sections of these megakaryocytes were examined carefully in the electron microscope. Records were made of the types of cell present, any unusual morphological features and any particles inside or outside the cells which could possibly have been virus particles. A special search was made of cell membranes and of platelet membranes for any evidence of budding type-C particles. While this process was very time consuming it must be remembered that the material examined represents only a very small fraction of the total material available. Only particles which were relatively numerous would have been detected.

RESULTS

Observations on virus-like particles

We did not record in either control material or leukaemic material any structure which we considered to be a virus. We did, however, note a number of structures which bear a superficial resemblance to virus but these were equally common in leukaemic material and control material. Some can readily be identified as well known cell organelles (Fig. 1) such as nuclear pores, micropinocytotic vesicles and small granules, while others are known to

be constituents of at least some types of normal cell although their precise function is unknown, e.g. perichromatin bodies which are discussed in detail below. In some cases, however, isolated particles were noted in the cytoplasm which fit neither of the above categories (Fig. 2). Such particles are very rare and do not seem to occur in any particular type of cell, either normal or leukaemic, but in every instance it was considered that the particle differed in some important respect from any known virus. In particular the location of the particle usually did not tally with the expected location of such a particle if it were virus; Fig. 2b shows an intracytoplasmic particle which resembles a herpes virus as it would appear in the nucleus. Alternatively the internal structure of the particle was not clear enough to enable one to say, in the absence of other evidence, that this was indeed virus.

One structure which can at first sight be confused with a vacuole containing virus particles is the compound vacuole which has a definite sub-structure composed of spherical sub-units (Fig. 3a). Similarly some polymorph granules may also have substructure of spherical units (Fig. 3b). Both entities can however easily be distinguished from known viruses.

We examined only three plasma pellets, one from an acute myeloid leukaemia, one from a chronic myeloid leukaemia and one from a control. The acute myeloid leukaemia and the control gave amorphous structureless pellets. The pellet from the chronic myeloid leukaemia case, however, contained an enormous number of membraneous structures of many shapes and sizes. Many of these were approximately circular in section and it was found possible to select areas which contained a number of particles similar to those described by Levine *et al.* [1] as virus-like particles (Fig. 4). Since these were only a few amongst a great mass of material of various shapes and sizes which was obviously not virus we believe that in the absence of supporting evidence it is more reasonable to consider these to be subcellular constituents of disrupted cells.

A comparison of our material with thin sections of platelets from the peripheral blood of a kitten inoculated with material from a case of feline lymphosarcoma* was made. In this material particles were seen budding from the cytoplasmic membrane of platelets while mature type-C particles were seen extra-cellularly and in the intra-cellular vacuoles of the platelets (Fig. 1b). This comparison leaves us convinced that there were no type-C particles in the material examined by us. In particular a special search of material from 8 cases of leukaemia failed to reveal any virus particles in 500 sections taken from approximately 75 megakaryocytes. This does not enable us to say that no virus was present in the peripheral blood or bone marrow of the patients whom we studied, but we can say that, if such particles were present in these tissues they were very much less numerous than in similar material from leukaemias of other species.

Observations on morphology

It is not the purpose of the present paper

*We are grateful to Dr. Helen Laird, Animal Leukaemia Research Unit, Glasgow, for giving us this material.

Key to abbreviations

C	Centriole	GO	Golgi apparatus	N	Nucleolus
F	Fibril	GL	Glycogen	NB	Nuclear body
FR	Free ribosomes	M	Mitochondrion	PG	Perichromatin granule
G	Granule	MV	Micropinocytotic vesicle	PV	Pinocytosis vacuole
				V	Vacuole

Fig. 1*a*. *A micropinocytosis vesicle invaginating into the cytoplasm of the cell on the right. These vary in size but the one shown is approximately* 110 *mμ in diameter* (×80,000).

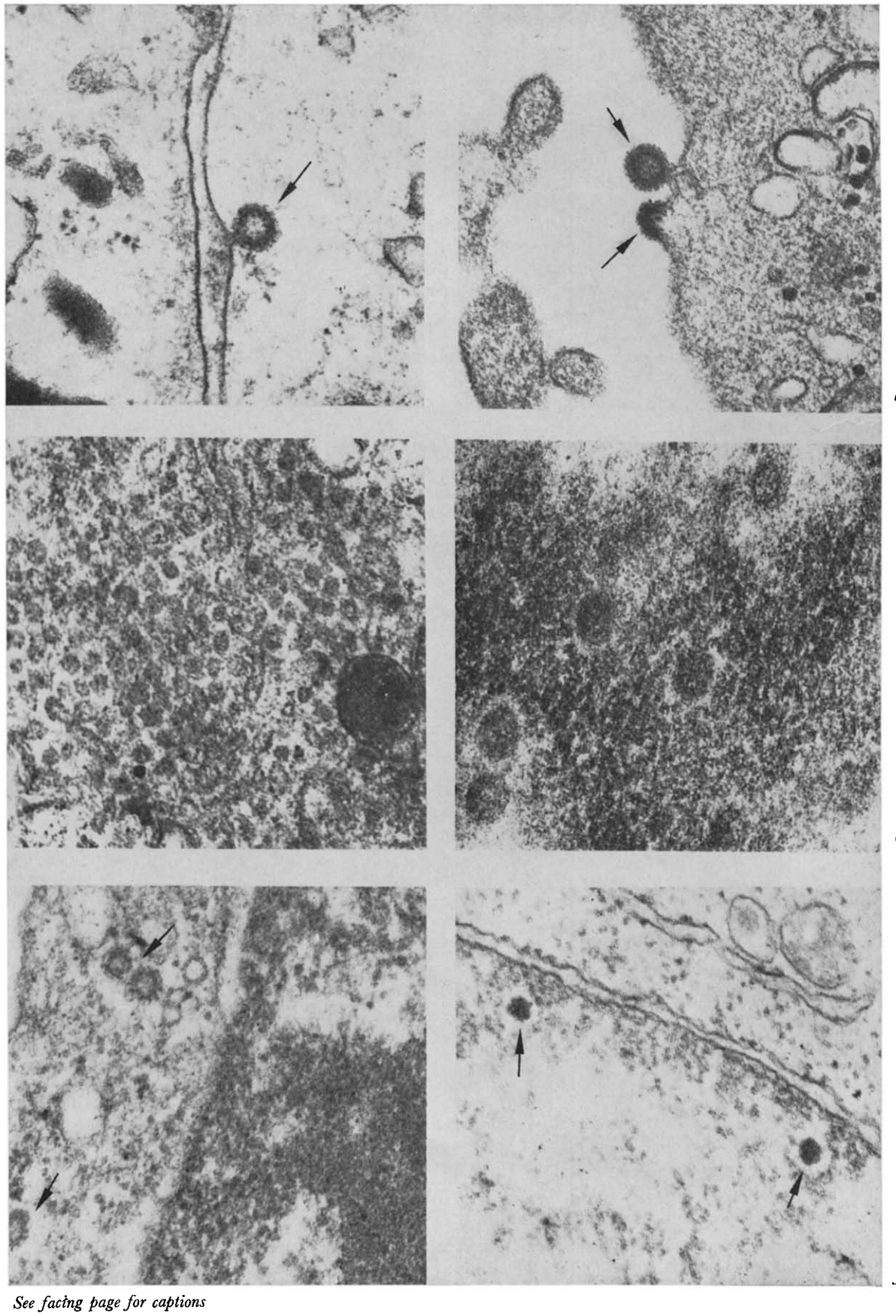
Fig. 1*b*. *C type particles budding from a platelet of a kitten injected with tissue extract from a case of feline lymphosarcoma. They measure approximately* 100 *mμ in diameter* (× 80,000).

Fig. 1*c*. *Section of a platelet showing an area of microtubules cut in cross section. They range in size from* 30–70 *mμ in diameter* (×80,000).

Fig. 1*d*. *Nuclear pores in cross section. They are approximately* 100 *mμ in diameter* (×80,000).

Fig. 1*e*. *Micropinocytosis vesicles in the cytoplasm of a red cell precursor. They are approximately* 75 *mμ in diameter* (×80,000).

Fig. 1*f*. *Perichromatin bodies in the nucleus of a blood cell. These are electron dense particles approximately* 50 *mμ in diameter surrounded by a halo of less electron dense material which separates the dense core from the nuclear chromatin. The overall diameter is approximately* 85 *mμ* (×80,000).



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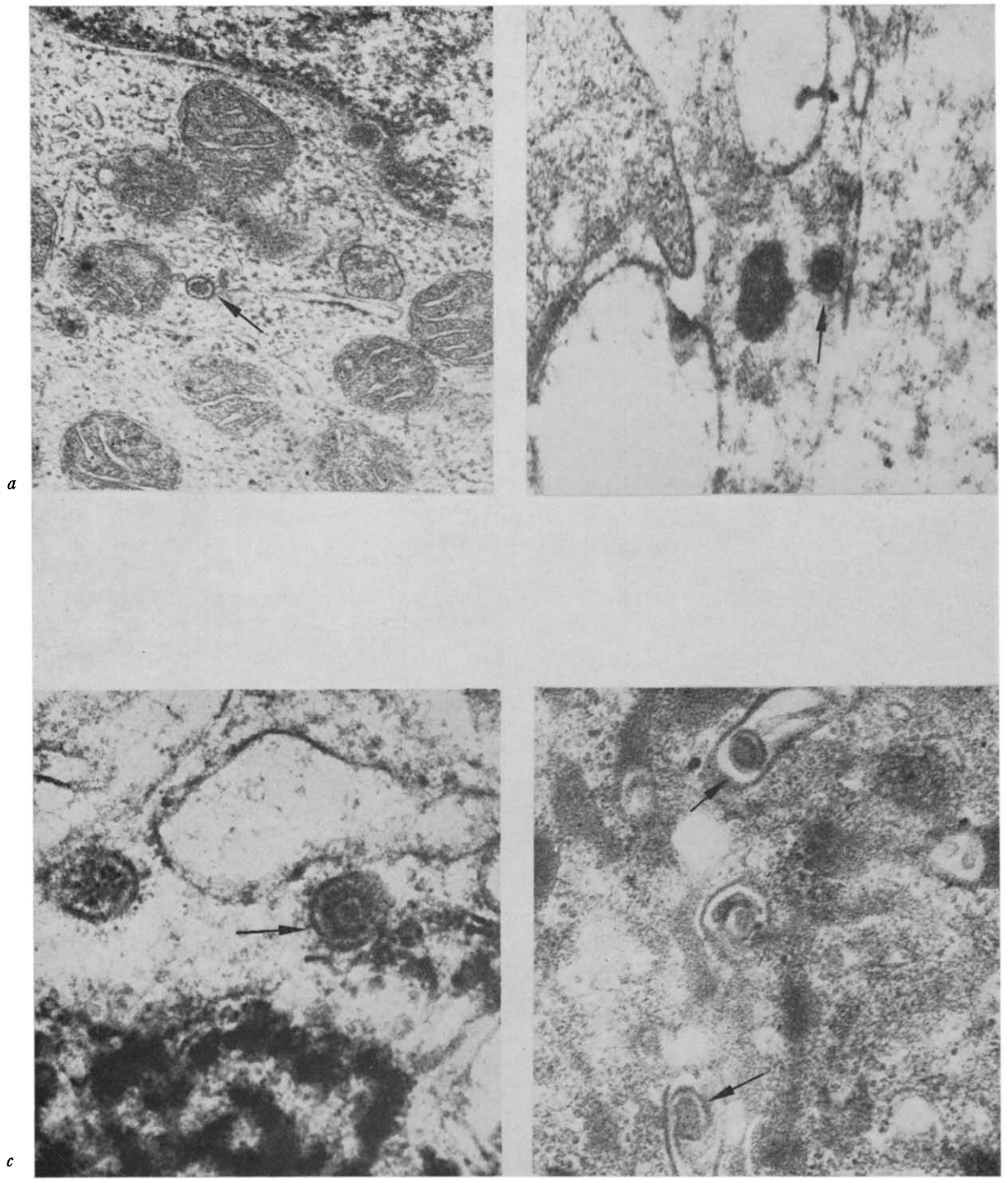


Fig. 2a. Particle found in the cytoplasms of a blast cell in case 8. It has an electron dense core 25 mμ in diameter; the overall diameter is 50 mμ (×80.000).

Fig. 2b. Particle in the cytoplasm of an immature myeloid cell in case 4. It has an electron dense core 35 mμ in diameter and an overall diameter of 65 mμ (×80,000).

Fig. 2c. Particles in a macrophage from normal bone marrow. Such particles are thought to contain iron pigment bounded by a membrane. The diameter varies from 50 to 150 mμ (×80,000).

Fig. 2d. Particles found in cytoplasmic vacuoles of a myeloid precursor cell from case 21. The electron dense core is approximately 40 mμ in diameter with an overall diameter of 75 mμ (×80,000).

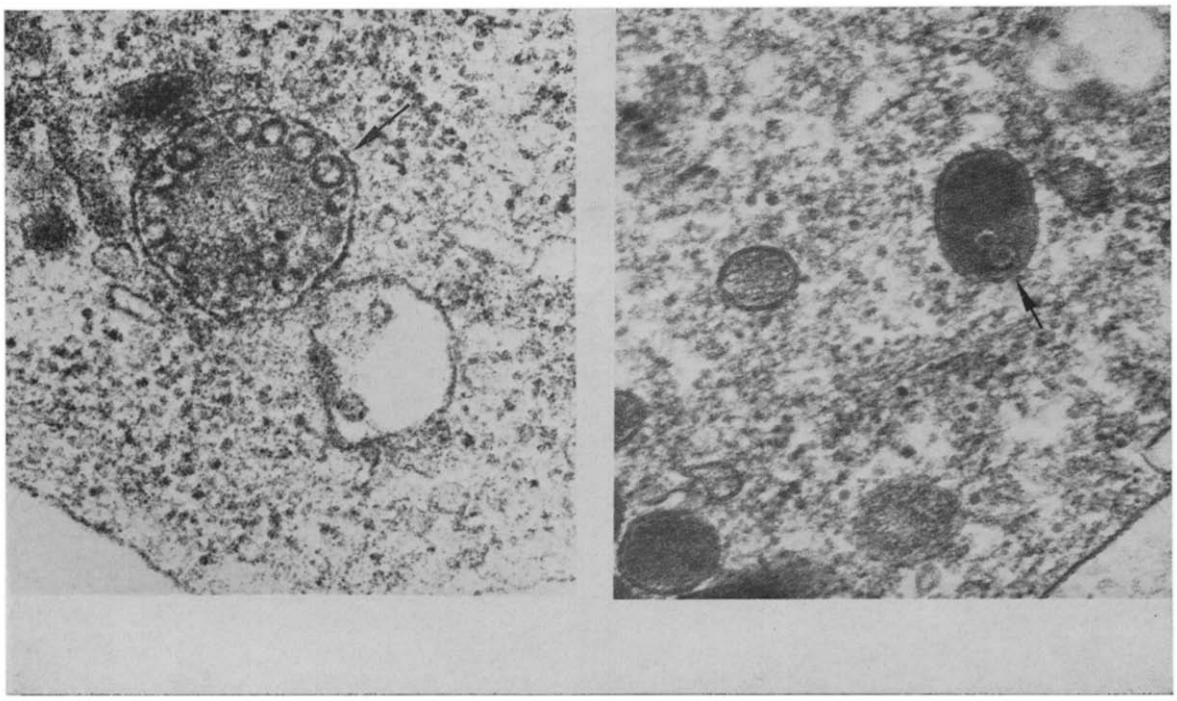


Fig. 3a. Compound vacuole in the ctyoplasm of a lymphocyte from case 28. It contains a number of small particles approximately 50 mμ in diameter (×80,000).

Fig. 3b. Neutrophil granule from a normal polymorph showing a double membrane which contains particles ranging from 20 to 40 mμ in diameter (×80,000).

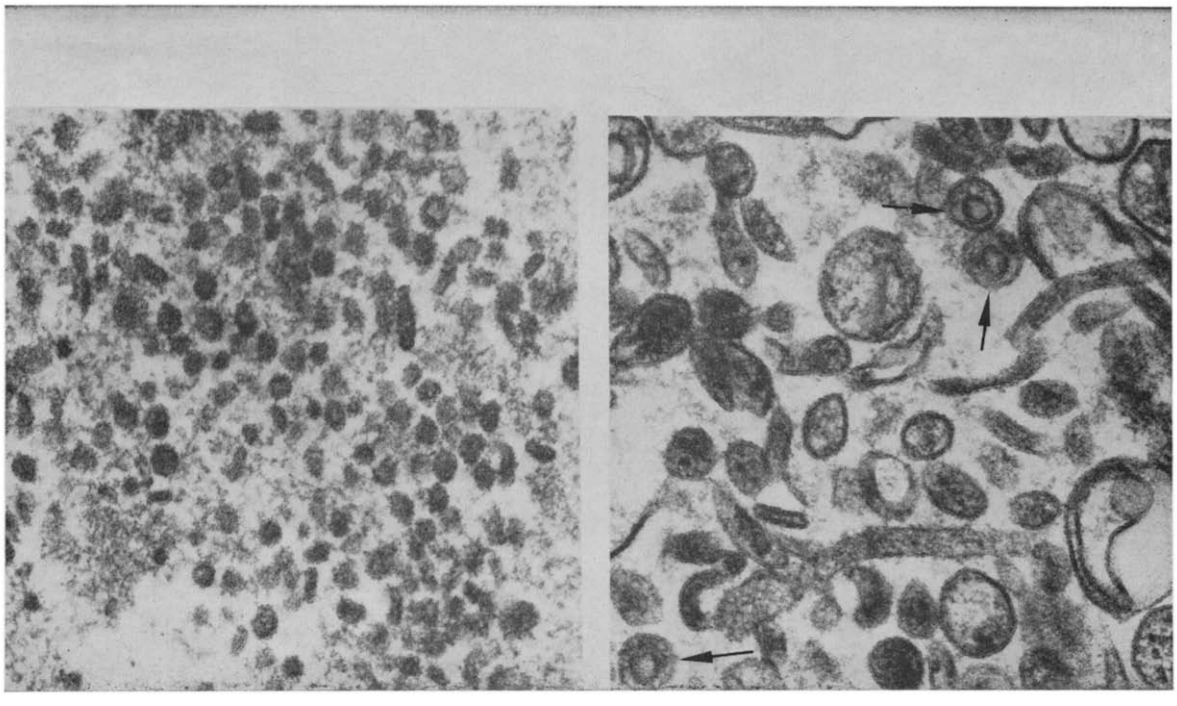


Fig. 4. Plasma pellet prepared from case 13 and sectioned at two different levels.
(a) Small particles ranging from 25 to 50 mμ in diameter.

(b) Particles of a larger size three of which resemble a mature C type particle. They contain a core approximately 50 mμ in diameter with an overall diameter of 90 mμ (×80,000).

Fig. 5. *Normal neutrophil showing perichromatin granules in the nucleus* ($\times$ 19,500).

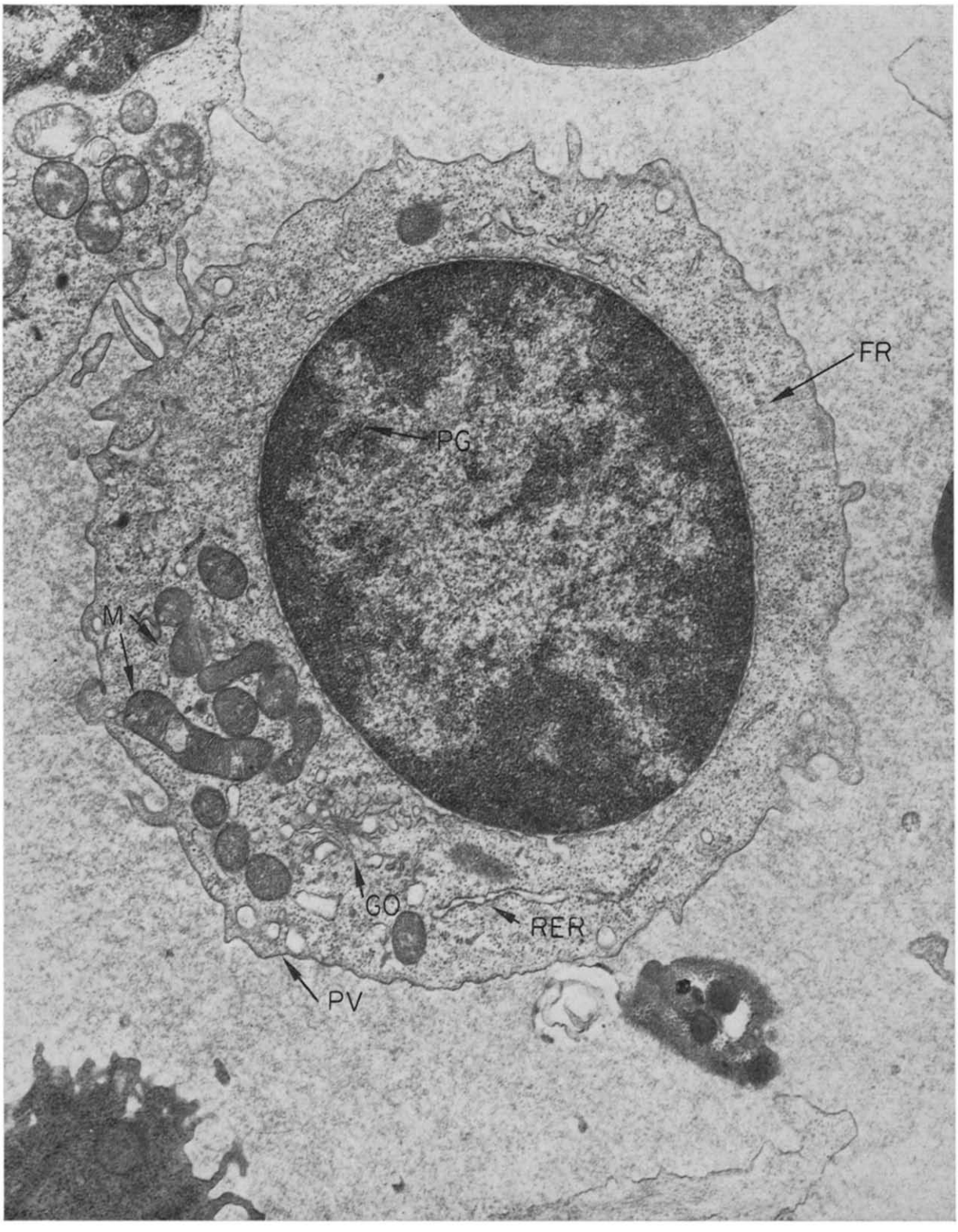


Fig. 6. *Normal large lymphocyte showing a single perichromatin granule in the nucleus* (×19,5000).

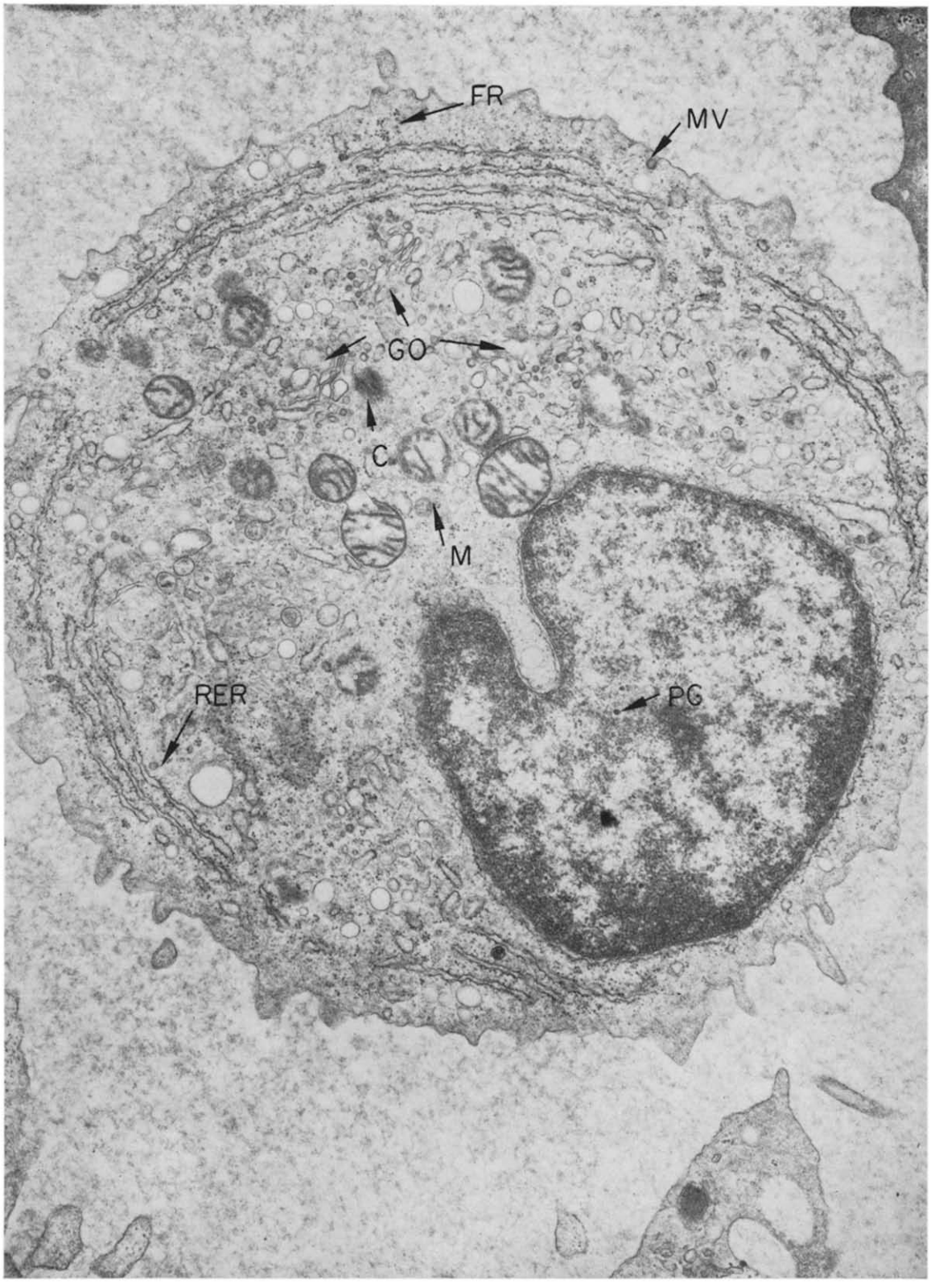


Fig. 7. Agranulocyte.

Occasionally a cell of this type is found in normal blood. It contains a single nucleus and its cytoplasm contains a large amount of rough endoplasmic reticulum in the form of concentric cisternae so that it resembles the cytoplasm of a plasma cell. No plasma cells were encountered in the differential counts of normal bloods. Note the perichromatin granule in the nucleus ($\times$ 19,500).

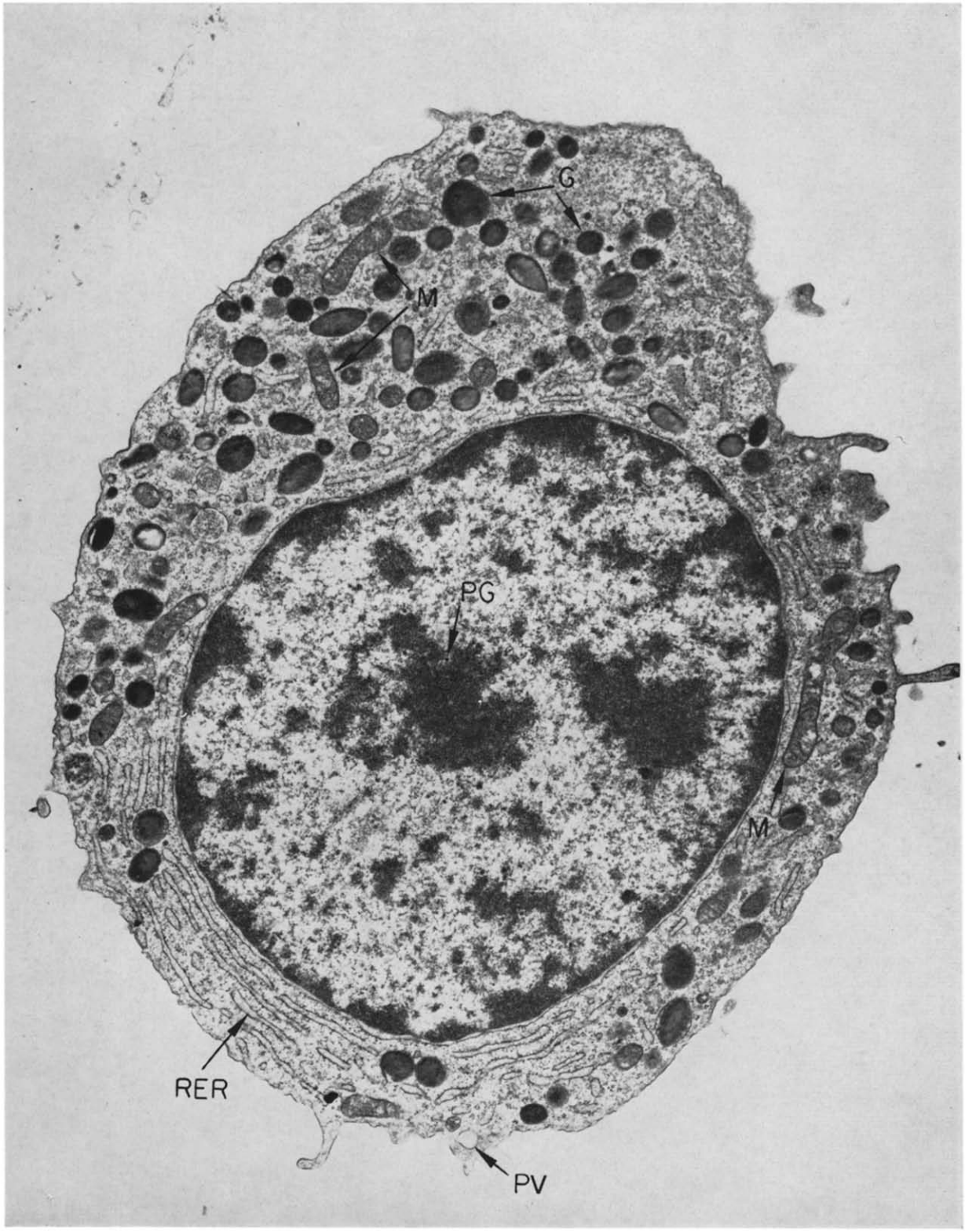


Fig. 8. *Normal promyelocyte showing a single perichromatin granule in the nucleus* (×15,000).

Fig. 9. *Normal blast cell showing prominent nucleoli* ($\times$ 12,500).

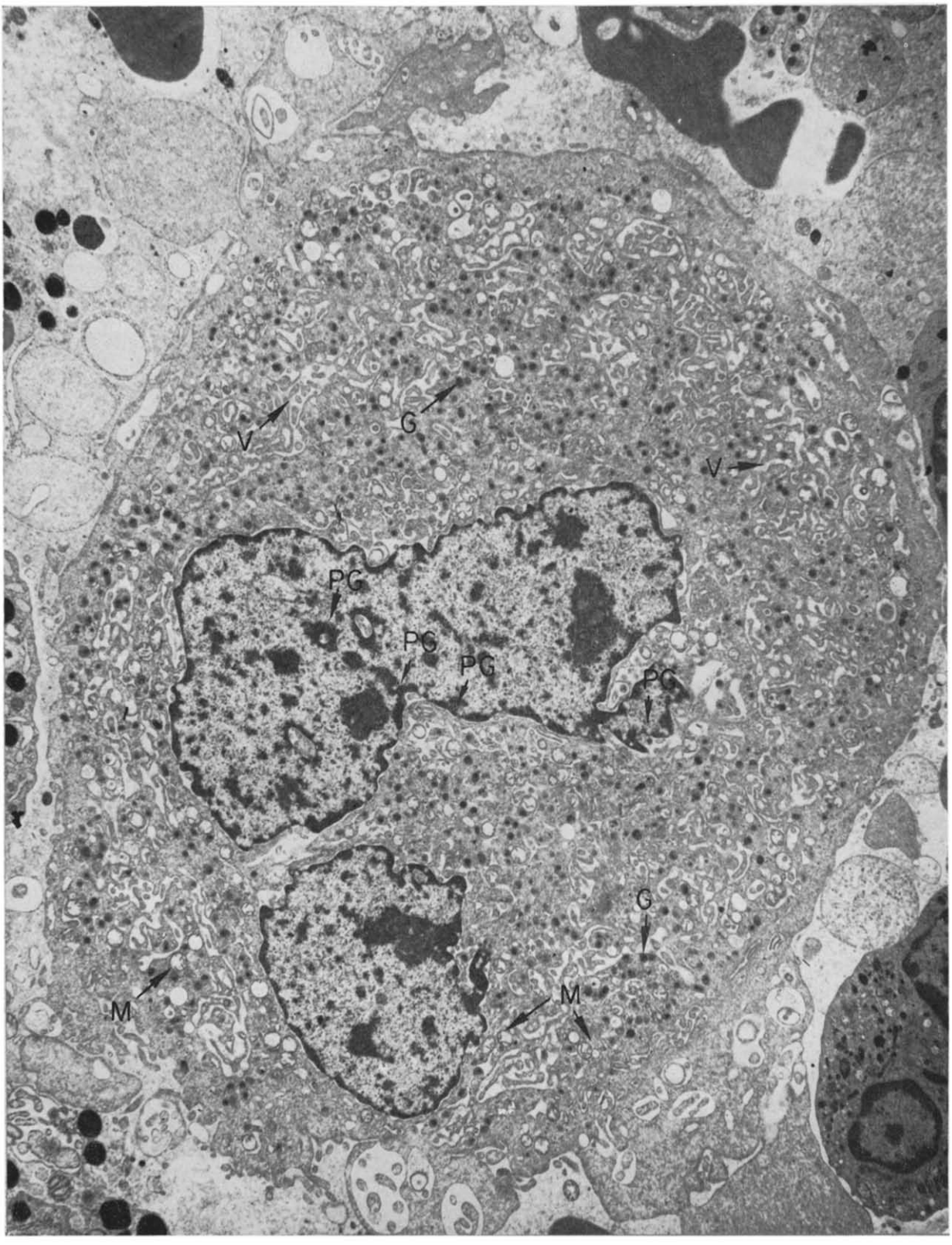


Fig. 10. *Normal megakaryocyte. Note the perichromatin granules in the nucleus* (×9,000).

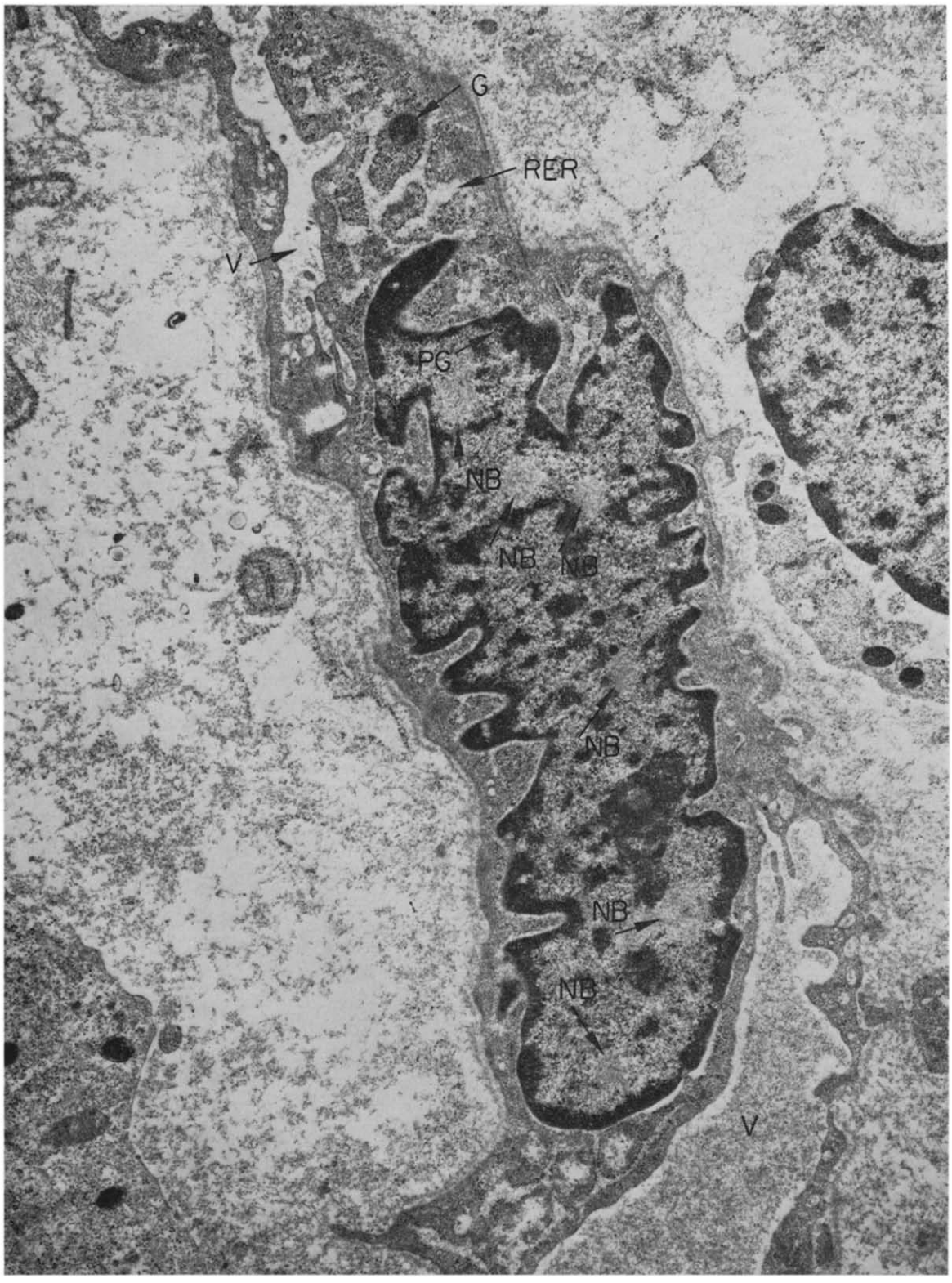


Fig. 11. *Normal reticulum cell from bone marrow. Note the nuclear bodies and perichromatin granules present in the nucleus* ($\times$ 15,000).

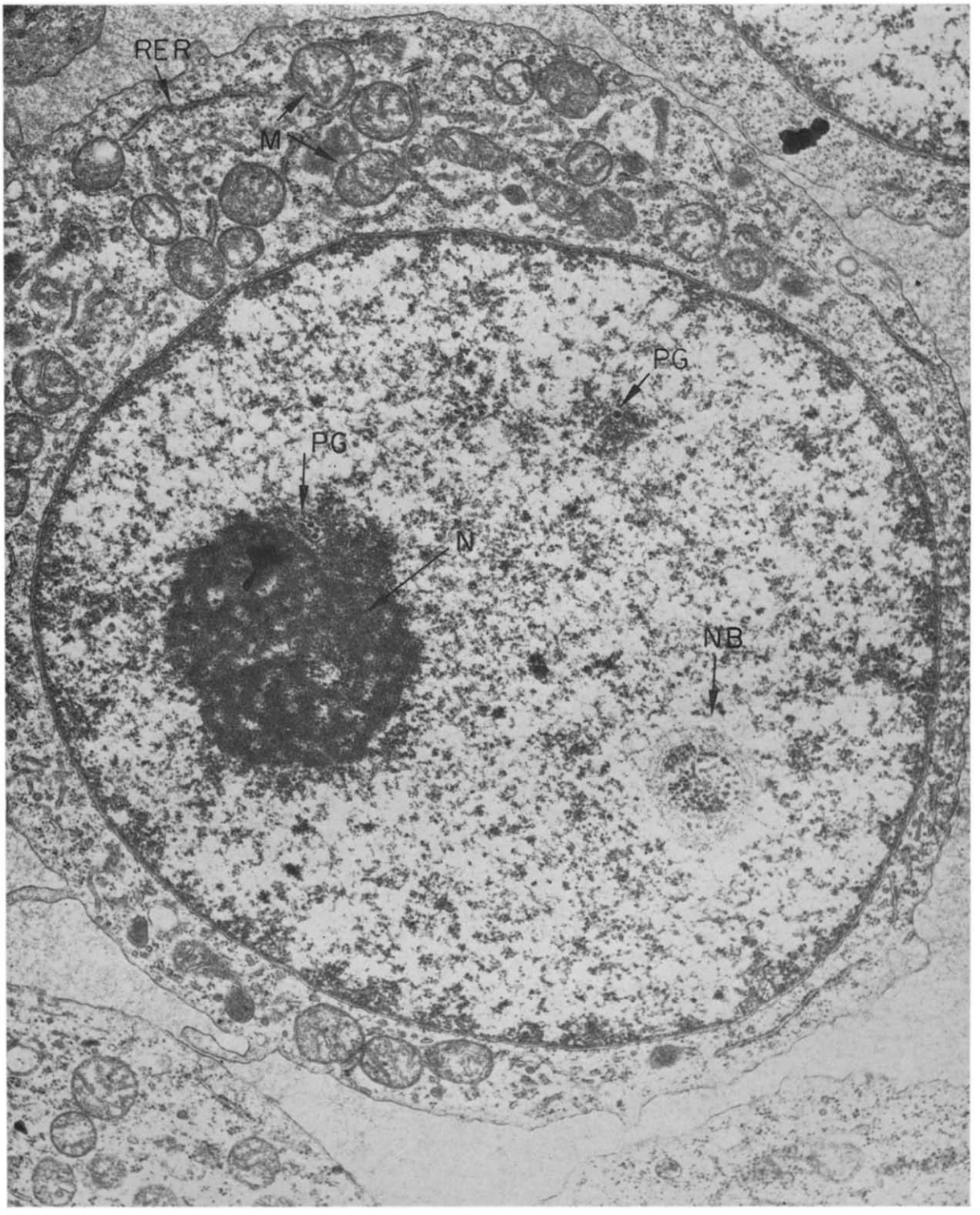
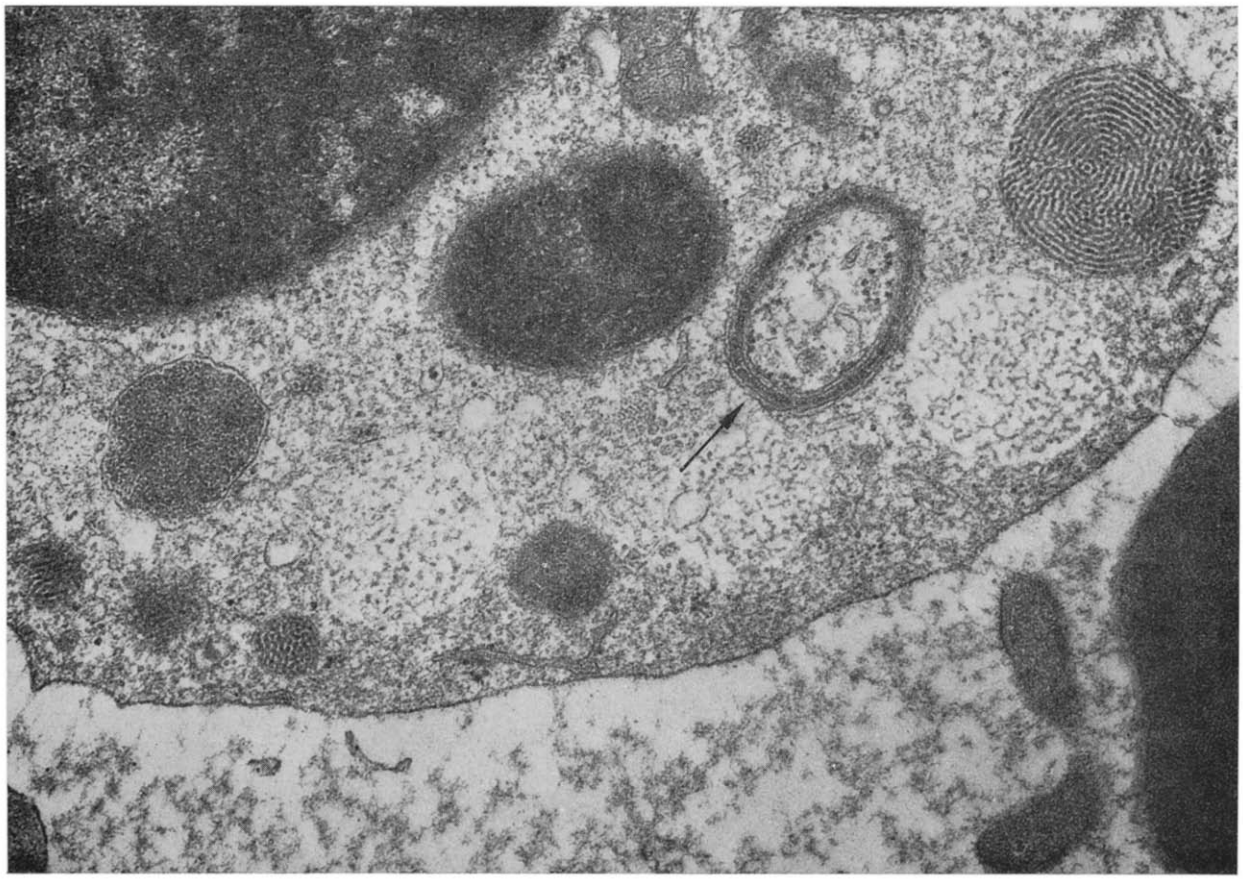


Fig. 12. *Blast cell from case* 20 *showing a nuclear body of the Bouteille type* 3 *in the nucleus* (×20,000).

Fig. 13. (*a*) *Blast cell showing a nuclear bleb from case* 18 (×37,500).



(*b*) *Basophil showing a nuclear ring from case* 8 (×37,500).

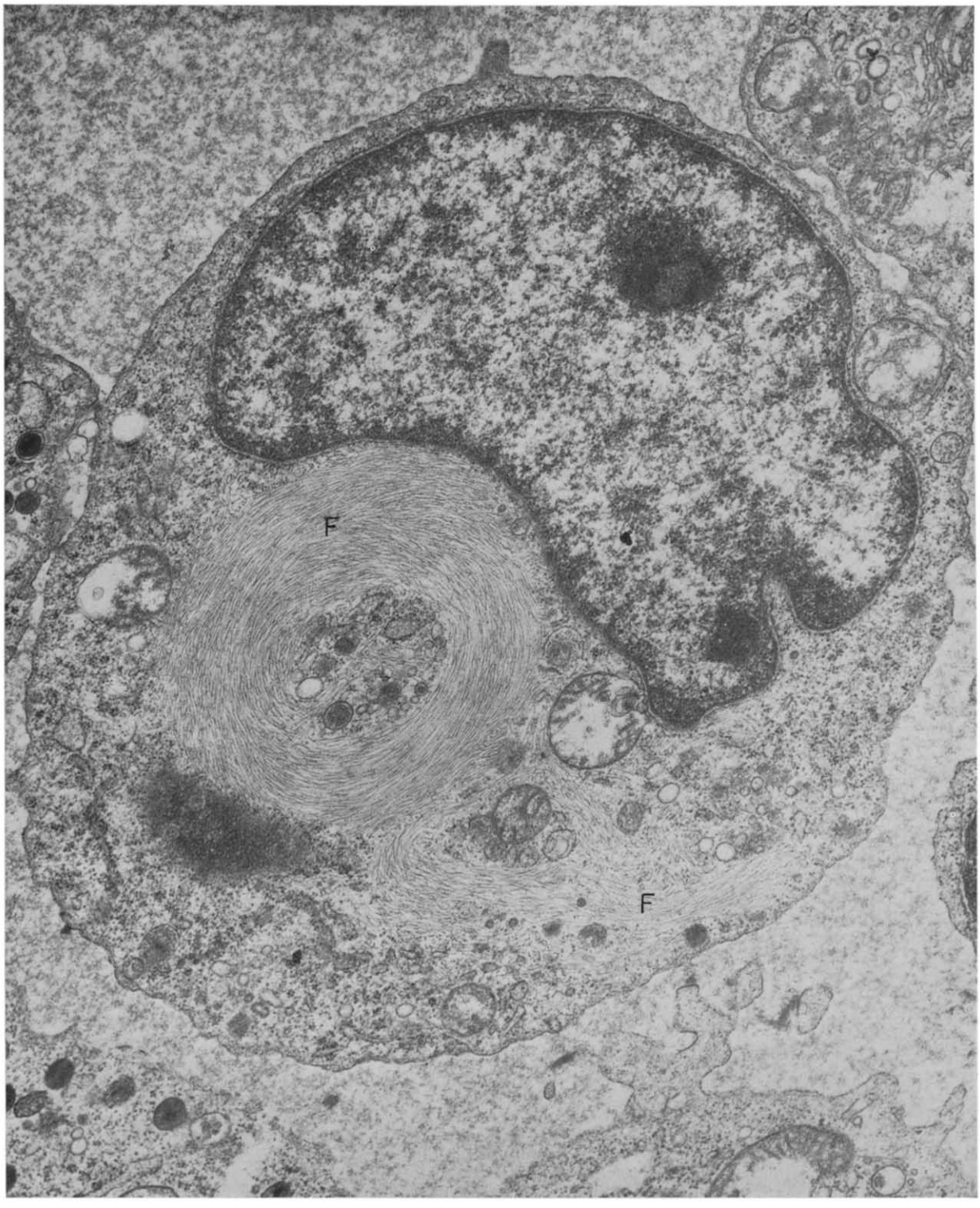


Fig. 14. *Blast cell from case* 33 *showing a large amount of fibrillar material in the cytoplasm arranged in a whorl* (×20,000).

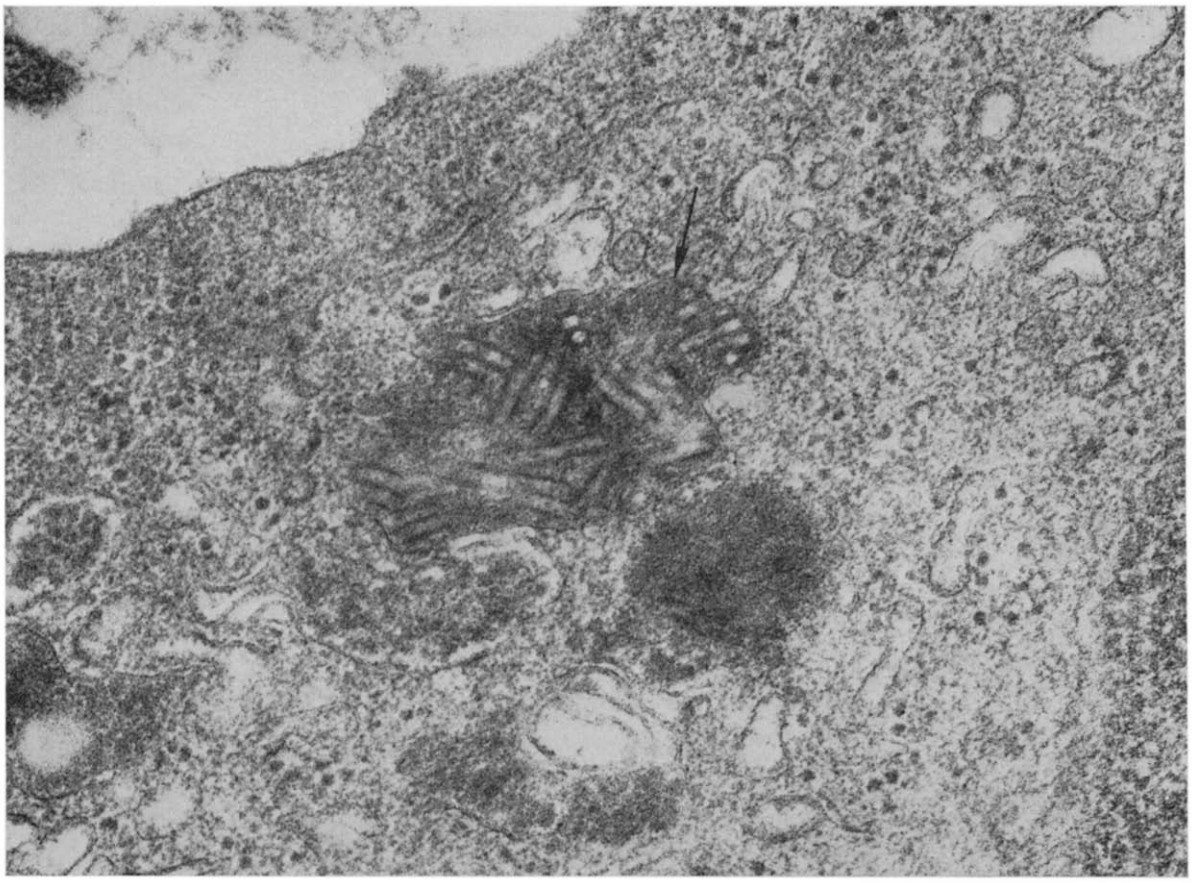


Fig. 15. *Tubular inclusion found in an occasional lymphocyte from normal peripheral blood* ($\times$ 108,000).

to describe the morphology of normal or leukaemic blood and bone marrow in detail. The general morphology of normal human blood has already been described [8, 9] and our observations are in good accord with these previous reports. A neutrophil, lymphocyte and an agranulocyte are shown in Fig. 5–7. There are several previous reports which deal with normal bone marrow [e.g. 10, 11] and many deal with various aspects of leukaemic cell structure. These latter observations are reviewed by Anderson [8] and Kakefuda [12]. In normal marrow the various cell lineages could be readily indentified and a promyelocyte, blast cell and megakaryocyte are illustrated in Figs. 8–10. The uniformity of cell type in the leukaemic marrow as compared with normal marrow was a striking feature. Two cell types, monoblasts and lymphoblasts which are not seen in normal peripheral blood or bone marrow were observed in material from patients with acute monocytic and acute lymphatic leukaemia respectively.

A number of features have already been described in the light microscope which are specific for certain types of leukaemic cells and the ultrastructure of these features is already well documented, e.g. Freeman [13, 14] and White [15] have described Auer rods in detail and are in general agreement on their structure and probable origin. There are, however, a number of features which have only been noted since the introduction of electron microscopic techniques and some of these have been claimed as specific for leukaemic cells or to be the result of treatment. There are also a number of features which have been claimed to be more numerous in malignant cells than in normal cells. We present here our observations on these features and record some other unusual features of normal and leukaemic haematopoietic cells.

1. *Perichromatin granules.* These are electron dense circular bodies approximately 25 mμ in diamater which are found in the nucleus separated from the chromatin by a clear halo 75mμ in diameter (Fig. 1f). These were originally described [16, 17] in cells of several different tissues of the rat and the mouse including eosinophil polymorphs but they have not previously been reported in human haematopoietic cells. In the present study they were observed in all types of cell in normal and leukaemic peripheral blood and normal and leukaemic bone marrow. The frequency, which was roughly the same in all cases, averaged five granules per section of nucleus. Megakaryocytes however frequently had more than this and it is suggested that the number of bodies may be related to nuclear size. It has been suggested by Lynn *et al.* [18], who examined 102 tumour specimens and 60 non-neoplastic specimens that these granules are increased in number in malignant cells, but our observations on leukaemic cells do not support this view.

The nature of these bodies is obscure. Some authors have suggested that they may bear some relationship to virus particles [17, 18]. Because of their ubiquitous nature it seems much more probable that they are normal nuclear organelles and that if their number is related to nuclear size, this could suggest some direct relationship with the number of chromosomes in the nucleus.

2. *Nuclear bodies.* These are intranuclear fibrillar structures sometimes enclosing groups of granules, first reported by de Thé *et al.* [19] and described in detail by Bouteille *et al.* [20] and by Krishan *et al.* [21]. The bodies are of several types which have been classified by Bouteille *et al.* [20] and by Popoff and Stewart [22] who also summarise the present state of observations. It is agreed that these bodies occur in both normal and malignant tissue of a variety of different species but that they are commoner in malignant tissue and also in other tissues which are hyperactive. They are also said to be common in cells of patients with diseases of proved or supposed viral etiology [20]. It appears further that types 3 and 4 of Bouteille are commoner in malignant cells and in virus infected tissues.

In the present study nuclear bodies were not observed in normal peripheral blood, but they were found in all other tissues examined. In normal bone marrow they were only rarely observed and were normally of the type 1 of Bouteille. They were observed in both rib marrow samples and in aspirated marrows. Although they do not appear to be specific for one cell type they occur more commonly in reticulum cells, which may have several bodies in a single nucleus (Fig. 11). In leukaemic marrow they occur at roughly the same frequency as in normal marrow but in leukaemic peripheral blood, of all cell types, they appear to be relatively common. Further, in these cells the bodies are more often of types 2 and 3 (Fig. 12). Since these are more easily observed this may have contributed something to the impression of increased incidence but they are nevertheless still considered more common in leukaemic blood than in any other tissue examined.

Two points emerge from these observations.

Firstly, we are not aware of nuclear bodies being described previously in normal human haematopoietic cells. Secondly, these observations tend to support the view that nuclear bodies are more common in the cells of leukaemic patients than in normal individuals.

The nature of these bodies is not known. There has been much speculation but little fact has emerged. In particular there is little at present to support the view that they are virus associated inclusion bodies [23] but rather that they are associated with specific types of normal (and possibly abnormal) cellular hyperactivity [20].

Nuclear blebs are small protrusions of the nucleus enclosing an area of cytoplasm (Fig. 13a). In some instances the outfolded portion of the nucleus appears to be separated from the main portion of the nucleus so that it forms a ring-like structure (Fig. 13b). These structures were first described [24] in the immature granulocytes of patients with neoplastic disease and also in bone marrow cells of cases of pernicious anaemia treated with fluorouracil. They have since been described in normal and malignant cells of several different species. They have been found in cultured cells of Burkitt's lymphoma [25], cells from patients with leukaemia [8, 26, 27] and in normal human blood cells [28–30]. Ahearn *et al.* [31] suggested an association with treatment with cytosine arabinoside. Davies and Small [32] have described the ultrastructure of rings and blebs in detail and show that they appear to be composed of two layers of nuclear membrane enclosing a single layer of the ordered structure which is found immediately below the nuclear membrane of the main part of the nucleus and are therefore closely related to normal nuclear structure.

In the present study blebs and rings were noted in a small number of normal blood and bone marrow cells. In leukaemic material blebs and rings were again found in both blood and bone marrow. In some leukaemic cases they were present in large numbers in peripheral blood cells but there appeared to be no correlation with any type of leukaemia, but they do occur more commonly on polymorphs than on lymphocytes.

They were observed more frequently in leukaemic peripheral blood than in any other material. They were observed in both treated and untreated cases and we have found no correlation with any particular type of treatment. Our observations therefore tend to support the view that blebs are more common in malignant tissue than in normal tissue. A further study of the factors contributing to the formation of blebs could yield useful information on normal nuclear structure and on the behaviour of malignant cells.

Micro fibrils have been reported [8] to be present in some cells of both normal and leukaemic patients. This finding was confirmed in the present study and in general the quantity of fibrillar material was no greater in leukaemic patients than in controls. In one case of acute myeloid leukaemia however many blast cells were found to contain large amounts of fibrous material. This material was always arranged in whorls (Fig. 14) enclosing cytoplasmic material which appeared in some cases to be degenerating endoplasmic reticulum. It may be relevant that this was the only case in which Auer rods were observed.

Tubular inclusions. In a small number of cases occasional lymphocytes in normal blood were found to contain in the cytoplasm aggregates of tubules which were cut at a variety of different angles (Fig. 15). The aggregates were irregular in shape, variable in size and were not enclosed in a membrane. Individual tubules were approximately 25 mμ in diameter. Somewhat similar structures have been seen in lymphocytes of a patient with chronic rheumatoid arthritis and leukopaenia [33] but in that case the tubules were sometimes contained in a vacuole and the aggregates were much larger in size. The nature of these structures is not known and they were only noted in normal blood cells.

DISCUSSION

There have been many reports of virus-like particles being found in electron microscopic studies of material from human leukaemias. The situation has, however, been complicated by the changing standards of technology in electron microscopy during the period when these studies have been made. Haemopoietic cells, particularly those of the peripheral blood, have in the past proved to be difficult to fix and section to give good preservation of the ultra structure and it is only within the last few years that thin sections of good quality could be produced with regularity from peripheral blood cells. For this reason many of the early reports which claimed to demonstrate the presence of virus particles in human leukaemic material should be considered with the greatest caution. This problem was recognised by the WHO subcommittee [34] which, in 1966, laid down criteria which should be fulfilled before particles observed in leukaemic material are claimed to be virus particles.

Even in recent work there is a wide divergence of opinion on whether or not virus particles can be seen. There are many reports which described particles which may be virus but which in the absence of supporting evidence cannot categorically be stated to be virus. A very small number of reports have clearly demonstrated structures which, even in the absence of infectivity studies, would be accepted by most workers as virus particles [35]. But even in these cases the question remains as to what is the relationship between the virus and the leukaemia.

There are two particular areas of interest, the herpes-like viruses and the type-C particles. Herpes-like viruses, first described by Epstein *et al.* [3] in cultured cells from Burkitt's lymphoma have now been detected in many different lines of these cells by many different authors. Similar particles have been demonstrated in cultured blood cells from patients with leukaemia of several different cell types [36, 37, 39] from patients with other malignancies [37, 39], from patients with infectious mononucleosis [38, 39] and also from normal subjects [39]. It has been shown that contact with virus-containing nonproliferating cells can confer on normal lymphocytes the property of indefinite proliferation in suspension culture [40]. This change is associated with the transfer of the herpes-like virus. There is serological evidence that the virus is common in many different areas of the world [41, 42] and that it may be the causal agent of infectious mononucleosis [38]. Its relationship to Burkitt's lymphoma and to human leukaemia is still obscure but the view which Dameshek [43] has held for some time, that infectious mononucleosis is a leukemia which undergoes spontaneous regression, takes on a new significance in the light of the present observations. Reports of herpes-like virus in uncultured human leukaemic material are rare but Carper *et al.* [44] has recently reported a herpes virus in peripheral blood cells of a leukaemic child and its unaffected mother. Interest in viruses of this type as possible causal agents in human leukaemia has been sharpened by the demonstration that a herpes-virus may be the causal agent of Marek's disease in chickens where one important feature is the occurrence of lymphosarcoma [4].

Most attention has, however, been directed to attempts to demonstrate type-C particles in human leukaemia, since such particles are known to be associated with many different types of leukaemia in the mouse, with rat leukaemia, guinea pig leukaemia, feline lymphosarcoma and the avian leukoses. Experience with these animal leukaemias suggests that certain sources of material harbour larger numbers of virus particles. In both avain and murine leukaemia, ultraspun pellets of peripheral blood plasma are a particularly rich source of virus particles. In the mouse leukaemias particles are also commonly seen in megakaryocytes and platelets [5, 45, 46]. Particles have, however, been observed in other blood cells with a low frequency, e.g. lymphoblasts, normoblasts and eosinophils [5].

The search for virus-like particles in man is to some extent governed by the nature of the material available. The majority of studies that have been made so far have been carried out on plasma pellets, using negative staining techniques or thin sections [1, 2, 5, 7, 47–51]. Studies on thin sectioned cellular material are less common but positive findings have been reported [5, 52, 53] while de Harven [54] and Arnoult and Hagenau [51] make negative reports. All the reports are agreed that virus-like particles are present but the contradictions arise at the level of interpretation. The only type of evidence which it is generally agreed would be conclusive proof of the presence of virus particles of the murine leukaemia type is the demonstration of budding particles. The few reports of budding in human material come almost exclusively from Dmochowski. His recent pictures are of great interest but they do not yet show the same detail as his own pictures of budding murine leukaemia particles. Further work in this field will be watched with great interest. The recent work of Laird *et al.* [55, 56] on the presence of budding particles in platelets of peripheral blood from kittens infected with a tissue extract from a case of feline lymphosarcoma strengthens the conviction that only the demonstration of budding in human leukaemia will be acceptable as evidence for the presence of type-C particles in these cases.

In the present study a detailed search has been made for virus, virus-like particles or budding particles in thin sectioned blood and bone marrow material from a variety of different types of leukaemia and from normal individuals. Most of the virus-like particles which were observed can be identified as specific cell structures while a small number remain unidentified. None of these particles, however, fits any category of virus particle known to us and in particular they do not resemble either herpes virus or the type-C particles of the murine, avain and feline leukaemias.

This result is therefore at variance with the previous observations on thin sectioned leukaemic cells [5, 52, 53, 57] all reporting the finding of virus particles in a low proportion of the cases examined. On the other hand it agrees with the work of de Harven [54] and of Arnoult and Hageneau [51] who considered that none of the particles which they observed in thin sectioned material was virus.

Clearly there is still much work to be done in this field but already a number of facts are beginning to emerge. Although we found no virus particles we would not claim that this rules out their presence in human leukaemic material. Similarly the vast majority of authors who claim to find virus in human leukaemic material find it only with difficulty in a low proportion of the cases examined. It follows therefore either that virus is present in human leukaemic material in very much smaller quantities than in the animal leukaemias or it is in a state which renders it not easily demonstrable by the present techniques or it is not there at all and the positive reports are either artefactual or else represent the accidental presence of viruses not relevant to the aetiology of the disease.

Much of the work that has been done in this field has been influenced by the earlier studies on animal leukaemias. It now seems clear that the hypothesis of a direct parallel is no longer tenable. This is hardly surprising since work with mouse leukaemias has been done with selected stocks of virus in highly inbred animals. Similarly avain leukosis has been studied with virus which has been passed many times in experimental animals and selected domestic stocks of birds. In human leukaemia, one is dealing with an outbred population and any virus, if it is present, will not have been subjected to artificial selection. The situation may, therefore, appear to be quite different when using the approaches that have been developed for the study of the animal leukaemias. Consideration is now being given to factors which might interplay with virus in the initiation of the disease, e.g. it is quite clear that genetic factors can influence the susceptibility of animals to leukaemogenic viruses [58, 59]. A study of physiological host factors which influence the development of virus leukaemias, whether these are environmentally or genetically determined, may show that the difference between human and animal leukaemias is more apparent than real.

One other possible approach is the development of new techniques for looking for evidence of virus infection. One could try to develop systems of pretreatment which might render a virus more readily detectable, e.g. Henle and Henle [60] have shown that the percentage of cells from cell lines cultured from Burkitt's lymphoma in which virus can be readily detected may be increased dramatically by growing the cells in deficient media.

From the ultrastructural point of view one could also look for morphological features of leukaemic cells which might be indicative of virus infections although virus particles themselves are not observed. Several of the unusual morphological features described in this paper have been claimed to be associated with the presence of virus in the cell, e.g. perichromatin bodies [18], nuclear bodies [23] or nuclear blebs [25]. While there is at present no convincing evidence that such structures are indicative of a virus infection the evidence does warrant further investigation of the conditions which control the appearance of these morphological features, e.g. the resemblance between nuclear blebs and some of the abnormal structures which appear during infection with herpes simplex are too striking to be discarded lightly.

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RESUME

La présence de particules virales fut recherchée, au microscope électronique, dans des coupes fines de cellules sanguines et de cellules médullaires provenant de malades atteints de leucémie et de sujets normaux. On ne trouva aucune particule qui pût être considérée comme étant un virus. Les particules ayant l'aspect de virus furent identifiées soit comme

des organelles cellulaires normales, soit comme différant, pour l'une ou l'autre raison importante, de virus connus. De ces observations, et d'autres travaux récents, on conclut que le concept d'un parallélisme direct avec les leucémies murines et avaires ne peut être défendu. La présence de particules virales, dans les cellules leucémiques humaines, ne peut être formellement écartée; mais, si ces particules existent, elles doivent se trouver en quantité beaucoup plus faible que dans les leucémies des animaux.

Les autres caractéristiques ultrastructurelles des cellules normales et leucémiques furent étudiées. On en conclut que les granules périchromatiniens, les corps nucléaires et les vésicules nucléaires s'observant à la fois dans les cellules hématopoïétiques normales et dans les cellules leucémiques. Cependant, alors que les granules périchromatiniens sont également fréquents dans tous les cas étudiés, les corps et les vésicules nucléaires sont plus abondants dans les cellules leucémiques que dans les cellules normales.

SUMMARY

Thin sections of peripheral blood cells and bone marrow cells from patients with leukaemia and from normal subjects were examined in the electron microscope for the presence of virus particles. No particles were seen which were thought to be virus. Virus-like particles were either identified as normal cell organelles or else differed in some important respect from known viruses. On the basis of these and other recent observations it is concluded that the concept of a direct parallel with murine and avian leukaemias is no longer tenable. Although the presence of virus particles in human leukaemic cells is by no means ruled out, their frequency, if present at all, must be very much lower than in the animal leukaemias so far studied.

Other unusual ultra structural features of normal and leukaemic cells were also studied. It is concluded that perichromatin granules, nuclear bodies and nuclear blebs are found in both normal and leukaemic haematopoietic cells but that while perichromatin granules are equally common in all cases studied, nuclear bodies and nuclear blebs are more common in leukaemic cells than in normal cells.

ZUSAMMENFASSUNG

Dünne Schnitte peripherer Blutzellen und Knochenmarkzellen von Leukämiepatienten und von gesunden Individuen wurden elektronenmikroskopisch auf das Vorhandensein von Viruspartikeln untersucht. Keine Partikel wurden gefunden, die als Viren angesehen werden konnten. Virusähnliche Partikel erwiesen sich entweder als normale Zellorganellen oder unterschieden sich in wesentlichen Punkten von bekannten Viren. Diese und andere, kürzlich gemachten Beobachtungen führen zu der Schlußfolgerung daß das Konzept einer direkten Parallele zwischen der Leukämie der Nager und der Vögel nicht länger aufrecht erhalten werden kann. Obwohl die Gegenwart von Viruspartikeln in menschlichen Leukämiezellen keineswegs ausgeschlossen werden darf, mußihre Häufigkeit, falls überhaupt vorhanden, sehr viel geringer sein als in der Tierleukämie, soweit sie bis jetzt bekannt ist.

Es wurden auch andere ungewöhnliche Ultrastrukturen normaler und leukämischer Zellen untersucht. Folgende Schlüsse wurden gezogen: Perichromatingranula, Kerneinschlüsse und Kernausstülpungen sind sowohl in normalen wie in leukämischen hämatopoetischen Zellen vorhanden. Perichromatingranula jedoch erscheinen gleichmäßig in allen untersuchten Fällen, während Kerneinschlüsse und Kernausstülpungen häufiger in leukämischen Zellen auftreten als in normalen Zellen.

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Interference Between two Strains of the Mouse Mammary Tumour Virus in the GR Mouse Strain

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INTRODUCTION

IN THE GR mouse strain neoplastic lesions of the mammary gland appear at an extremely early age in breeding females [1–3]. These lesions resemble the so-called plaques which have been described by Foulds [4] for the RIII mouse strain, and which have also been observed in the DD strain [5]. The histological appearance of these lesions and their pregnancy-dependency are controlled to a great extent by the GR-strain mammary tumour virus [3, 6] as has also been observed for the mammary tumour virus in the RIII strain [7, 8].

The GR-strain virus, which has the same morphology as the classical Bittner mammary tumour virus (B-particles) [2, 3, 6], is also called the Mühlbock mammary tumour virus [3, 9–11].

If the Mühlbock virus is introduced into other mouse strains, neoplastic lesions appear much later than in the GR-strain [3, 6]. They appear even later than true mammary carcinomas induced by the Bittner virus in those mouse strains. Genetical analysis revealed that this extreme susceptibility of the GR-strain to the action of its mammary tumour virus is controlled by a single Mendelian factor [3].

The most remarkable feature of the GR-strain with regard to the Mühlbock virus is the vertical transmission of this virus. It is not only transmitted by the milk [1–3, 6] but also by the ovum [6] and by the male [1–3,6]. If the Mühlbock virus is introduced into other mouse strains, only milkborne transmission will be found [3, 6]. Genetical analysis demonstrated that the remarkable mode of transmission in the GR-strain is controlled by a single Mendelian factor, which is identical with the factor for extreme susceptibility to the virus [3, 6]. It has been postulated in several publications that the Mühlbock virus is transmitted as a genetical factor of the GR host [3, 6, 10, 11] as has also been assumed for the C3Hf-strain mammary tumour virus [12] and the AKR-strain (Gross) leukaemia virus [11, 13, 14].

Latarjet [15] inoculated AKR mice with cell-free extracts of radiation-induced leukaemias of the C57BL mouse strain and got a significant decrease in leukaemia incidence. The offspring of the non-leukaemic mice, however, had a high incidence of leukaemia. A decrease was also achieved by exposing the Gross virus, from AKR mice, to water, which has been irradiated (100,000 rad). Lieberman and Kaplan [16] observed that inoculation of C57BL mice with the Gross virus made these mice less susceptible to the leukaemogenic action of the radiation-induced leukaemia virus of the C57BL-strain.

The aim of the present investigation was to achieve, in similar ways as described above, a decrease in the incidence of neoplastic lesions of

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the mammary gland of the GR-strain by inoculations with the Bittner virus.

MATERIALS AND METHODS

Bittner virus B particles were isolated from mammary tumours, which arose in BALB/cfC3H forced breeding females. Mühlbock virus B particles were isolated from tumours, which arose in forced breeding GR females and which were in the pregnancy-independent state. For the isolation, the procedure of Calafat and Hageman [17] was followed in which tumour-homogenates are subjected to ultracentrifugation on a discontinuous four-steps Ficoll-D_2O gradient followed by ultracentrifugation on a continuous Urografin gradient. One milligram of purified B particles was dissolved in 1 ml phosphate buffered saline (PBS) (pH 7·4; 8·0 g NaCl; 0·2 g KCl; 0·914 g Na_2HPO_4 anhydride; 0·4 g bovine serum albumin per liter). The virus preparations were stored at −196°C until they were used.

Virgin GR mice were injected intraperitoneally at several ages with 0·5 ml of different dilutions of the stock virus preparations. In some experiments the mice were also injected intramuscularly with Freund's adjuvant (2 parts complete Freund's adjuvant emulsified in 5 parts PBS). After the injections, two females were caged together with one hybrid male, free of either Bittner or Mühlbock virus. Each day the cages were inspected for the presence of newborn litters, which were immediately removed. Twice a week the females were palpated for the presence of a neoplastic lesion of the mammary gland. Tumour ages are based upon the first moment that a palpable lesion was observed. When the lesion had occurred several times the animals were sacrificed. Tumours were fixed in Susa fluid, paraffin sections were made and stained with haematoxylin and eosin.

Control GR mice were subjected to forced breeding at several ages. In other control experiments, GR mice were injected with Freund's adjuvant at various ages and then subjected to forced breeding. In all control experiments hybrid males, free of either Bittner or Mühlbock virus, were used.

In some experiments GR mice were injected with Bittner virus preparations, which we have attempted to inactivate. The following procedures of inactivation were followed:

(a) Adding 1 ml undiluted B-particle preparation to 0·1 ml ether and shaking it on a Vortex for 30 sec, thereafter diluting it 100× in PBS and waiting 30 min at 4°C for evaporation.

(b) Keeping an undiluted B-particle preparation at 60°C in a waterbath for 30 min.

(c) Putting a B-particle preparation in formalin (0·1%), buffered in PBS, for 21 days at 4°C.

These treated preparations were also tested for their carcinogenic activity in 4 weeks old BALB/c female mice, which were subjected to forced breeding. The amount of B particles was comparable to a 100× diluted stock solution of live Bittner virus. As controls, BALB/c mice were injected with various dilutions of live Bittner virus.

Mühlbock virus was inactivated by H_2O_2 in the following way: 0·2 ml of a stock preparation was diluted to 20 ml in a 3% H_2O_2 solution. After 15 min exposure, H_2O_2 was removed by dialysis against PBS overnight at 4°C. The removal was controlled by adding 1 ml preparation to 1 ml mouse blood with known catalase activity. No oxygen was formed.

RESULTS

When GR females were caged with a male at four weeks of age, 90% of them became pregnant at five weeks of age. Between pregnancies often irregular intervals were observed. If females were caged with a male at later ages, often it took several weeks before the females became pregnant.

The incidence of neoplastic lesions of the mammary gland, and the average number of pregnancies needed to get such a lesion are reported for control GR mice in Table 1. All

Table 1. *Rate of development of neoplastic lesions of the mammary gland in forced breeding GR mouse strain females*

Expt. No.	Age beginning of forced breeding	No. of animals	No. with neoplastic lesions	Average No. of pregnancies +S.D.
1	4 weeks	26	26	2·5±1·1
2	11 weeks	15	15	2·3±0·8
3	22 weeks	7	7	2·6±1·3
4	45 weeks	5	5	1·2±0·4

Table 2. The influence of complete Freund's adjuvant upon the rate of development of neoplastic lesions of the mammary gland in GR mouse strain females

Expt. No.	Age at inoculation of Freund's adj. and beginning forced breeding	No of animals	No. with neoplastic lesions	Average No. of pregnancies +S.D.
5	4 weeks	9	9	2·7±1·1
6	11 weeks	8	8	2·2±1·0
7	22 weeks	7	7	2·0±1·7
8	45 weeks	4	4	1·0

mice developed a lesion. The number of pregnancies needed to get such a lesion is the same in the three first experiments, 2–3 pregnancies. This indicates that the breeding stimulus, which is needed to get a tumour, is independent from the age at which the experiment is started. Tumours appear in the first pregnancy only when mice are mated for the first time at the extreme late age of 45 weeks.

Since in several experiments Freund's adjuvant has been used, the effect of this substance upon the rate of neoplastic transformation in the GR-strain has been tested (Table 2). No differences in incidences and average number of pregnancies are observed between untreated controls and the mice treated with Freund's adjuvant.

The effect of inoculations with Bittner virus in different concentrations at various ages, whether or not in combination with Freund's adjuvant, can be concluded from Table 3. Contrary to the work of Latarjet [15] and Lieberman and Kaplan [16] on murine leukaemia viruses, no significant decrease in the incidence of tumour-bearing animals is found. More striking is the increase in the number of pregnancies needed to get a tumour (exp. 9–14), especially when a high dose of virus or a low dose in combination with Freund's adjuvant is injected at four weeks of age.

For a statistical analysis it was computed in the various experiments how many animals got a tumour before four pregnancies were undergone. In the control experiments 1, 2 and 3 these numbers were 23, 6 and 12 respectively. In the control experiments with Freund's (exp. 5, 6 and 7) these numbers were 6, 7 and 5 respectively. This gives a total ratio of 59 positive to 13 negative. In the experimental groups 9–14 these numbers were 7, 3, 3, 7, 3 and 5 respectively. This gives a total ratio of 28 positive to 29 negative. The chi-square test gives a χ^2 of 15, with one degree of freedom corresponding with a $p<0{\cdot}001$. When animals are injected at seven weeks of age no significant delay can be observed unless Freund's has been used. At eleven weeks of age or later even the addition of Freund's does not cause a delay.

The carcinogenic activity of the Bittner virus preparation has been tested in several

Table 3. Effect of inoculation with Bittner mammary tumour virus on the development of neoplastic lesions of the mammary gland in GR females

Expt. No.	Dilution Bittner virus	With simultaneous injection Freund's adjuvant	Age at injection and beginning forced breeding	No. of animals	No. with neoplastic lesions	Average No. of pregnancies +S.D.
9	10^{-2}	+	4 weeks	15	14*	4·2±2·0
10	10^{-2}	−	4 weeks	7	7	4·3±1·5
11	10^{-3}	+	4 weeks	9	9	4·8±1·4
12	10^{-3}	−	4 weeks	12	10†	3·5±1·4
13	10^{-3}	+	7 weeks	7	7	3·9±1·4
14	10^{-3}	−	7 weeks	7	7	3·3±1·8
15	10^{-3}	+	11 weeks	6	6	1·8±0·1
16	10^{-3}	+	18 weeks	9	9	2·3±1·2
17	10^{-4}	+	11 weeks	8	8	1·7±0·8
18	10^{-4}	−	11 weeks	8	8	3·2±1·9

*One animal died from a lymphatic leukaemia without having developed a mammary tumour. It had undergone eight pregnancies.

†At one year of age two animals were still tumourless. One had undergone 9 pregnancies, the other seven.

Table 4. Biological activities of different dilutions of a Bittner virus preparation in BALB/c mice

Expt. No.	Dilution	No. of animals	No. with tumours at 300 days of age	Average latency period from birth in days +S.D.
19	10^{-2}	22	22 (100%)	189±47
20	10^{-3}	56	47 (84%)	161±29
21	10^{-4}	43	33 (77%)	178±31
22	10^{-5}	50	25 (50%)	192±44

dilutions in BALB/c mice (Table 4). As has already been concluded by Hageman *et al.* [18] there is no correlation between average tumour age and virus dose. Just like it can be concluded from their data, the results in Table 4 indicate a correlation between tumour incidence at 300 days and virus dose.

In Table 5 is presented the effect upon the rate of tumour-development in the GR mouse strain of Bittner virus preparations, which we attempted to inactivate. Ether-treated virus causes a similar delay as live Bittner virus, but heat-inactivated virus does not. Formalinised Bittner virus does not cause a delay if injected at two weeks of age, but causes a significant delay with regard to pregnancies if injected at four weeks of age. At eight weeks of age no delay has been found.

At four weeks of age 30 GR mice were injected with formalinised virus together with Freund's adjuvant and were mated for the first time when they reached the age of 22 weeks, all developed neoplastic lesions, for which 1·4 ±0·6 pregnancies were needed. Obviously the delay will only be found if animals are mated as soon as possible.

It has been tested whether the various treatments indeed inactivated the biological activity of the B particles (Table 6). As untreated B particles can be diluted 100,000× and still retain high carcinogenic activity, it seems that formalinization and heat-treatment lead to complete inactivation as has also been found for the Rauscher leukaemia virus [19]; ether-treatment does not inactivate the viral activity completely as has been found before [20], but it neverthelesss reduces the activity considerably.

If autochthonous virus (Mühlbock virus) is injected into GR mice at 4 weeks of age, neither acceleration nor delay in the rate of neoplastic transformation could be observed (see Table 7). This excludes the possibility that the effect of the Bittner virus is due to so-called auto-interference. In order to simulate in some way the experiments of Latarjet [15] on the effect of injection into AKR mice of Gross virus, which have been exposed to water which has been irradiated with 100,000 rad before, Mühlbock virus has been exposed to H_2O_2. Such treated Mühlbock virus also causes a delay in the development of tumours (Table 7).

The Mühlbock virus is not as effective in causing mammary tumours in BALB/c mice as the Bittner virus (Table 8). Tumours appear in a lesser incidence and at a somewhat later age than with the Bittner virus (Table 4).

*Table 5. Influence of inactivated Bittner virus on the development of neoplastic lesions of the mammary gland in GR females**

Expt. No.	Treatment Bittner virus	Combined with Freund's adjuvant	Age at injection	No. of animals	No. with neoplastic lesions	Average No. of pregnancies +S.D.
23	Ether	+	4 weeks	5	5	4·2±2·0
24	Ether	—	4 weeks	5	5	3·8±1·9
25	60°C	+	4 weeks	5	5	2·4±1·0
26	60°C	—	4 weeks	5	5	2·4±0·8
27	Formalin	—	2 weeks	10	10	2·2±0·9
28	Formalin	—	4 weeks	14	14	4·6±1·5
29	Formalin	—	8 weeks	27	27	2·6±1·2

*The amount of virus is in all experiments comparable to a 100× diluted stock solution of live Bittner virus.

*Table 6. Biological activity of treated Bittner virus in BALB/c mice**

Expt. No.	Treatment	No. of animals	No. with tumours at 300 days	Average latency period in days +S.D.
30	Formalin	19	0	—
31	60°C	21	0	—
32	Ether	14	6	163±27

*The amount of virus in the three experiments is comparable to a 100× diluted stock solution of live Bittner virus.

*Table 7. Effect of inoculations with Mühlbock virus on the development of neoplastic lesions of the mammary gland in GR-strain females**

Expt. No.	Treatment	Combined with Freund's adj.	No. of animals	No. with neoplastic lesions	Average No. of pregnancies +S.D.
33	None	—	18	18	2·2±1·3
34	H_2O_2	+	8	8	4·1±1·7
35	H_2O_2	—	8	8	4·4±1·2

*All animals were injected with a 100× diluted stock solution at four weeks of age.

The H_2O_2-treatment inactivates the biological activity of the Mühlbock virus completely, as can be concluded from Table 8

In Table 9 is reported the histological classification of the neoplastic lesions in all experiments. In most cases the animals were sacrificed several months after the lesions were first observed. The classification includes: only carcinoma; carcinoma in plaque; only plaque. In spite of the fact that we often sacrificed the animals several months after they first develop tumours, the majority of the lesions was plaques although several lesions contained carcinomatous foci.

DISCUSSION

The typical neoplastic lesions of the GR-strain mammary gland (plaques) arise only under influence of pregnancies. The number of pregnancies needed to get such a lesion may therefore be regarded as a measure of the carcinogenic stimulus. Our data clearly demonstrate that the number of pregnancies is a reliable parameter, which is in general not influenced by the age at which the experiment is started. Latency period, however, is influenced by the age at which a female may get pregnant and by irregular intervals between pregnancies. In our experiments latency period is therefore an unreliable criterium.

Negative viral interference has been observed on several occasions in the mouse mammary tumour system. Mouse leukaemia viruses are known to interfere with the Bittner virus under experimental conditions [21–23]. It has been assumed that such an interference between the two groups of viruses also plays a role in so-called natural infections [22, 24]. Riley [25] detected that the LDH agent may interfere with the production of mammary tumours. Nandi and DeOme [26] assume that the C3Hf-strain mammary tumour virus, the so-called Nodule Inducing Virus (NIV) interferes with superinfecting Bittner Virus. However, relative resistance of the C3H-strain to the Bittner virus may be genetically controlled [3].

Negative interference may be explained by: (1) competition between two viruses for replication sites in the same cell; (2) the production of interferon [27]; (3) blocking by

*Table 8. Infectivity of normal and H_2O_2-treated Mühlbock virus in BALB/c mice**

Expt. No.	Treatment	No. of animals	No. with tumours	Average latency period +S.D.
36	None	25	17	238±41 days
37	H_2O_2	18	0	—

*A 100× diluted stock preparation was used.

Table 9. Histological classification of the neoplastic lesions of the mammary gland in all experiments

Expt. No.	No. of lesions	Average age at autopsy in days+S.D.	Plaques	Plaques+ carcinoma	Carcinomas
1	36	156±18	28	7	1
2	22	177±23	17	5	0
3	8	265±33	6	1	1
4	5	422±18	4	0	1
5	15	143±27	12	3	0
6	13	190±32	10	1	2
7	11	283±11	6	4	1
8	5	403±16	2	3	0
9	20	188±21	16	3	1
10	10	195±44	7	1	2
11	14	223±24	8	4	2
12	13	165±37	12	1	0
13	9	184±42	5	3	1
14	12	173±22	10	2	0
15	13	199±36	7	5	1
16	11	286±51	3	6	2
17	15	162±17	11	4	0
18	12	194±43	6	4	2
19	23	208±42	0	0	23
20	47	172±32	0	0	47
21	38	193±29	0	0	38
22	25	203±37	0	0	25
23	7	172±23	5	2	0
24	9	194±48	6	1	2
25	5	163±50	4	1	0
26	8	182±39	4	2	2
27	14	188±10	12	2	0
28	23	212±44	17	3	3
29	41	200±21	31	5	5
32	6	171±24	0	0	6
33	25	152±18	21	4	0
34	8	134±27	6	2	0
35	11	162±22	7	3	1
36	21	261±43	9	6	6

one virus of the early phases of infection of the second virus, presumably at the level of the cell membrane [28]; (4) the induction of antibodies by one virus which will cross-react with the second virus [15], and (5) in the same way sensitization of lymphocytes, which will attack virus-producing cells.

Our results with inactivated virus preparations exclude competition as a possible explanation for the observed delay in the development of neoplastic lesions of the mammary gland. If interferon is involved, it is then hard to understand how H_2O_2-treated Mühlbock virus induces a delay, whereas untreated Mühlbock virus fails to do so. The third possibility of blocking at the membrane level is also not very tenable because the Mühlbock virus, which is the victim of interference, is present before the Bittner virus is inoculated. Par exclusionem we arrive then at the conclusion that inoculation with the Bittner virus or H_2O_2-treated Mühlbock virus induces immunity to virus antigens which also will attack the Mühlbock virus.

The GR strain is assumed to be completely tolerant to its eggborne Mühlbock virus [3, 11] as has been described for an eggborne avian leukosis virus [29]. The presumably antigenically different Bittner virus or the antigenically altered H_2O_2-treated Mühlbock virus may then cause a breakthrough in this tolerance. That we are indeed dealing with an immunological reaction is supported by our finding that Freund's adjuvant can enhance the weak delaying effect of low doses of virus. Pilot experiments indicated the presence of precipitating antibodies, which react with an insoluble antigen of both the Mühlbock and the Bittner virus, in the serum of GR-mice treated with the Bittner virus, whereas antibodies are absent in the serum of untreated controls.

Blair *et al.* [30] found in some C3H mice precipitating antibodies directed against Bittner virions despite neonatal infection with the virus. We observed in our laboratory that tumour-bearing C3H and BALB/cfC3H mice contained

antibodies against the whole virion and a soluble antigen of the Bittner virus. We could not find it in normal mice of these strains (Daams and Bentvelzen, unpublished results). This indicates the existence of an 'underground' immunity in such mice which becomes manifest after 'booster' injections with B particles into the blood due to the presence of the tumour. Yunis and Stutman [31] found that injection of C3H spleen cells into neonatally thymectomised C3H mice interferes with the development of mammary tumours, whereas an injection with C3Hf spleen cells does not give such a reaction. This strongly indicates the presence of immunity to the Bittner virus in C3H mice. In over a hundred tumour-bearing GR mice, we examined we could not find precipitating antibodies to the Mühlbock virus which is in accordance with the concept of true tolerance to this virus in the GR strain [3, 11].

Although we favour the hypothesis of a break-through in tolerance to the Mühlbock virus by inoculations with Bittner virus, it is still difficult to imagine how immunity would cause a delay in the development of neoplastic lesions. With the aid of completely mammectomized mice, Dux and Mühlbock [32, 33] demonstrated that the Bittner virus can multiply in the absence of the mammary gland. The work of Nandi *et al.* [34–37] strongly suggests that, after inoculation, the Bittner virus first replicates in the haemopoietic tissues in which no mature virions are formed, and that a small form of the virus then infects the mammary gland [36]. This small form of the virus seems to be associated with H-2 antigens [38] and it is very likely that this viral entity will not be attacked by antibodies directed against the mature virion [39]. Therefore, it can be understood why blood of BALB/cfC3H can infect old BALB/c mice, while mammary tumour extracts fail to do so [4]. In our experiments, it is not likely that antibodies against the Bittner virions also neutralise blood-borne Mühlbock virus.

The possibility may not be overlooked that, due to the presumed genetical transmission of the Mühlbock virus [3, 6, 10, 11], another mode of infecting mammary cells exists other than through life cycles of the milkborne Bittner virus, as postulated by Nandi [36], or Yunis and Stutman [31]. In our experiments lymphocytes or antibodies may interfere with this mode of infection.

Another possibility is that antibodies or lymphocytes attack virus-producing cells and in this way delay the development of a palpable tumour. We favour this hypothesis and then especially that lymphocytes, sensitized against the Bittner virus, will attack mammary cells, which produce Mühlbock virions.

Most lesions we observed were plaques, even if we sacrificed the animals several months after we first observed the lesion. Often carcinomatous foci were present in these plaques. In general, our manipulations lead to a delay of two pregnancies before plaques appear. It is possible that the transformation of plaques into carcinoma is also delayed. Medina and DeOme [41] demonstrated that the Bittner virus not only induces hyperplastic nodules but may also cause transformation of nodules into carcinomas. Therefore, it seems very likely that the Mühlbock virus also plays a role in the development of carcinomas from plaques and that immunity towards the virus can interfere with this development. Our material does not give an answer to this problem. Right now we do not know a satisfactory method to measure the time needed for such a change.

An interesting phenomenon is the time range in which Bittner virus has to be injected in GR mice in order to get a delay in plaque appearance. The failure at two weeks can be explained by a too weak immunological response at that age. The failure at ages older than seven weeks can be explained by such an increase of Mühlbock virus in the mammary gland within seven weeks, that immunity will be ineffective.

We have observed more than once that mammary tumour incidence is dependent on the dose of injected virus [18] as has also been found for Rous sarcoma virus [42]. A similar relationship has been found for the amount of virus ingested during nursing [3, 39, 43] but not for injections of several dilutions of a tumour extract [44]. Our results indicate that during the establishment of an infection an interfering mechanism is involved which acts proportionally to the amount of virus given. This mechanism can be the rapid development of an immune response and, more likely, the production of interferon. Experiments are under way to elucidate this mechanism.

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RESUME

Toutes les femelles de la souche consanguine de souris GR, qui se reproduisent de façon répétée, développent des tumeurs mammaires après une moyenne de trois grossesses; ce phénomène ne dépend pas de l'âge auquel l'expérience a débuté. Dans le cas, uniquement, où l'accouplement commence à l'âge de 45 semaines, ces tumeurs ce développent au cours de la première grossesse.

Des inoculations de virus de Bittner, isolé des tumeurs mammaires de la souche BALB/cfC3H, soit vivant, soit formolé ou traité à l'éther, retarde de deux grossesses le développement des tumeurs. Pour obtenir ce retard, il est nécessaire d'inoculer le virus entre les âges de 4 et 7 semaines.

L'inoculation du virus de Mühlbock vivant, isolé des tumeurs mammaires de la souche GR, n'a pas d'effet sur le développement des tumeurs; cependant, si le virus est traité à l'H_2O_2, on constate un retard de deux grossesses.

On suppose que le virus de Bittner et le virus de Mühlbock, traités à l'H_2O_2, induisent l'immunité contre les cellules infectées du virus de Mühlbock. Il est probable que c'est à une différence antigénique entre le virus de Bittner et le virus de Mühlbock que l'on doit attribuer l'immunisation de la souche GR par le virus de Bittner, alors que cette souche est tolérante au virus de Mühlbock.

Ces virus sont très carcinogènes pour les souris de la souche BALB/c. Le chauffage, la formolisation, ou le traitement à l'H_2O_2, causent l'inactivité complète du virus, alors que le traitement à l'éther n'occasionne qu'une réduction de l'activité de celui-ci. La fréquence de la tumeur mammaire, chez les souris BALB/c, est liée à dose de virus injectée. On peut donc conclure, d'après cette réponse, que l'immunité et l'interféron peuvent empêcher l'apparition de tumeurs, chez certaines souris, suivant la dose de virus injectée.

SUMMARY

Forced breeding GR mouse strain females get 100% *neoplastic lesions of the mammary gland (so-called plaques) within an average of three pregnancies, irrespective of the age at which the experiments are started. Tumours develop in the first pregnancy only, if females are mated for the first time at* 45 *weeks of age. Inoculations with either live or formalinised or ether-treated Bittner virus, isolated from BALB/cfC3H mouse strain mammary tumours, cause a delay in the development of these neoplastic lesions of two pregnancies, if injected at the age of* 4–7 *weeks. Injections at two or eight weeks of age fail to induce such a delay. Heated Bittner virus did not cause such a delay either.*

Live Mühlbock virus, isolated from GR mouse strain mammary tumours, had no effect upon the rate of neoplastic transformation, but H_2O_2-treated Mühlbock virus also caused a delay of two pregnancies.

It is assumed that the antigenic difierence between the Bittner and the Mühlbock virus and the antigenic alterations in the Mühlbock virus, caused by the H_2O_2-treatment, induces immunity to the Bittner virus which is also directed against the Mühlbock virus to which GR mice are ordinarily completely tolerant.

Bittner and Mühlbock viruses were highly carcinogenic in BALB/c mice. Formalinization, heating, or H_2O_2-treatment leads to complete inactivation, but ether-treatment leads only to a reduction of activity. Mammary tumour incidence in BALB/c mice is correlated with the dose of virus injected. From the observed dose-response relationship it has been concluded that immunity and interferon can prevent the establishement of an infection in some mice, proportional to the amount of virus given.

ZUSAMMENFASSUNG

Weibchen des Inzuchtmäusestammes GR entwickeln in 100% *der Fälle Tumoren der Milchdrüse, wenn sie dauernd in Schwangerschaft gehalten werden. Diese Tumoren entstehen im Durchschnitt im Laufe von drei Schwangerschaften, unabhängig vom Alter, bei dem die Züchtung begonnen wurde. Nur wenn sie erst in der* 45. *Woche anfing, entwickeln sich die Tumoren schon während der ersten Schwangerschaft.*

Impfung mit lebendem, mit Formol oder mit Aether vorbehandeltem, aber nicht erhitztem Bittner-Virus, das aus Milchdrüsentumoren des BALB/cfC3H Stammes isoliert wurde, verursacht eine Verzögerung des Entstehens der Tumoren um zwei Schwan-

gerschaften. Um diese Verzögerung zu erreichen, müssen die Injektionen zwischen der vierten und siebenten Woche gegeben werden.

Verwendet man lebendes Mühlbock-virus aus GR-Milchdrüsentumoren, so findet man keinen verzögernden Effekt, außer wenn es mit H_2O_2 vorbehandelt war.

Es wird angenommen, daß das Bitter-Virus sowie das Mühlbock-Virus nach H_2O_2-Behandlung Immunität gegen mit Mühlbock-Virus infizierte Zellen induzieren. Wahrscheinlich ist der Antigenunterschied zwischen dem Bittner- und dem unbehandelten Mühlbock-Virus der Grund, warum das Bittner-Virus immunisert, aber das Mühlbock-Virus, für das die GR Weibchen von Geburt tolerant sind, nicht.

Das Bittner- und das Mühlbock-Virus sind für BALB/c Mäuse sehr karzinogen. Erhitzung, Formolvorbehandlung oder Behandlung mit H_2O_2 führen zu kompletter Inaktivierung des Virus wie aus der Abwesenheit von Tumoren bein BALB/c Mäusen hervorgeht. Behandlung mit Aether gibt nur eine Verringerung der Aktivität. Die Frequenz der Tumoren ist abhängig von der Dosis des injizierten Virus. Aus diesem Dosis-Wirkungs-Verhältnis kann geschlossen werden daß Immunität und Interferon in manchen Mäusen die Entwicklung von Tumoren, in Abhängigkeit von der Virusdosis, verhindern können.

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

I. Effects of Single Exposures

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INTRODUCTION

DISCUSSIONS of cellular aspects of radiotherapy have been stimulated greatly by insights obtained during the past decade with respect to responses of mammalian cells to ionizing radiation [1]. In particular results of experiments concerning biological effects produced by different types of ionizing radiations have induced several investigators to re-evaluate the use of fast neutrons for the treatment of solid tumours [2–5]. This interest is based on information about differences between dose–effect relations, with respect to impairment of the reproductive capacity of mammalian cells, measured for low-LET radiations, e.g. X- and γ-rays, and high-LET radiations, e.g. fast neutrons, respectively [6–11].

The studies of responses of experimental tumours which will be reported in the present paper can be considered as an extension of investigations with cultured mammalian cells [3, 5]. The experiments were started to evaluate whether the differences between effects of various radiations observed *in vitro* correspond directly to differences between effects in tumours [5, 10, 12, 13]. It will be shown that the results of these experiments are not only of interest for an assessment of the possibilities of fast neutron radiotherapy, but that in addition various aspects of growth properties of tumours and their reactions to treatments with conventional low-LET radiations have been elucidated.

Studies with cultured mammalian cells have shown that three main factors must be considered in investigations of differences between effects of low-LET and high-LET radiations, namely the relative biological effectiveness (RBE), the extent of repair of sub-lethal damage in irradiated cells and the oxygen enhancement ratio (OER) [3–5, 10].

The RBE for impairment of the capacity for unlimited proliferation by mammalian cells is known to depend on the dose employed [8, 11]. With low-LET radiations, dose–survival curves generally exhibit an initial region of low slope followed by a more rapid decrease of the surviving fraction at high doses. With high-LET radiations, the shoulder of the curve at low doses is generally small or absent, i.e. the survival curve closely approximates an exponential curve [6, 7]. As a consequence of this difference between the shapes of the survival curves, the RBE-values of high-LET radiations attain the highest values at low doses and decrease with increasing dose [8–10, 14].

The second factor is concerned with recovery of sub-lethal damage in irradiated cells [15]. The shapes of the survival curves for low-LET radiations indicate that at higher doses part of the cell killing is due to accumula-

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tion of damage. By inference, sub-lethal damage is present in irradiated cells which survive after a given dose. These cells are known to recover from this sub-lethal damage within a few hours after irradiation. After this interval, they respond to a subsequent irradiation as if they had not been irradiated at all [15]. As a consequence, fractionated irradiation with low-LET radiation is less effective than irradiation with single doses if the same total dose is employed. It has been shown that with high-LET radiation the effect of fractionation is greatly reduced or absent [8, 9]. Consequently, the RBE of high-LET radiation depends on the fractionation regime, i.e. for a given total dose the RBE increases with increasing number of fractions [2, 5, 9]. This phenomenon has led to the suggestion that the adverse late effects on normal tissues, observed by Stone, Aebersold and Lawrence after clinical applications of cyclotron-produced fast neutrons between 1937 and 1943, may have been caused by over-exposure as a result of the application of RBE factors, which were derived from single exposures and were too low for the case of fractionated treatments [2–4, 16].

A third important difference between effects of low-LET and high-LET radiations is the smaller dependence of the effectiveness of the latter type of radiation on various modifying factors, notably oxygen [3, 5, 9, 17]. The presumed importance of this phenomenon in radiotherapy is based on evidence from experimental tumours, indicating that the curability of tumours with X-rays, γ-rays and fast electrons may be limited by the radio-resistance of a proportion of hypoxic cells [18, 19]. Consequently the use of radiations with an oxygen enhancement ratio that is considerably smaller than the factors between 2·3 and 3·2, generally measured for X- and γ-rays, may provide a significant advantage in radiotherapy. Although little is known about other modifying factors present in tumours, their influence may be expected to be smaller for high-LET radiations [17].

In a series of experiments with cultured cells of human origin, survival curves have been obtained for neutron beams with mean energies ranging from about 1 MeV to 15 MeV. The results demonstrated that the OER-values vary only little with neutron energy, ranging from 1·5 to 1·6 in this energy interval [20]. Since 15 MeV neutrons have a more favourable penetration in tissue than neutrons of lower energies, they offer the best prospects for the clinical application to deep-seated tumours which contain fractions of anoxic cells [3, 20, 21]. The studies reported in the present paper deal with a comparison of dose–survival relations of tumour cells, of growth delay and of cure rates measured after irradiation of a transplantable rhabdomyosarcoma in the rat with 300 kV X-rays and 15 MeV neutrons, respectively.

MATERIAL AND METHODS

(a) Characteristics of the tumour and its constituent cells

The transplantable solid tumours employed in our studies were derived from a rhabdomyosarcoma, which originated in December 1962 in the mandibule of an irradiated rat of the inbred WAG/Rij strain. This tumour was first propagated for nine passages, by subcutaneous injection of cell suspensions, prepared by the method of Reinhold [22]. Subsequently, a selection procedure was applied in order to obtain a tumour with reproducible growth characteristics, which would allow various types of investigations to be made. This selection procedure consisted of several steps. First a suspension of tumour cells was cultured *in vitro* in December 1963. The medium consisted of Hanks' salt solution, Eagle's amino acids and vitamins (MEM), 10% newborn calf serum and 100 IU penicillin/ml. A fraction of the cultured cells attached to the bottom of a Petri dish and developed into a monolayer. This cell culture has been employed for cloning experiments. Cell suspensions were prepared by gentle trypsinization of cultures growing in glass bottles. Inoculation of known numbers of unirradiated cells in Petri dishes with a Melinex* bottom, yielded plating efficiencies of 80±20% [5, 14].

From this established cell line, a single clone of cells was isolated, by cutting from the bottom of the dish the area of thin Melinex, on which it was located. These cells were inoculated in the flank of a WAG/Rij rat, where they developed into a tumour. Subsequently, a cell suspension was prepared from this tumour and the cells were cultured *in vitro*. This transfer from the tumour to the *in vitro* culture system and the inoculation of a single clone back into the animal, was repeated several times and finally resulted in December 1966 in a tumour denoted R-1, which can be studied both *in vivo* and *in vitro* by various techniques to be described.

Transplantation of the tumour in rats of the WAG/Rij strain can be performed by implan-

*Polyethylene terephthalate, ICI, Herts, England.

tation of small pieces of a tumour or by subcutaneous inoculation of a cell suspension, either obtained from a cell culture or from a tumour in a rat. The tumours have shapes which approximate closely to a sphere. The number of cultured cells required to produce tumours at the site of inoculation in 50% of the cases was equal to about 100. However, this number depends to a considerable extent on the method and location of inoculation. The cause of the failure of smaller numbers of cells to give rise to a tumour has not been elucidated. Reinhold has shown that for the original rhabdomyosarcoma, from which the R-1 rhabdomyosarcoma was derived by selection, antigenic differences could not be detected by various immunization techniques [23].

The tumours arising from inoculated R-1 cells grow as solid masses, in which the cells are arranged in intertwining strands. The cells are spindle-shaped with an elongated nucleus. The cytoplasm is usually scanty. The stroma is not abundant and consists of tiny bundles of collagenous and reticular fibres, which form a small-meshed network in close contact with the cells. The cells show only minor morphological variations. In the tumours only scattered pyknotic cells are observed, which tend to be more abundant in the centre. At the start of experiments, tumours are selected with volumes between 0·5 and 1·0 cm^3.

The growth rate of the tumours, growing in the flank of rats, decreases with increasing volume as shown in Fig. 3 curve 1. Tumours of 0·5–1·0 cm^3 have a volume doubling time of 4 ± 1 days. Measurements of the cell cycle time of the proliferating cells in these tumours, using pulse labelling with tritiated thymidine and autoradiographic analysis, yielded a value of about 20 hr [24]. From semi-continuous labelling and pulse labelling experiments, a growth fraction of proliferating cells of about 0·4 could be derived [24, 25]. In addition, considerable cell loss occurs [25].

The R-1 cells in cell culture conditions have a doubling time of the mean number of cells per clone of 17 ± 2 hr. This value is not significantly different from the cell generation cycle of 16 hr determined from autoradiographic analysis of pulse labelled cultures [25]. This implies that in small clones the growth fraction is 1·0 and cell loss is negligible [25].

(b) Assay of fractions of clonogenic cells in irradiated tumours

The selection technique described in the preceding section has resulted in tumours containing cells which, after application of Reinhold's dispersion technique, can be cultured directly in Petri dishes with a Melinex bottom. These cells produce clones with a plating efficiency of $35\pm10\%$ for unirradiated tumour cells. At 12 days after plating, clones have developed containing a few hundreds to a few thousands of cells. The number of clones can be scored after fixation with Bouin and staining with haematoxylin. If cells from a suspension prepared from irradiated tumours are cultured *in vitro*, the spread in clone size is considerably larger than for cells derived from unirradiated controls [46, 47]. Clones containing 50 or more cells at 12 days after plating are considered to have originated from a clonogenic tumour cell that has retained the capacity for unlimited proliferation. The use of conditioned medium and of 10,000 lethally irradiated cells per dish as a feeder layer, has been found to improve the reproducibility of the plating efficiency [26, 27].

Using this plating technique for cells derived directly from a tumour, fractions of clonogenic cells can be measured for tumours which have been irradiated with various single and fractionated doses of radiation. With this assay method, the advantages of the cell culture technique, with respect to speed and accuracy of experiments, can be employed to derive survival curves for tumour cell populations irradiated *in vivo*. This method is less elaborate and far more economical than the quantitative titration *in vivo*, by the end-point dilution technique, since each clone, scored after 12 days on a Petri dish, provides approximately the same amount of information as a rat in which a given inoculum of cells grows into a tumour within 150 days. Furthermore this assay is not complicated by host factors operating against the tumour cells. A disadvantage of this technique is that accurate and reproducible results are obtained only for doses where the surviving fractions of cells range between 1 and 10^{-4}. If more than 40,000 cells have to be plated in a dish in order to obtain a few clones, the numbers of clones which develop per dish show wide variations between dishes plated with the same number of cells. The causes of these difficulties have not been resolved. The use of larger and more dishes could probably extend the range to surviving fractions of 10^{-5}, but no attempt has been made to achieve this.

(c) Measurements of tumour growth delay and cure rates

After irradiation of a R-1 rhabdomyosar-

coma with a dose in excess of 2000 rad of X-rays, the volume of the tumour continues to increase during the first two days, but at a reduced growth rate relative to controls. Starting on the third day the volume decreases. If the dose is not sufficiently large to attain a cure, the tumour will start to grow again after a certain time interval [5, 25]. The time interval after irradiation, required for a tumour to regrow to its original size, depends on the radiation dose. In studies of tumour growth delay as a function of the radiation dose, tumour volumes were determined from measurements of three dimensions of the tumours with vernier callipers at 2 or 3 days intervals. The tumours can be easily palpated through the skin and true volumes can be derived by calibration with extirpated tumours. Tumour growth delay after irradiation has been assessed from the time interval required for the tumour to attain twice the volume at the time of irradiation and subtraction of the doubling time of unirradiated tumours. In this way growth delay can also be estimated for tumours irradiated with low doses, which do not cause a temporary decrease in volume, but only induce a temporary reduction in the rate of growth (cf. Fig. 3 curve 2). This method for the measurement of growth delay is similar to the technique used extensively by Thomlinson [28, 29].

Tumour cure rates were estimated by the observation of groups of 10 or more animals during periods up to 6 months after irradiation. No further recurrences have been observed after 150 days. Tumour cure rates have therefore been calculated as the fraction of tumours that have not recurred within 150 days.

(d) Irradiation techniques and dosimetry

Irradiations of tumours with 300 kV X-rays were carried out with a Philips–Müller X-ray generator operated at 10 mA. The rats are anaesthetized by injection of 0·45 mg of Veterinary Nembutal per gram body weight and placed on a horizontal platform of 4 mm thick lead, with a centre hole of 25 mm diameter. The tumours growing in the flanks of the rats were positioned over the hole, through which the X-ray beam passed vertically. Characteristics of the irradiation conditions are: HVL 2·0 mm Cu, FSD 20 cm, dose rate 340 rad/min.

Mono-energetic neutrons of 15 MeV energy were produced by bombarding tritium, absorbed in a titanium layer on a copper backing, with 400 keV deuterons at a current of 250 μA, accelerated with a Van de Graaff accelerator, made by High Voltage Engineering Corporation. Due to the limited output of this generator, equal to 2×10^{10} neutrons/sec, the tumours had to be irradiated at a rather short distance of about 6 cm from the target in order to obtain a dose rate of 20 rad/min. As a consequence, only a 5 cm thick collimator could be provided in front of the animals. Studies of shielding properties of different materials have shown that steel is the most suitable material for collimation of 15 MeV neutrons [30–32]. The steel collimator of 5 cm thickness used in the present experiments absorbs only 50% of the dose, i.e. part of the intestines close to the tumour receive 50% of the dose delivered to the tumour, which is positioned in front of a circular hole of 24 mm diameter in the collimator. Absorbed doses were derived from measurements with tissue-equivalent ionization chambers. The dose distribution in the tumour was investigated by irradiating sulphur pellets mounted behind slabs of tissue-equivalent plastic of various thickness [33]. Dose variations over the tumour amounted to 20% of the mean value.

The experiments reported here were limited to relatively low doses of up to 800 rad, since at higher doses the animals died from the total body irradiation, due to neutrons passing through the shield.

EXPERIMENTAL RESULTS

(a) Survival curves of R-1 rhabdomyosarcoma cells, cultured in vitro, *assayed* in vitro *or* in vivo

In Fig. 1, the fractions of cultured R-1 cells, which after irradiation have retained the capacity for unlimited proliferation *in vitro*, are presented as a function of doses of 300 kV X-rays and of 15 MeV neutrons respectively. Cells derived by trypsinization of a stock culture were plated in dishes with a Melinex bottom. After a 4 hr interval, required for attachment to the bottoms of the dishes, the medium was removed and the cells were equilibrated with air or with nitrogen containing less than 50 ppm of oxygen, for a period of 10 min prior to and during irradiation [34]. The data presented are mean values derived from the numbers of clones on at least four dishes irradiated with the same dose and the standard errors were calculated from the variation between the numbers of clones on these dishes [9].

The X-ray survival curves have shapes similar to those commonly observed for mammalian cells irradiated with low-LET radiation, i.e. an initial region of low slope is followed at higher doses by a more rapid decrease of the

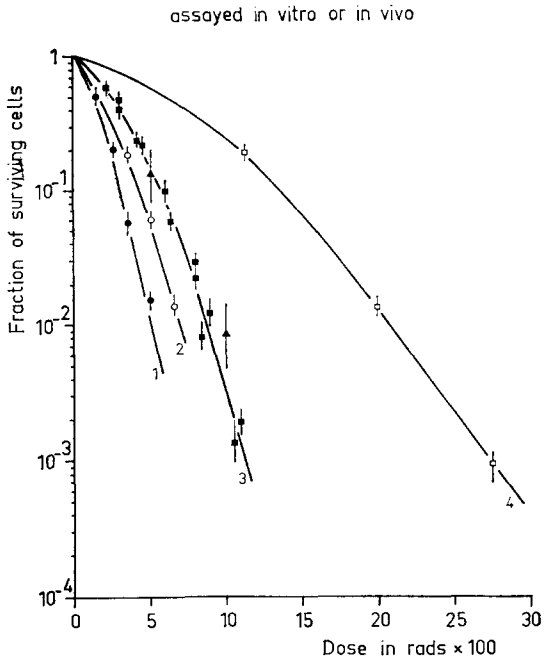

Fig. 1. *Survival curves obtained by the clone technique after irradiation of cultured R-*1 *rhabdomyosarcoma cells with* 15 *MeV neutrons or* 300 *kV X-rays.*

Curves 1 *and* 2*: Cells in equilibrium with air (closed circles) and nitrogen (open circles) respectively, irradiated with* 15 *MeV neutrons.*

Curves 3 *and* 4*: Cells in equilibrium with air (closed squares) and nitrogen (open squares) respectively, irradiated with* 300 *kV X-rays.*

*The two closed triangles were obtained by the end-point dilution assay after X-irradiation of cultured R-*1 *rhabdomyosarcoma cells in equilibrium with air.*

surviving fraction with the dose. For the region between surviving fractions of 0·1 and 0·001, the X-ray survival curve 4 of Fig. 1, measured for cells in equilibrium with nitrogen, can approximately be represented by a mean lethal dose D_0 of 295 rad and an extrapolation number N equal to 10. The survival curve 3, for cells in equilibrium with air, can be described by a D_0 of 120 rad and an N equal to 10. The OER derived from these curves for 300 kV X-rays is $2{\cdot}4 \pm 0{\cdot}2$.

In order to compare the assay technique for clone formation *in vitro* with the transplantation technique, cells from the same stock culture were irradiated with different doses of X-rays and subsequently inoculated in serial dilutions in rats*. Surviving fractions were calculated from the numbers of cells required for 50% tumour takes by the method of Hewitt and Wilson [23, 35]. It is clear from the two triangular points shown in Fig. 1 that the results obtained with the two assay techniques are not significantly different.

The survival curves obtained with 15 MeV neutrons have a considerably smaller curvature in the low dose region as compared with the X-ray survival curves. The high dose region of curve 2, for cells in equilibrium with nitrogen, can approximately be described by a D_0 of 120 rad and an N of 3 and curve 1 for cells in equilibrium with air by a D_0 of 85 rad and an N of 3. The OER value derived from these curves for 15 MeV neutrons is $1{\cdot}4 \pm 0{\cdot}1$.

*(b) Survival curves of R-*1 *rhabdomyosarcoma cells, irradiated in the tumour and assayed* in vitro

In Fig. 2, survival curves are presented for cells from tumours irradiated in flanks of animals, which were either alive during irradiation or had been killed 10 min prior to irradiation. This latter condition produces severe hypoxia of the cells [23, 36]. The tumours were excised after irradiation and a cell suspension was prepared by the dispersion technique described by Reinhold [22]. The concentration of cells in this suspension was determined with a haemocytometer and known numbers of cells were plated in at least 5 dishes. Clones were scored after 12 days of incubation. Fractions of cells capable of unlimited proliferation were calculated from the mean numbers of clones per dish, relative to the numbers of cells plated. Standard errors, calculated from the variation of the numbers of clones per dish, were usually less than 10%.

*We are indebted to Dr. H. S. Reinhold for his co-operation in this assay.

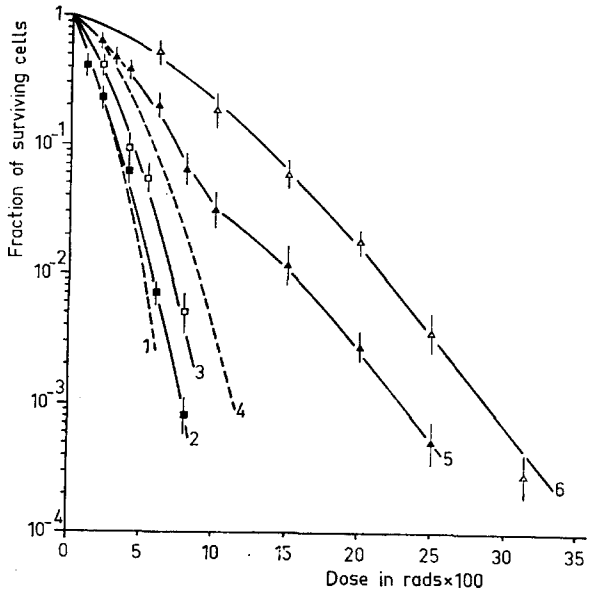

Fig. 2. Survival curves obtained by irradiation of R-1 rhabdomyosarcomas growing in the flanks of rats, followed by excision of the tumour and plating of cells in vitro.

Curves 2 *and* 3: *Tumours irradiated with* 15 *MeV neutrons in living anaesthetized and dead rats respectively (closed and open diamonds).*

Curves 5 *and* 6: *Tumours irradiated with* 300 *kV X-rays in living anaesthetized and dead rats respectively (closed and open triangles).*

Curves 1 *and* 4: *Included for comparison, represent the survival curves for cultured R-1 cells in equilibrium with air, irradiated with* 15 *MeV neutrons and* 300 *kV X-rays respectively.*

This error is generally small compared to differences observed between values obtained for individual tumours irradiated with the same dose. These latter variations may reflect differences in oxygenation and growth fraction within a population of tumours. As noted earlier, the mean plating efficiency of cells derived from unirradiated tumours is $35 \pm 10\%$. The standard deviation of this value was calculated from the variation in the plating efficiency of about 100 assays performed within a period of 2 years. If a number of assays of unirradiated tumours is made within a period of 2 weeks, the standard deviation is found to be smaller by a factor of about 2. This indicates that long-term variations occur, either with respect to the number of clonogenic cells present in the tumour or with respect to the efficiency of the plating procedure. Therefore, fractions of cells surviving after irradiation of a tumour were calculated as the ratio between the fraction of clonogenic cells and the mean plating efficiency obtained for control tumours assayed in the preceding interval of 3 weeks. The data presented in Fig. 2 are mean values and standard errors derived from values of at least three tumours assayed separately after irradiation with equal doses.

Curve 6 of Fig. 2 has been obtained with 300 kV X-rays for cells from tumours irradiated in dead animals. Its shape is not significantly different from the curve for cultured cells, irradiated in equilibrium with nitrogen, presented in Fig. 1, curve 4. The high dose region, beyond doses of 1500 rad, can approximately be represented by a D_0 of 305 rad and an N of about 10. It can be concluded that R-1 cells grown in culture and in the tumour respectively, exhibit closely similar sensitivities to X-rays if they are irradiated in severely hypoxic conditions.

Curve 5 of Fig. 2 for cells irradiated in tumours in living animals has a shape which is characteristic for a population of cells consisting of a large fraction of oxygenated cells and a smaller fraction of hypoxic cells, as will be discussed later. At doses in excess of 1500 rad, this curve is parallel to curve 6 for hypoxic cells and can approximately be represented by a D_0 of 305 rad and an N of 1·5.

The dotted curve 4 of Fig. 2, included for comparison, represents the survival of cultured R-1 cells irradiated in equilibrium with air as shown in curve 3 of Fig. 2.

Curve 3, obtained with 15 MeV neutrons for cells from tumours irradiated in dead rats, has a shape which does not differ significantly from the corresponding curve for cultured cells in equilibrium with nitrogen shown in Fig. 1. The region corresponding to surviving fractions of less than 0·1 can be represented by a D_0 of 120 rad and an N equal to 3. Curve 2 has

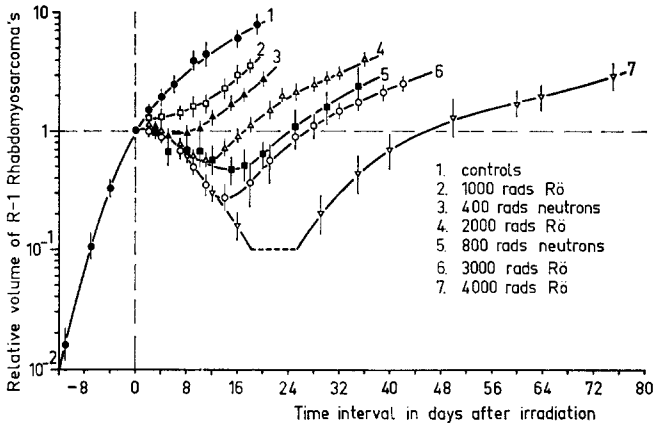

Fig. 3. *Volume changes of R-*1 *rhabdomyosarcomas as a function of the time interval after irradiation. The points indicate mean values of the ratios of the volume measured at a given time and the volume at the time of irradiation.*
Curve 1 *represents the growth of unirradiated tumours.*
Curves 2, 4, 6 *and* 7 *were obtained after irradiation with* 1000, 2000, 3000 and 4000 rad of 300 *kV X-rays respectively.*
Curves 3 *and* 5 *were obtained after irradiation with* 400 *and* 800 *rad of* 15 *MeV neutrons respectively.*

been obtained with 15 MeV neutrons for cells from tumours irradiated in living rats. The region between 0·1 and 0·001 survival can approximately be described by a D_0 of 110 rad and an N equal to about 1·5. Curve 1, which represents curve 1 of Fig. 1 for cultured R-1 cells, irradiated in equilibrium with air, has been included for comparison.

(*c*) *Tumour growth delay and tumour cure rates*

In Fig. 3, tumour volume changes, induced by different doses of 300 kV X-rays and 15 MeV neutrons, are presented as a function of the time interval after irradiation. For each curve, 5 or more tumours with volumes between 0·5 and 1·0 cm^3, growing in the flanks of rats, were irradiated. From the three measured dimensions, tumour volumes were calculated relative to the volume at the time of irradiation [24]. As mentioned earlier, a correction has been applied for the effect of the skin thickness, using a calibration curve obtained from measurements of excised tumours. The points shown in Fig. 3 represent mean values. The vertical lines represent standard errors computed from the variation between values derived for at least 5 tumours. Curve 1 is the growth curve for unirradiated tumours.

The curves 2, 4, 6 and 7 show that the growth

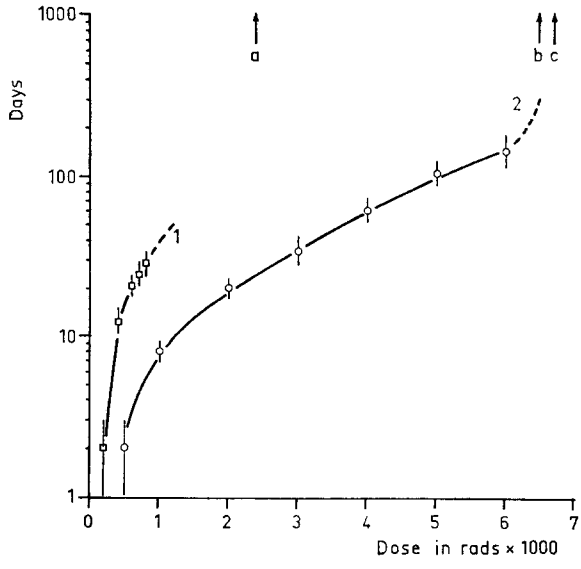

Fig. 4. *Growth delay of R-*1 *rhabdomyosarcomas as a function of the dose of* 15 *MeV neutrons (curve* 1*) and* 300 *kV X-rays (curve* 2*). Arrows* a *and* c *indicate the doses of* 15 *MeV neutrons and* 300 *kV X-rays respectively, calculated on the basis of survival curves to be required for attatining cure rates of* 90 *per cent. Arrow* b *indicates the observed TCD* 90 *for* 300 *kV X-rays.*

delay, defined as the time interval relative to control tumours required to grow to a volume equal to twice the volume at the time of irradiation, increases with the dose of X-rays, but at doses of up to 5000 rad, no cures have been obtained. At a dose of 6000 rad, 3 out of 9 animals were cured, at a dose of 6500 rad, 1 out of 10 tumours recurred, and at 7000 rad no recurrences have been observed in ten animals within 150 days. The dose required for 50% cures (TCD 50) can be estimated at 6150 ± 200 rad.

For 15 MeV neutrons, growth delay could be measured only up to doses of 800 rad, due to the limited amount of shielding which could be provided for the animals. Consequently, with the present generator no cures could be obtained*. In a number of earlier studies a different arrangement was used with the tumour in a position directly adjacent to the target [5]. However, the dose distribution in the tumour was unfavourable and the results obtained with this arrangement have not been included in the present discussion.

In Fig. 4, tumour growth delay has been presented as a function of the doses of 15 MeV neutrons or 300 kV X-rays respectively. In this figure are also indicated the doses required for attaining 90% cures, calculated by extrapolation of the survival curves (arrows *a* and *c*, respectively), and the observed TCD 90 for 300 kV X-rays (arrow *b*).

(d) Changes of the numbers of clonogenic cells in a tumour as a function of the time interval after irradiation

Measurements of fractions of surviving cells, of tumour growth delay and of cure rates after single doses, provide only a correlation of the amount of damage induced, with the gross responses of the tumours observed after many days. In order to investigate quantitatively the proliferation of surviving cells in irradiated tumours during the period of regression and recurrence, measurements of fractions of clonogenic cells were carried out as a function of the time interval after treatment. In these experiments a number of tumours with volumes between 0·5 and 1·0 cm^3, growing in the flanks of rats, were irradiated with doses of 300 kV X-rays or 15 MeV neutrons, respectively and at different time intervals after irradiation, tumours were excised. From each tumour a cell suspension was prepared and assayed for the fraction of clonogenic cells by the *in vitro* plating technique.

The results of these measurements for tumours irradiated with 1000 rad or 2000 rad of 300 kV X-rays are presented in Fig. 5C. Each point of curve 1d and 2d represents the fraction of clonogenic cells relative to the total number of cells present in a tumour, excised and assayed at a given time interval after irradiation. All values have been divided by the plating efficiency of cells from unirradiated tumours, which served as a base line value. This implies that the increase of the fraction of clonogenic cells to the value 1·0 demonstrates a return of this parameter to a value equal to that of unirradiated tumours. Although cells from a given tumour cell suspension are always plated on 5 dishes, the standard errors, which can be calculated from the variation of the number of clones per dish, are not presented in Figs. 5 and 6, because they are usually less than 10% of the value measured and are small compared to differences observed between several tumours treated identically. These differences between tumours which were irradiated with the same dose and assayed after the same time interval may be due to various factors, e.g. variations of the fractions of anoxic cells in the tumours at the time of irradiation, differences in growth fractions, related to tumour volume and differences in the supply of blood and nutrients. In addition the removal of dead cells which possibly influences the onset of repopulation of the tumours by cells surviving after irradiation may occur at different rates in different tumours.

Figure 5C shows that doses of 1000 and 2000 rad of X-rays reduce the fractions of clonogenic cells in these tumours to about 3·5% and 0·27% respectively. The data demonstrate further that during the first few days after irradiation with doses of 1000 rad and 2000 rad of X-rays, the ratio of the number of clonogenic cells and the total number of cells in the tumour changes only slowly, but after about 4 days a rapid increase is observed which continues until the fraction of clonogenic cells approaches the value measured for unirradiated tumours. These large changes measured with respect to the proliferative capacity of cells surviving after irradiation, can be compared with the much smaller gross response of the tumours, shown in Fig. 5A. From a comparison of curve 2a, which demonstrates the volume change of the tumours after a dose of 2000 rad of X-rays, with curve 2d, it can be concluded that the rapid increase of the fraction of clonogenic cells starts at approxi-

*A neutron generator with a higher output providing 250 kV, 5 mA deuterons on a rotating tritium target has been put into operation recently and will be used in future experiments with higher doses.

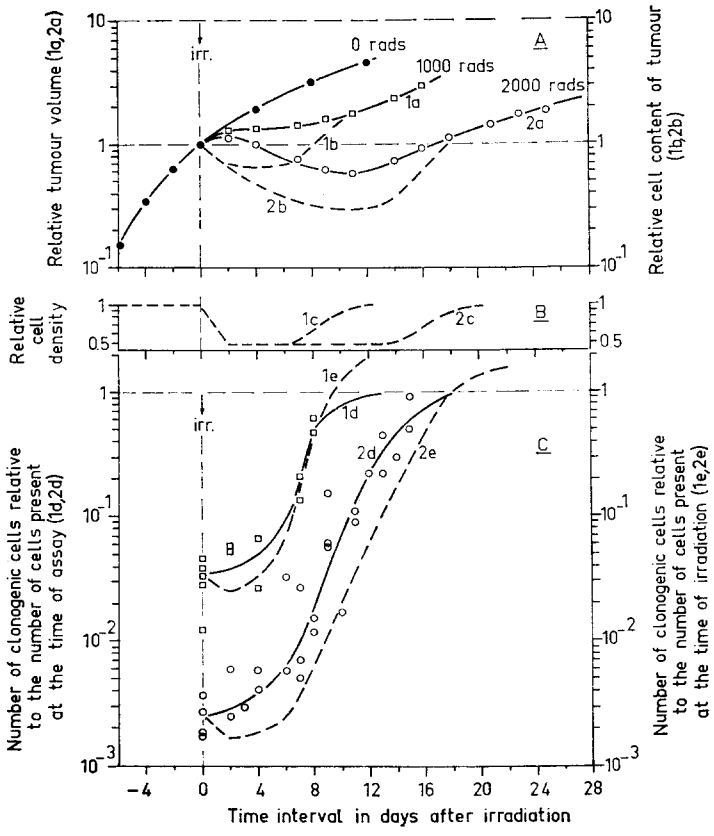

Fig. 5. *Gross volume responses and cell proliferation of R-*1 *rhabdomyosarcomas after irradiation with* 1000 *rads and* 2000 *rads of* 300 *kV X-rays respectively.*

A. Curves 1*a and* 2*a: Volume changes as a function of the time interval after irradiation, relative to the volume measured at* t=0.

Curves 1*b and* 2*b: Changes in the total numbers of cells in the tumours as a function of the time interval after irradiation, relative to the number present at* t=0.

B. Curves 1*c and* 2*c: Changes in cell density of the tumours as a function of the time interval after irradiation, relative to the cell density at* t=0.

C. Curves 1*d and* 2*d: Changes in the fraction of clonogenic cells in the tumours as a function of the time interval after irradiation, relative to the numbers of cells present in the tumour at the time of assay. The squares and circles represent values measured for individual tumours excised at a given time interval.*

Curves 1*e and* 2*e: Changes of the numbers of clonogenic cells in the tumours as a function of the time interval after irradiation, relative to the number of cells present at* t=0.

mately the same time interval after irradiation at which the tumour volume begins to decrease. Furthermore it is shown that the tumour starts to grow again at the time at which the fraction of clonogenic cells is approximately 20% of the value for an unirradiated tumour.

After a dose of 1000 rad of X-rays, the tumour growth rate decreased only temporarily, and at about day 7 after irradiation growth is resumed. This resumption of the growth occurs at the time interval after irradiation at which the fraction of clonogenic cells is also shown to be approximately 20% of the value for an unirradiated tumour.

In Fig. 6, results are presented of measurements of tumour volume and of the fraction of clonogenic cells as a function of the time interval after irradiation with a dose of 600 rad of 15 MeV neutrons. The dose of 600 rad of fast neutrons reduces the clonogenic fraction of cells to about 0·7%. This fraction changes only slowly during the first 6 days after irradiation, but starting at about the sixth day a rapid increase is observed and the value of 1·0 is approached on day 16. The volume of the tumour changes only slowly after irradiation. A minimum value equal to about 0·65 times the value at the time of irradiation is measured between days 10 and 12. Growth is resumed at approximately the twelfth day. This resumption of growth is observed at the time interval after irradiation at which the fraction of clonogenic cells is equal to about 20% of the value for an unirradiated tumour.

In order to correlate the data presented in Fig. 5 A and C, it is necessary to estimate the changes of the total numbers of cells in the

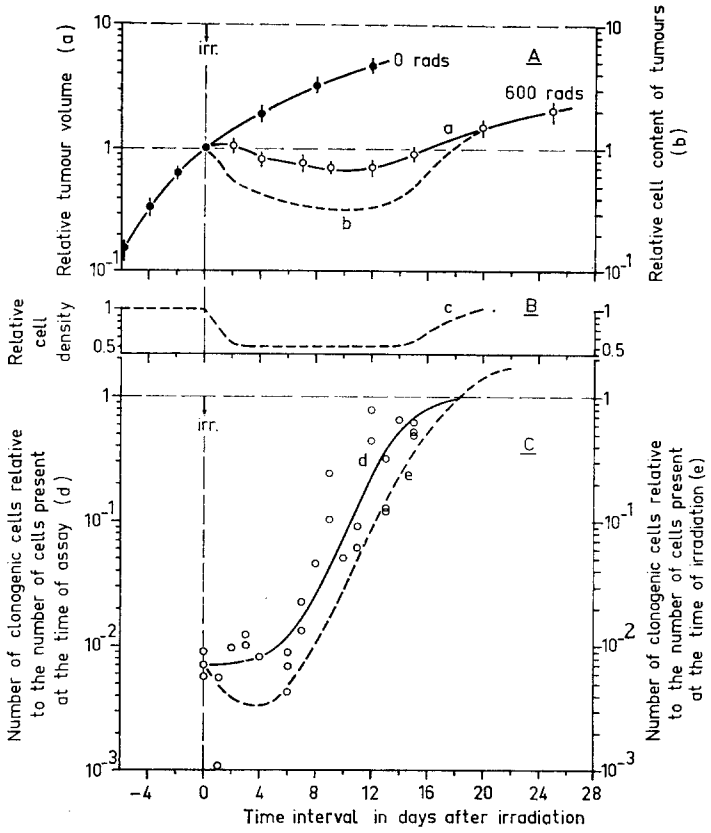

Fig. 6. *Gross volume response and cell proliferation of R-1 rhabdomyosarcomas after irradiation with* 600 *rads of* 15 *MeV neutrons.*

A. Curve a: Tumour volume as a function of the time interval after irradiation, relative to the volume measured at t=0.

Curve b: Variation of the total number of cells in the tumours as a function of the time interval after irradiation, relative to the number of cells present at t=0.

B. Curve c: Cell density of the tumours, relative to the cell density at t=0.

C. Curve d: Changes in the fraction of clonogenic cells in irradiated tumours, relative to the number of cells present in the tumour at the time of assay. The circles represent values measured for individual tumours excised at a given time interval.

Curve e: Changes of the numbers of clonogenic cells in the tumours as a function of the time interval after irradiation, relative to the number of cells present at t=0.

tumours as a function of the time interval after irradiation. This relation can be obtained from the changes in volume and in cell density of the tumours. Volume changes are presented in Fig. 5A. The relative cell density has been derived from measurements of the yields of the dispersion technique and counting of cells in histological preparations. The mean yield for unirradiated tumours obtained with the dispersion technique was equal to $(25\pm10^*)\times10^6$ cells per gram of tumour. The mean yield for irradiated tumours, assayed immediately after irradiation, was $91\pm25^*\%$ of the control value, i.e. the irradiation does not alter the yield significantly. From the yields of the dispersion technique for tumours assayed at different time intervals after irradiation, ratios have been calculated relative to the control value. Considerable variation of these ratios by a factor of about 2 is observed for tumours assayed at the same time interval after irradiation and this hampers an accurate estimation of the cell density as a function of the time interval after irradiation. Nevertheless, the data are sufficiently consistent to deduce that after doses of 1000 rad and 2000 rad 300 kV X-rays, as well as after a dose of 600 rad 15 MeV neutrons, the mean cell density decreases during the first 2 days after irradiation to a value which is about a factor of 2 smaller than in control tumours. Subsequently, the cell density remains approximately constant for a time interval which depends on the dose, and finally an increase to the density of control tumours is observed at about the same time at which the tumour starts to grow again. This variation of the cell density as a function of the time after irradiation, has been presented in Fig. 5, curve 1c

*S.D. derived for about 100 tumours assayed.

and 2c and Fig. 6, curve c, respectively. At two time intervals after a dose of 2000 rad of 300 kV X-rays, the cell densities calculated from the yields obtained with the cell dispersion technique, were compared with data derived by counting cell densities in histological sections of known thickness. At 4 days after a dose of 2000 rad of X-rays, the mean cell density was a factor of $0{\cdot}5\pm0{\cdot}1$ times the density in control tumours, while at 10 days after 2000 rad a factor of $0{\cdot}4\pm0{\cdot}1$ was obtained.

A more accurate evaluation of the cell density in irradiated tumours by chemical methods or by incorporation of labelled DNA precursors would have required the analysis of a separate series of tumours. In view of the fact that in the present experiments the cell density does not appear to vary by more than a factor of about 2, these more accurate estimates would be of limited value for the analysis of the results concerning clonogenic cells, because this factor is small compared to the factors by which the numbers of clonogenic cells in the tumours change during the same time interval.

From the tumour volumes and the cell densities it is possible to calculate the numbers of cells in the tumours as a function of the time interval after irradiation relative to the number present at the time of irradiation. This relation is presented in Fig. 5, curve 1b and 2b, and Fig. 6, curve b, respectively. Furthermore, from the total numbers of cells in the tumours and the fractions of clonogenic cells presented in Fig. 5, curves 1d and 2d, and Fig. 6, curve d, it is possible to calculate the variation of the number of clonogenic cells as a function of the time interval after irradiation.

These relations are presented in Fig. 5, curves 1e and 2e and Fig. 6, curve e, for doses of 1000 rad and 2000 rad of 300 kV X-rays and a dose of 600 rad of 15 MeV neutrons, respectively.

DISCUSSION

(a) Comparison of dose–survival curves measured for cells irradiated in vitro *and* in vivo, *respectively.*

From the curves presented in Fig. 1 for cultured R-1 cells, the RBE of 15 MeV neutrons, relative to 300 kV X-rays, can be estimated for cells equilibrated with air and nitrogen respectively. Due to the differences between the shapes of the curves obtained with neutrons and X-rays, the RBE is not a constant factor, but varies with the level of damage considered. For cells in equilibrium with air for instance, the doses required to obtain 50% survival are equal to 255 rad of 300 kV X-rays and 120 rad of 15 MeV neutrons. From these values an RBE of 15 MeV neutrons equal to 2·1 can be calculated. Figure 1 shows further that in order to decrease the fraction of surviving cells to 1%, doses of 870 rad of 300 kV X-rays and 520 rad of 15 MeV neutrons are required. Consequently, the RBE of 15 MeV neutrons at this level of damage is about 1·7. Corresponding RBE-values for cells in equilibrium with nitrogen are given in Table 1. The latter values are larger than the values calculated for cells in equilibrium with air, because the OER-values of 15 MeV neutrons and 300 kV X-rays differ by a factor of 1·7.

From Fig. 2 it can be concluded that the survival curves for R-1 cells, irradiated in tumours shortly after the rats were killed, are not significantly different from the corresponding curves for cultured cells irradiated in equilibrium with nitrogen, which are shown in Fig. 1. This demonstrates that R-1 rhabdomyosarcoma cells growing in the tumour and R-1 cells cultured *in vitro* are equally sensitive if irradiated in severely hypoxic conditions. Consequently, the RBE-values of 15 MeV

Table 1. *RBE-Values of* 15 *MeV Neutrons Relative to* 300 *kV X-rays for Different End-points*

Condition of exposure	RBE-values at 50% cell survival	at 1% cell survival	other end-points
R-1 cells in culture in equilibrium with air	2·1	1·7	
R-1 cells in culture in equilibrium with nitrogen	3·4	3·0	
R-1 tumours irradiated in living rats	2·4	2·7	
R-1 tumours irradiated in dead rats	3·4	3·0	
90 per cent cure rate of R-1 tumours (calculated)			2·?
R-1 tumour growth delay of 20 days			3·3

neutrons are equal for both systems, as shown in the second and fourth row of Table 1. The survival curve 5 of Fig. 2, for tumour cells irradiated in living rats, is parallel to curve 6 for severely hypoxic cells, in the region of less than 1% survival. It may be concluded that a fraction of the cells in this tumour has a radiosensitivity which is equal to that of severely hypoxic cells, and the most likely interpretation is that this low sensitivity is indeed due to poor oxygenation. As discussed originally by Hewitt and Wilson [36], dose-survival curves for mixtures of anoxic and oxygenated cells, for low doses, will depend predominantly on the killing of oxygenated cells, while at intermediate doses a transition will be observed to a slope equal to that of anoxic cells. At high doses where the number of radiosensitive oxygenated cells becomes negligible, curve 5 is closely parallel to curve 6.

The fraction of severely hypoxic cells in a mixture can be shown to correspond directly to the factor of displacement in vertical direction of the survival curve of the mixture, relative to the survival curve for a population consisting entirely of severely hypoxic cells [36, 37]. Comparison of the curves 4, 5, and 6 of Fig. 2 shows that the shape of curve 5 can be interpreted by the assumption that about 15% of the cells in these tumours are severely hypoxic. It is noteworthy, however, that in this R-1 rhabdomyosarcoma no distinct necrotic areas can be observed histologically [25].

Curve 2 of Fig. 2, obtained by irradiating tumours with 15 MeV neutrons, is shown to be intermediate between the curves 1 and 3 for oxygenated and severely hypoxic cells respectively. The relation of these curves can be interpreted in the same way as the relation of curve 5 to curves 4 and 6 for 300 kV X-rays, by assuming that a fraction of approximately 15% of the cells in the R-1 rhabdomyosarcoma is severely hypoxic. Due to the smaller OER of 1·4 for 15 MeV neutrons as compared with 2·4 for 300 kV X-rays, the deviation of curve 2 from curve 1 occurs at a lower level of survival and the change in slope is less distinct. As a consequence, the slope of curve 2 in the dose region where only severely hypoxic cells survive, could not be measured with sufficient accuracy to determine the fraction of hypoxic cells, on the basis of the neutron data alone.

Due to the difference in OER between neutrons and X-rays and the presence of a fraction of severely hypoxic cells, the RBE of 15 MeV neutrons for damage to tumour cell irradiated in living rats, is a complicated function of the level of damage. At doses corresponding to 50% survival, the RBE is close to the value observed for oxygenated cells. Beyond a transition region corresponding to fractions of between 10^{-1} and 10^{-2} surviving cells, the RBE gradually approaches the value measured for anoxic cells. In Table 1, the RBE-values for 50% and 1% survival are tabulated.

(b) Comparison of cell survival curves and cure rates

It is of interest to compare the dose at which a given cure rate is observed, with the dose at which this cure rate can be predicted on the basis of survival curve parameters. For this comparison a number of assumptions must be made which have been discussed by various authors [38–42]. The main assumption implies that for local regrowth of the tumour after irradiation, a minimum number of cells in the tumour must have retained the capacity for unlimited proliferation. Thus, if the number of viable cells in an unirradiated tumour is known, extrapolation of the relevant measured survival curve can be used to estimate the dose at which the probability that sufficient cells have survived is, e.g. equal to 0·1 corresponding to a cure rate of 90%.

The R-1 rhabdomyosarcoma contains about 5×10^8 cells per gram [23, 25]. The mean volume of the tumours used in this study is 0·8 cm^3 and the specific gravity was not significantly different from 1·0. Consequently the mean cell content per tumour is about 4×10^8 cells. Not all of these cells may be assumed to be capable of unlimited proliferation, however. Although the low yield of the cell dispersion technique, which varies from 5 to 10%, introduces considerable uncertainty, it will be assumed that the mean *in vitro* plating efficiency of 35%, determined for tumour cells from unirradiated tumours, is a measure of the fraction of cells in the tumour, capable of unlimited proliferation. Thus, according to this reasoning, the unirradiated tumours contained $1{\cdot}4 \times 10^8$ cells capable to cause a recurrence.

The number of viable cells required to cause regrowth of the tumour is difficult to evaluate, since it depends presumably on the conditions present in an irradiated tumour, whereby various factors may influence the probability of recurrence. The observation mentioned earlier that with subcutaneous inoculation a number of viable cells of the order of 100 is required to obtain 50% tumour takes, cannot be taken as evidence that the same number of cells is required in an irradiated tumour to start regrowth, because the two environments are vastly different, with respect to the blood

and nutrient supply, the concentration of cells etc. [41]. Since no exact data are available the assumption will be made that the minimum number of cells is equal to 1·0. It should be noted, however, that this is a major uncertainty.

On the basis of the assumptions discussed, it can be stated that in order to obtain a cure rate of 90%, the mean number of $1{\cdot}4 \times 10^8$ viable cells in the tumour must be reduced to 0·1, i.e. the dose required should reduce the fraction of surviving cells to $0{\cdot}1/1{\cdot}4 \times 10^8 = 0{\cdot}7 \times 10^{-9}$. Extrapolation of curve 5 of Fig. 2 to higher doses according to an exponential relation, shows that the required dose is equal to about 6700 rad. As mentioned earlier, results of experiments showed that at 6500 rad, one out of ten tumours recurred. It can be concluded that the experimental data and the calculated estimate for a cure rate of 90% are in very close agreement. A similar result has been obtained with another technique by Reinhold [41]. It should be noted, however, that this agreement is perhaps fortuitous, because it is not certain that the extrapolation of the survival curve according to an exponential curve is justified. In Fig. 4, the measured and calculated doses, required for 90% cure rates, are represented by the arrows *b* and *c*.

As mentioned earlier, with 15 MeV neutrons no cures could be attained because of the low output of the generator employed. Extrapolation of the survival curve 2 of Fig. 4 results in a calculated dose of 2400 rad, required for a cure rate of 90%. This value is indicated by arrow *a* in Fig. 4. From the ratio of the doses required for a cure rate of 90%, calculated from the survival curves, an RBE of 2·8 can be derived for 15 MeV neutrons.

(c) The growth rate of recurrent tumours after high doses

In Fig. 4, curve 2, the number of days of tumour growth delay is presented as a function of doses of 300 kV X-rays up to a dose of 6000 rad. As noted earlier after a dose of 6000 rad, not all tumours recur and the mean growth delay was calculated from the 6 tumours which were not cured. Although the tumours, which recurred after 6000 rad, could always be detected within 150 days, the growth delay was frequently longer than 150 days because we have defined this quantity as the difference between the time interval required by the irradiated and control tumours, respectively, to attain a volume equal to twice the volume at the time of irradiation. The tumours which recurred after high doses have considerably smaller growth rates than unirradiated tumours of equal volume. This is illustrated for instance in Fig. 3, curve 7, for a dose of 4000 rad. After the volume decrease, induced by this dose, the tumours regrow and attain a mean volume equal to the volume at the time of irradiation, on the 46th day. The time interval in which these recurring tumours increase in volume by a factor of two, is equal to 18 days, while unirradiated tumours of the same size double their volume in 4 to 5 days. This difference is of great importance for the interpretation of radiation effects and cell population kinetics of these late recurrences are being investigated. At doses of 6500 rad, the number of recurrent tumours is too small to derive an estimate of the growth delay. The dotted extrapolation of curve 2 in Fig. 4 merely serves to indicate that beyond 6000 rad the growth delay increases rapidly to an infinite value.

(d) Cell survival and growth delay

From curves 1 and 2 of Fig. 4, RBE-values of 15 MeV neutrons can be derived for growth delay. For a growth delay of 20 days, for instance, doses of 600 rad of 15 MeV neutrons and 2000 rad of 300 kV X-rays are required. From these data an RBE of 3·3 can be calculated, which is presented in Table 1. It is important to note that this RBE-value for a growth delay of 20 days is larger than the RBE-value of 2·8 which can be derived from the survival curves 2 and 5 of Fig. 2 at a surviving fraction of 0·7%, measured at a dose of 600 rad of 15 MeV neutrons. The difference between the RBE-values for cell survival and growth delay is even larger for lower doses than 600 rad of 15 MeV neutrons. A dose of 400 rad of neutrons reduces the surviving fraction to about 5 per cent (curve 2 of Fig. 2). The equivalent dose of 300 kV X-rays is 880 rad (curve 5, Fig. 2). Thus the RBE for this effect is 2·2. The growth delay induced by 400 rad of 15 MeV neutrons is equal to about 12 days, corresponding to a dose of 1400 rad of 300 kV X-rays (curves 1 and 2 of Fig. 4). The RBE-value derived from these data is 3·5. It must be concluded that doses of 15 MeV neutrons and 300 kV X-rays which reduce the surviving fraction of cells in the tumour by the same factor, cause different tumour growth delays. This implies that the relation between the surviving fraction of cells and growth delay is not a unique correspondence, but may be dependent on the type of radiation. The cause of this difference will be shown to be correlated with the post-irradiation proliferation kinetics of cells in the tumour, as discussed in the next section.

(e) Proliferation of surviving cells in tumours after irradiation with doses of 15 MeV neutrons and 300 kV X-rays

To a first approximation it might be expected that the time interval in which an irradiated tumour regrows to its pre-irradiation volume, depends on the fraction of cells which have retained the capacity for unlimited proliferation and the rate at which their number increases. The preceding discussion has indicated already that the cellular phenomena occurring during regression and recurrence are quite complex. As noted earlier, the doubling time of tumours of 0·5–1·0 cm^3 volume is approximately 4 days. If the most simple assumption is made that the production of new cells in a tumour, by cells that have not been killed, continues after irradiation at the same rate as prior to irradiation, then a dose which reduces the surviving fraction to 0·5 would be expected to cause a growth delay of 4 days. A reduction of the surviving fraction to 0·25 would then cause a growth delay of 8 days, etc. With some exponentially growing tumours, this type of relation appears to be applicable. In such cases, back extrapolation of the growth curve of the recurrent tumour to the day of irradiation, can be used to calculate a "surviving tumour volume" [39]. With the R-1 rhabdomyosarcoma, however, this simple relationship is not observed. As shown in Fig. 3, a dose of 1000 rad of X-rays causes a growth delay of 8 days, which on the basis of the simple assumption mentioned, should correspond to a surviving fraction of 0·25. From Fig. 2 it can be deduced however that the fraction of cells which has retained the capacity for unlimited proliferation is reduced to about 0·032. Conversely, a reduction of the surviving fraction to 0·032, i.e. by a factor of about 2^5, would be expected, on the basis of the simple assumption mentioned, to cause a growth delay of about $5 \times 4 = 20$ days, instead of the observed growth delay of only 8 days. Similar differences can be deduced for other doses. It must be concluded that the tumour cells which after irradiation have retained the capacity for unlimited proliferation, repopulate the tumour more rapidly than expected on the basis of the gross response of the tumour volume [5]. A similar conclusion has been derived by Malaise and by Hewitt [44, 48]. A more direct experimental method is required however to elucidate this phenomenon. This is provided with our data.

The results presented in Figs. 5 and 6, which demonstrate the changes of the fractions of clonogenic cells in the tumours as a function of the time interval after irradiation, provide experimental evidence that cells, which after irradiation have retained the capacity for unlimited proliferation, do indeed repopulate the tumour at a very rapid rate. As indicated earlier, the experimental data plotted in the curves 1d and 2d in Fig. 5 and curve d in Fig. 6 represent ratios of the number of clonogenic cells to the total number of cells present in the tumour at the time of assay. Consequently, the rates at which the numbers of clonogenic cells in the tumours change with the time interval after irradiation have to be deduced from these curves by applying a correction for the variation of the total numbers of cells present in the tumour at the time of assay, as discussed in the preceding section. The curves 1e and 2e of Fig. 5 and curve e of Fig. 6 show that during the first two days after irradiation the number of cells capable of unlimited proliferation decreases by a factor of between 1·5 and 2·0.

The cause of this decrease cannot be resolved directly from the data available at present but it may be noted that even in unirradiated tumours, a fraction of the cells produced per division do not continue to proliferate but become part of a non-proliferating compartment [43, 25]. Not all cells in this compartment retain the capacity for unlimited proliferation, but a fraction dies after a finite life span [25]. If it is assumed that this movement of cells present in the non-proliferating compartment, into a sub-compartment of dying cells, continues during the first 2 days after irradiation, then the small decrease in the number of clonogenic cells in the first 2 days after irradiation could be explained, because the production of new clonogenic cells is temporarily inhibited due to mitotic delay. For cells removed from the tumour and cultured *in vitro*, the completely different environment prevents cells from moving into a non-proliferating compartment as demonstrated by the fact that *in vitro* the growth fraction is equal to 1·0 [25]. This interpretation must be considered as tentative however.

The curves 1e and 2e of Fig. 5 and curve e of Fig. 6, show further that, starting at the third day post-irradiation, the number of cells capable of unlimited proliferation increases very rapidly. Curve 2e of Fig. 5 demonstrates that between 6 and 14 days after a dose of 2000 rad of 300 kV X-rays, the rate of increase corresponds to a doubling time of about 30 hr. As noted earlier, the volume doubling time of unirradiated tumours with a volume between 0·5 and 1·0 cm^3, is about 4 days. The number of clonogenic cells in these unirradiated

tumours increases at the same rate because the ratio of the number of clonogenic cells and the total number of cells does not change significantly with the volume of the tumour. It can be concluded that in irradiated tumours, after a short lag period of about two days, the cells which have retained the capacity for unlimited proliferation increase in number at a rate which is much larger than in unirradiated tumours.

Measurements by autoradiographic techniques, which were reported earlier, have shown that the proliferating cells in unirradiated R-1 rhabdomyosarcomas have cell cycle times of about 20 hr [24, 25].

It was demonstrated further that at 4 days after a dose of 2000 rad X-rays, the mean cell cycle time of the proliferating cells was equal to about 12 hr. This indicated an increased proliferation activity. However, at 4 days after irradiation with a dose of 2000 rad, more than 99% of the cells in the tumour are capable of only a limited number of divisions and only about 0·27% of the cells are clonogenic, i.e. are capable of unlimited proliferation. Consequently the cell cycle times measured with autoradiographic techniques at 4 days after irradiation with a dose of 2000 rad, pertain to cells which proliferate during a limited period and die subsequently. The data obtained do not show definitely that the 0·27% of the cells which are capable of unlimited proliferation also proliferate with a cell cycle time of 12 hr, because the proliferation characteristics of such a small fraction of the total cell population cannot be investigated separately by autoradiographic techniques. It will be clear, however, that the rate of increase of the number of clonogenic cells corresponding to a doubling time of 30 hr is not incompatible with data obtained with respect to the cell population kinetics in these irradiated tumours.

With respect to the proliferation of clonogenic cells in tumours irradiated with a dose of 1000 rad of X-rays, a similar conclusion can be drawn as discussed for a dose of 2000 rad, i.e. after an initial decrease of the number of clonogenic cells during the first 2 days after irradiation, a rapid repopulation of the tumour occurs. This rate of increase corresponds, in the steepest part of curve 1e of Fig. 5, with a doubling time of 24 hr.

The pattern of proliferation of clonogenic cells in tumours irradiated with a dose of 600 rad of 15 MeV neutrons, shown in Fig. 6, curve e, is very similar to that observed after a dose of 2000 rad of X-rays, i.e. a decrease in the number of clonogenic cells during the first 2 days after irradiation and a subsequent rapid increase corresponding to a doubling time of about 30 hr. Comparison of curve 2e of Fig. 5 and curve e of Fig. 6 shows a small difference however, with respect to the time interval between irradiation and the onset of the rapid repopulation. This interval is about 1·5 days longer in the case of 600 rad of 15 MeV neutrons, as compared with 2000 rad of X-rays. It can be concluded that, although after a dose of 600 rad of 15 MeV neutrons 0·7% of the cells has retained the capacity for unlimited proliferation compared to 0·27% after a dose of 2000 rad of 300 kV X-rays, the time intervals required for the number of clonogenic cells to reach a value equal to 10% of the number present prior to irradiation are approximately equal, namely 12·5 days and 13·0 days respectively, as a result of the longer delay in the onset of repopulation. This longer lag period causes the difference noted earlier between the RBE of 2·8 for a dose of 600 rad of 15 MeV neutrons with respect to impairment of the capacity for clone formation, as compared to the RBE of 3·3 with respect to a tumour growth delay of 20 days. It is evident, however, that with respect to the probability of attaining a cure of the rhabdomyosarcoma, it is not the larger RBE-value for tumour growth delay, but the RBE factor for cell killing which is relevant.

CONCLUDING REMARKS

The results presented in this paper demostrate that relatively large RBE-values for single doses of 15 MeV neutrons compared to 300 kV X-rays are obtained with respect to survival of cells in the tumour and tumour growth delay. These larger RBE-values are due at low doses to the reduced shoulder of the survival curve obtained with 15 MeV neutrons as compared with X-rays, while at large doses they are caused by the presence of hypoxic cells and the fact that the OER-values of 15 MeV neutrons and 300 kV X-rays differ by a factor of about 1·7.

Comparison of these data with RBE-values of between 1·1 and 1·5 measured for various effects in normal tissues, e.g. rat skin, mouse bone marrow and mouse intestine, indicates that, at least for single doses, fast neutrons would provide an advantage in the radiotherapy of tumours containing cells which are either anoxic or have survival curve characteristics, demonstrating a broad shoulder and a correspondingly large capacity for repair of sublethal damage [5, 45].

The comparison of RBE-values measured

for different end points has shown that the phenomena occurring in the tumours after irradiation are more complicated than have been demonstrated thus far, especially with respect to a rapid repopulation of the tumours by cells which have retained the capacity for unlimited proliferation. It is obviously not enough to measure RBE-values of fast neutrons for single and fractionated exposures but it is necessary to obtain a better insight into the responses of tumours to different types of radiations. This can only be attained if not one but various methods of analyses including studies of cell cycle parameters, of cell survival, of tumour growth delay and of tumour repopulation, are applied. Studies of effects of fractionated exposures obtained with these techniques will be reported in a subsequent paper.

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RESUME

Les auteurs ont étudié la sensibilité d'un rhabdomyosarcome transplantable du rat à diverses doses de rayons X de 300 *kV et de neutrons de* 15 *MeV. Les courbes de survie furent établies par une technique de formation de clones* in vitro, *pour les cellules irradiées* in vitro *et* in vivo. *De plus, le retard de la croissance et les taux de guérison furent déterminés. Enfin, la vitesse de repopulation de la tumeur, par les cellules survivantes, fut mesurée.*

Les résultats ont montré que, pour les cellules irradiées in vitro, *les courbes de survie, pour les rayons X de* 300 *kV, sont caractérisées par une dose léthale moyenne* D_0 *égale à* 120 *rad et un nombre d'extrapolation* $N=10$ *lorsque les cellules sont au contact de l'air, et* $D_0=295$ *rad et* $N=10$ *pour des cellules en anoxie. Avec les neutrons de* 15 *MeV les valeurs correspondantes sont* $D_0=85$ *rad, et* $N=3$, *pour les cellules bien oxygénées, alors que* $D_0=120$ *rad et* $N=3$ *pour les cellules en anoxie.*

Pour les tumeurs irradiées dans de mauvaises conditions d'oxygénation, les valeurs avec les rayons X de 300 *kV sont* $D_0=305$ *rad et* $N=10$ *rad, tandis qu'avec les neutrons de* 15 *MeV,* $D_0=120$ *rad et* $N=3$. *Les courbes du survie, établies pour les cellules de tumeurs irradiées* in vivo, *montrent qu'environ* 15% *des cellules se trouvent dans des conditions d'anoxie sévère.*

Les taux de guérison permettent de calculer que la dose requise pour obtenir 90% *de guérisons (TCD* 90) *est de* 6.700 *rad pour les rayons X de* 300 *kV, ce qui correspond bien à la valeur de* 6.500 *rad obtenue par extrapolation de la courbe de survie. Pour les neutrons de* 15 *MeV, les taux de survie ne purent être mesurées à cause du débit trop faible du générateur, mais l'extrapolation de la courbe de survie donna un TCD* 90 *de* 2.400 *rad. L'efficacité biologique relative (EBR) des neutrons de* 15 *MeV pour un TCD* 90 *est égale à* 2,8. *Les mesures du retard de croissance montrent que l'EBR des neutrons rapides est d'environ* 3,3 *pour grande variété de doses.*

Les mesures de la régression du nombre de cellules proliférantes, dans les tumeurs irradiées, montrent que, après une latence de 4 *jours, le rythme moyen de multiplication des cellules survivantes est plus rapide que celui des cellules des tumeurs non irradiées de même volume. Le temps de doublement des cellules des tumeurs irradiées est de* 30 *heures, alors qu'il est de* 96 *heures pour les cellules des tumeurs non irradiées.*

SUMMARY

Experiments have been carried out to evaluate various responses of a rhabdomyosarcoma transplantable in an inbred strain of rats to different single doses of 300 *kV X-rays and* 15 *MeV neutrons. Survival curves were derived by a direct* in vitro *cloning technique for cells irradiated* in vitro *or* in vivo. *In addition, growth delay and cure rates were determined and the rate of repopulation of the tumour by surviving cells was measured. The results demonstrate that for cells irradiated* in vitro *the survival curves for* 300 *kV X-rays can be described approximately by a mean lethal dose* D_0 *equal to* 120 *rad and an extrapolation number* $N=10$ *for cells in equilibrium with air, while a* D_0 *of* 295 *rad and* $N=10$ *were derived for cells in hypoxic conditions. For* 15 *MeV neutrons the corresponding values are* $D_0=85$ *rad,* $N=3$ *and* $D_0=120$ *rad,* $N=3$ *for oxygenated and hypoxic conditions, respectively. For tumours irradiated in hypoxic conditions, the*

survival curves of the constituent cells can be represented by D_0=305 *rad,* N=10 *and* D_0=120 *rad,* N=3 *for* 300 *kV X-rays and* 15 *MeV neutrons, respectively. The survival curves for cells from tumours irradiated in living animals indicate that approximately* 15% *of the cells are severely hypoxic.*

The measurements of proportion of tumours cured indicate that a TCD 90 *is attained at* 6700 *rad of* 300 *kV X-rays, in good agreement with an expected value of* 6500 *rad obtained by extrapolation of the survival curve. For* 15 *MeV neutrons proportion of tumours cured could not be measured due to the low output of the present generator, but extrapolation of the survival curve yields a TCD* 90 *of* 2400 *rad. The RBE of* 15 *MeV neutrons for a TCD* 90 *is equal to* 2·8. *Measurements of growth delay show that the RBE of fast neutrons is equal to about* 3·3 *over a wide range of doses.*

Measurements of changes in the number of clonogenic cells in irradiated tumours show that after an initial lag period, surviving cells repopulate the tumour at a high rate, corresponding to a doubling time of 30 *hr, which is a factor of* 3 *larger than the rate of increase of cells in an unirradiated tumour, corresponding to a doubling time of about* 96 *hr.*

ZUSAMMENFASSUNG

An einem auf einem Inzucht-Rattenstamm transplantablen Rhabdomyosarkom, wurden die Wirkungen verschiedener Einzeldosen von 300 *kV Röntgenstrahlen und* 15 *MeV Neutronen studiert. Unter Anwendung einer direkten* in vitro *cloning-Technik für* in vitro *und* in vivo *bestrahlte Zellen wurden Überlebenskurven angelegt. Zusätzlich wurde die Wachstumsverzögerung und Erholungsrate bestimmt und die Wiederherstellung der Tumorzellpopulation durch die überlebenden Zellen festgestellt. Die Ergebnisse zeigen, daß für* in vitro *bestrahlte Zellen in normaler Atmosphäre die Überlebenskurven für* 300 *kV Röntgen eine durchschnittliche* D_0 *von* 120 *rad zeigen und eine Extrapolationszahl* N=10, *während unter Sauerstoffmangel die* D_0=295 *rad bei* N=10 *betrug. Die bei* 15 *MeV Neutronen entsprechenden Werte sind* D_0=85 *rad,* N=3 *unter Zutritt von Sauerstoff und* D_0=120 *rad,* N=3, *unter hypoxischen Bedingungen. Für unter Sauerstoffmangel bestrahlte Tumoren zeigen die Überlebenskurven der verbliebenen, regenerierenden Zellen* D_0=305 *rad,* N=10 *bei* 300 *kV Röntgen und* D_0= 120 *rad,* N=3 *bei* 15 *MeV Neutronen. Die Überlebenskurven im lebenden Tier bestrahlter Tumoren deuten darauf hin, daß ungefähr* 15% *der Zellen unter starkem Sauerstoffmangel stehen.*

Aus Berechnungen des Anteils der geheilten Tumoren ergibt sich eine TCD 90 *bei* 6700 *rad und* 300 *kV Röntgen, ein Ergebnis, daß in guter Übereinstimmung steht mit den, durch Extrapolation der Überlebenskurve erhaltenen* 6500 *rad. Der Anteil der geheilten Tumoren bei* 15 *MeV Neutronen konnte nicht bestimmt werden, da die Leistung des derzeitigen Generators nicht ausreichte, jedoch ergab die Extrapolation der Überlebenskurve eine TCD* 90 *bei* 2400 *rad. Die RBW von* 15 *MeV Neutronen beträgt* 2·8 *für eine TDC* 90. *Messungen der Wachstumsverzögerung zeigen, daß die RBW schneller Neutronen gleich* 3,3 *ist und sich über einen breiten Dosisbereich erstreckt.*

Der Wechsel in der Anzahl koloniebildender Zellen in bestrahlten Tumoren zeigt, daß nach einer anfänglichen Verzögerungsperiode die überlebenden Tumorzellen sich mit einer Verdopplungszeit von 30 *Stunden vermehren, d.h. um den Faktor* 3 *schneller als die Zellen des unbestrahlten Tumors, die eine Verdopplungszeit von ca.* 96 *Stunden haben.*

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Comparative Effects of Vinblastine and Procarbazine in Advanced Hodgkin's Disease

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THE THERAPEUTIC program recently adopted for Hodgkin's disease in several centres involves the use of high energy radiation therapy in Stages I, II and III [1, 2]. The administration of radical and prophylactic irradiation as an exclusive initial therapeutic tool as long as the disease appears confined to the lymph nodes has limited in recent years the indication for chemotherapy to patients with visceral or bone involvement (Stage IV) and to some cases with adenopathies above and below the diaphragm but in which concomitant severe systemic symptoms and poor general conditions are present (Stage III B). While five or more years will be necessary to prove with certainty that the use of radiation therapy alone can either cure or markedly improve the survival of patients with Stage III disease, some investigators have already demonstrated that the single intravenous administration of nitrogen mustard in addition to radiotherapy for localized Hodgkin's disease (Stage I and II) did not significantly contribute to the increase of survival time [3–6].

Several drugs are useful in the treatment of Hodgkin's disease. The most widely used in the past decade have been the nitrogen mustard (HN2) and the related polyfunctional alkylating agents, the vinca alkaloids (especially vinblastine) and the corticosteroids. In the past few years several authors have described consistent objective results with procarbazine, while some demonstrable but not yet well established therapeutic activity has been reported with methotrexate [7, 8] methyl ester of streptonigrin [9], actinomycin D [8] and 1,3-bis(β-chloroethyl)-1-nitrosourea [10].

About 80–90% of patients in relatively good general conditions responded to their first course of intravenous alkylating agents (HN2, 0·4, mg/kg or thioTEPA, 0·8 mg/kg) and improvement following short interrupted courses may average 2–3 months [11]. Remissions can be continued for longer periods of time by maintaining oral chemotherapy (chlorambucil 0·1–0·2 mg/kg/day or cyclophosphamide 2–3 mg/kg/day) [12, 13]. Corticosteroids when administered to high doses (prednisone or methylprednisolone 2–8 mg/kg/day) produce objective responses in about 60% of the cases for a median duration of 10 weeks and an average duration of 16 weeks [14].

Between the two alkaloids of Vinca rosea Linn vinblastine (0·10–0·15 mg/kg/week i.v.) seems to be more active in Hodgkin's disease than vincristine and produces prompt and prolonged relief of symptoms as well as objective improvement. These responses can also be obtained in a number of patients no longer responding satisfactorily to an alkylating agent but were usually reported to be of short duration. The summary of experiences reported in the literature with the use of intravenous vinblastine is listed in Table 1. This table includes only those series of patients previously treated with radiation therapy and/or alkylating agents and improved for a period

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Table 1. *Subjective and objective improvement of greater than* 1 *month duration* (*Category* 1) *after intravenous vinblastine therapy in Hodgkin's disease as well as could be estimated from the published case material.*

No. patients treated	Previous therapy (No. cases) Yes	No	Responsive cases Category 1	(%)	Duration of response in months Mean	Median	Reference	
45	27 alkyl. agents	18	29	64	not specified		Todd	[31]
35	RT, alkyl. agents		20	61		7	Sohier	[32]
32	alkyl. agents		15	47	not specified		Carbone	[33]
29	RT, alkyl. agents		18	62	not specified		Bernard	[34]
28	RT, alkyl. agents		18	65	not specified		Fairley	[35]
28	RT, alkyl. agents		23	82	not specified		Cooperative study	[36]
23	alkyl. agents		10	43	7		Bleehen	[37]
22	1RT, 2 alkyl. agents	19	16	67	not specified		Mathé	[38]
21	RT, alkyl. agents (most cases)		7	33	4		Wright	[39]
14	not specified		9	64	5		Hodes	[40]
14	RT, alkyl. agents		12	85	2·9	2·5	Ezdinli	[41]
9	RT, alkyl. agents		6	66	not specified		Marmont	[19]

longer than one month as it could be estimated from the published case material.

Procarbazine, a methylhydrazine derivative has been tried in a number of patients at the initial dose of 3–4 mg/kg/day and lowered in maintenance therapy to 1·5–2·5 mg/kg/day.

Although remarkable subjective and objective responses in far advanced Hodgkin's disease have been documented, procarbazine seems, from the published reports, to have a less certain therapeutic effect and less well established clinical indications as those for the alkylating agents and for vinblastine [11]. The summary of experiences reported on the treatment with procarbazine in Hodgkin's disease is listed in Table 2. As for vinblastine we included only those series of patients which appeared from the published case material to have been treated previously with other forms of therapy (irradiation and/or chemotherapy) and responding to the administration of procarbazine for more than 1 month.

Prior treatment with radiotherapy and more than one drug as well as duration of disease seem to have an adverse effect on response to further chemotherapy [7]. Nevertheless a cross resistance among the different classes of chemotherapeutic agents useful in Hodgkin's disease and in lymphoreticular sarcomas has not been demonstrated [15]. This study reviews our results obtained in a number of patients with Hodgkin's disease treated initially with radiation therapy in association to alkylating agents, and when the disease became refractory, with vinblastine and then with procarbazine. The purpose is to analyze first whether the previous heavy treatment with radiomimetic agents can produce an adverse

Table 2. *Subjective and objective improvement of greater than* 1 *month duration* (*Category* 1) *after procarbazine therapy in Hodgkin's disease as well as could be estimated from the published case material.*

No. patients treated	Previous Therapy (No. cases) Yes	No	Responsive Cases Category 1	(%)	Duration of response in months Mean	Median	Reference	
33	RT, 13 alkyl. VLB		23	69	3		Kenis	[42]
33	20 RT, chemotherapy	13	23	69	not specified		Fiorentino	[43]
24	RT, chemotherapy		15	62·5	3·3		Todd	[31]
22	Some with RT, alkyl. agents, VLB	7	15	68	not specified		Mathé	[44]
20	18 chemotherapy	2	12	60	4·5		Brunner	[20]
18	14 RT, chemotherapy	4	10	55	not specified		Obrecht	[45]
17	RT, alkyl. agents, VLB		10	59	not specified		Fairley	[35]
14	11 RT, chemotherapy	3	14	100	2		Martz	[46]
11	RT, alkyl. agents, VLB		8	72		4	Samuels	[15]

effect on the response to vinblastine or procarbazine and second whether the type of regressions obtained with procarbazine administered as a third drug after alkylating agents and vinblastine could be consistently compared to those observed in the same patients after vinblastine.

MATERIAL AND METHODS

Patient group. A total of 44 adult patients (25 males and 19 females) admitted to the National Cancer Institute of Milan from 1963 to 1966 were evaluated. In all cases the diagnosis of Hodgkin's disease was made after a nodal biopsy. The initial diagnostic work-up had included chest X-ray with mediastinal tomograms, skeletal survey, lower extremity lymphangiography, bone marrow aspiration, liver function tests, serum uric acid, calcium and phosphorous levels, as well as serum electrophoresis. The patients have been staged according to the International Staging Classification proposed at Rye in 1965 [16]. According to the treatment policy adopted at the Institute from January 1963 to June 1965 [17] those patients classified as Stage I received a course of irradiation with ^{60}Co to the site of disease and most of the times to the adjacent lymphoid regions. Those in Stages II and III were given radiation therapy above the diaphragm and endolymphatic radiotherapy to the retroperitoneal nodes [18] in association with a single intravenous dose of mechloretamine (HN2) 0·4 mg/kg. Stage IV patients were treated with HN2, endolymphatic radiotherapy and with palliative irradiation to bulky nodes. In Stages III and IV a maintenance chemotherapy with oral chlorambucil, 0·1–0·2 mg/kg, was administered after bone marrow recovery from HN2.

In few patients classified as Stage I when the disease after different lengths of time recurred outside the lymph nodes (lungs, liver, bones) a course of HN2 was administered and followed with maintenance therapy (chlorambucil). In these patients as well as in those initially classified as Stages II, III and IV, when the disease was not responding any more to alkylating agents, a course of vinblastine was tried. When the disease progressed again vinblastine was stopped and procarbazine started. In presence of severe systemic symptoms indomethacin and sometimes prednisone were given, the latter mostly in presence of hemolytic anemia. Therefore all patients but 1 (43/44), since a 27-year old female who being 6 months pregnant was treated with ^{60}Co above the diaphragm and vinblastine rather than HN2, were treated with radiomimetic agents (radiation therapy and polyfunctional alkylating agents) before receiving a course of vinblastine. Furthermore all patients but 1 (27/28) receiving procarbazine were previously treated with vinblastine. In this patient procarbazine was started when the disease was no more responsive to radiation therapy and chlorambucil.

The mean duration of Hodgkin's disease in the group of patients reported in this study from the initial treatment to the start of vinblastine therapy was 29·3 months (range 3–120 months).

Drug administration. Vinblastine was administered intravenously at the initial dose of 0·10–0·15 mg/kg/week for 3–5 weeks, then every 2–4 weeks mostly to avoid bone marrow depression during the treatment in the outpatient clinic. Procarbazine was given mostly by mouth and only in a few patients initially by intravenous injection at the dose of 3–4 mg/kg/day for 30–40 days. The total daily dose never exceeded 250 mg. Maintenance therapy was always administered orally at the dose of 1·5–2·5 mg/kg/day. During the first week of treatment the dose of procarbazine was gradually increased by 50 mg every 2 days to avoid as much as possible the signs of gastric intolerance.

Adequacy of treatment. In this study the drug administration has been considered adequate to detect an antineoplastic effect if the period of continuous treatment exceeded 3 weeks for each drug or if therapy resulted in tumor regression or bone marrow depression prior to two weeks. The duration of response was measured from the time of maximum tumor regression to the time of unequivocal objective evidence of progression or relapse.

Response categeories. The therapeutic effects of vinblastine and procarbazine have been classified according to response categories proposed by Karnofsky [20]. This classification is reprinted in Table 3.

RESULTS

A total of 38 patients (22 males and 16 females) met the criteria of adequate treatment for vinblastine and 28 patients (14 males and 14 females) for procarbazine. Six patients in the vinblastine treated group were not included in the therapeutic evaluation either because they did not receive an adequate dose of the drug, or because retrospectively the objective

Table 3. Categories of response

Category	Drug effect on disease
0	No clinically useful effect on the course of the disease
0–0	Disease progresses—no objective or subjective benefit
0–A*	Subjective benefit without favourable objective changes
0–B*	Favourable objective changes without subjective benefit
0–C	Subjective benefit and favourable objective changes in measurable criteria, but of less than 1 month duration; then the disease progresses
1	Clinical benefit with favourable objective changes in all measurable criteria of the disease
1–A*	Distinct subjective benefit with favourable objective changes in all measurable criteria for 1 month or more
1–B*	Objective regression of all palpable or measurable neoplastic disease for one month or more in a relatively asymptomatic patient who is able to carry on his usual activities without undue difficulty. The observed tumor regression should be unequivocal and it is suggested that all lesions be reduced at least 50% in bulk. This category applies as long as the regresison persists and ends if any old or new lesion recurs.
1–C	Complete relief of symptoms, if any, and regression of all manifestations due to the active disease for 1 year or more. The relation to the frequency of therapy is not relevant if the disease does not recur between courses of therapy.

*Categories apply as long as improvement from baseline persists. Superscript: time in months of duration of response. Example: 0–A^4 or I–B^3.

parameters were not judged to be clearly measurable. Since these cases have been rejected from the study only 22 patients received procarbazine after vinblastine and are therefore available for the study of this sequential treatment.

The types of response observed after treatment with vinblastine and procarbazine are described in Table 4 while the percentage of response and the duration of objective improvement after each drug are respectively reported in Table 5. One can see from these tables that in both groups the majority of responses were classified in the category I and that a large variety as far as time regression ranging from a mimimum of 1 month to a maximum of 17 months was observed. Furthermore while the total percentage of response (categories 0 and 1) was practically equal for vinblastine (65·7%) and for procarbazine (64·2%) the real percentage of objective benefit influencing the course of disease (category 1 only) as well as the time spent in remission appears slightly higher in the vinblastine (55·2% with a median of 5 months) rather than in the procarbazine (46·4% with a median of 4 months) treated group. This difference however is not statistically significant ($P>0·05$). At the time of this report 4 patients treated with vinblastine and 5 with procarbazine are still in regression.

Table 6 shows the results obtained in the 22 patients in which the sequential therapy vinblastine—procarbazine was administered when the disease was no more responsive to radiation therapy and alkylating agents. Only 3/22 patients did not show any subjective or objective benefit (categories 0 and 1) from either vinblastine or procarbazine, while 11 improved after treatment with both drugs, 4 only after vinblastine and respectively 4

Table 4. Type of response after vinblastine and procarbazine in generalized Hodgkin's disease.

Drug	Dose	No. cases	Categories of response* 0–0	0–A	0–B	0–C	1–A	1–B	1–C
Vinblastine	0·10–0·15 mg/kg/week 3–5 then every 2–3 weeks	38	13	2(1, 1)	2(1, 1)		9(2, 2, 3, 3, 4, 5, 6, 6, 7)	9 (1, 2, 3, 4, 5, 6, 8, 10, 17)	3 (13, 13, 21)
Procarbazine	3–4 mg/kg/day × 30–40 days, then 1·5–2·5 mg/kg/day	28	10	5(1, 2, 5, 6, 8)			4 (4, 4, 6, 1, 7)	9 (2, 3, 3, 4, 4, 4, 6, 8, 10)	
			23	7	2		13	18	3

*In parentheses, time in months of duration of response.

Table 5. Percentage of response and duration of regressions after treatment with vinblastine and procarbazine in generalized Hodgkin's disease.

Drug	No. cases	Total responsive cases Category 0 and 1	Category 1	Duration of regression in months (category 1) Mean	Median
Vinblastine	38	25 (65·7%)	21 (55·2%)	6·7	5
Procarbazine	28	18 (64·2%)	13 (46·4%)	5·7	4

only after procarbazine. By analysing only those patients with a category 1 response the total of cases responding to both drugs is lower (8 patients). Of the other 3 cases 2 had a category 0 response to vinblastine and procarbazine and 1 showed a category 1 regression only after vinblastine (total 5 patients) but a category 0 after procarbazine (total 4 patients). The total percentage of cases responding to either drug was identical for category 0 and 1 (68·1%) and very close for category 1 only. Again the difference in percent for category 1 response is not statistically significant.

Side effects were minimal. Transitory leukopenia was observed in 22 patients treated with vinblastine (in 16 after the first or second dose, in 4 and in 2 respectively after the third and the fourth dose). Procarbazine produced only nausea and vomiting usually during the first 7–10 days of treatment in about half of the patients. Then the signs of gastric intolerance gradually disappeared without stopping the drug. Despite prolonged administration, even for many months, no signs of bone marrow depression detectable in the peripheral blood counting were seen nor neurological disturbances [20].

DISCUSSION

The analysis of the results obtained with the sequential treatment vinblastine–procarbazine allows some considerations which can be summarized as follows:

1. Previous extensive treatment with radiation therapy and alkylating agents does not prevent or limit *per se* the possibility to obtain subjective and objective benefits to subsequent treatment first with vinblastine and then with procarbazine. The percentage of total (subjective and objective) improvement for both drugs is above 60% and the mean duration is approximately 6 months while few cases can hold a remission with maintenance therapy for more than one year.

2. Procarbazine is an effective agent in the treatment of Hodgkin's disease. The number and the quality of regressions produced with this drug is comparable on a statistical basis to those obtained with vinblastine. The equal percentage of response appears also from the published case material (Tables 1 and 2). Furthermore procarbazine, despite prior extensive and prolonged myelosuppressive therapy, did not produce at the doses employed in the present study leukopenia or thrombocytopenia. Therefore this drug, which can produce a remarkable rate of regressions without employing high doses, should be given after alkylating agents and vinblastine which, on the contrary, are often myelosuppressive.

3. The fact that a considerable number of patients can respond to vinblastine and then to procarbazine after prior therapy with radio-

Table 6. Evaluation of 22 patients treated sequentially with vinblastine and with procarbazine.

Response	Total responsive cases Category 0 and 1	Category 1	Duration of regression in months (category 1) Mean	Median
None	3	5		
Only to vinblastine	4	5	3·4	2
Only to procarbazine	4	4	7·5	5
To vinblastine and to procarbazine	11	8	10·3	10·5
Total cases responding to vinblastine	15 (68·1%)	13 (59·0%)		
Total cases responding to procarbazine	15 (68·1%)	12 (54·5%)		

mimetic agents confirms the absence of cross resistance among the different classes of antiproliferative agents useful in Hodgkin's disease.

As a general rule prior treatment and duration of disease lowers the percentage of improvement to further radiotherapy [21] or chemotherapy [7] for recurrent neoplastic disease. Therefore any given drug usually produces a different response rate whether it is administered as the first chemotherapeutic agent or after previous treatment with radiotherapy and/or other growth inhibiting compounds. The mechanism of this different response rate has not been clearly defined although it has been postulated that prior chemotherapy or radiotherapy may select resistant cell lines or produce a mutation of the original tumor. No statistical evidence in the response rate between vinblastine and procarbazine was evident in our case material. Nevertheless one may not exclude that a greater percentage of objective improvement could be obtained with either vinblastine or procarbazine administered in untreated patients in earlier phase of disease.

We believe it is of great importance to plan a proper treatment for Hodgkin's disease not only in localized cases [1, 2] but also in patients with a generalized process. The program of treatment adopted since 1964 at the National Cancer Institute for Hodgkin's disease [16], not suitable any more for radical radiation therapy, includes a first course with HN2 followed by chlorambucil. If and when the disease becomes refractory to the alkylating agents vinblastine is tried next and then procarbazine if symptoms persist or recur. When all these agents have been adequately tried and chemotherapy is still indicated because of further progression vincristine or cyclophosphamide or thioTEPA are given. Adrenal steriods are administered mostly in presence of bone marrow depression, hemolitic anemia, fever, anorexia and cachexia. The use of localized radiotherapy is nevertheless not neglected while the patient is managed by chemotherapy mainly to cause regression of bulk nodal masses especially if not responding to the drug therapy.

The judicious use of all these drugs, provided they are adequately administered with a proper sequential schedule, by producing several consistent tumor regressions, can keep the patient active, relatively asymptomatic and in a good general condition for long periods of time.

In recent years considerable efforts have been made to increase the frequency, the magnitude and the duration of regression in Hodgkin's disease by giving more drugs in combination [7, 22–28]. In our opinion the published results do not yet provide sufficient evidence to state that the combination chemotherapy is definitely superior to single sequential chemotherapy. Lacher and Durant [26] treated 16 patients with vinblastine plus chlorambucil and although remissions occurred in 13 cases (81%) their mean duration was 7·5 months. De Vita and Serpick [24] treated 30 patients with a quadruple combination (vincristine, HN2, procarbazine and prednisone): 27 cases (90%) improved but for a median duration of only 6 months. The same drug combination was administered by Bernard *et al.* [23] to a group of 67 patients with an 87% regression rate and an average duration of 2–19 months. A similar quadruple therapy has been designed by Mathé *et al.* [22] with corticosteroids, vinblastine, TEM and procarbazine. Although an 86·2% remission rate was achieved at the expense of severe leukopenia, relapses occurred within a short period of time (of the 15 patients who had a complete remission 8 relapsed within 4·2 weeks and 4 within 14 weeks). The quadruple combination proposed by Frei *et al.* [7, 25, 27] employs ciclophosphamide, vincristine, Methotrexate and prednisone. Chemotherapy was given to 14 patients (7 with Stage I or II and 7 with Stage III). Patients with localized disease were also treated with radiation therapy and all but one entered a complete remission unmaintained for more than 30 months. Of the 7 patients with Stage III 2 showed an induction failure, 2 died after 2 and 12 months respectively and 3 had a complete unmaintained remission for 32, 33 and 35 months (2 patients received also X-ray treatment above the diaphragm).

The present data indicate that combination chemotherapy induces in patients with generalized Hodgkin's disease, especially if not previously treated, a high degree of complete remission (80–90%). A similar response rate in untreated patients can also be achieved with HN2 alone [11], but a lesser number of cases shows a complete disappearance of all adenopathies. The real advantage of concurrent vs sequential drug combination should be, besides the frequency of regressions, the magnitude and the duration of unmaintained remissions. The median duration of remissions after HN2 and maintenance therapy with chlorambucil was 8 months [13], while the total response rate to the sequence of vinblastine–cyclophosphamide or cyclophosphamide–vin-

blastine was approximately 80% with a median duration of maintained remission close to 10 months [28]. The trials with combination chemotherapy, as could be estimated from the published case material with the exception of few patients with Stage III disease treated by Frei *et al.* [25, 27], do not yet provide a sufficient evidence that the duration of unmaintained remissions obtained with this method of treatment is greater than that achieved with single agents given in sequence and with maintenance therapy.

Furthermore, the high regression rate obtained by Frei *et al.* [25, 27] in the 7 patients with localized Hodgkin's disease treated with radiotherapy and quadruple chemotherapy can be presently achieved with radical and prophylactic irradiation alone without the addition of chemotherapy [29, 1, 30]. This association did not seem from previous experiences to have a useful adjunctive role in affecting survival time [3–6].

In conclusion the question whether in advanced Hodgkin's disease it is better to use chemotherapeutic agents in sequential or in concurrent combination still deserves more long term studies. At the present moment eradication of Hodgkin's disease can be achieved only by intensive irradiation although prolonged remissions may be expected in a certain number of cases after intensive combination chemotherapy. Nevertheless, we believe that a proper sequential rotation of useful drugs, as shown in the present study, can allow the control of the disease for long periods of time in the majority of patients. Furthermore, sequential chemotherapy can be administered on ambulatory bases while this is not usually feasible with combination chemotherapy because bone marrow depression almost invariably occurs after one or two cycles [22, 25, 27].

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RESUME

Les auteurs analysent les effets d'un traitement chimiothérapique séquentiel à la vinblastine–procarbazine dans un groupe de patients atteints de maladie de Hodgkin généralisée. Avant de recevoir la vinblastine, les malades avaient été traités par radiothérapie et administration d'agents alkylants, et ils avaient reçu la vinblastine avant l'administration de procarbazine. Une rémission objective, de plus d'un mois, fut observée dans 55,2% des cas, après traitement à la vinblastine, chez 38 malades, et de 46,4%, après administration de procarbazine, chez 28 malades. La différence entre les pourcentages de réponses positives n'est pas statistiquement significative. Aux doses administrées, la procarbazine n'a pas produit de dépression de la moelle osseuse, malgré les traitements myélo-suppressifs, intensifs et prolongés, qui avaient été administrés précédemment. Les auteurs en concluent que la procarbazine, même administrée après des agents radiomimétiques et de la vinblastine, se montre un agent efficace dans le traitement de la maladie de Hodgkin à un stade avancé, et que le nombre et la qualité des régressions obtenues par ce médicament sont comparables à celles que provoquent la vinblastine. Les effets d'un traitement chimiothérapique séquentiel, par opposition à un traitement chimiothérapique combiné intensif, sont également discutés.

SUMMARY

The authors analyze the responses obtained in a group of patients with generalized Hodgkin's disease in which a sequential chemotherapy with vinblastine–procarbazine has been administered. Patients before receiving vinblastine had been treated with radiotherapy and alkylating agents and with vinblastine before treatment with procarbazine. A 55·2% objective improvement for more than 1 month was obtained after treatment with vinblastine in 38 patients and 46·4% after procarbazine in 28 patients. The difference in the response rate is not statistically significant. At the doses administered procarbazine did not produce depression of bone marrow despite prior extensive and prolonged myelosuppressive treatment. The authors conclude that procarbazine even if administered after radiomimetic agents and vinblastine is an effective agent in the treatment of advanced Hodgkin's disease and that the number and the quality of regressions produced by this drug is comparable to those obtained with vinblastine. Sequential vs intensive combination chemotherapy in Hodgkin's disease is also discussed.

ZUSAMMENFASSUNG

Die Autoren analysieren die Reaktion einer Gruppe von Patienten mit generalisierter

Hodgkinscher Krankheit bei denen eine Nachbehandlung mit Vinblastin-Procarbazin durchgeführt worden war. Die Patienten waren vor der Applikation von Vinblastin mit Strahlen und alkylierenden Substanzen behandelt worden, beziehungsweise mit Vinblastin vor Procarbazin. 55,2% *objektive Besserung für länger als einen Monat wurde nach der Behandlung mit Vinblastin bei* 38 *Patienten festgestellt und* 46,4% *nach Procarbazin in* 28 *Patienten. Der Unterschied in der Erfolgsrate ist nicht statistisch signifikant. Bei den angewandten Dosen unterdrückte Procarbazin das Knochenmark nicht, trotz vorausgegangener intensiver und verlängerter myelosuppresiver Behandlung. Die Autoren kommen zu dem Schluß, daß Procarbazin, selbst nach vorausgegangener Behandlung mit radiomimetischen Agentien und Vinblastin, eine in der Behandlung der Hodgkinischen Krankheit wirksame Substanz ist, und daß die Anzahl der durch sie erzielten Regression–den mit Vinblastin erreichten vergleichbar ist. Aufeinanderfolgende Anwendung der verschieden-Therapeutika vs intensive Kombinationstherapie bei Hodgkinscher Krankheit wird diskutiert.*

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Pharmaco-biochemical Studies on Cytotoxic Polyol derivates

I. Effects of 1-6-Dibromo-1-6-Dideoxy-dulcitol on Sensitive, Resistant and Refractor Tumours

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INTRODUCTION

THE SYNTHESIS of hexitols substituted with halogens and the tumour inhibitory effect of some representatives of this series was reported by Institoris *et al.* [1]. The chemotherapeutic action on some sorts of experimental tumours of one of these compounds, namely of 1-6-dibromo-1-6-dideoxy-dulcitol (DBD) was established by Kellner *et al.* [2, 3] and this derivative also proved to be a valuable drug in human leukemias.

Comparison of the metabolic activities in sensitive and resistant lines of the Yoshida tumour influenced by DBD could throw light on the processes which might be responsible for the chemotherapeutic action of the drug. We suppose DBD is able to alter a number of metabolic pathways in sensitive as well as in resistant tumours. But the alterations of cellular metabolism essential in the therapeutic action of the drug, occurring on sensitive tumour cells but not in the acquired, nor in natural resistant tumours, are of special interest. A more detailed study of such differences in cellular processes might lead to a better knowledge concerning the site of attack and selective action of DBD.

We carried out investigations on the nucleic acid content of normal bone marrow and both lines of tumour cells influenced by DBD. Subsequently, using the ascitic forms of both types of Yoshida tumours, we compared the rates of DNA, RNA and protein synthesis in the cells by following the incorporation of ^{14}C-thymidine, ^{14}C-orotic acid and ^{14}C-leucine into the cells in the absence and presence of DBD. Finally, we studied the thermal denaturation properties of DNA treated with DBD.

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MATERIAL AND METHODS

The antitumour effect of DBD was measured on 8–10-week old CB Wistar rats transplanted bilaterally with Yoshida sensitive and nitrogen mustard resistant tumours [4]. DBD was suspended in saline containing 5% Tween 80 and injected in logarithmically increasing doses intraperitoneally into several groups of animal (one comprising 5 animals) when the tumours were palpable.

The *in vitro* and *in vivo* toxicity and the tumour inhibitory effect were determined according to the method of Ball *et al.* [5]. In experiments where Yoshida ascites cells were used the cells were collected 7–8 days after transplantation.

The nucleic acid content of bone marrow was assayed as described previously [6].

For the isolation of phosphorous fractions of the solid tumour tissues the procedure of Schimdt–Thannhauser [7] was used and the P content was measured according to Robinson's method [8].

The *in vitro* incorporation of ^{14}C-thymidine,

^{14}C-orotic acid and the ^{14}C-leucine into the 7–8-day-old ascites tumour cells was studied in a salt medium described by Hölzel [9]. Fifteen ml of 10^7/ml cells were preincubated for 2 hr with DBD in a siliconised glass vessel and mixed with a motor driven propeller. After adding the labelled precursors the samples were removed at various times and put in cold 10% trichloracetic acid (TCA). The precipitates were collected on a Millipore filter (MF 50), washed with 5 vol. cold 5% TCA containing the non-radioactive precursors, and plated on alumina planchettes. The radioactivity was measured in a windowless gas flow-counter.

For the study of *in vivo* effect of DBD on ^{14}C-orotic acid incorporation, rats with 10 day-old solid Yoshida tumour were treated with 50 mg/kg DBD intraperitoneally, and 18 hr later 15 μC/100 g ^{14}C orotic acid (spec. activity 10·52 mc/mM) was injected intraperitoneally. After 1 hr the animals were killed, the tumours and livers removed and the RNA were isolated in the following way: 40 ml of 0·015 M nahpthalene-1:5-disulphonic acid sodium salt (NDS) was added to 5 g tissue and homogenized at 0°C in a Waring Blender for 3 min. After addition of an equal amount of 90% phenol (containing 0·1% 8-hydroxy-quinoline) the homogenization was continued for 20 min. The suspension was centrifuged at 0°C with 3000 **g**. The supernatant I was removed and the same amount of NDS was added. This mixture was homogenised at 65°C for 30 min. Then the supernatant II was removed from the centrifuged suspension and 0·5% sodium-laurylsulfate was added, homogenized at room temperature for 30 min. After centrifugation the supernatant III and the phenol layer were removed and the interphase was incubated twice in 5 ml 0·5 M $HClO_4$ at 70°C to release all the remaining RNA. These two supernatants were combined (IV) and neutralized with KOH. The RNAs from the supernatant obtained by the phenol extraction procedure were precipitated at −20°C after the addition of 2 vol. ethanol, washed several times with ethanol, separated from DNA with alkali hydrolysis and dissolved in a small amount of water.

The Tm point of calf thymus DNA, prepared by T. Valyi-Nagy and A. Zsindely [10], was measured according to the method of Marmur and Doty [11] but using 0·015 M NaCl+ 0·0015 M sodium citrate as solvent for DNA. The temperature was raised gradually (1°C in 5 min) and the optical density was read at 260μ in a Spectromon spectrophotometer equipped with thermal spacer connected to a temperature controlled circulating water bath.

EXPERIMENTS

Effect of DBD *on normal bone marrow*

Bone marrow of all normal tissues is the most susceptible to the toxic actions of cytostatics. Therefore, we estimated the extent of damage caused by DBD on bone marrow by measuring its nucleic acid content. From the work of Conners *et al.* [12] it is well known

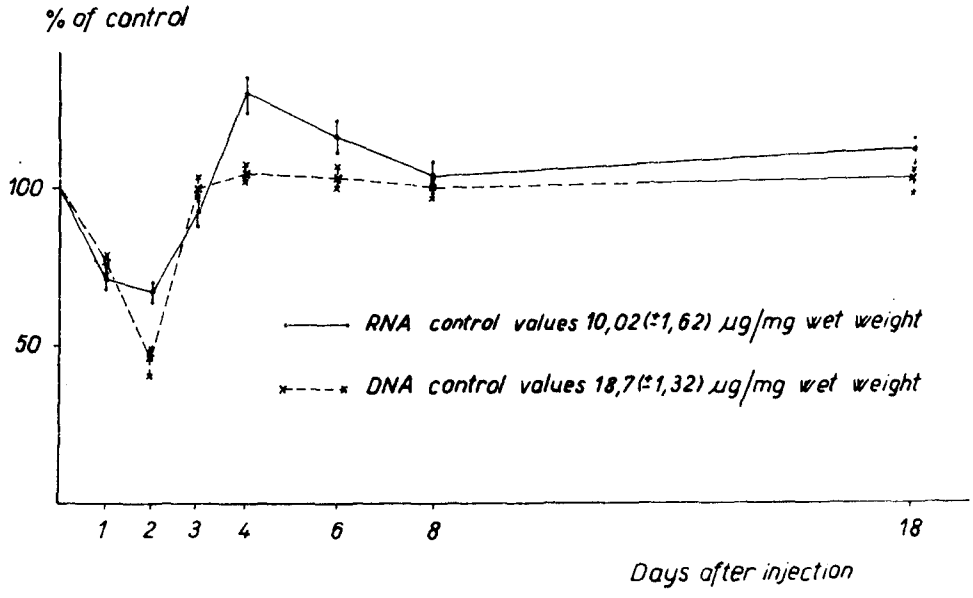

Fig. 1. *The effect of* DBD *on the* DNA *and* RNA *content of bone marrow. CB Wistar rats* (160–200 g) *were treated with a single dose of* 250 mg/kg DBD *intraperitoneally. At times indicated on the abscissa the animals were killed and the bone marrow of both femurs removed.* DNA *and* RNA *were estimated as indicated in* Materials and Methods.

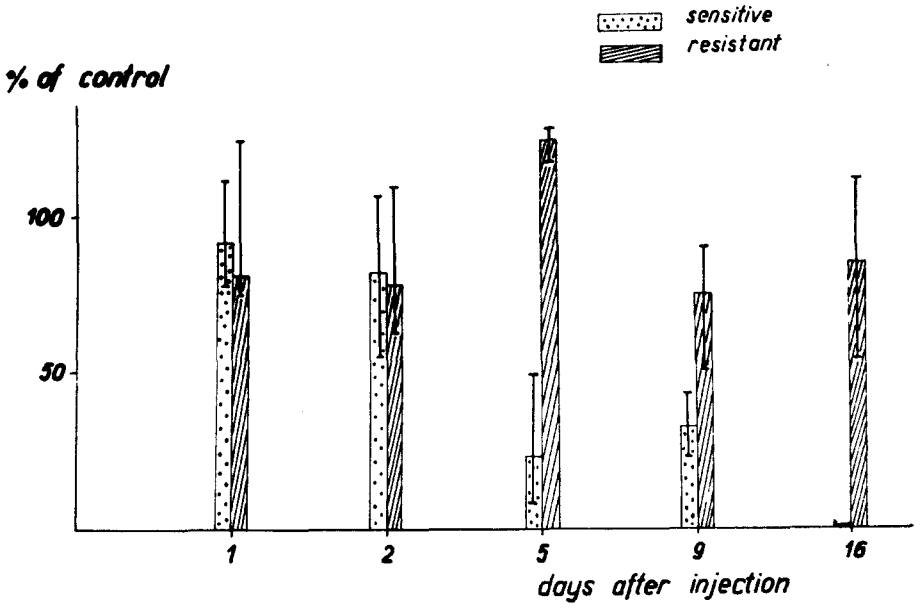

Fig. 2. The effect of DBD *on the daily change of weight of sensitive and resistant Yoshida tumours growing bilaterally in the same animal.*
A single dose of 50 mg/kg DBD *was administered intraperitoneally at the time when the tumours were palpable.*

that the decrease of nucleic acid content of bone marrow shows a definite parallelism to the degree of damage on this tissue brought about by Merophan. Figure 1 shows the action of DBD on the nucleic acid content of bone marrow.

According to the results of the experiment illustrated in Fig. 1 the DNA content of bone marrow shows a decrease of nearly 60%, while the decrease in RNA content is much smaller on the second day of DBD treatment. This damage of the bone marrow represented by the decrease of its nucleic acid content is reversible as demonstrated by the fact that the DNA content of bone marrow reaches the control value again on the third or fourth day after DBD treatment.

The effect of DBD *on the sensitive and resistant Yoshida tumours*

In addition, we have studied the action of DBD on the growth rate on sensitive and resistant line of solid Yoshida tumours (see Fig. 2).

The points of the curve shown in Fig. 2 represent the average relative weight of tumours. The animals bearing the tumours were treated with a single dose of 50 mg/kg of DBD injected intraperitoneally on the 7th day after transplantation. At the time indicated on the abscissa 10 animals were killed and their tumour weights were expressed in per cent of the control (untreated) tumour weights shown in the figure. It appears that nearly a 20% decrease occurs in the weight of sensitive as well as in resistant tumours on the 1–2 days following DBD treatment. On the 5th day, however, a marked deviation occurs in the growth behaviour of sensitive and resistant tumours. After a rapid initial decrease in the weights of the sensitive tumours, the further regression is slower but finally, a sac filled with necrotic tissue is to be found in the place of the tumour on the 16th day after DBD treatment. On the other hand, the average weight of resistant tumours increased in comparison with the control values after the 2nd day and then from the 10th to the 16th day their weights were lower as compared to those of untreated control tumours.

Effect of DBD on DNA and RNA content

Seeking a general picture of the changes of nucleic acid contents of sensitive and resistant Yoshida tumours treated with DBD we estimated the DNA and RNA contents of tumour tissue using the method of Schmidt–Thannhauser [7].

The most marked changes were found in the RNA fractions of sensitive tumours (Fig. 3a). They showed a transient rise at the 6th hour and then a marked drop of about 75% as compared to the control value, on the first day after DBD treatment. On the subsequent days the values of RNA content of sensitive tumour showed a tendency to gradual increase and on the 9th day it reached the level of control values. The DNA content of sensitive tumours

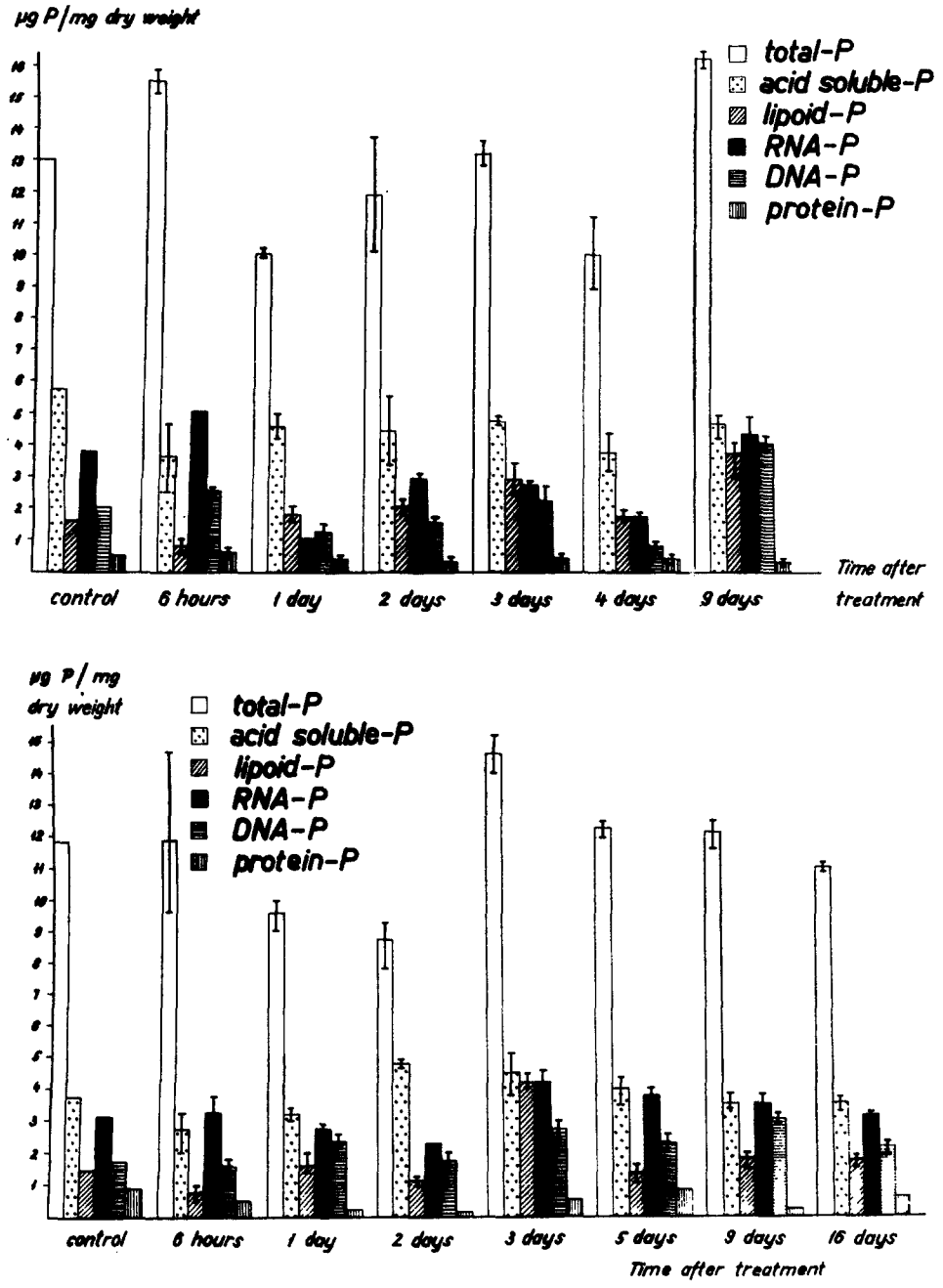

Fig. 3a and 3b. The action of DBD *on the phosphorous fractions of sensitive* (3a) *and resistant* (3b) *solid Yoshida tumours growing bilaterally in the same animal, measured by the method of Schmidt and Thannhauser.*

CB Wistar rats (160–200 g) *were treated with a single dose of* 50 mg/kg DBD *i.p. At the times indicated on the figures the animals were killed and the tumours removed, weighed and homogenized. Fractionation of the homogenate was carried out by the method of Schmidt and Thannhauser* [7]. *The* P *content of the fractions was analysed by the method of Robinson* et al. [8] *and the data were calculated on the dry weight basis of the homogenates.*

also decreased after the treatment but the overall changes of this fraction were not so marked.

The analysis of the same fractions originating from resistant Yoshida tumours did not show essential changes (Fig. 3b). The total Phosphorus content, however, rose to a high level on the 3rd day following DBD treatment. This change immediately preceded the increase in weight of resistant tumours as compared to the controls (see Fig. 2). The rise in total P content originated from the substantial increase in both the lipid P and DNA-P fractions. It can be concluded from the data of experiments presented in Figs. 3a and 3b that the phosphorus fractions in the sensitive cells initially

Table 1. *The viability of tumour cells treated with* DBD.

Treatment	No. of transplanted cells/animal	Takes after 30 days (%)		
		Yoshida tumour sensitive	Yoshida tumour resistant	Sarcoma 180 tumour
Control	10^7	100	100	100
	10^6	100	100	67
	10^5	60	60	4
DBD 25 µg/ml	10^7	73	100	100
50 µg/ml	10^7	20	100	100
100 µg/ml	10^7	10	100	77

The tumour cells were incubated in a salt medium [9] with or without DBD for 2 hr and transplanted into 150–200 g Wistar rats. The appearance of the tumours was verified with biopsy 30 days after transplantation.

decrease after treatment and then re-establish themselves, while there is little effect on resistant cells.

Effect of DBD *concentration on growth of cells* in vitro

By incubating sensitive, resistant or refractory tumour cells in various concentrations of DBD we were able to estimate the percentage take of tumour cells in animals. The results are summarized in Table 1.

From the results of these experiments we established that a dose of 25 µg/ml DBD inhibits the take of sensitive Yoshida cells in 27% in comparison to control cells which had been incubated for the same period of time in physiological solution containing no DBD. The degree of inhibition, as seen in Table 1 is gradually increasing with higher doses. The specificity of the action is verified by the fact that neither the cells of resistant Yoshida tumour, nor those of the refractor Sarcoma 180 were altered in their viability after the DBD treatment. Another characteristic feature is that the drug cannot be washed out from the cells once they have been treated with it, or that its action is irreversible.

Effect of DBD *on rate of* DNA *synthesis*

The rate of synthesis in the sensitive and resistant Yoshida cells, as measured by the ^{14}C-thymidine incorporation, was not influenced by the presence of 50 µg/ml DBD. On the other hand, 100 µg/ml of the drug inhibited in 90% the ^{14}C-thymidine uptake into the DNA of the sensitive cells. No inhibition by

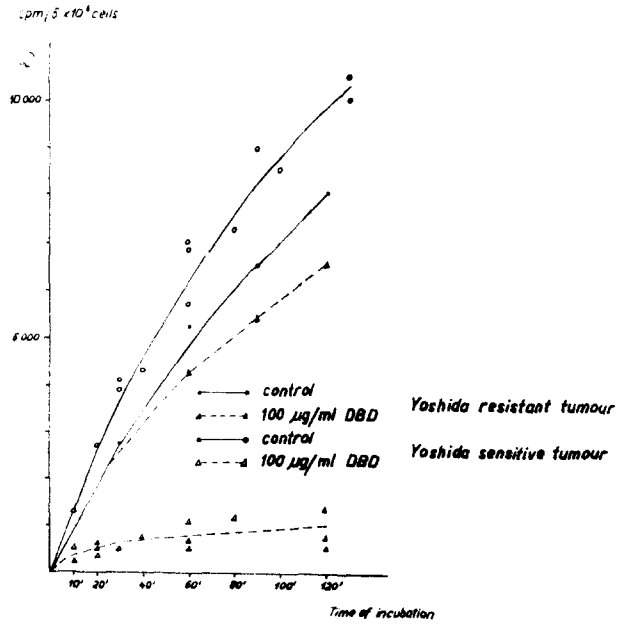

Fig. 4. *The* in vitro *incorporation of* ^{14}*C-thymidine into sensitive and resistant Yoshida tumour cells in the presence of* 100 µg/ml DBD.
Fifteen ml of 10^7*/ml Yoshida ascites cells were preincubated in a salt medium* [9] *for 2 hr with* DBD *and then* 10 µC ^{14}*C-thymidine (spec. act.* 35·9 *mC/mM, Radiochemical Centre, Amersham) was added. Samples were removed at various times, the cold* TCA *precipitates were collected on Millipore filter and the radioactivity was measured in a windowless gas flow counter.*

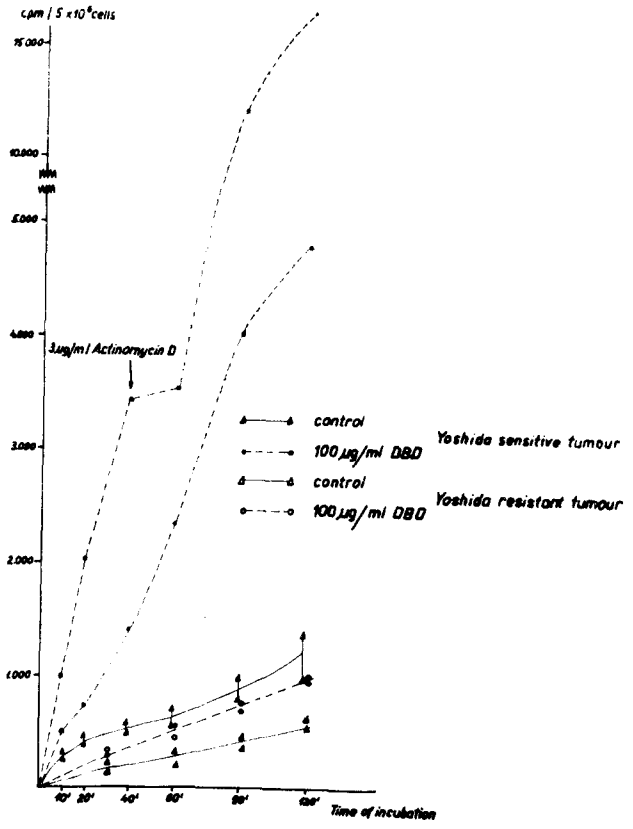

Fig. 5. *The* in vitro *incorporation of* C^{14}*-orotic acid into sensitive and resistant Yoshida tumour cells, in the presence of* 100 µg/ml DBD.
Fifteen ml of 10^7/ml *Yoshida ascites cells were preincubated in a salt medium* [9] *for* 2 *hr with* DBD, *then* 10 µC ^{14}C-*orotic acid* (*spec. act.* 44·5 mC/mM, *Radiochemical Centre, Amersham*) and 40 *min later* 3 µg *actinomycin D were added. Samples were removed at various times, the cold* TCA *precipitates were collected on Millipore filter and the radioactivity measured in a windowless gas flow counter.*

DBD could be detected using cells originating from the resistant line of Yoshida tumour (Fig. 4).

Effect of DBD *on synthesis of* RNA

At a concentration of 100 µg/ml DBD has a capacity to increase ^{14}C-orotic acid incorporation into the RNA of sensitive Yoshida tumour cells. The rate of this increase varied in samples between 4–6 fold related to the control (Fig. 5).

Actinomycin D at 3 µg/ml concentration could not suspend the enhanced RNA synthesis induced by DBD when added 40 min after the addition of the ^{14}C-orotic acid. In another experiment—not published in this paper—the same concentration of actinomycin brought about a complete inhibition of RNA synthesis in the Yoshida tumour cells. If resistant Yoshida cells were used a much smaller degree of stimulation of the ^{14}C-orotic acid incorporation was observed.

Effect of DBD *on protein synthesis*

Using ^{14}C-leucine for measuring its incorporation rate into the proteins of tumour cells treated with DBD we found that enhanced RNA synthesis was not followed by an increase in protein synthesis. As it appears from Fig. 6 the rate of ^{14}C-leucine incorporation showed a moderate decrease as compared to the controls, in the presence of 100 µg/ml of DBD.

Comparison of the effect of DBD *on orotic acid incorporation into tumour and liver cells* in vivo

The following experiment was performed to ascertain if a similar enhancement of RNA synthesis also occurred *in vivo*.

The data of Table 2 shows that 18 hr after a dose of 50 mg/kg of DBD the RNA fractions of the tumour tissue are not uniformly affected. The specific activity of RNA extracted with cold phenol altered very slightly. On the contrary, the RNA fractions extracted with lauryl sulfate and especially with hot phenol, showed a very marked decrease. In the residual RNA, i.e. the RNA released by using 0·5 N perchloric acid, the incorporation of ^{14}C-orotic acid increased twice of the control level.

In the liver tissue, the alterations in the specific activity of the RNA fractions are not so obvious. The incorporation into the liver

was much higher as compared to the tumour tissue, which finding is in agreement with the observation of Matsudaira [13].

Effect of DBD *on the thermal denaturation profil of* DNA

It is well known that biological alkylating

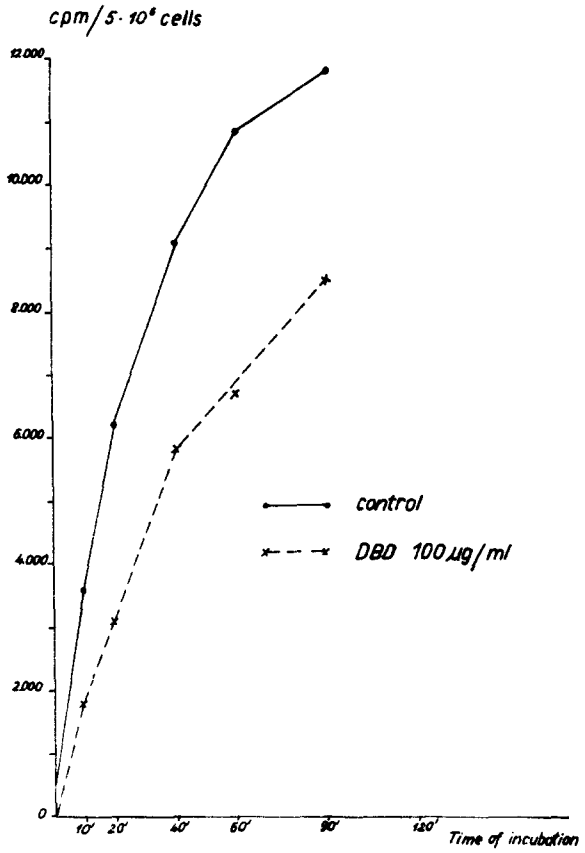

Fig. 6. *The effect of* 100 µg/ml DBD *on the* in vitro *incorporation of* C^{14}*-leucine into Yoshida tumour cells.*
Fifteen ml *of* 10^7/ml *Yoshida ascites cells were pre-incubated in a salt medium* [9] *for* 2 *hr with* DBD *then* 10 µC ^{14}C*-leucine (spec. act.* 1·60 mC/mM, *Central Research Institute for Physics, Budapest) was added. Samples were removed at various times, the cold* TCA *precipitates were collected on Millipore filter and the radioactivity was measured in a windowless gas flow counter.*

Table 2. *The effect of* DBD *on the* in vivo *incorporation of* ^{14}C*-orotic acid in the* RNA *fractions of Yoshida sensitive tumour tissue and liver*

Tissues		I. RNA 1	I. RNA 2 (%)	II. RNA 1	II. RNA 2 (%)	III. RNA 1	III. RNA 2 (%)	IV. RNA 1	IV. RNA 2 (%)
Control	Tumour	381	85	2834	10	633	2	2370	3
	Liver	10691	91	60840	5	91100	2	107638	2
50 mg/	Tumour	237	87	162	9	260	2	4875	2
kg DBD	Liver	10977	90	42400	6	54555	1	72272	3

CB Wistar rats bearing Yoshida sensitive tumour were treated with 50 mg/kg DBD i.p. After 18 hr 15 µC/100 g body weight ^{14}C-orotic acid (spec. act. 10·42 mc/mM, Central Research Institute for Physics, Budapest) were administered i.p. to the animals. One hour later the animals were sacrificed, RNA extracted as described in Materials and Methods.

RNA fractions:

I =extracted by cold phenol
II =extracted by hot phenol
III =extracted by sodium lauryl sulphate
IV =released with perchloric acid

The results representing averages of two experiments are expressed in cpm/mg RNA [1] and in percentages of the total RNA content [2].

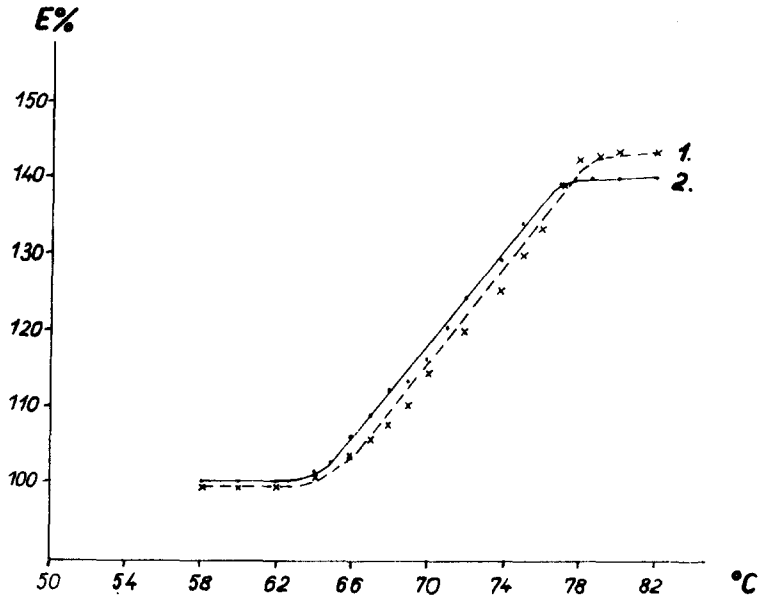

Fig. 7. The effect of DBD *on the thermal denaturation profile of* DNA.
Abscissa: temperature in centigrade
Ordinates: relative absorbance at 260 mμ
Curve 1*:* DNA *alone,* Tm 72°C; *Curve* 2*:* DNA+DBD, Tm 71; 5°C.
Thymus DNA *and* DBD *were dissolved in a concentration of* 12·5 μg/ml *in a buffer containing* 0·015 M NaCl *and* 0·0015 M *Na citrate at* pH 6·8.

agents alter very markedly the physicochemical properties of DNA. For this reason we investigated the thermal denaturation profile of DNA in the presence of DBD.

As is shown in Fig. 7, DBD does not influence the Tm point and the hyperchromic properties of DNA. By using this method it was concluded that DBD did not damage the native DNA.

DISCUSSION

In the experimental part of our study we made attempts to obtain information on the biochemical mechanism which might be involved in the therapeutic action of DBD. In the first part of our investigations we followed the action of DBD on the nucleic acid contents of various sensitive and resistant tissues. The changes in the nucleic acid contents reflect the time required for the regression of sensitive tumours induced by a single dose of DBD.

It is to be noted that the drug administered in a single dose is excreted in the first 24 hr [14]. It is therefore quite obvious that such tumour cells which are sensitive to DBD are exposed to the drug for a relatively short period of time. On the other hand, the fact that the regression period lasts approximately 15–16 days suggests that cell division ceases immediately after exposure to the drug, but time is required for the subsequent degeneration or lysis of the cells. On the other hand, the observations of Kellner *et al.* [3] that incomplete mitoses and giant cells occur in the late period of DBD action suggest that the damage caused by this drug may be manifest only in subsequent cell cycles. These—and probably other mechnisms—may be possible explanations of the delayed action of DBD.

Comparing the alterations in the nucleic acid contents of the Yoshida tumours with the bone marrow, two kinds of differences could be revealed. Firstly, the time required for rearrangement of nucleic acid content is considerably shorter in the bone marrow than in the sensitive tumour tissue following DBD treatment. Secondly, the damage of bone marrow is characterized by a more conspicuous decrease in DNA than in the RNA content. On the other hand, in the sensitive tumour the marked change in the RNA content was manifested in a sudden rise followed by a gradual decrease. At the same time no essential changes were found in the nucleic acid content of the resistant Yoshida tumour treated with DBD.

Supposing that a relatively short exposure of tumour cells to DBD is essential for the drug action we characterized this period of drug action in the sensitive cells by looking for differences which might occur in the rate of synthesis of macromolecules. However, our studies on the action of DBD on the synthesis of nucleic acids in tumour cells showed that the concentrations needed to inhibit the nucleic acid synthesis are substantially higher than those which proved to be sufficient to inhibit

tumour *in vivo* or to depress the viability of tumour cells *in vitro*. In spite of this, it is surprising that the marked increase in the incorporation of ^{14}C-orotic acid was not observed in the resistant Yoshida tumour not in the refractory Sarcoma 180 tumour cells. Furthermore our results—not presented in this paper—that the chemically closely related 1-6-dichloro-1-6-dideoxy-D-mannitol and 1-6-dichloro-1-6-dideoxy-D-dulcitol, which have no tumour inhibitory effects, do not alter the RNA synthesis in the Yoshida sensitive cells refers to the significance of the enhanced ^{14}C-orotic acid incorporation in the chemotherapeutic action of DBD.

Unfortunately, we have insufficient knowledge of the character of that kind of RNA in which the incorporation of ^{14}C-orotic acid is enhanced shortly after DBD treatment. DBD brought about a manifold elevation of ^{14}C-orotic acid incorporation measured in the *in vitro* experiments, while *in vivo* this elevation could be detected only in one fraction of RNA. This difference could be explained by the observation of Watson *et al.* [15], who found that the characteristics of RNA synthesis occurring in mammalian cells are different if investigated *in vitro* or *in vivo*.

The study of the nature of RNA synthesized in the presence of DBD is handicapped by difficulties encountered at the isolation of this fraction. Despite this, there is accumulating information in the literature indicating the existence of an unusual RNA in the cells, e.g. Klenow *et al.* [16] found in the interphase obtained from Ehrlich ascites tumour cells after treatment with phenol, an RNA fraction unextractable by lauryl sulfate and resistant to actinomycin D. Other authors also demonstrated that the synthesis of some RNA species cannot be antagonized by actinomycin D. So Revell *et al.* [17] showed that ^{14}C-orotic acid incorporation into the s-RNA-pseudouridine was not inhibited after actinomycin treatment. Furthermore, Dubin [18] could demonstrate a difference in the susceptibility of RNA synthesis occurring in the cytoplasm on the one hand, and in mitochondria, on the other. In the presence of actinomycin D only 4s-RNA was synthesized in the former but the synthesis of 17s-RNA also could be demonstrated in the latter. Recently, Martin and Brown [19] observed an undisturbed synthesis of 12–20 s-RNA in cultures of hamster kidney cells when the culture medium contained 1 μg/ml actinomycin D. Martin and Brown established that the existence of the synthesis responsible for the production of this kind of RNA depended on the age of culture.

We think that the RNA species synthesized only in the sensitive Yoshida tumour cells due to the action of DBD might have a similar nature to the above mentioned RNA. This is supported by the fact that the increase in ^{14}C-orotic acid incorporation induced by DBD could not be depressed in the presence of actinomycin D. Our experiments now in progress tend to reveal the mode of isolation and the characteristics of that unique RNA synthesised rapidly and resistant to actinomycin, in the Yoshida tumour cells treated with DBD.

It seems to be less likely that chemotherapeutically active concentrations of DBD would bring about profound changes in the physicochemical nature of DNA. This is demonstrated by the fact that the Tm point of the DNA remains the same after incubation with DBD. This property of DBD is in contrast to that of other alkylating agents, mainly of those containing chloroethylamino groups which produced marked alterations in the thermal denaturation profile of DNA [20–22]. Consequently, it is unlikely that DBD would alter the template function of DNA.

The results of our experiments described in this report cannot explain the ultimate reason for the tumour inhibitory properties of DBD. It seems likely that the changes observed in the nucleic acid metabolism induced by the drug are the consequences rather than the true cause of the action. The fact that certain changes occur exclusively in the sensitive tumour cells and not in the resistant or refractory tumour cells following DBD treatment can be considered as a characteristic attribute of the chemotherapeutic properties of this drug.

Continuation of this work (to be published later) on the interaction of deoxyribonucleoprotein complex with DBD led us to assume that this drug brings about damage to the regulatory system of nucleic acid synthesis.

RESUME

Le 1-6-*dibromo*-1-6-*dideoxy-dulcitol* (DBD) *exerce une influence curative sur la tumeur de Yoshida. Par contre, la même substance s'est avérée complètement inefficace contre une lignée résistante de tumeurs de Yoshida, et également contre un certain nombre d'autres tumeurs transplantables.*

Les auteurs ont examiné, dans le cas de tumeurs sensibles au DBD comme dans le cas de tumeurs résistantes, les altérations du contenu en acides nucléiques sous l'action du DBD. Les modifications—en particulier en ce qui concerne le contenu en RNA —sont apparues comme nettement plus importantes dans les tumeurs sensibles que dans les tumeurs résistantes.

Dans la moelle osseuse, contrairement à ce qui se passe dans le tissu de la tumeur, nous avons constaté une diminution plus nette du contenu en DNA, alors que les altérations du contenu en RNA étaient moins marquées. En plus, le laps de temps nécessaire à la régénération était plus court pour la moelle osseuse que pour le tissu des tumeurs.

Dans le cas des cellules in vitro *de la tumeur de Yoshida sensible au DBD,* 100 µg/ml *de cette substance inhibent l'incorporation de la* ^{14}*C-thymidine et intensifient celle de l'acide* ^{14}*C-orotique dans les acides nucléiques correspondants. On ne constate aucune altération,* in vitro, *en présence de DBD, dans le taux d'incorporation des précurseurs marqués, lorsqu'il s'agit de tumeurs résistantes ou réfractaires. La synthèse accélérée du RNA, se produisant dans les cellules des tumeurs sensibles, ne peut pas être inhibée par l'actinomycine D. Ces modifications de l'activité synthétique dans la tumeur sensible ne constituent certainement pas le point d'attaque de DBD, puisqu'une concentration plus faible de cette substance,* 25–50 µg/ml, *s'est avérée suffisante pour diminuer la viabilité des cellules* in vitro *provenant de tumeurs sensibles.*

Une dose thérapeutique de DBD intensifie la synthèse d'une fraction du RNA dans les cellules in vivo *de tumeurs sensibles. La nature de cette espèce de RNA est encore inconnue.*

Le DBD n'altère pas la courbe de thermodénaturation du DNA; il paraît donc relativement peu vraisemblable que cette substance trouble, comme d'autres agents alkylants, la fonction de matrice du DNA.

SUMMARY

1-6-*dibromo*-1-6-*dideoxy-dulcitol* (*DBD*) *has a curative effect on the Yoshida tumour. On the other hand, the same drug proved to be completely ineffective on a resistant line of Yoshida tumour and on a number of other transplantable tumours.*

The action of DBD on the alterations in nucleic acid contents of sensitive and resistant tumours was investigated. The changes especially in the RNA content were much more characteristic in sensitive tumours than in resistant ones.

Contrary to tumour tissue, a more marked decrease in the DNA content of bone marrow was observed, while the changes in the RNA content were not so extensive. In addition, the time necessary for regeneration was shorter in the bone marrow than in the tumour tissue.

100 µg/ml *DBD inhibited the* ^{14}*C-thymidine, and increased the* ^{14}*C-orotic acid incorporation into the nucleic acids of sensitive Yoshida tumour cells* in vitro. *However, no alteration occurred in the rate of incorporation of the labelled precursors in resistant or refracter tumour cells in the presence of DBD* in vitro. *The increased RNA synthesis occurring in sensitive tumour cells could not be depressed by actinomycin D. These changes in the synthetic activities taking place in the sensitive tumour cannot be considered as the site of action of DBD because lower concentration of the drug* (25–50 µg/ml) *proved to be sufficient to decrease the viability of sensitive tumour cells* in vitro.

Therapeutic dose of DBD increased the synthesis of a RNA fraction in the sensitive tumour cells also in vivo. *The nature of this RNA species is so far unknown.*

DBD does not alter the thermal denaturation profile of DNA so it seems unlikely that the drug interferes with the template function of DNA, like other alkylating agents.

ZUSAMMENFASSUNG

1-6-*Dibromo*-1-6-*Dideoxy-Dulcitol* (*DBD*) *unterdrückt das Wachstum des Yoshida-Tumors. Andererseits war diese Substanz unwirksam bei einer resistenten Linie des Yoshida-Tumors und ebenso bei einer Anzahl von Impftumoren.*

Die Rolle von DBD bei Änderungen im Hukleinsäuregehalt sensitiver und resistenter Tumoren wurde untersucht. Der Wechsel im RNS Gehalt war in sensitiven Tumoren sehr viel charakteristischer als in resistenten.

Im Gegensatz zum Tomorgewebe wurde im Knochenmark ein deutliches Absinken des

DNS Gehaltes beobachtet, während die Veränderungen im RNA Gehalt nicht so ausgesprochen waren. Zusätzlich war die Erholungszeit im Knochenmark kürzer als im Tumorgewebe. 100 µg/ml *DBD hemmten den* C^{14}*-Thymidineinbau und rehöhten den Einbau von* C^{14}*-Orotsäure in die Nukleinsäuren sensitiver Yoshida-Tumorzellen* in vitro. *Jedoch bei resistenten oder refraktären Tumorzellen* in vitro *trat in Gegenwart von DBD keine Veränderung der Einbaurate markierter Vorläufer auf. Die erhöhte RNS Synthese bei sensitiven Tumorzellen konnte durch Actinomycin D nicht herabgesetzt werden. Dieser Wechsel, der in der Synthesetätigkeit sensitiver Tumoren auftritt, kann nicht als der Angriffspunkt von DBD angesehen werden, da geringere Konzentrationen der Substanz* (25–50 µg/ml) *genügten, um die Lebensfähigkeit sensitiver Tumorzellen* in vitro *herabzusetzen.*

Eine therapeutische Dosis erhähte auch in vivo *die Synthese einer RNS Fraktion in sensitiven Tumorzellen. Die Natur dieses RNS Types ist noch unbekannt.*

DBD ändert Thermodenaturierungspunkt der RNS nicht. Es ist also unwahrscheinlich, daß die Substanz die Templatefunktion der DNS stört wie andere alkylierende Substanzen.

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Carcinoma of the Colon in a Rat

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INTRODUCTION

SPONTANEOUS carcinoma of the colon appears to be exceedingly rare in rats. Only five reports are to be found in the literature despite the large numbers of rats used every year in experimental work. Bullock and Curtis [1] reported a single, mucus-secreting adenocarcinoma in the caecum of a 13 month old male rat, among 521 neoplasms found in 31,000 autopsies carried out on rats over a period of 9 years. Willis [2] described a mucoid adenocarcinoma of the proximal colon with metastases in the regional lymph nodes in two white rats (unspecified strain), 36 weeks old. Crain [3] gave an account of a colon adenocarcinoma in a Wistar Rochester rat 18–24 months old, which had invaded the gut wall and mesenteric fat. Mitra [4] described a moderately well differentiated adenocarcinoma of the proximal colon in a 20-week old rat which had infiltrated the muscle coat of the intestine and in some areas had reached the serous layer.

The carcinoma of the colon reported in this communication occurred in a 20-week old albino Wistar rat. It seems appropriate to draw attention to this rare condition in rats in view of the increasing number of compounds that have been shown to induce this type of tumour readily under experimental conditions.

CASE REPORT

The tumour was an incidental finding in a female rat during routine necropsy examination of a group of rats killed at the termination of a 90-day toxicity test on Howflex GBP (diethylene glycol bisphthalate). The animal formed one of a group fed the test compound at 1% of the diet. At the termination of the test the average age of the animals was approximately 19–20 weeks.

Examination of weight records of individual animals revealed that the affected animal showed no impairment of growth at any stage during the 90-day period of observation.

At necropsy, an annular growth, about 0·9 cm long and 1 cm in diamater was found in the ascending colon beyond the caecum, infiltrating the mesentery and posterior abdominal wall (Fig. 1). The peritoneal surface of the tumour exhibited numerous cysts containing a gelatinous fluid which projected from the surface like tiny glass beads. There was no ascites and no metastases either in the regional lymph nodes or in any other organ.

Histologically, the tumour mass consisted of irregular, but well defined acinar formations lined by tall, columnar, mucus-secreting epithelial cells showing considerable variability in size and nuclear structure (Figs. 2a, b). Mitotic activity was considerable and randomly scattered throughout the tumour. The tumour stroma was made up of oedamatous connective tissue, infiltrated with a variable amount of inflammatory cells.

The tumour infiltrated all layers of the colon, separating and destroying the smooth muscle coat (Fig. 3). Cystic formations made up of malignant cells and infiltrated by inflammatory cells could be seen on the surface of the tumour.

The mucus in the tumour cells and cysts was positive with periodic Acid-Schiff before and after diastase digestion, Alcian blue 8GS, Alcian green 3BX, Mucicarmine (Southgate), Thionine and Tripas, carried out by the methods described by Culling [5]. These

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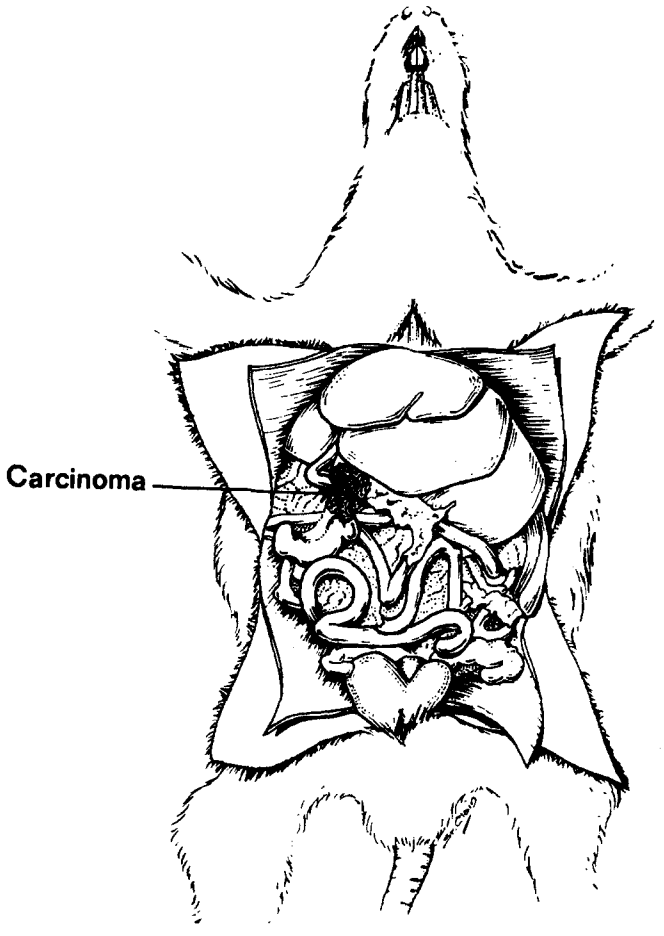


Fig. 1. *Anatomic position of tumour in ascending colon at necropsy.*

results were identical with those given by the mucus formed by the goblet cells of normal rat colonic mucosa.

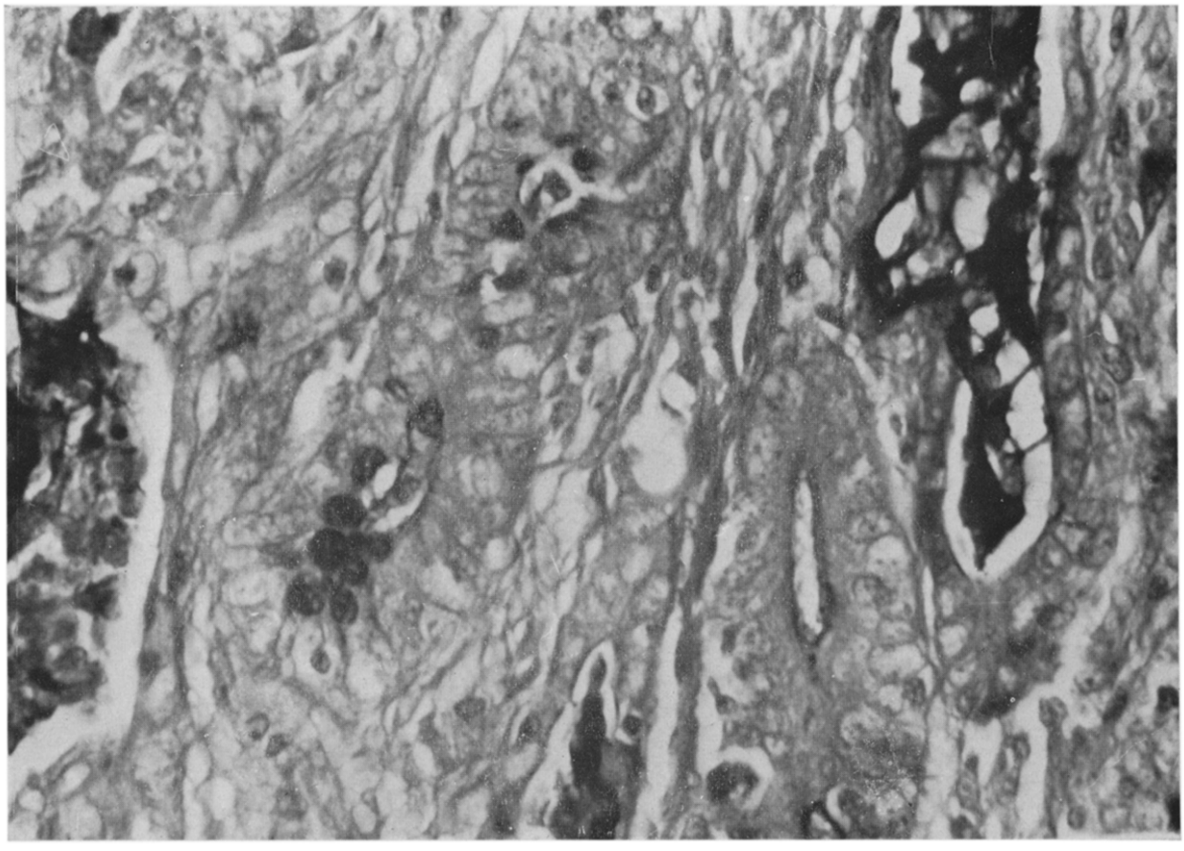
DISCUSSION

The colonic adenocarcinoma reported in this paper was the only tumour observed in a group of animals (40 male and 40 female) used for a 90-day toxicity test on Howflex GBP. Since no other malignancies or proliferative lesions suggestive of precarcinomatous conditions were observed at any of the dose levels tested (0·25%, 1% and 2·5%), the tumour was considered to be of spontaneous origin. This lesion was of particular interest as it was the only intestinal malignancy observed in our laboratories over a period of 5 years in rats used in toxicity studies on food additives. During this period, post mortem examinations were carried out on approximately 4000 rats about 19 weeks old and 1000 rats aged between 70 and 115 weeks.

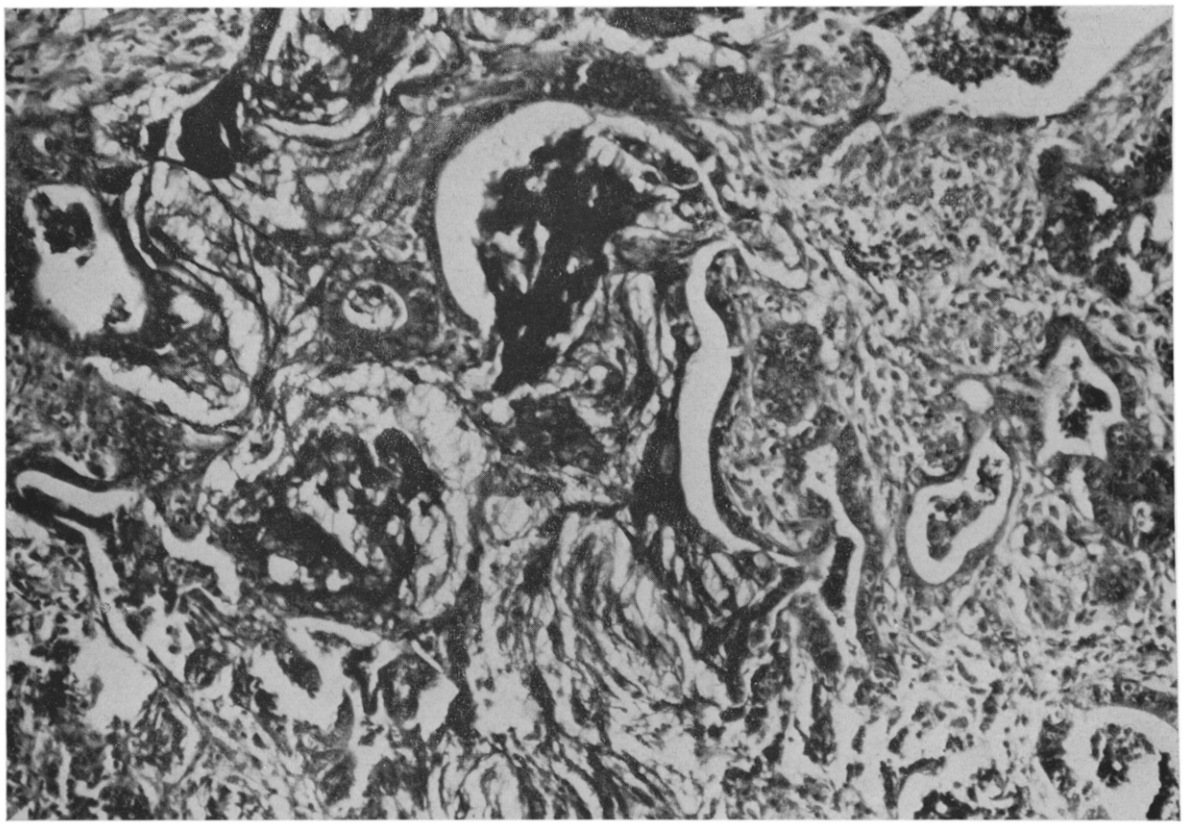
All of the six reported cases of colonic adenocarcinoma had invaded the muscle coat and neighbouring tissue, but only two showed metastases in the regional lymph nodes. This would indicate that, as in man, metastasis tends to occur after the primary growth has spread by direct continuity to neighbouring structures [6].

Under laboratory conditions, the rat leads a carefully controlled existence, and it may be thought that the unusually low incidence of this type of tumour is due to an absence of sufficient contact with external sources of carcinogenic agents. The low incidence of this tumour in the wild rat, however, which is not so protected, contradicts such an explanation [7].

This exceedingly rare natural incidence of malignancy is in contrast to the readiness with which colonic tumours appear following the administration, by a variety of routes, of a number of carcinogenic agents. Laqueur *et al.* [8–10] found such tumours, among others, after feeding crude cycad meal or methylazoxymethanol glycoside (thought to be the active carcinogenic principle of cycad nuts), for periods of 13 and 21 days and observing the animals for a total period of 12 months. Feeding of 2-acetylaminofluorene daily for 30 weeks also produced intestinal neoplasms [11]. Subcutaneous injections in rats of 4-aminobiphenyl, 3,2-dimethylaminobiphenyl [12], 1,2-dimethylhydrazine [13] or benzidine



(*a*) (*Thionine* × 400)



(*b*) (*PAS* × 100)

Fig. 2. *Acinar formations in the well differentiated part of the tumour lined by tall columnar cells some of which are secreting mucus. Pools of mucin present in some of the acini.*

(to face p. 416)

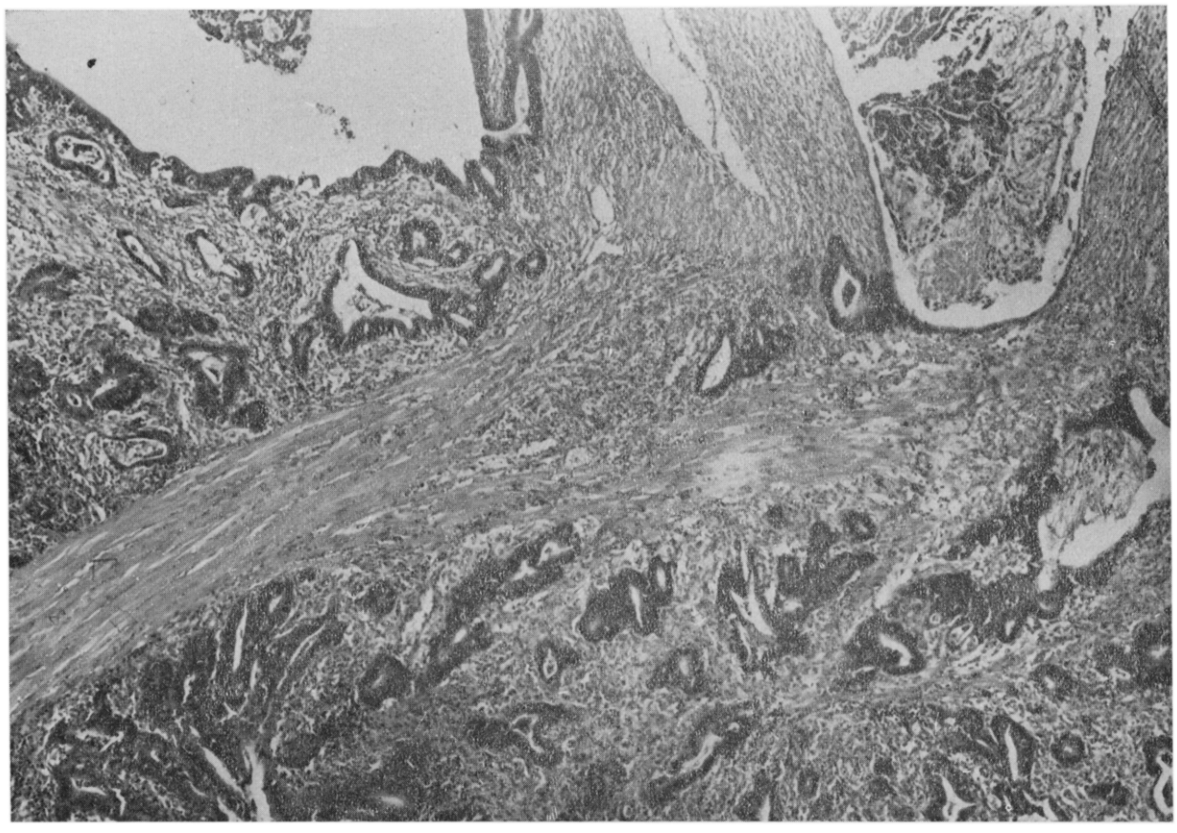


Fig. 3. Adenocarcinoma infiltrating and destroying intestinal muscle coat.
(Haematoxylin and eosin × 40).

throughout their life span [14], gave rise to distant neoplastic lesions, including colon carcinomas. Schoental and Bensted [15] found that malignancies in various parts of the intestine, including the colon, resulted after 1–3 i.p. injections of *n*-methyl and *n*-ethyl-*n*-nitrosourethane. Horava and von Haam [16] in experiments on 20-methylcholanthrene produced a high incidence of colon carcinomas by the intraluminal application of thick cotton string heavily impregnated with the hydrocarbon. Later, disappointing results by Stevenson and von Haam [17], working in the same laboratory, were explained by the fact that the strings became coated with hard faeces minimising the apparently necessary intimate contact between carcinogen and mucosa.

Colonic carcinoma is rare in mammals except man [18]. In a review of the incidence of colonic carcinoma in a number of species, Wells *et al.* [19] quote the incidence to be about ½–3% of all malignancies in mice, cats, horses, dogs and cows. Despite the increasing attention that has been given to these species in recent years, there is no suggestion that the incidence of intestinal tumours is higher than the figures quoted by Wells. The incidence of colonic neoplasms in man, however, is much higher and is generally quoted at about 20% of carcinomas [18].

It is difficult to account for this considerable discrepancy. Local pathological conditions could account, at least in part, for this high incidence. In man the colon is frequently the site of pathological damage involving the mucosal epithelium, such as polyposis or ulcerative colitis [20, 21]. These conditions are associated with a high incidence of colonic carcinoma and are thought to be predisposing factors.

Although under natural conditions the rat colonic mucosa appears to be relatively free from such pathological conditions, studies of preneoplastic lesions have shown that polyps and ulcers of the colon occur in the course of tumour induction by 3:2-dimethyl-4-aminodiphenyl [22] and by polycyclic hydrocarbons [16]. It has also been shown in mice that tumours of the large bowel occur readily in cases of rectal prolapse [19] and are thought to be due to the ensuing irritation of the mucose.

Genetic relationships appear also to play a part in determining the incidence of these tumours [19]. It was shown that inbreeding of a particular strain of mice (Strain 90) enhanced greatly the incidence of intestinal tumours. The known importance of genetic factors in the human in the transmission of intestinal polyposis, a condition known to predispose to malignancy [23, 24], would suggest that an hereditary predisposition might exist in man for the development of this type of cancer.

Recently, McVay [25] showed an inverse relationship between the height of the ambient environmental temperature and death rate from colon carcinoma in humans and suggested that these correlations could be related, at least in part, to the prevalence of endemicity of an environmental agent possibly of a viral nature involved in the causation of these neoplasms.

It would appear that the colonic mucosa in the rat is not subject to the same degree as that of the human to the development of pathological lesions or to the genetic or environmental influences that might be responsible for the more frequent incidence of this type of tumour. The rarity of the spontaneous incidence of these tumours in rats, together with their apparently closely similar biological behaviour with the human neoplastic disease, emphasises the necessity for considering with concern reports of the experimental production of these tumours in terms of human hazard.

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RESUME

Les auteurs ont découvert un carcinome spontané du côlon chez le rat, au cours d'un test de toxicité provoquée par un additif alimentaire, le Howflex GBP. *Les carcinomes du côlon constituent une très petite proportion des néoplasmes spontanés observés chez le rat. Cependant, sous certaines conditions carcinogéniques expérimentales, de telles tumeurs apparaissent facilement. Chez l'être humain, la proportion de carcinomes spontanés du côlon est considérablement plus élevée que chez les autres mammifères. On pense que cette différence peut être due à une prédisposition de l'homme à la polypose et à la colite ulcéreuse, d'origine génétique ou autre, qui, ainsi qu'on a pu le constater, peuvent favoriser la formation d'un carcinome. Les auteurs suggèrent également que, sous certaines conditions, des agents viraux peuvent être impliqués dans l'étiologie des lésions humaines.*

SUMMARY

A spontaneous carcinoma of the colon in a rat was encountered during a routine toxicity test on a food additive (Howflex GBP). *Colonic carcinomas form a very small proportion of the spontaneous neoplasms reported in rats. However, under conditions of experimental carcinogenesis, such tumours occur readily. In the human, the proportion of spontaneous colon carcinoma is considerably higher than in other mammals. It is thought that this difference may be due to the susceptibility of man to polyposis and ulcerative colitis, of genetic or other origins, which are known to predispose to carcinoma. It is also suggested that, under certain conditions, viral agents may be involved in the aetiology of the human lesion.*

ZUSAMMENFASSUNG

Während eines routinemäßigen Toxicitätstests bei einer Zusatznahrung (Howflex GBP) *wurde ein spontanes Dickdarm-Carcinom einer Ratte festgestellt. Dickdarm-Carcinome bilden einen sehr kleinen Anteil der bei Ratten beobachteten Spontantumoren. Jedoch bei der experimentellen Carcinogenese treten solche Tumoren leicht auf. Beim Menschen ist der Anteil der spontanen Dickdarm-Carcinome erheblich höher als bei anderen Mammaliern. Dieser Unterschied könnte in Zusammenhang stehen mit der aus genetischen oder anderen Gründen bestehenden Anfälligkeit des Menschen für Polyposis und ulzerierende Colitis, deren Neigung zum Carcinom bekannt ist. Es wird auch angenommen, daß unter gewissen Bedingungen virale Agentien an der Aetiologie der menschlichen Erkrankung beteiligt sein könnten.*

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Induction d'une Immunité vis-à-vis de la Greffe de Plasmocytosarcomes chez la Souris BALB/c

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INTRODUCTION

L'EXISTENCE d'antigènes spécifiques dans des tumeurs provoquées par des carcinogènes chimiques a été mise en évidence par plusieurs auteurs [1–3], grâce à des expériences de transplantation; une résistance vis-à-vis de la greffe de ces tumeurs peut être obtenue par l'injection préalable de cellules tumorales, modifiées ou non, chez des animaux isogéniques.

Nous avons cherché à obtenir une immunité vis-à-vis de tumeurs à plasmocytes provoquées par l'injection de Bayol F chez la souris BALB/c. Ces tumeurs ont été provoquées et gardées en lignée de transplantation dans notre laboratoire; les paraprotéines, présentes dans le sérum des animaux porteurs de ces tumeurs, ont déjà fait l'objet d'études immunochimiques [4–6].

L'immunisation par les cellules tumorales, qui ne doit pas donner lieu au développement d'une tumeur, peut se faire à l'aide de doses faibles de cellules, ou de cellules irradiées; plus récemment, Appfel *et al.* [7] ont proposé l'incubation des cellules dans l'iodoacétate de sodium qui laisse les cellules viables, tout en bloquant leur multiplication. C'est cette méthode que nous avons utilisée.

MATERIEL ET METHODES

Les tumeurs primaires ont été obtenues selon la méthode de Potter [8] par injection intrapéritonéale de Bayol F, à raison de 3 fois 0,5 ml, à 1 mois d'intervalle. Les tumeurs furent ensuite transplantées par voie sous-cutanée.

Toutes les expériences ont été faites sur des souris BALB/c, de sexe mâle, élevées en colonie fermée, dont l'isogénie a été vérifiée par greffes cutanées.

Préparation des suspensions cellulaires

Les suspensions de cellules tumorales sont préparées à partir des tumeurs solides, dans une solution saline tamponnée à pH 7,4, additionnée de 1% de polyéthylène glycol de poids moléculaire 6.000. La tumeur prélevée est coupée, aux ciseaux, en petits morceaux, dissociée dans le tampon et passée sur double épaisseur de gaze. Les cellules sont lavées trois fois dans le tampon, par centrifugation de 5 min à 500 tours/min; les cellules non colorées par le Bleu Trypan sont comptées à l'hématimètre et ajustées à la concentration désirée.

Immunisation

Les cellules sont incubées pendant 1 hr, à 37°C, dans une solution d'iodoacétate de sodium 10^{-3}M en tampon salin additionné de polyéthylène glycol, puis lavées trois fois dans ce tampon. Trois séries ont été faites avec les lignées transplantables de plasmosarcomes appelées E_2, E_8, A_1 et K_{10}.

Dans la première série, 3 lignées tumorales ont été étudiées E_2, A_1 et K_{10}. Pour chacune de ces lignées tumorales, 30 souris ont été immunisées, chaque animal a reçu 3 injections

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sous-cutanées de 5×10^5 cellules, séparées par un intervalle de 2 semaines. Pour tester la résistance des animaux vis-à-vis de la greffe tumorale, chacun des animaux immunisés a reçu, 2 semaines plus tard, une injection sous-cutanée de 5×10^5 cellules de la même lignée tumorale, n'ayant pas été incubées dans l'iodoacétate de sodium. Dix animaux témoins, de même âge et de même sexe, n'ayant reçu aucune injection préalable, ont été inoculés, en même temps, avec la même dose de cellules.

Dans la deuxième série, on a étudié l'effet de l'adjuvant de Freund et des voies d'inoculation différentes. Chaque souris a reçu, par voie sous-cutanée, 10^5 cellules incorporées dans l'adjuvant de Freund complet; 2 semaines plus tard, une injection sous-cutanée de 10^5 cellules et une injection intrapéritonéale de 10^5 cellules et, une semaine plus tard, 2×10^5 cellules par voie sous-cutanée et 2×10^5 cellules par voie intrapéritonéale. Pour chacune des trois lignées E_2, A_1 et E_8, 20 animaux immunisés avec la tumeur, 20 témoins immunisés de la même façon avec des cellules de rate normale en même nombre, traitées par l'iodoacétate de sodium, et 20 animaux n'ayant reçu aucun traitement, ont reçu la greffe tumorale homologue, comme dans la première série.

Dans la troisième série, on a recherché l'existence de réactions croisées entre 3 tumeurs E_8, A, et K_{10}. Celle-ci comportait 3 groupes d'animaux, chacun de ces groupes était divisé en 5 lots:

Animaux témoins
Animaux recevant des cellules de rate normale
Animaux immunisés avec la tumeur E_8
Animaux immunisés avec la tumeur A_1
Animaux immunisés avec la tumeur K_{10}

Tous les animaux du 1er groupe ont été greffés avec la tumeur E_8, ceux du 2e groupe avec la tumeur A_1 et ceux du 3e groupe avec la tumeur K_{10}.

Ainsi, chacune de ces trois lignées tumorales a été greffée à des animaux immunisés vis-à-vis de la tumeur homologue, et à des animaux immunisés vis-à-vis des deux autres lignées tumorales.

Le mode d'immunisation a été le même que pour la deuxième série, les animaux recevant cependant une injection supplémentaire de 4×10^5 cellules par voie sous-cutanée et de 4×10^5 par voie intrapéritonéale.

La même dose de cellules tumorales, soit 5×10^5, a été utilisée pour tester la résistance des animaux. Après cette greffe, les tumeurs E_2 et E_8 sont décelables à la palpation plus précocement (2–4 semaines après la greffe) que A_1 (3–6 semaines) et K_{10} (6–10 semaines). Quand aucune nouvelle tumeur n'était apparue depuis 2 semaines dans un groupe d'animaux, ceux qui ne présentaient pas de tumeur décelable à la palpation étaient considérés comme résistants à la greffe tumorale: la surveillance portant sur les 2 mois suivants n'a jamais décelé de développement plus tardif de la première greffe tumorale. Après deux semaines sans apparition de nouvelle tumeur, les animaux ne présentant pas de tumeur ont reçu une deuxième greffe, de la même lignée, à la même dose, 5×10^5 cellules, en un autre point d'inoculation.

RESULTATS

Immunisation vis-à-vis de la tumeur homologue

Les expériences ont été faites avec les lignées transplantables de plasmosarcomes créées et entretenues dans notre laboratoire et appelées E_2, E_8, A_1 et K_{10}.

Les tumeurs E_2 et E_8 ont subi de plus nombreux passages en transplantation que A_1 et K_{10} (voir le Tableau 1). Elles évoluent plus rapidement, apparaissant plus précocement et entraînant la mort de l'animal dans un délai plus court, ceci pour le même nombre de cellules inoculées, soit 5×10^5. Cette dose donne, chez les témoins, pour les 4 lignées, un pourcentage élevé de prise de tumeur, 60–100%, avec des différences statistiquement non significatives, d'une série à l'autre pour la même lignée, et d'une lignée à une autre lignée.

Tableau 1. *Caractéristiques des plasmosarcomes*

Tumeur	Nombre de passages	Paraprotéine produite	Durée moyenne d'évolution (jours)
E_2	38–48	IgG_2	46
E_8	44–55	IgA	52
A_1	6–15	IgA	80
K_{10}	3–10	IgG_2	94

Nombre de passages en transplantation lors de l'immunisation.
Type de l'immunoglobuline à laquelle se rattache la paraprotéine produite par la tumeur.
Temps moyen en jours écoulés entre la greffe de la tumeur et la mort de l'animal, chez les témoins, pour 5×10^5 cellules.

Le Tableau 2 rassemble les résultats des première et deuxième séries, ainsi que la partie de la troisième série concernant l'immunisation avec la tumeur homologue.

Tableau 2. Immunisation avec les tumeurs homologues

Immunisation / Tumeur test	— (+)	Rate normale	E_2		A_1		K_{10}		E_8	
1ère série			I	II						
E_2	10/10		15/30	5/15						
	100%		50%	33%						
					I	II				
A_1	7/10				9/29	5/20				
	70%				31%	25%				
							I	II		
K_{10}	10/10						12/29	2/17		
	100%						41%	12%		
2e série			I	II						
E_2	18/20	14/18	4/17	3/13						
	90%	78%	23%	23%						
					I	II				
A_1	15/20	13/17			5/19	3/14				
	75%	76%			26%	21%				
									I	II
E_8	16/20	14/20							7/20	4/1
	80%	70%							35%	31%
3e série									I	II
E_8	16/20	13/20							12/38	4/2
	80%	68%							32%	15%
					I	II				
A_1	12/18	13/18			5/36	5/31				
	68%	72%			14%	16%				
					I	II				
K_{10}	15/20	12/18			3/38	4/35				
	75%	68%			8%	12%				

Rapport entre le nombre d'animaux ayant présenté une tumeur et le nombre total d'animaux.
I. 1ère greffe tumorale.
II. 2e greffe faite chez les animaux ayant résisté à la 1ère.
(+) Pas d'immunisation

Tableau 3.

Immunisation / Tumeur test	— (+)	Rate normale	E_8	A_1	K_{10}
E_8	16/20 80%	13/20 68%	12/38 32%	← NS → 5/19 26%	12/20 60%
			*		
		NS			
A_1	12/18 68%	13/18 72%	3/16 19%	← NS → 5/36 14%	8/18 44%
			**		
		NS			
K_{10}	15/20 75%	12/18 68%	8/18 44%	← NS → 9/21 43%	3/38 8%
			**		
		NS			

N.S. Ne diffèrent pas significativement entre eux.
* différent au risque de 5%.
** différent au risque de 2%.
(test de comparaison des pourcentages par le χ^2 de Pearson).
3e série d'immunisation, pour l'étude des réactions croisées.
(+) Pas d'immunisation

Les deux types de témoins, animaux n'ayant rien reçu avant la greffe tumorale et animaux ayant reçu des injections de cellules de rate normale incubées dans l'iodoacétate de sodium, ont développé des tumeurs dans la même proportion. Les différences observées entre ces deux groupes de témoins ne sont statistiquement pas significatives.

Les animaux prétraités avec des cellules tumorales modifiées ont présenté, après injection de cellules malignes, des tumeurs dans un pourcentage de cas beaucoup plus faible que les témoins. Mais, dans tous les groupes, un certain nombre d'animaux immunisés développaient une tumeur, dans une proportion allant de 8% (K_{10} dans la troisième série) à 50% (E_2, première série).

L'addition d'adjuvant de Freund complet aux cellules traitées par l'iodoacétate de sodium ne modifie pas le degré de résistance, comme le montrent les résultats comparables entre la première série dans laquelle les animaux n'ont pas reçu d'adjuvant, et la deuxième série dans laquelle, pour la première injection, les cellules ont été incorporées à l'adjuvant de Freund complet.

Nous n'avons pas trouvé de corrélation entre l'importance de la résistance et le type de paraprotéine synthétisée par la tumeur (IgA pour E_8 et A_1, IgG_2 pour E_2 et K_{10}).

Les animaux n'ayant pas présenté de tumeur après la première greffe sont, pour la plupart, résistants à une deuxième greffe de la même tumeur; cependant, dans chaque groupe, quelques animaux développent une tumeur.

Immunisation croisée (Tableau 3)

Les animaux présentent le même degré de résistance vis-à-vis de la tumeur A_1, qu'ils aient été immunisés avec A_1 ou E_8. Réciproquement, qu'ils aient été immunisés avec A_1 ou E_8, ils présentent le même degré de résistance vis-à-vis de la tumeur E_8. A_1 et E_8 sont également efficaces pour une immunisation réciproque.

Les animaux immunisés avec la tumeur K_{10}, et greffés avec la tumeur A_1 ou la tumeur E_8, développent des tumeurs dans la même proportion que des animaux non immunisés. Les animaux immunisés soit avec A_1, soit avec E_8, ne présentent pas de résistance vis-à-vis de la greffe de la tumeur K_{10}. Les différences avec les animaux témoins ne sont pas significatives. Il n'y a pas de réaction croisée entre K_{10} d'une part, A_1 et E_8 d'autre part.

Les deux tumeurs A_1 et E_8 produisent le même type de paraprotéine (IgA), alors que la tumeur K_{10} synthétise une paraprotéine de type IgG_2. Y a-t-il une relation entre le type de paraprotéine lié à la tumeur et la résistance croisée? Il est impossible de répondre sur un seul exemple, et d'autres tumeurs devront être étudiées.

DISCUSSION

La résistance obtenue vis-à-vis des tumeurs à plasmocytes peut être attribuée à une immunité dont sont responsables des antigènes spécifiques de tumeur, à condition d'éliminer un certain nombre de facteurs non spécifiques. Le principal d'entre eux est l'existence d'un hétérozygotisme résiduel dans la souche de souris utilisée. Pour vérifier l'isogénie de la souche, nous avons pratiqué des greffes cutanées qui ne présentaient aucun signe de rejet au bout de 3 mois. Par ailleurs, les témoins ayant reçu des cellules de rate normale, incubées dans l'iodoacétate de sodium, ont développé des tumeurs dans la même proportion que des animaux neufs. Ceci permet d'éliminer aussi un éventuel rôle non spécifique de l'iodoacétate de sodium ou des produits formés par celui-ci à la surface des cellules.

Le niveau de résistance obtenu par nous pour les tumeurs à plasmocytes apparaît plus faible que celui obtenu pour des tumeurs provoquées par des carcinogènes chimiques. Pour les tumeurs provoquées par le méthylcholanthrène, on obtient une résistance chez la plupart des animaux, avec des doses faibles de cellules vivantes, ou avec des cellules irradiées [1–3].

Nous avons tenté d'obtenir une immunisation vis-à-vis des tumeurs à plasmocytes à l'aide de cellules vivantes ou irradiées. Nous avons observé un allongement discret du temps de survie des animaux traités par rapport aux témoins, sans observer de différence dans le pourcentage d'apparition de la tumeur. Nous avons aussi répété l'expérience de Whisson et Connors [9]: ces auteurs ont obtenu la régression de tumeurs à plasmocytes de la souris par le N–N di 2 chloroéthylaniline, et, après une deuxième inoculation de la même tumeur, ils observent une apparition retardée et un développement plus lent de celle-ci. Nous avons obtenu le même résultat, mais sans différence significative du pourcentage de prise de la tumeur greffée entre animaux témoins et traités.

Nos résultats semblent plus proches de ceux obtenus avec les tumeurs spontanées, pour lesquelles il est difficile d'obtenir une immunité par l'injection de cellules vivantes ou irradiées. Plusieurs auteurs signalent l'échec à susciter

une résistance vis-à-vis de tumeurs spontanées par ces méthodes [2, 10–12]. Pour les adénocarcinomes mammaires, on obtient une immunité, mais modérée, après greffe tumorale puis exérèse de la tumeur [13, 14]. Baldwin [15] a mis en évidence une immunité vis-à-vis d'une tumeur spontanée du rat, après ligature de la tumeur, alors que l'injection de cellules irradiées était inefficace. Pour cet auteur, les tumeurs spontanées, du moins certaines d'entre elles, sont porteuses d'antigènes spécifiques de la tumeur, mais faiblement immunisants. Les résultats que nous obtenons suggèrent que les tumeurs à plasmocytes sont, de ce point de vue, plus comparables aux tumeurs spontanées qu'aux tumeurs provoquées par des carcinogènes chimiques.

Les tumeurs à plasmocytes ne sont pas spontanées, mais la période de latence après l'injection de Bayol F est longue [6–10 mois]. Pour Prehn [16], il existe une relation entre le degré de résistance et la période de latence. Plus le carcinogène est puissant, plus la période de latence est réduite, et plus grandes sont les probabilités d'apparition d'antigènes nouveaux permettant d'induire une résistance solide. Pour cet auteur, si les tumeurs qui surviennent après une longue période de latence possèdent des antigènes spécifiques, ce sont des antigènes faibles.

Les méthodes d'immunisation utilisées pour les tumeurs provoquées par des carcinogènes puissants ne donnent que des résultats très modéré pour les tumeurs possédant des antigènes spécifiques faibles, comme c'est le cas pour les tumeurs spontanées ou à longue période de latence.

L'incubation des cellules tumorales dans l'iodoacétate de sodium permet l'injection de doses fortes de cellules viables, incapables de se multiplier. Par ailleurs, pour Appfel *et al.* l'alkylation des groupes thiol, modifiant la surface des cellules, pourrait permettre à une antigénicité discrète de s'exprimer, peut-être en stabilisant les antigènes de surface.

Cette méthode, utilisée dans des systèmes isogéniques pour des tumeurs peu antigéniques, permet de mettre en évidence une immunité difficile à obtenir, point de départ pour une étude plus poussée de la nature et de la spécificité de ces antigènes.

RESUME

Des souris BALB/c ont été immunisées vis-à-vis de la greffe de plasmosarcomes, à l'aide de cellules tumorales incubées dans l'iodoacétate de sodium, selon la méthode décrite par Appfel et al. *Après greffe de la tumeur, le pourcentage d'apparition d'une tumeur est diminué de manière significative chez les animaux immunisés par rapport aux témoins. L'immunisation a été étudiée pour quatre lignées de tumeurs à plasmocytes, avec des résultats comparables pour chacune des quatre. Pour trois d'entre elles, on a recherché s'il existait une immunité croisée. Celle-ci a été trouvée pour deux d'entre elles, mais non entre la troisième et les deux précédentes.*

SUMMARY

Mice of the BALB/c strain were immunized against plasma cell sarcoma graft by means of tumour cells incubated in sodium iodoacetate, according to the method described by Appfel et al. *The percentage of takes was significantly diminished in immunized animals as compared with controls. Immunization has been studied in four strains of plasmacell tumours and the results were found equivalent for each strain. For three out of these four strains the possibility of cross immunization was investigated. Cross reactions were found in two of them but not between the third and the two preceding ones.*

ZUSAMMENFASSUNG

Mäuse BALB/c wurden gegen ein Plasmosarkomtransplantat immunisiert mit Hilfe von Tumorzellen in Natriumjodacetat nach der Methode von Appfel u. Mitarb. Nach der Transplantation ist der Prozentsatz des Tumorangehens bei den immunisierten Tieren signifikant erniedrigt gegenüber den Kontrolltieren. Die Immunisierung wurde an vier Passagen von Plasmozytentumor untersucht mit vergleichbaren Ergebnissen für jede der vier Passagen. Bei drei davon wurde dann noch das Vorhandensein einer Kreuzimmunität geprüft. Diese wurde bei zweien gefunden, aber nicht zwischen der dritten und den beiden vorhergehenden Passagen.

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Effect of Lysine Polypeptides on the Surface Charge of Normal and Cancer Cells

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INTRODUCTION

MOST cells in suspension in an electrolyte solution such as saline, when subjected to an electric field, exhibit an anodic electrophoretic mobility, i.e. a net negative charge, which arises as a result of the ionization of the various groups present on the cell surface. Surface problems in neoplasia have been discussed in many publications [1–8]. The surface charge of tumour cells has been examined in connection with their invasiveness [9], decreased mutual adhesiveness and lack of contact inhibition [3, 10, 11]. Electrokinetic studies on hamster kidney cells and their stilboestrol-induced neoplastic transformation showed that, at least for this system, the tumour cells had a significantly higher surface charge density than the normal cells from which they were derived [12, 13]. Work on embryonic [15] and regenerating liver cells [14, 16] suggested that probably increased surface charge density is associated with growth in general and not specifically with malignancy. No correlation between electrophoretic mobility and the 'malignancy' of a small range of mouse normal and tumour cells was found [17]. Experiments on normal and neoplastic human cells showed that mesenchymal tumour cells had a higher surface charge density than carcinoma cells but normal and malignant epithelium did not have significantly different surface charge densities [6].

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Marked inhibitory action of positively charged diamines on spontaneous tumours were reported many years ago; the aliphatic bases which were found to inhibit tumour growth were aliphatic diamines or hydroxy amines expressed by the general formula P–X–P′, where P and P′ are polar groups [18]. In the series of aromatic bases also the maximum activity was obtained with compounds containing two terminal amino groups [18]. However, in the case of β-alanine, where P is NH_2 and P′ is a COOH group, there is no activity [18]. The synthetic polypeptides of lysine such as positively charged poly-DL-lysine inhibit Ehrlich ascites tumour (EAT) development in Swiss mice and TA3 ascites carcinoma in BAF1 mice [19]. Shah and Reilly [20] reported that the inhibitory effect on the growth of mouse mammary tumours was obtained by incubation of the tumour cells with polylysine and lysine-rich histone fractions before inoculation. Polylysine readily combines with the surface of erythrocytes (which behave as polyanions [21]) reducing the net charge on the surface of erythrocytes to zero [22]. Polyethylene imine interacts specifically with Walker tumour cells [23]. Recently, Larsen and Olsen [24] reported that polyornithine inhibited the transplantability of mouse leukaemia whereas arginine-rich protamine sulphate did not do so. These authors also found that the inhibitory effect of polyornithine was reversed by (negatively charged) heparin [24]. Thus the antitumour action has not been observed with compounds in

which the positive charge is less dense as in arginine-rich protamine sulphate or a neutral amino acid, β-alanine [19, 24]. The effectiveness of polycations with *high* charge density (polylysine, polyornithine) in inhibiting tumour development probably involves electrostatic interaction with the surface charge of tumour cells. It was therefore decided to examine the surface charge of tumour cells treated with the following three types of compounds: (1) tumour-inhibiting polycations, (ii) polycations which do not possess antitumour activity and (iii) a neutral amino acid. Electrophoretic mobilities of Ehrlich ascites tumour cells treated with polyornithine, polylysine, arginine-rich protamine sulphate, β-alanine and heparin were determined. Electrokinetic data on human epidermoid cancer cells (HeP ≠2 in flask culture), human blood platelets and erythrocytes treated with polyornithine are also presented.

MATERIAL AND METHODS

Analytical grade NaCl, NaOH, HCl and $NaHCO_3$ were used. Poly-L-α ornithine HBr (mol. wt. 90,000), (lot 107B-2610), poly-L-lysine HBr (mol. wt. 175,000), (lot 78B-0890), β-alanine, heparin-Na salt and protamine sulphate (mol. wt. *ca.* 6000) with an arginine content of 65%, essentially histone free, prepared from salmon were all supplied by Sigma, London. An aqueous solution of NaCl (0·145M) using doubly distilled water from an all 'Pyrex' glass unit, was made 10^{-4}M with respect to $NaHCO_3$. The pH of this standard saline [21] is 7·2±0·2 and this simple medium was used for preparing the solutions of polypeptides and cell suspensions. To alter the pH of the solutions of peptides or cell suspensions, 0·145N-NaOH or 0·145N-HCl were used.

Two to three months old Tuck no. 1 male mice, bearing Ehrlich ascites tumour were killed 6–8 days after transplantation of the tumour. The ascitic fluid was removed, mixed with saline in a volume ratio of 1:10 and the suspension centrifuged at 60 **g** for 5 min. The cells were washed once more and cell suspensions containing $1·5–3·5 \times 10^6$ cells per ml were prepared (haemocytometer counts of 600–1000 cells).

The strain of human epidermoid cancer cells (cancer of the larynx), designated HeP ≠2 [25] was established in flask culture in 1963 and has been passed serially since that time and stock maintained in this Department. The cells were removed from the glass surface mechanically—with the aid of 'a clean glass rod with rubber at the end', often called a 'policeman'. The cells were washed twice with saline to remove the culture medium.

Freshly withdrawn venous blood from healthy individuals was defibrinated to obtain erythrocytes which were washed with saline and resuspended in saline ($2–3 \times 10^6$/ml). Citrated-blood was centrifuged at 200 **g** for 15 min at 18°C to obtain platelet-rich plasma which in turn was centrifuged at 200 **g** for 15 min to obtain a platelet button. These platelets were gently suspended in saline in a volume ratio of 1:50 and washed twice with saline. Suspensions were prepared to contain $2–4 \times 10^8$ platelets per ml.

The solutions of PO, PL, PS, βA and heparin were prepared in saline and added to the cell suspensions. Examination of the cells treated with the various reagents was commenced within 15 sec of the addition of the reagent and completed within 6–10 min. The cells were examined in the electrophoresis chamber well before any cell membrane damage could be seen. The cell suspensions were examined under phase contrast at intervals to judge any cell death, agglutination and vacuolation etc. The suspensions which contained damaged cells were discarded for electrophoretic examination. Data on cells with intact cell membrane —as judged by phase contrast microscopy— are presented. Air-dried smears of chemically treated and control cells were fixed in methanol and stained with May-Grünwald/Giemsa stain to confirm the observations made under phase contrast.

Electrophoretic mobility, zeta potential and surface charge density

A cell electrophoresis apparatus, equipped with a chamber of circular cross section and Ag/AgCl/KCl (Molar) electrodes, described elsewhere [26, 27], was used to determine the electrophoretic mobilities of cells at 25± 0·1°C. The reliability of the apparatus was checked repeatedly with saline-washed erythrocytes (human). Since the basic polyelectrolytes are irreversibly adsorbed on glass [22] and alter its characteristics leading to spurious results, it was found necessary to remove the adsorbed layers from the electrophoresis chamber. Between experiments the electrophoresis chamber was soaked with 1N-NaOH for a few minutes, thoroughly rinsed with saline and distilled water and the reliability of the apparatus checked.

Electrophoretic mobilities of saline-washed

EAT* cells, cultured human cancer cells, human blood platelets and erythrocytes treated with increasing concentrations of PO, PL, PS, βA and heparin and control cells were converted into zeta potentials (ξ, mV) by the use of the Helmholtz–Smoluchowski equation for large particles [e.g. see reference 21]:

$$\zeta = \frac{4\pi\eta}{D} \cdot u \qquad (1)$$

where η and D are the bulk viscosity and dielectric constant respectively of the suspending medium and the electrophoretic mobility u is expressed as μ/sec/V/cm. For aqueous solutions of NaCl at 25°C (1) reduces to

$$\zeta = 12{\cdot}85 \,.\, u \qquad (2)$$

The surface charge density (σ in e.s.u./cm^2) at 25° was evaluated from a modified form of the Gouy–Chapman equation [28]

$$\sigma = [1+(1-\alpha)^{\frac{1}{2}}]\, 2 \,.\, \left[\frac{NDkT}{2000\pi}\right]^{\frac{1}{2}} \,.\, I^{\frac{1}{2}} \,.\, 10^{4} \sinh\left(\frac{e\zeta}{2kT}\right) \qquad (3)$$

where N is the Avogadro number, k the Boltzmann constant, T the absolute temperature, e the electronic charge and α is the fraction of the total space within the surface which is not available to counter ions. It is assumed that α does not vary within the distance from the surface and that only uni-univalent electrolyte is present. For aqueous solutions of NaCl of the ionic strength I= 0·145 at 25°C, equation (3) for the surface charge density evaluation from the zeta potential (ζ, mV) becomes

$$\sigma = [1+(1-\alpha)^{\frac{1}{2}}]\, 13410 \sinh(\zeta/51{\cdot}3). \qquad (4)$$

It should be noted that the surface charge density evaluated from (4), for the value of α=1, is the same as without the correction factor α which takes into account the penetrable peripheral region of a cell [28]. For surfaces which are penetrable, there will be free ions both inside and outside the surface and the electrokinetic charge density may be higher by a factor of up to 2 than that calculated with the original Gouy–Chapman equation [28]. The surface charge density was therefore computed for the various values of α=0, 0·25, 0·5, 0·75 and 1, using (4). The calculations are tedious and time consuming. Autocode programmes were written incorporating (1) and (4) and the apparatus constants and a digital computer (Titan) used routinely for accuracy, speed and convenience (see *Appendix*).

The diameters of about 200 saline-washed EAT cells, using the hanging drop method, were very kindly determined by Dr D. B. Cater. The EAT cell was assumed to be a sphere for calculating the surface area of the cell. Since there is a large variation in the size of the EAT cell, the values of the diameters were programmed for the computer to obtain the histogram plots of the cell diameters and cell surface areas; the mean values of the cell diameter and the mean value of the surface area with the standard deviation were also obtained and are as follows: mean diameter: 11·2±1·9 (s.d.) μ; mean surface area: 899± 307 (s.d.) μ^2.

The total surface area of cells treated with a reagent (in μg) was worked out in cm^2. Now, the electrophoretic mobility of a cell is dependent upon the surface charge density (in e.s.u./cm^2) rather than the total charge. To compare the effect of the various reagents on the surface charge densities of four cell types of varying sizes, the material (in μg) per cm^2 of the cell surface area was calculated and is

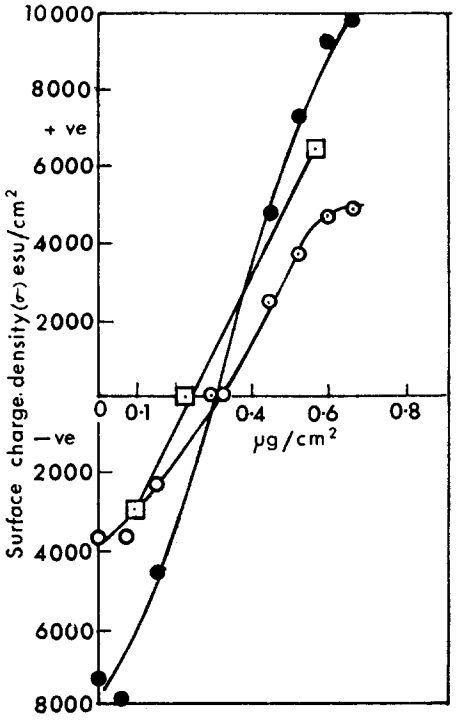

Fig. 1. Amount of polypeptide adsorbed per cm² of Ehrlich ascites tumour cells surface area (μg/cm²) vs. surface charge density (σ, e.s.u./cm²), calculated, using equation (4) [28]. Polyornithine treated EAT cells: o—o surface charge density calculated for the value of α=1; ●—● surface charge density calculated for the value of α=0. Polylysine treated EAT cells: □—□ surface charge density calculated for the value of α=1.

*The abbreviation EAT stands for Ehrlich ascites tumour cells.

Table 1. *Effect of some polyelectrolytes and* β*-alanine on the electrophoretic mobility* (μ/sec/V/cm), *zeta potential* (mV), *surface charge density* (e.s.u./cm²) (±S.D.) *of saline-washed Ehrlich ascites tumour cells, human cancer cells (in flask culture)* (HeP≠2), *human blood platelets and erythrocytes in saline* (pH: 7·2±0·2) *at* 25°C

Cell type	Chemical	Chemical added per cm² of cell surface area (μg/cm²)	Electrophoretic mobility (μ/sec/V/cm)	Zeta potential (mV)	Surface charge density (eqn. 4; α=1 [28]) (e.s.u./cm²)
Ehrlich ascites tumour (surface area: 900 μ²)	CONTROL	—	−1·08±0·08	13·9	3664±85
	Polyornithine mol. wt. 90,000	0·37	ZERO*	—	—
	Polylysine mol. wt. 175,000	0·24	ZERO*	—	—
	Protamine sulphate (arginine content 65%) mol. wt. 6000	0·20–2·71	−0·79±0·05†	10·2	2563±120
	β-Alanine	1–15	−0·97±0·06†	12·5	3249±150
	Heparin	0·2–2·0	−1·14±0·17†	14·7	3906±614
HeP ≠2 Human cancer cells	CONTROL	—	−1·07±0·07‡	13·7	3600±90
	Polyornithine	1·78	ZERO‡	—	—
Human blood platelets (diam. 3μ [29]; surface area: 28,27μ²)	CONTROL	—	−0·91±0·05	11·8	3105±93
	Polyornithine	0·88	ZERO§	—	—
Human erythrocytes (surface area: 163μ² [29])	Polyornithine	0·35	ZERO	—	—
		—	−1·08±0·04	13·9	3664±35

*Twenty four animals.
†12 animals.
‡12 cultures.
§ 3 experiments.
The number of control samples of EAT cells, platelets and erythrocytes runs into hundreds.

expressed as μg/cm² required to influence the charge density in e.s.u./cm² (see Fig. 1, Table 1).

RESULTS

Polyornithine and polylysine tend to change the normally anodic mobility of Ehrlich ascites tumour cells, cultured human cancer cells (HeP ≠2), human blood platelets and erythrocytes first to smaller anodic values, then to zero and finally to high positive values. Figure 1 shows the plots of surface charge densities of polyornithine-treated ascites cells evaluated by (4) for the values of α=1 (open circles) and α=0 (closed circles) and polylysine-treated ascites cells, for the value of α=1 (squares) vs the increasing concentration of the polypeptide (expressed as μg/cm² of the cell surface area).

These changes were found to be reversible: if the polyornithine and polylysine-treated cells were washed with saline, the original electrokinetic behaviour of the cells was restored [22]. If small amounts of heparin (3–5 μg/10⁶ cells) were added to the cell suspension before the addition of polyornithine or polylysine, the cellular electrophoretic mobility did not become zero or positive. If heparin was added to the polylysine or polyornithine-treated cells with zero electrophoretic mobility, the cells exhibited a net negative charge.

The amounts of polyornithine, polylysine required to change the anodic mobility of

Ehrlich ascites tumour cells to zero, the amounts of polyornithine required to change the mobilities of HeP ≠2 cells, human blood platelets and erythrocytes to zero and the electrokinetic parameters of Ehrlich ascites cells treated with varying amounts of protamine sulphate, β-alanine and heparin are given in Table 1.

It is clear from the data presented in Table 1 that even very high concentrations of arginine-rich protamine sulphate or β-alanine do not change the electrophoretic mobility (zeta potential, surface charge density) of Ehrlich ascites tumour cells to zero.

DISCUSSION

The electrophoretic behaviour of the Ehrlich ascites tumour cells appears to arise as a result of the presence of several types of ionizable groups on the surface: two dissociable acid functions, a carboxyl group of sialic acid, pK about 2·8; an unidentified acidic group(s), pK about 4·0; and basic group(s), pK about 10, suggested to be an amino group [30] and recently established to be (lys, arg) amino groups [31]. The presence of sulphydryl groups also on the Ehrlich ascites tumour cell surface has been suggested [32]. Based on the ribonuclease treatment of Ehrlich ascites cells, it has been suggested that these cells have a marked contribution of RNA groups to their electrokinetic or surface charge [33]. The human cancer cells (HeP ≠2, flask culture) exhibit a net negative charge with a mobility of $-1{\cdot}08\pm0{\cdot}06$ μ/sec/V/cm at 25°C. The human blood platelet surface has been reported to have a cationogenic group (pK greater than 9, shown to be an amino group [34]), an anionogenic group attached to a neuraminidase-susceptible structure, the carboxyl group of a sialic acid and one or more unidentified anionogenic groups of pK approximately 4 [34]. It has already been pointed out that erythrocytes behave as polyanions [21]. At pH $7{\cdot}2+0{\cdot}2$ and ionic strength 0·145, the polylysine, polyornithine molecule is fully ionized, i.e. say for polylysine, every lysine residue carries a positive charge, the fully extended chain is coplanar with the C=O oxygen atoms and the N–H hydrogen atoms in the plane of the chain [22]. Orientation of the side chains attached to the carbon atoms, pointing alternately to the left and upward from the plane of the chain, and to the right downward or an interlaced arrangement with each side chain of one molecule lying between two side chains of its neighbour have been discussed fully by Nevo *et al.* [22]. When the cells in suspension are treated with polyornithine or polylysine, adsorption of the polycations on the cell surface leads to a reduction in the net negative charge. If all the positive groups of the polypeptide are not utilised and are operative in the electrophoretic plane of shear, further reduction in the net negative charge results. When the amount of the adsorbed polycations on the cell surface increases to cover up approximately half of the cell surface negative groups and the number of unutilised side chain amino groups of the adsorbed polycations becomes equal to the number of unblocked negative groups on the cell surface, the cell exhibits a zero mobility. Since a small number of groups on the cell surface before the chemical treatment are present, a small cathodic mobility may be observed. The contribution of these basic groups in question would give rise to an electrophoretic mobility of about 0·25 mobility units [30, 31] which corresponds to a surface charge density of 850 ± 150 (s.d.) e.s.u./cm². However, in this state of the cell, the positive sign of the cell surface charge is only just discernible and is not measurable with adequate precision. When all the negative groups are blocked by the adsorbed polycations, the cell acquires a high positive charge (see Fig. 1).

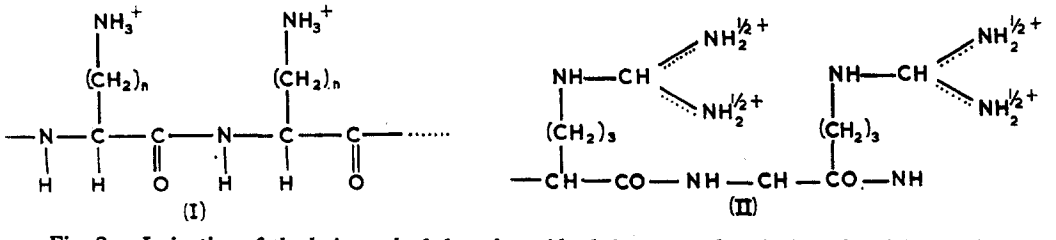

Fig. 2. Ionization of the lysine end of the polypeptide chain compared with that of arginine residues.
n=4: polylysine.
n=3: polyornithine (see reference 22 for models).

PL *and* PO, *having the anti-parallel pleated sheat structure and ease of rotation of the side chain amino groups, fold on the cell surface easily, whereas in polyarginine the rotation of the amino groups is somewhat restricted.*

Human cancer cells appeared to need about five times the amount of polyornithine to reduce the net charge to zero than was required for the Ehrlich ascites cell membrane charge to become zero. The mean surface area of the ascites cells and HeP ≠2 cells is nearly the same, i.e. 899±307 (s.d.) μ^2. No explanation for the observation can be offered at present.

At pH 7 β-alanine exists as a zwitter ion with no net charge and, as expected, this simple neutral amino acid possessing no antitumour activity [18] has now been shown not to affect the surface charge density of the tumour cells (Table 1). It seems highly significant that polycations polylysine, polyornithine in which the charge density of the polyions is high, possess antitumour activity and change the surface charge of tumour cells to zero or to positive values, whereas protamine sulphate with 65% content of arginine, in which the charge density of the polyion is much less dense, does not possess anti tumour activity and does not change the surface charge to zero or positive values (see Fig. 2; Table 1; references 19, 24).

The cytotoxic polypeptide(s) isolated by Holmberg [35] from different sources such as mouse ascites and ascites from human cancer patients are qualitatively similar in amino acid composition and exhibit a positive charge at neutral and acid pH, indicating that the *arginine* residues predominate over the glutamic acid ones [36]. Holmberg [37] has recently published some data on the electrophoretic mobilities of human red blood cells and Chang liver cells treated with this (arginine-rich) polypeptide derived from ELD mouse ascites fluid [36]. In agreement with my *in vitro* observations that arginine-rich protamine sulphate does not alter the cellular electrophoretic mobilities, Holmberg [37] has reported that there is no reaction between the arginine-rich polypeptide and the cell surface. It would be naive to make broad generalisations about the correlation between the antitumour activity and the ability of the chemicals to render the surface charge of tumour cells to zero or to positive values. No claims are laid as to the direct applicability of these *in vitro* studies to *in vivo* conditions.

The findings reported in this paper support the view that the *lysine* polypeptides polylysine, polyornithine which are known to possess the antitumour activity *in vivo* [19, 20, 24] perhaps interact by electrostatic and polar forces with other peptides or protein molecules of the cell surface/membrane. When such an interaction due to the high charge density of the polyion in the *lysine* polypeptide occurs, the surface charge density and physiological activity of the tumour cell change. The role of heparin in the reversal of the inhibitory effect of polyornithine on tumour transplantability observed by Larsen and Olsen [24] appears to consist in restoring the net negative charge on the tumour cell membrane. The net negative charge of the tumour cell membrane may well play a role in its highly invasive properties.

Acknowledgements—I should like to thank Professor J. S. Mitchell, F.R.S. for constant encouragement and help, Dr P. Dendy for a generous supply of HeP ≠2 cells, Mrs. I. Simon-Reuss for discussions about the cytology of the cells, Dr. C. Barker and Mr. E. King for supplying the inoculated mice and Miss Janice Wright for excellent technical help. Thanks are also due to Mr. David Juett for considerable help in programming and to Mrs. Beryl Gardiner for checking the bibliography and processing some of the data on the computer. Financial support from the British Empire Cancer Campaign and Professor Mitchell's Research Fund is gratefully acknowledged.

RESUME

On a étudié les modifications de la mobilité électrophorétique (potentiel zeta–densité de charge superficielle) des cellules de la tumeur ascitique d'Ehrlich, en milieu salin, sous l'effet de la polylysine, HBr (PL), de la polyornithine, HBr (PO), du sulfate de protamine (SP) (un polypeptide de faible poids moléculaire à forte teneur en arginine), de la β-alanine (βA) et de l'héparine. Seuls les polypeptides de la lysine (PL et PO), que l'on sait capables d'inhiber la transplantation et la croissance des tumeurs, diminuent la charge négative nette des membranes des cellules tumorales, pouvant même l'annuler, ou la transforment en valeurs fortement positives à la suite de leur adsorption à la surface des cellules. L'héparine inhibe cette réaction et inverse l'action des polypeptides PL et PO. Lorsque les cellules tumorales sont traitées par de hautes doses de sulfate de protamine ou de β-alanine, de façon à disperser les charges positives, la charge négative nette des cellules ne peut être annulée, ni changée en valeurs positives. L'action de PO sur la charge de

surface des cellules épithéliomateuses humaines (HeP≠2), cultivées en flacon, sur des plaquettes et des érythrocytes humains, est semblable à celle observée sur les cellules tumorales.

Le traitement des cellules tumorales par PL et PO ayant pour effet d'inhiber la transplantation et de diminuer la charge superficielle, on en conclut que la charge négative des surfaces cellulaires pourrait être une condition sine qua non de propriété invasive de la cellules tumorale.

SUMMARY

The effect of polylysine, HBr (PL), polyornithine, HBr (PO), protamine sulphate (PS) (low molecular weight basic polypeptides with 65% content of arginine, no tryptophan or tyrosine), β-alanine (βA) and heparin on the electrophoretic mobility (zeta potential—surface charge density) of saline-washed Ehrlich ascites tumour cells have been investigated. Only the lysine polypeptides (PO, PL) which have been reported to inhibit tumour transplantation/development change the net negative charge of the tumour cell membrane to zero or high positive values, presumably because of adsorption on the cell surface. Heparin inhibits this reaction and reverses the effect of the polypeptides, PO and PL, on the cells. When the tumour cells were treated with high doses of protamine sulphate or β-alanine, the net negative charge of the tumour cells was not altered to zero or positive values. Polyornithine changed the surface charge of human epidermoid cancer cells (HeP ≠2 in flask culture), human blood platelets and erythrocytes to zero and positive values.

Since the PO-, PL-treated tumour cells inhibit tumour transplantation and also change the cell surface charge to zero or to positive values, it is suggested that the negative charge of the tumour cell surface may well play a role in its highly invasive properties.

ZUSAMMENFASSUNG

Der Effekt von Polylysin-Hydrobromid (PL), Polyornithin-Hydrobromid (PO), Protaminsulfat (PS) (ein basisches Polypeptid mit hohem Arginingehalt und von niederem Molekulargewicht) β-Alanin (βA) und Heparin auf die elektrophoretische Beweglichkeit (Zeta-Potentiallandungsdichte der Oberfläche) von mit Salzlösung gewaschenen Ascites-Tumor-Zellen ist untersucht worden. Nur die Lysin-Polypeptide (PO und PL), von denen bekannt ist, dass sie die Tumortransplantation und -Entwicklung hemmen, setzen die negative Ladung der Tumorzellmembran auf null herab oder verändern diese durch Adsorption zu hohen positiven Werten. Heparin hemmt diese Reaktion und verhindert den Effekt der Polypeptide, PO und PL, auf Zellen. Wurden Tumorzellen mit hohen Dosen von Protaminsulfat oder β-Alanin behandelt, so dass die positiven Ladungen ausgeglichen wurden, wurde die reine negative Ladung der Tumorzellen nicht zu null oder positiven Ladungen verändert. Der Effekt von PO auf die Oberflächenladung von menschlichen epidermalen Krebszellen (HeP≠2 in Flaschenkulturen), menschlichen Blutblättchen und Erythrocyten ist dem der Tumorzellen ähnlich.

Da Die PO-, PL-behandelten Tumorzellen die Tumortransplantation hemmen und auch die Oberflächenladung nach null oder zu positiven Werten ändern, wird vermutet, dass die negative Ladung der Zelloberfläche eine Voraussetzung für die invasiven Eigenschaften der Tumorzellen ist.

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APPENDIX

For rapid handling of an enormous amount of data on cells which show a large variation in the various parameters I have been using a digital computer routinely. I have found the use of the computer efficient, convenient and time saving. Programmes in Autocode and Fortran 4 for the Cambridge University computer Titan and Atlas have been written incorporating the apparatus constants such as the size of the graticule (in microns, μ) mounted in the ocular of the electrophoresis microscope, the length of the electrical path in the electrophoresis cell (in cm), the applied voltage (V in volts) and the other constants of the equations (1) and (4). The observed times of migration of cells (in sec) following the application of the field strength (X=V/cm) are fed into the computer which processes the data to print out the values of the electrophoretic mobilities (μ in μ/sec/V/cm), zeta potential (ζ in mV) (equation 2) and electrokinetic charge densities (σ in e.s.u./cm²) for the individual cells evaluated by equation (4) for various values of α ranging from 0 –1. For the present work an electrophoresis chamber of a circular cross section has been used and the experiments carried out at constant voltage. For calculation of the electrokinetic parameters from observations of the times of migration in an electrophoresis chamber of a rectangular cross section at constant current, progammes are easily edited to incorporate the specific conductivity of the electrolytic medium and current etc. The second programme, which incorporates the standard statistical formulae, processes the individual values of μ, σ and ζ to print out the mean values together with the standard deviations and standard error of the mean. The third programme processes the data to plot histograms from the individual values. The computer-processed histogram plot for the surface area of the Ehrlich ascites tumour cells is reproduced in Fig. 3. When required, the processed data can be run through paired or unpaired t-tests and the computer prints out the degrees of freedom, variances, t-values and the results of the tests of significance at 0·1, 1 and 5% levels. Appropriate tables are incorporated in the programmes.

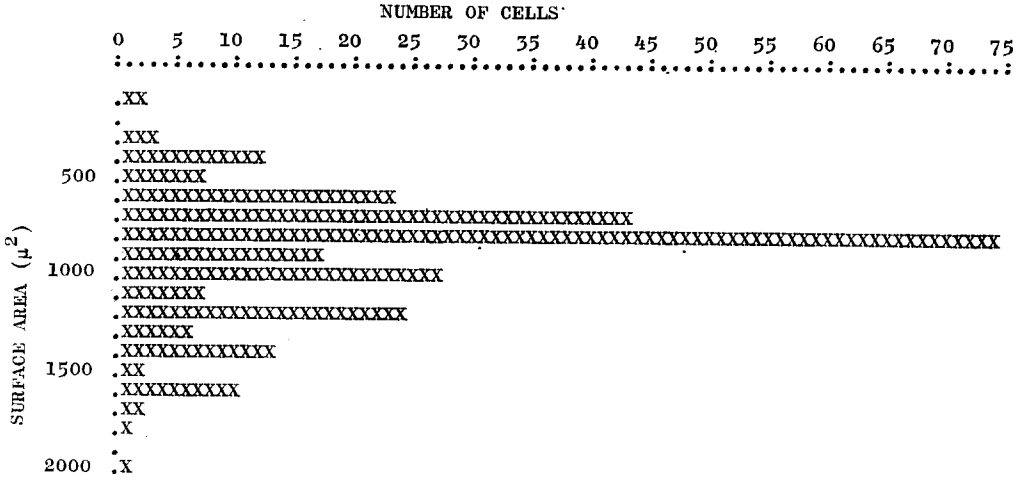

Fig. 3. Computer print out of the histogram showing the variation of the EAT cell size in terms of μ² of the cell surface area. The EAT cell is assumed to be a sphere. Diameters were determined by the hanging drop method.

The computer programme facilities will be discussed elsewhere [38] and may be made available to other workers on application.

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Differential Effects of Nitrogen Mustard, Melphalan and Vinblastine on the Metabolism of Mouse Tumour and Spleen Slices

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INTRODUCTION

THERE is now considerable evidence that malignant disease can be accompanied by reactive changes in local lympho-reticular tissues [1] and it is widely accepted that such changes represent the operation of immunological mechanisms. Since most cytotoxic drugs used in cancer chemotherapy suppress immunological reactions to varying extents [2] it is possible that under certain conditions the use of such drugs may accelerate the progress of malignancy.

The relative effects of such drugs on tumour and host reticulo-endothelial tissue should thus be considered in the selection of a drug for cancer chemotherapy. This argument has formed the basis for the design of an *in vitro* test to aid in drug selection comprising two new features. Firstly the use of a common metabolic precursor, glucose-6-^{3}H for the simultaneous assessment of RNA, DNA and protein synthesis in tissue slices [3]. The broad metabolic field covered by this method allows the use of drugs whose mechanism of action is unknown. The second feature is the application of the test to both tumour and host reticulo-endothelial tissue in order to detect possible differential susceptibilities, which could be a guide to drug selection for therapy.

The spleen was chosen as the reticulo-endothelial organ of study because of the importance previously attached to it as an index of host immunological resistance to animal tumours [4] and because it provides amounts of tissue sufficient for metabolic studies in the small rodents required for an isologous system.

The *in vitro* biosynthetic activity of tumour was compared with spleen from tumour-bearing animals and the effects of varying concentrations of nitrogen mustard, melphalan or vinblastine added to the incubation media studied in order to detect possible differential effects. *In vivo* correlation was attempted by measuring tumour and spleen sizes in animals receiving intraperitoneal injections of these drugs.

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MATERIALS AND METHODS

Chemicals

Glucose-6-^{3}H was obtained from the Radiochemical Centre (Amersham, Bucks, U.K.); Nitrogen mustard (mustine HCl) was obtained from Boots Pure Drug Co. Ltd., (Nottingham); melphalan ('Alkeran') from Burroughs Wellcome & Co. (London) and vinblastine sulphate ('Velbe') from Eli Lilly & Co. Ltd. (Basingstoke, Hants, U.K.). All other chemicals came from British Drug Houses Ltd. (Poole, Dorset, U.K.).

Animals and tissues

Female mice of DBA 2J strain (Jackson

Table 1. *Mean of all the control values* for RNA, DNA and protein synthesis in slices of spleen and tumour used in the* in vitro *drug experiments*

Tissue	Specific activity (μμc/100 μg)		
	RNA	DNA	Protein
Spleen	167·7±11·7 (19)	397·4±51·3 (21)	194·1±11·6 (21)
Tumour	516·1±36·7 (20)	616·5±37·2 (21)	392·4±21·3 (24)

*The Mean±S.E. is given with the number of determinations in parentheses.

Laboratory, Maine) were used (8–12 weeks old, 18–22 g) and the isologous mammary adenocarcinoma CaD_2 (originally spontaneous in DBA 2J mice) maintained by serial solid implantation. Both tumour and spleen were removed from mice receiving tumour implants 11 days previously. At this stage the tumour mass was between 300 and 500 mg. The pooled tissues from several mice (7–20 according to the experiment) were used.

In vitro *test system*

Incorporation studies using glucose-6-^{3}H as a common metabolic precursor were carried out as described previously [3]. In the present studies incubation was for 60 min at 37°C and the cytotoxic drugs were included in the incubation medium at the concentrations shown in the figures. Aliquots of the same tissue incubated without drugs were taken as controls in individual experiments.

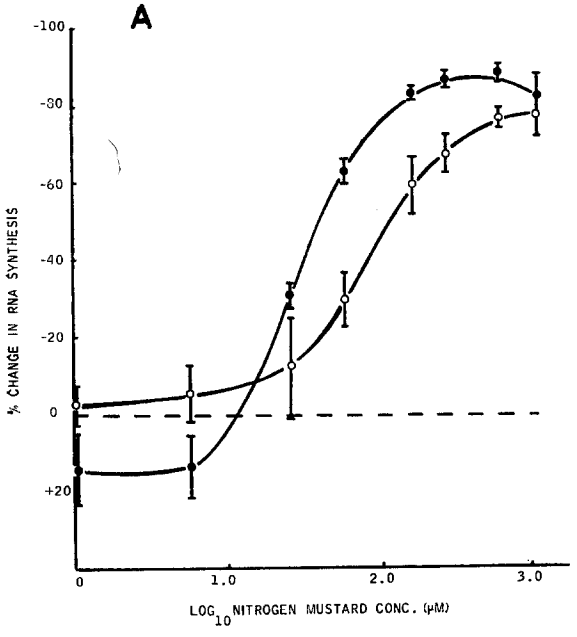

Fig. 1a.

Fig. 1. *Effects of varying concentrations of nitrogen mustard on mouse tumour* (●——●) *and spleen* (o——o) *slices using glucose-6-^{3}H for synthesis of RNA* (A), *DNA* (B) *and proteins* (C). *Each point is the mean*±S.E. *of* 8 *determinations.*

In vivo *studies*

Mice receiving drugs *in vivo* were given i.p. injections of various concentrations of drug in 0·1 ml isotonic saline. Controls received 0·1 ml saline. A total of 4 injections were given at 5, 8, 11 and 13 days after tumour implantation. The mice were killed 18 days after tumour implantation and the wet weight of tumour and spleen determined.

RESULTS

Table 1 shows the comparative *in vitro* metabolic activities of the two tissues studied. The spleen slices showed high levels of biosynthesis, especially of DNA, when compared with spleens from non-tumour bearing mice [3]. The metabolic activity of the tumour slices was nevertheless higher than that of spleen particularly in regard to RNA synthesis.

The effects of varying concentrations of nitrogen mustard on incorporation of tumour and spleen slices are shown in Figs. 1 a–c. The percentage change in RNA synthesis (Fig 1a) shows that there was a different dose response

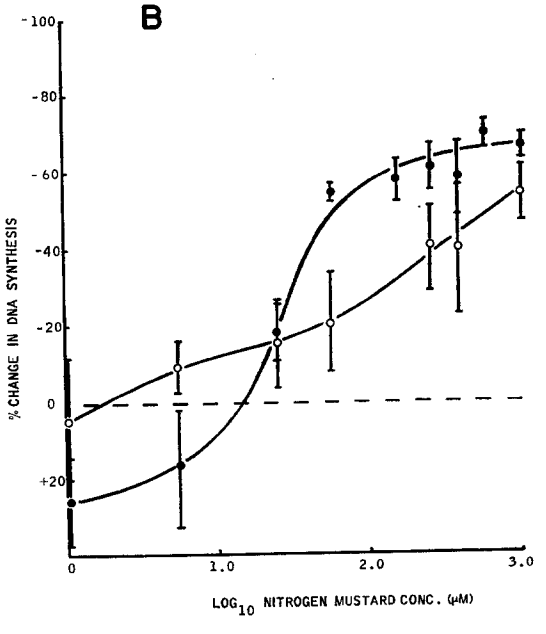

Fig. 1b.

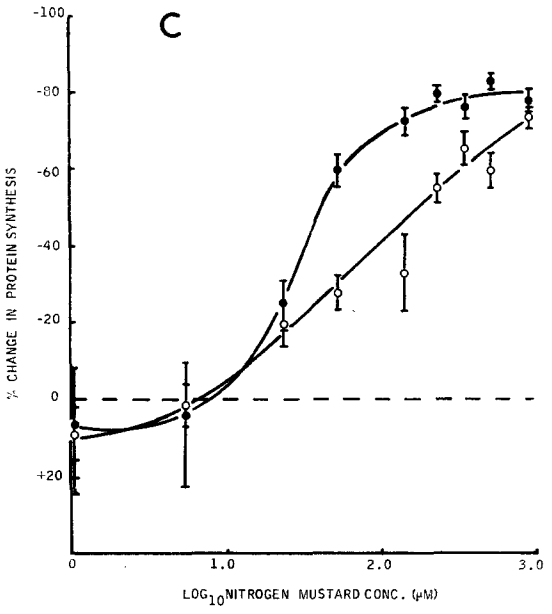

Fig. 1c.

by the two tissues. Thus, tumour slice response was much steeper than spleen and at concentrations above 16μM tumour RNA synthesis was inhibited more than spleen. An optimum difference between the tissues was reached at about 100μM, above this concentration the difference between the two tissues diminished. Statistically, these differences in response were highly significant (e.g. at 56μM, $P<0{\cdot}001$). Another feature of these curves was the slight difference in RNA synthesis in tumour at low

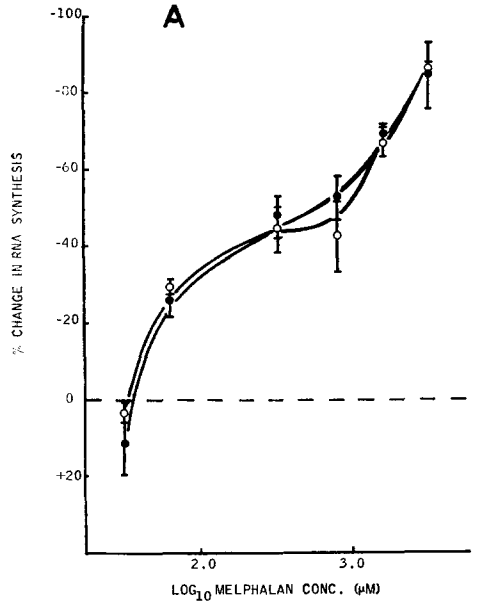

Fig. 2a.

Fig. 2. *Effects of varying concentrations of melphalan on mouse tumour* (●——●) *and spleen* (o——o) *slices using glucose-6-*^{3}*H for synthesis of RNA* (A), *DNA* (B) *and protein* (C). *Each point is the mean* ±S.E. *of* 6 *determinations.*

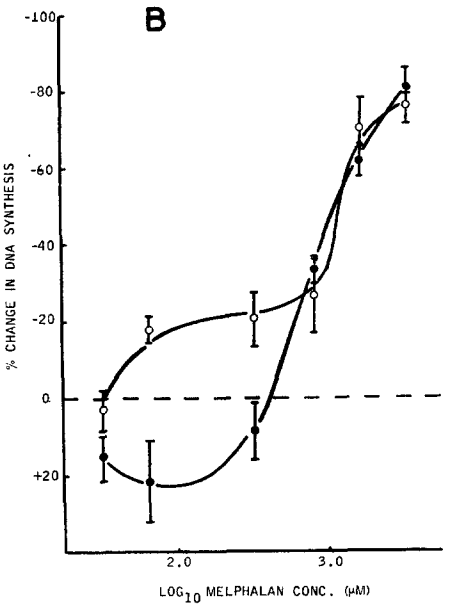

Fig. 2b.

doses, although here the differential effect was far less significant ($P=0{\cdot}1$ at 5·6μM). Similar results were obtained when examining incorporation into DNA (Fig. 1b). Again there was an optimum concentration of about 100μM for maximum relative inhibition of tumour to spleen, a crossover of inhibition curves at about 25μM and evidence of stimulation of tumour DNA synthesis at concentrations below 10μM. Dose response curves of protein synthesis (Fig. 1c) were similar to RNA and DNA at higher concentrations but showed no differential effect at lower doses.

The dose response curves using melphalan are shown in Figs. 2a–c. The curves for spleen

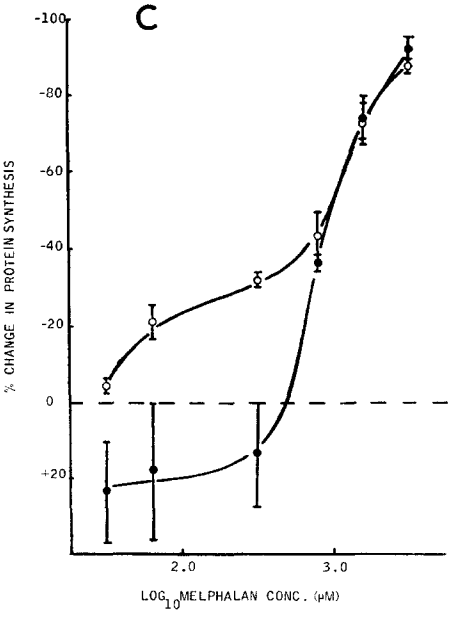

Fig. 2c.

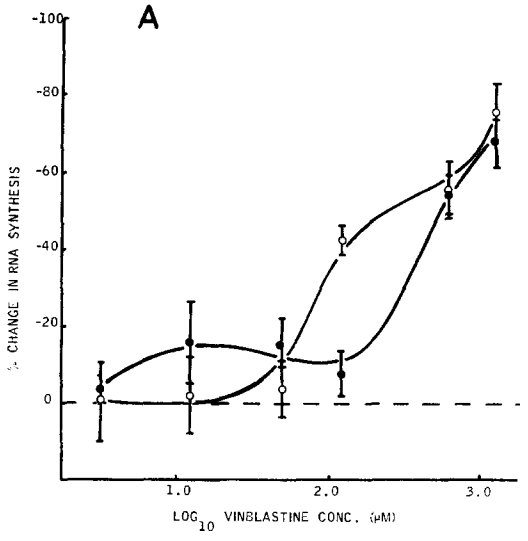

Fig. 3a.

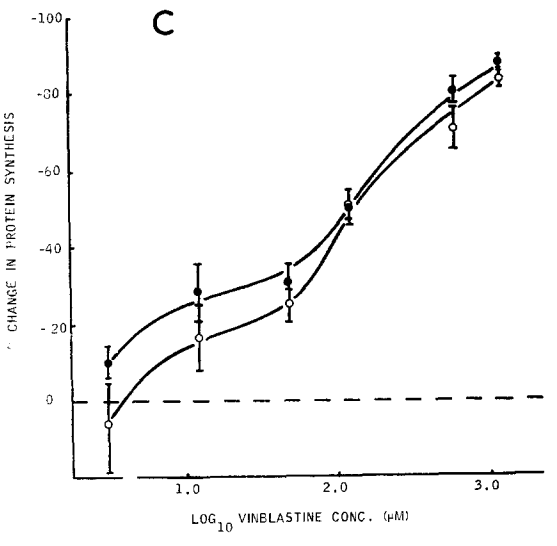

Fig. 3c.

Fig. 3. *Effects of varying concentrations of vinblastine on mouse tumour* (●——●) *and spleen* (o——o) *slices using glucose-6-³H for synthesis of RNA* (A), *DNA* (B) *and protein* (C). *Each point is the mean* ±S.E. *of* 6 *determinations.*

and tumour RNA synthesis (Fig. 2a) were virtually superimposed showing no differential dose effect. DNA synthesis studies, however, (Fig. 2b) showed a different result. Above 500μM melphalan inhibited at concentrations that produced stimulation of incorporation into tumour DNA. This differential response between the tissues was stastically significant ($P<0{\cdot}02$ at 300μM and $P<0{\cdot}01$ at 50μM). A very similar result was obtained in the case of incorporation into protein (Fig. 2c).

A differential dose effect was observed when using vinblastine (Figs. 3a–c). Little change in RNA synthesis of tumour was noted until concentrations of vinblastine exceeded 200μM whereas spleen RNA synthesis was over 40% inhibited at 100μM (Fig. 3a). Greater inihbition of spleen DNA synthesis was observed in comparison with tumour (Fig. 3b) over a large concentration range but protein synthesis was inhibited to a similar extent in the two tissues (Fig. 3c).

The *in vivo* organ wet weight studies also revealed some tendency towards a differential response between tumour and spleen. Figure 4 shows the *in vivo* effect of melphalan and demonstrates a considerable similarity in dose response of tissue weights *in vivo* with the *in vitro* response of DNA and protein synthesis to melphalan as shown in Figs. 2b and 2c. At higher concentrations, change in weight of both tissues was similar whereas at lower concentrations splenomegaly was inhibited

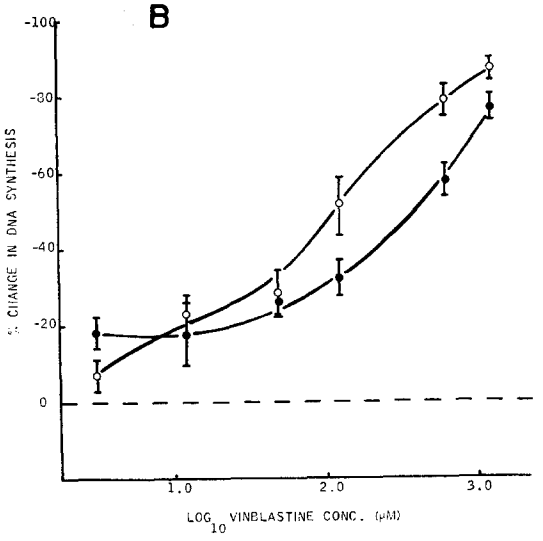

Fig. 3b.

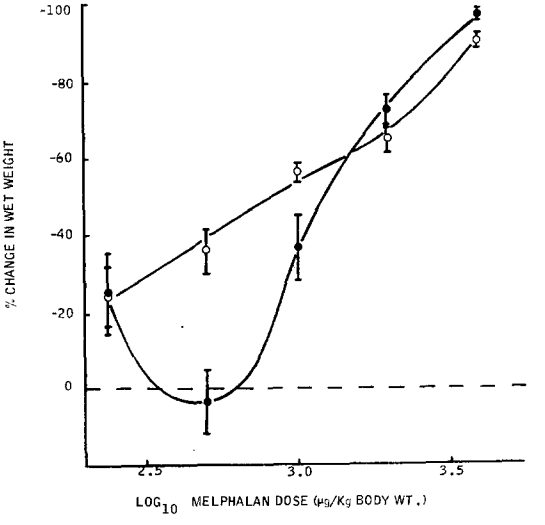

Fig. 4. *Percentage change in wet wt. of tumour* (●——●) *and spleen* (o——o) *of mice after* in vivo *administration of melphalan. Each point is the mean* ±S.E. *of determinations using* 8 *mice.*

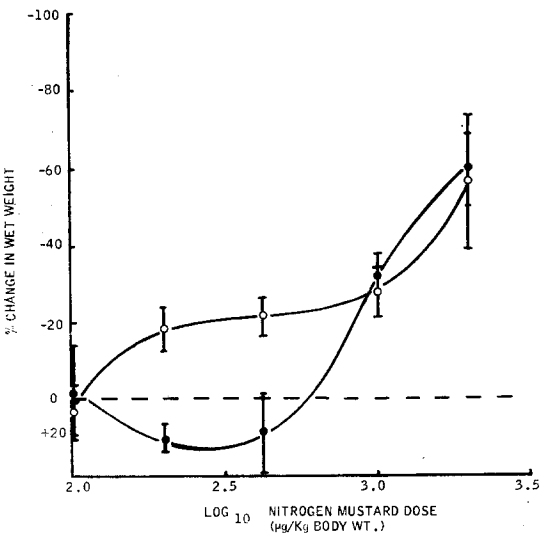

Fig. 5. Percentage change in wet wt. of tumour (●——●) and spleen of mice after in vivo *administration of nitrogen mustard. Each point is the mean* ±S.E. *of determinations using 7 mice.*

more than tumour growth. This difference was highly significant ($P<0{\cdot}01$) at a dose of 0·5 mg/Kg body weight. At the lowest concentration used, inhibition of the two tissues was again the same. Similar determinations were carried out using nitrogen mustard (Fig. 5). Once again inhibition of spleen was greater at lower concentrations than that of tumour which showed some evidence of stimulation of growth. At higher concentrations there was similar inhibition of the two tissues. Figure 6 shows the differential response of tumour and spleen weight to vinblastine *in vivo*. At lower concentrations no significant difference was obtained but at the

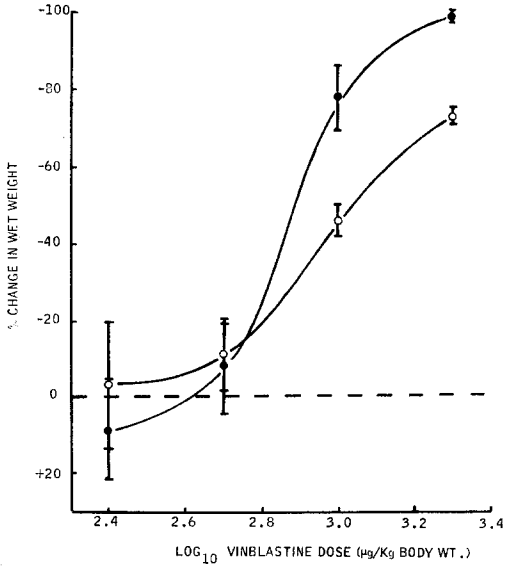

Fig. 6. Percentage change in wet wt. of tumour (●——●) and spleen of mice after in vivo *administration of vinblastine. Each point is the mean* ±S.E. *of determinations using 5 mice.*

two higher concentrations, tumour was inhibited more than spleen.

DISCUSSION

There have been numerous attempts to design an *in vitro* sensitivity test as a guide to drug selection in cancer chemotherapy [5–8]. These tests in general, however, determine the effects of such drugs on tumour tissue only and consideration of host tolerance are limited to total toxicity as measured by conventional pharmacological standards of lethality. The effective concentration range *in vitro* is often very different to that *in vivo* and such tests are therefore extremely difficult to interpret and to correlate with *in vivo* effectiveness. A test which determines the relative effect of drug on tumour and some host tissue has obvious advantages; the problem is to choose an appropriate host tissue. Since there is mounting awareness that immunological mechanisms play a role in the progress of human cancers [9, 1] the tissues involved in host defence appear to present a logical choice. However, for such a relative *in vitro* test to be of any value, two questions must first be answered: (a) are there any differences in the response of tumour and reticulo-endothelial tissue metabolism *in vitro* to any one anti-tumour agent? (b) If such differences exist do they reflect the *in vivo* effect of the agent? An attempt has been made to answer these questions using initially an experimental animal model consisting of an isologous tumour transplanted into mice of the strain of origin of the tumour.

In order to be sure of detecting any possible differences in metabolic response to different anti-tumour agents, glucose-6-^{3}H has been used as a common precursor for nucleic acid and protein synthesis. The reasons for believing that this forms a more physiological approach than the use of traditional nucleoside and amino acid precursors are discussed elsewhere [3]. So as to compare the inhibition of the two tissues whose control levels of activity differed, the results were presented as a percentage inhibition. In this way it is possible to detect differential effects between the tissues with the various drugs under test.

Evidence that differences exist in the metabolic response of spleen and tumour slices to nitrogen mustard was adduced from the three dose responses with this drug. The most dramatic difference was the greater steepness of the curves using tumour tissue. This was common to all three biosynthetic pathways as were the differential effects in general at higher concentrations of nitrogen mustard. The

close similarity of the inhibition curves of RNA, DNA and protein synthesis with the higher concentrations of nitrogen mustard suggests that the drug may be acting at an early point in the metabolic pathway of glucose utilization, common to all three biosynthetic pathways, and that the differential effects on spleen and tumour at higher concentrations are due to differential susceptibility of such a point of attack in the two tissues. One may speculate that the phosphorylation of glucose by hexokinase is this point of attack since it has been shown that nitrogen mustard is strongly inhibitory to hexokinase from adenocarcinoma [10]. At the lower concentrations shown in the dose response curves the inhibition of spleen protein synthesis tends to differ from that of spleen nucleic acid synthesis whereas the tumour curves show similar patterns in all three pathways. The significance (if any) of this difference in spleen response is not known.

A differential effect was also obtained using melphalan. It was, however, only seen at the lower concentrations used and was absent in the response of RNA synthesis. No explanation for this can be given without further data but the results suggest that tumour RNA synthesis is more sensitive to melphalan at lower concentrations than tumour DNA and protein synthesis whereas the similarity in response of the three parameters in spleen suggests again a common site of action in this tissue.

No very clear differential pattern emerged from the vinblastine studies. Higher concentrations, however, appeared to be more inhibitory to RNA and DNA synthesis of spleen than of tumour and the drug in general was more inhibitory to DNA and protein synthesis than to RNA synthesis, except at the highest concentrations used. Richards *et al.* [11] showed in studies on the effects of vinblastine on labelled formate incorporation in thymus cells that RNA synthesis was less susceptible than DNA synthesis. It is of interest that the present results show the effect to be more marked in tumour than splenic tissue, particularly since tumour RNA synthesis in the controls was high relative to that in spleen. In the thymus cell studies [11] it was also shown that labelled serine accumulated in the acid soluble fraction of the cells. This may represent inhibition of serine utilization for protein synthesis and would readily explain the marked inhibition of protein synthesis in the present studies since serine is the most highly labelled amino acid incorporated into protein when glucose-6-^{3}H is used as a precursor [3]. Glutamate (also labelled in protein when using glucose-6-^{3}H) has been implicated in the action of vinblastine. Johnson *et al.* [12] showed a reversal of the *in vivo* effects of this drug by administration of glutamate, and Cutts [13] was able to show a modification of metaphase arrest with glutamic acid or tryptophan. The similarity of the protein synthesis response curves of tumour and spleen in the present study suggest, however, that no differential effect of vinblastine on serine or glutamate metabolism exists in these two tissues.

The question of *in vivo* correlation was tackled by comparing the *in vitro* dose response curves with inhibition curves of tumour and spleen weight after an arbitrary *in vivo* administration schedule. The most striking agreement was obtained when using melphalan, where there was a marked similarity in the differential response observed *in vivo* with the *in vitro* effects on DNA and protein synthesis. Correlation was also good over the lower concentration ranges using nitrogen mustard. Here the similarity was between *in vivo* response and the effects on RNA and DNA synthesis *in vitro*. Vinblastine *in vivo* produced a differential effect at high concentrations that was not predicted by the *in vitro* results. There was, however, similar lack of differential at lower concentrations of vinblastine by both *in vitro* and *in vivo* methods. It thus seems that where correlations exist, they are most marked when using lower concentrations of anti-tumour agents for *in vitro* studies. The higher concentrations are in any case several orders of magnitude larger than the lethal *in vivo* doses. With the drugs and the experimental model studied so far it also appears that the most consistent correlations are obtained using DNA synthesis *in vitro* as the parameter for comparison. In comparing the inhibition of tumour and spleen on a percentage of control basis the quantitative differences that exist in these two tissues are eliminated and by examining a range of drug concentrations it has been possible to demonstrate qualitative metabolic differences in response to three cytotoxic drugs and relate them to *in vivo* response.

Acknowledgements—We wish to thank Mr. C. G. Francis for skilful technical assistance and Professor G. W. Taylor for the facilities of his department. We are also indebted to Miss M. Sumner for the transplantation of tumours. The work was supported by a generous grant from the Board of Governors of St. Bartholomew's Hospital.

RESUME

On a entrepris l'étude préliminaire d'un test in vitro *permettant une sélection de composés antitumoraux. Ce test était basé sur la comparaison de l'effet inhibiteur de différents composés sur le tissu tumoral et sur le tissu réticulo-endothélial de la souris. En effet, si une substance présente une activité plus intense sur le système réticulo-endothélial, cette substance a peu de chance de posséder une activité thérapeutique valable. Afin de détecter,* in vitro, *des variations métaboliques de ces tissus, des coupes de tumeurs de glande mammaire et de rate ont été incubées à des concentrations variées de méchloréthamine, de melphalan ou de vinblastine.*

Le pourcentage de l'inhibition de la synthèse des protéines, de l'ARN, et de l'ADN, a été déterminé, in vitro, *en utilisant le glucose-6-^3H comme précurseur commun. Les courbes de réponse, en fonction de la dose, montrent qu'il existe un effet différentiel sur le tissu tumoral et sur le tissu réticulo-endothélial, et que cet effet varie aussi bien avec le médicament qu'avec les paramètres métaboliques envisagés.* In vivo, *avec le méchloréthamine et le melphalan, on retrouve une bonne corrélation avec les résultats obtenus* in vitro, *surtout aux faibles concentrations, et plus particulièrement si l'on considère la synthèse de l'ADN, la vinblastine ne montrant une corrélation qu'aux plus faibles concentrations utilisées.*

SUMMARY

Preliminary studies on an in vitro *test to guide antitumour drug selection were undertaken in a mouse model. It is based on a comparison between inhibitory effect of these drugs on host reticulo-endothelial and tumour tissue. If the drug shows greater inhibition of reticulo-endothelial tissue than tumour tissue it may lack therapeutic effectiveness. In order to detect possible differences in the* in vitro *metabolic response of such tissues, slices of mouse mammary tumour or spleen were incubated with varying concentrations of either nitrogen mustard, melphalan or vinblastine. Percentage inhibition of protein, RNA and DNA synthesis* in vitro *was determined using glucose-6-^3H as a common precursor. The dose response curves show that differential effects are present and these vary both with the drug and with the metabolic parameter studied.* In vivo *effects of nitrogen mustard and melphalan showed correlation with the result of the* in vitro *test, especially at lower concentrations and when considering inhibition of DNA synthesis. Vinblastine showed correlations only at the lowest concentrations used.*

ZUSAMMENFASSUNG

Um die Prüfung von Antitumormedikamenten zu erleichtern, wurden vorläufige Untersuchungen an einem Mäusemodell in vitro *durchgeführt. Es basiert auf einem Vergleich des Hemmeffektes dieser Substanzen am redikuloendothelialen Gewebe und am Tumorgewebe. Hemmt die Substanz redikuloendothelialen Gewebe stärker als Tumorgewebe, dann könnte sie wenig therapeutische Wirkung zeigen. Un mögliche Unterschiede in der Reaktion des Stoffwechsels dieser Gewebe* in vitro *nachzuweisen, wurden Scheiben vom Mammacarcinom der Maus oder von Milz mit wechselnden Konzentrationen von Stickstofflost, Melphalan oder Vinblastin inkubiert. Die prozentuelle Synthesehemmung von Eiweiss, RNS und DNS* in vitro *wurde bestimmt mit Hilfe von Glukose-6-^3H als Vorlaüfer. Die Dosiswirkungskurven zeigen den unterschiedlichen Effekt und beide variieren sowohl in Abhängigkeit von der Substanz wie in metabolischen Parameter.*

Die in vivo *Effekte von N-Lost und Melphalan zeigten einen Zusammenhang mit den Ergebnissen des* in vitro *Tests, besonders bei niederen Konzentrationen und hinsichtlich der Hemmung der DNS-Synthese. Bei Vinblastin bestand eine Korrelation nur bei der niedrigsten angewandten Konzentration.*

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Penetration of the Vascular Endothelial Barrier by Non-Neoplastic Thyroid Cells in Circulation*

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INTRODUCTION

TAKAHASHI [1] has proposed two stages in the formation of metastases following the intravenous injection of tumour cells in mice. (1) Attachment of tumour cells to the vascular endothelium after arrest in lung capillaries; (2) penetration of the endothelium and the development of extravascular growth. He also stressed the importance of the endothelial barrier as being the major obstacle to metastatic growth. Reports on the morphogenesis of blood-borne metastases to the lung are numerous [2, 3]. More recently Wood [4, 5] provided cine-photomicrographic evidence with experiments *in vivo* of sequences involved in the production of blood-borne secondary growths. Capillary arrest appears not to be the result of mechanical impaction. It seems to be related to a marked tendency of tumour cells to stick to the capillary endothelium even when the capillary diameter is 2 or 3 times the size of tumour cells and when there is a brisk flow of blood [5]. Recent concepts on metastasis have been discussed by Fisher and Fisher [6].

In experimental blood-borne metastases, outward penetration of the vascular endothelium by tumour cells may occur in two ways: (a) by production of an initial intravascular growth and subsequent penetration of the endothelial wall; (b) by initial migration of individual tumour cells to an extravascular position and subsequent growth.

In such experimental studies other investigators have almost invariably used tumour cells. No comparative studies have been reported in which dissociated non-neoplastic cells, of similar tissue origin as that of the tumour cells, were also used to find out if they could penetrate the endothelial wall and proliferate extravascularly.

The object of the present experiments was to test, by intravenous injection in mice, whether dissociated thyroid cells from groitrogen-induced thyroid tumours or from hyperplastic or normal thyroid glands were capable of endothelial penetration and extravascular growth.

Goitrogen-induced thyroid tumours in mice and rats are usually pituitary thyrotrophic hormone (TSH) dependent. For successful transplantation such tumours require at first thyroxine deficient hosts [7, 8]. In the present experiments some of the recipients used were thyroxine deficient and others were normal mice.

In earlier experiments [9], by intravenous injection of hyperplastic thyroid cells into thyroxine deficient hosts, it was found that: (1) 10^5 thyroid cells were required per mouse, for successful growth in the lung; and (2) the time required for the injected cells to evolve into microscopic foci of thyroid tissue was about 4–6 weeks.

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*This work was carried out at the Tumour Biology Research Unit, University of Otago Medical School, Dunedin, New Zealand.

Table 1. Treatment of donor mice

Group	No. of mice	Treatment	Duration of treatment (weeks)	Classification of thyroid glands
I	11	MTU*	50–70†	Neoplastic (metastasizing)
II	409	MTU	6–12	Hyperplastic
III	228	None	None	Normal
Total	648			

*0·05% methylthiouracil in their drinking water beginning at the age of 4 weeks.
†The donor mice of Group I were kept on MTU treatment until the occurrence of general signs of ill health, laboured respiration and persistent loss of weight.

MATERIAL AND METHODS

1. *Mice*

A total of 889 female NZO/B1 mice were used, inbred for more than 60 generations. The majority were 3–4 months of age. The animals were housed in galvanized iron boxes with perforated lids, no more than 4–7 animals per box, and were kept at a constant temperature of 22°C with free access to water and food. Their diet has been described elsewhere [10].

2. *Goitrogen*

0·05 % of 6-methyl-2-thiouracil in drinking water (MTU).

3. *Donor material*

Supensions of dissociated thyroid cells obtained by trypsinization of goitrous or normal thyroid glands from 648 animals. The trypsinization technique has been described previously [10]. The donor mice comprised 3 groups as shown in Table 1. Their thyroid glands were classified as *neoplastic*, *hyperplastic* or *normal* depending on the duration of MTU treatment and other criteria (see below). The lungs of donor mice with neoplastic thyroid glands contained large secondary thyroid growths invading bronchi (Fig. 1).

4. *Criteria for distinction between neoplastic and hyperplastic thyroid glands*

The term 'neoplastic' is used here to denote invasive behaviour and metastasis formation, both locally and in the lungs, irrespective of the degree of cellular pleomorphism and atypism present or the dependence for progression on elevated levels of thyrotrophic hormone [11].

The term hyperplastic represents degrees of benign goitrous enlargement. It is used here to denote mitotic proliferation of thyroid cells and hypertrophy of both their cytoplasm and nuclei, and also the formation of new follicles, but without evidence of invasive or metastatic growth. Such growth, in MTU treated NZO mice, usually occurs after 35–45 weeks of goitrogen treatment [12].

5. *Preparation of recipient mice before injection of thyroid cells*

Mice selected as recipients of thyroid cells were either thyroxine deficient or normal. Thyroxine deficiency was obtained mostly by MTU treatment. However, a small group of untreated mice were rendered thyroxine deficient by irradiation of the thyroid gland with ^{131}I.

Table 2. Summary of the experimental design

Donor material		Injected intravenously	Recipient mice
1. Stimulated cells	Neoplastic / Hyperplastic	to	Stimulated recipients
2. Stimulated cells	Neoplastic / Hyperplastic	to	Normal recipients
3. Normal cells		to	Stimulated recipients
4. Normal cells		to	Normal recipients
5. PBS* without cells		to	Stimulated recipients
6. Non-injected mice treated with MTU for periods up to 28 weeks.			

*Phosphate buffered saline solution.

Table 3. Results of intravenous injection of 10^5 thyroid cells into thyroxine deficient mice

Donor cells			Recipient mice			
Classification of thyroid cells	Group	No. of mice	Treatment of recipients before and after cell-injection	No. of recipients (in parentheses) killed at following times after injection (weeks)	No. of injected mice developing thyroid tissue in lungs (microscopically)	%
Neoplastic	1	48	MTU*	(16)6, (16)12, (16)18	48	100
Hyperplastic	2	54	MTU	(14)6, (14)12, (13)18, (13)26	51	94
Hyperplastic	3	48	MTU	(48)6	39	81
Normal	4	12	MTU	(6)6, (4)18, (2)26	12	100
Hyperplastic	5	10	^{131}I†	(10)6–26	8	80
Total		172				

*0·05% methylthiouracil in drinking water beginning 48 hr prior to cell-injection and continued until the animals were killed.
†See details of ^{131}I treatment in Table 5.

The MTU treatment began 48 hr prior to injection of thyroid cells and lasted until the animals were killed. The ^{131}I treatment was given subcutaneously at the age of 4 weeks. It was administered several weeks prior to injection of thyroid cells. Some mice were injected with 50μC carrier free ^{131}I in 0·2 ml normal saline and others with 42μC ^{131}I in 0·1 ml normal saline.

6. *Viability tests and dose of injected cells*

The viability tests are described elsewhere [10]. Cells were counted in a white cell counting chamber. Each recipient mouse was injected with 10^5 thyroid cells suspended in about 0·2 ml phosphate buffered saline (PBS). All injections were given into a dorsal or lateral tail vein.

7. *Experimental design*

Nomenclature. *Stimulated cells* are designated as those arising from goitrous glands of donor mice, as the result of MTU treatment. *Stimulated recipients* as those in a state of thyroxine deficiency as the result of MTU or ^{131}I treatment and, therefore, secreting an excess of TSH.

The experimental design is summarized in Table 2. Design no. 5 is designated as injected controls. Design no. 6 is designated as non-injected controls. The purpose of the last design was to demonstrate, by serial sections, that MTU administration for periods up to 28 weeks did not produce metastases of thyroid tissue to the lung.

8. *Division of recipient mice according to treatment*

A total of 241 recipients was used. Of these mice, 215 were arranged into 8 groups of stimulated or normal animals. These groups were killed at set times after i.v. injection, as shown in Tables 3–5.

Six other MTU-treated mice received i.v. 0·2 ml PBS without cells and were killed, three at 6 weeks and three at 26 weeks after injection.

Eighteen non-injected animals (comprising 9 pairs) remained on MTU treatment for periods varying from 20 to 28 weeks before being killed (Table 6).

Finally, two other mice were treated with MTU (for 23 and 57 weeks respectively), for electron microscopic examination of their pulmonary vascular endothelium.

The treatment of recipient mice is summarized as follows: (1) MTU+thyroid cells; (2) ^{131}I+thyroid cells; (3) thyroid cells only; (4) MTU+PBS without cells; and (5) MTU only.

Table 4. Results of intravenous injection of 10^5 thyroid cells into normal mice

Donor cells			Recipient mice		
Classification of thyroid cells	Group	No. of mice	Treatment of recipients before and after cell-injection	No. of recipients (in parentheses) killed at following times after injection (weeks)	No. of injected mice developing thyroid tissue in lungs (microscopically)
Neoplastic	6	10	None	(3)6, (3)18, (4)26	0
Hyperplastic	7	24	None	(8)6, (8)18, (8)26	0
Normal	8	9	None	(2)6, (3)18, (4)26	0
Total		43			

Table 5. Summary of pituitary weights and histological findings in the lungs and trachea of mice rendered thyroxine deficient by ^{131}I treatment and injected intravenously with 10^5 hyperplastic thyroid cells

No. of individual animal	Dose of ^{131}I* (μC)	Time interval between ^{131}I injection and cell injection (weeks)	Killed at following times after cell injection (weeks)	Minute thyroid remnants on trachea macroscopic	Minute thyroid remnants on trachea microscopic	Thyroid tissue in lungs macroscopic	Thyroid tissue in lungs microscopic	Pituitary gland wt. (mg)
1	50	7	6	—	+	—	+	2·4
2	50	7	6	—	+	—	—	2·6
4	50	28	10	—	+	—	+	3·0
5	50	28	12	—	—	—	+	4·0
7	50	28	16	—	+	—	+	15·2
9	42	42	13	+	+	—	+	21·7
12	42	42	13	+	+	—	+	3·8
13	42	42	18	+	+	—	—	3·2
14	42	42	26	+	+	+	+	88·5
6	42	46	26	+	+	+	+	5·0
Total 10				5	9	2	8	

*The ^{131}I was injected subcutaneously when recipients were about 4 weeks old.
+ = present.
— = absent.

9. *Preparation of specimens for light microscopy*

All recipient mice were killed with coal gas at the end of required treatment. Intratracheal fixation was applied before removal of the lungs, by slow injection (about 15 sec) of 1·5 ml Helly's fluid through the tracheal wall. Three minutes later, the lungs and heart were removed *en bloc*. After separation of the pulmonary lobes, each lobe was cut in half by an antero-posterior vertical section and the outer half of each lobe was retained and was transferred for further fixation into Helly's fluid. However, the pulmonary lobes of animals from experimental design no. 4 and 6 were kept *in toto* for further fixation. The thyroid gland and attached trachea, mediastinal tissue and any organ of abnormal appearance were removed and were fixed in Helly's fluid. Each thyroid gland was bissected in a horizontal plane through the isthmus before embedding. All pituitary glands were inspected and in selected groups were removed, weighed, fixed in sublimate–formol (9: v/v) and embedded. Histology of the pituitary glands was not carried out but the embedded glands were kept for histological examination at a future date. Sections were cut at 5μ and were mounted as two ribbons of 10–15 sections each. If the

Table 6. Summary of histological findings in the lungs of non-injected mice treated with MTU for periods varying from 20 to 28 weeks

No. of mice	Duration of MTU* treatment (weeks)	Histology of lungs†
2	20	Normal
2	21	Normal
2	22	Normal
2	23	Normal
2	24	Normal
2	25	Normal
2	26	Normal
2	27	Normal
2	28	Normal
Total 18		

*0·05% methylthiouracil in the drinking water beginning at the age of 12 weeks.
†Lungs sectioned serially.

required information was contained in the first or second slides no further sections were cut. Otherwise, further ribbon sections were prepared until the embedded tissue was exhausted. Sections were stained by haematoxylin and eosin and, in selected cases, also by periodic-acid Schiff (PAS), Van Gieson and Gomori's aldehyde fuchsin.

10. *Preparation of specimens for electron microscopy*

The purpose of the electron microscopic investigation was to find out whether the endothelium of small pulmonary vessels, in NZO mice treated with MTU, remained continuous or not. For example, what was the state of the endothelial-cell junctions and the underlying basement membrane after MTU treatment? Were there any endothelial discontinuities present which could have been responsible for the outward penetration of the endothelial wall?

For this purpose two mice were prepared as follows: the first was injected with hyperplastic thyroid cells and was treated with MTU for 23 weeks. The second mouse was not injected but was treated with MTU for 57 weeks. The mice were killed at the end of treatment by cervical dislocation. After exposing the trachea and removing the anterior chest wall 1 ml 2·5% glutaraldehyde, buffered at pH 7·4, was injected intratracheally as described by Karrer [13]. Two minutes later the lungs, trachea and heart were removed *en bloc*. Subsequently 3–4 pieces of lung tissue were dissected away and were transferred into a small petri dish containing 2·5% glutaraldehyde. There the lung tissue was sliced with a fine scalpel into small (about 2 mm) cubes and these were fixed for 5 hr. Glutaraldehyde-fixed cubes were washed for 16 hr in three changes of phosphate buffer, postfixed for 5 hr in phosphate buffered 1% O_8O_4, dehydrated in an ascending series of acetone from 30% to absolute over 1 hr, embedded in Vestopal W [14] and polymerized at 60°C for 4 days. From the total pool of cubes of lung a random sample of 14 pieces was embedded into consecutively numbered blocks.

Sections of about 0·25 mm^2 area and 500–700Å thickness were cut with a Porter–Blum ultramicrotome, using a glass knife, and were picked up on 200-mesh copper grids coated with a carbon-reinforced Formvar film. The sections were stained with 2% aqueous solution of uranyl acetate for about 30 minutes and were examined with a Philips EM 100B at 60 kV with an objective aperture of 25μ or a Hitashi EM HU11A at 75 kV with an objective aperture of 50μ.

RESULTS

A. *Intravenous injection of neoplastic, hyperplastic or normal thyroid cells into normal mice* (Table 4, Groups 6–8).

Lungs. No thyroid tissue developed in the lungs of these mice, irrespective of whether they were killed at 6, 18 or 26 weeks after cell injection. Macroscopically and histologically their lungs appeared normal.

Thyroid glands. The thyroid gland sections were those of normal-mouse thyroid gland with rather large peripheral follicles lined by cuboidal or low epithelium and containing an abundance of eosinophilic colloid.

B. *Intravenous injection of neoplastic, hyperplastic or normal thyroid cells into thyroxine deficient mice* (Table 3, Groups 1–5).

Lungs. In contrast to normal recipient mice, most of the thyroxine deficient animals injected with thyroid cells developed thyroid tissue in their lungs; and irrespective of whether the donor material was derived from neoplastic, hyperplastic or normal thyroid glands.

Macroscopically, the lungs of animals killed at 18 or 26 weeks after injection exhibited superficial nodules which varied in number and size. In some lungs only one or two minute nodules were present. In others, several nodules were noted, usually about 1 mm dia. and occasionally up to 2 or 3 mm dia. (Fig. 2). Histologically, all superficial nodules were identified as thyroid tissue. In addition, microscopic foci of thyroid tissue were observed deeper in the lung. The lungs of other animals, killed at 6 or 12 weeks after injection, contained only microscopic foci.

Histology of lungs containing thyroid deposits

I. After injection of *normal* or *hyperplastic* thyroid cells into MTU-treated mice (Table 3, Groups 2–4).

(a) General characteristics of such pulmonary deposits:

At 6 weeks after injection, the lungs of these recipients exhibited mostly small foci of thyroid tissue varying from one or two follicles (Fig. 3) to follicular aggregates of several follicles. At 12 weeks only follicular aggregates were present. Between 18 and 26 weeks many such aggregates consisted of larger foci of thyroid tissue some measuring 1 mm or more in diameter.

These lesions, some lying deep in the lung and others beneath the pleura, consisted of cuboidal or columnar epithelial cells forming

focal areas of irregularly shaped, colloid-containing follicles. Such follicles were often more numerous at the periphery of lesions. More centrally, the lesions consisted of aggregates of thyroid cells with less pronounced follicle formation but, occasionally, the follicular pattern predominated throughout. All colloid present was eosinophilic and PAS-positive. There was slight variation in the size of nuclei and mitoses were infrequent. Some of the nuclei appeared to be moderately vesicular. The presenting picture was that of simple hyperplastic thyroid tissue (Fig. 4).

Other features were the proliferation of thyroid cells and the formation of follicles within the pulmonary alveolar spaces and, also, the extension from one alveolus to another through the antra or the pores of Kohn. The intra-alveolar follicular structures were often avascular, their surface had no endothelial lining and appeared to be separated from the surrounding alveolar walls. Lymphocytic reaction was mostly absent but free vacuolated macrophages were occasionally seen. In general the morphology of pulmonary lesions after injection of normal or hyperplastic thyroid cells was similar.

(b) The position of the pulmonary deposits in relation to the pulmonary vasculature:

In the larger pulmonary deposits all trace of a vascular origin had been lost and only the intra-alveolar nature of the thyroid cells was discerned. But even in the lungs with small deposits of thyroid tissue (mostly from mice killed 6 weeks after injection) such deposits were observed as follicular or irregular structures (Fig. 3 and 5) predominantly in the air sacs. In serial sections, some of these deposits revealed no connection with intravascular growth.

The vascular origin of other small deposits was, occasionally, clearly seen. That is, they grew around engorged capillaries or other vessels with no evidence of intravascular growth (Fig. 6 and 7). However, intravascular growth was also observed in some capillaries (Fig. 8 and 9) and small arterioles (Fig. 10 to 13). The intravascular deposits grew beyond the endothelial wall and extended into the pulmonary alveolar space. In terminal arterioles such outward penetration and extension was observed to occur in the absence of dilatation of the vascular wall surrounding the intravascular part of the growth. It was also observed that these partly intravascular deposits formed a waist at the region of endothelial penetration (Fig. 12). No thyroid cells in either follicular or cluster forms were seen within large vessels of the lung. No thrombosis was found.

II. After injection of *neoplastic* thyroid cells into MTU-treated mice (Table 3, Group 1).

Lungs. Microscopically, the general features of thyroid growths present in the lungs of these animals were: (1) the large size of deposits even at 6 weeks after injection; (2) the invasion of bronchi (Fig. 14); and (3) a marked proliferation of thyroid cells with almost complete obliteration of acinar spaces (Fig. 15). Often a pulmonary lobe contained several desposits. The density of thyroid cells was at times remarkable. Epithelial hypertrophy, reduction of colloid and pronounced irregularities in follicle formation were predominant. Papillary infoldings within dilated cysts were occasionally seen. Sometimes, in follicular regions, nodules composed of closely packed thyroid cells were present. The nuclei in general, were vesicular and pale, giving the appearance of a decrease in the amount of chromatin. There was considerable variation in the size of nuclei and whereas mitoses in some areas were frequent in others they were seen occasionally. All colloid present was rather pale, but in some deposits, not all follicles contained PAS-positive colloid. Invasion of bronchi (Fig. 14), sometimes massive, was frequently noted as well as pulmonary macrophages engorged with clear droplets. Granular leucocytes were rarely seen but some lymphocytes were often present. In all pulmonary deposits of this group no trace of intravascular thyroid tissue was found.

Histology of thyroid glands of all MTU-treated recipients of thyroid cells

Thyroid-gland sections showed the typical effect of MTU treatment: diffuse follicular hyperplasia, epithelial hypertrophy, variations in the size and shape of follicles, increased vascularity, depletion of colloid and formation of some multinucleated epithelial cells.

In all sections examined there was no evidence of vascular or capsular invasion or metastasis of thyroid tissue to adjacent muscle tissue.

III. After injection of *hyperplastic* thyroid cells into ^{131}I*-treated mice* (Table 5).

Lungs. Histologically, almost all lesions seen in the lungs of this group consisted of follicular hyperplastic thyroid tissue residing extravascularly, in the air sacs (Fig. 16). However, in one animal, with a grossly enlarged pituitary gland weighing 88·5 mg, the pulmonary thyroid-tissue consisted of highly proliferative,

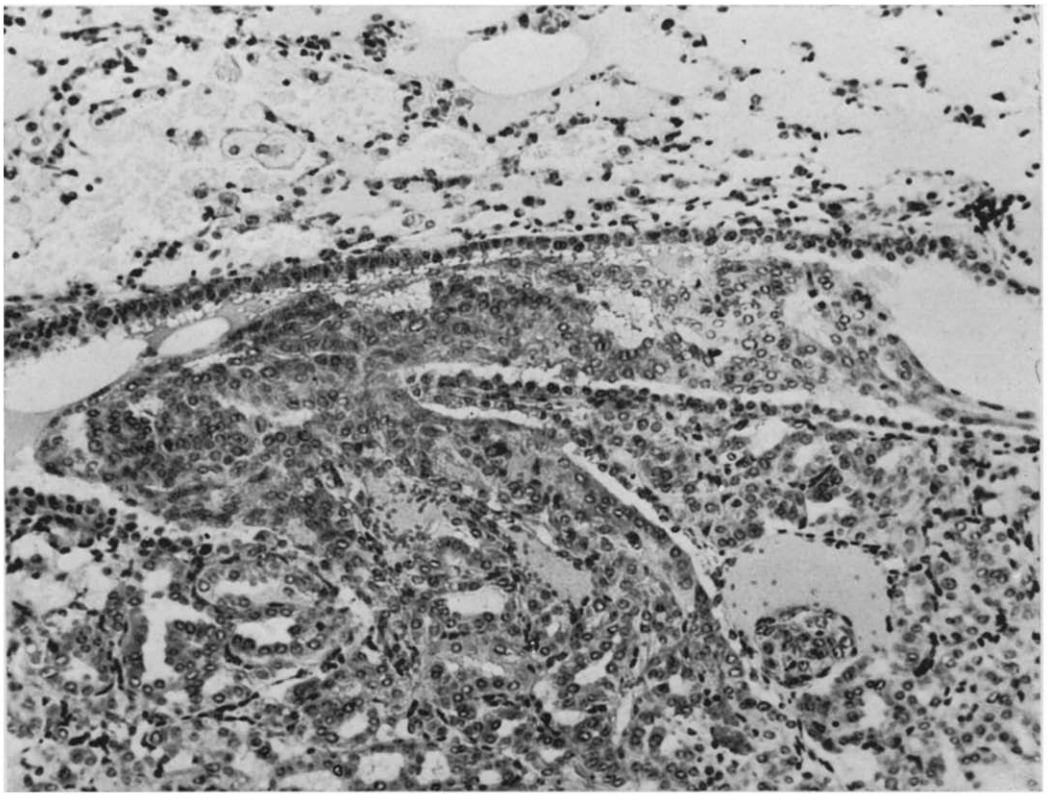


Fig. 1. *Lung section of donor mouse containing metastatic thyroid growth as the result of prolonged goitrogen treatment (administration of* 0·05% *methylthiouracil in the drinking water for a period of* 68 *weeks). Cellular details of thyroid tissue invading a bronchus are shown. H, E* × 200.

(to face p. 450)

Fig. 2. *Gross appearance of lungs (dorsal aspect) from mouse aged* 30 *weeks. At the age of* 12 *weeks the animal was injected i.v. with* 10^5 *normal thyroid cells. Two days prior to injection* 0·05% *methylthiouracil (MTU) was added in the drinking water. MTU treatment was continued for a period of* 18 *weeks. Note thyroid nodules on lung surface.* ×7·5.

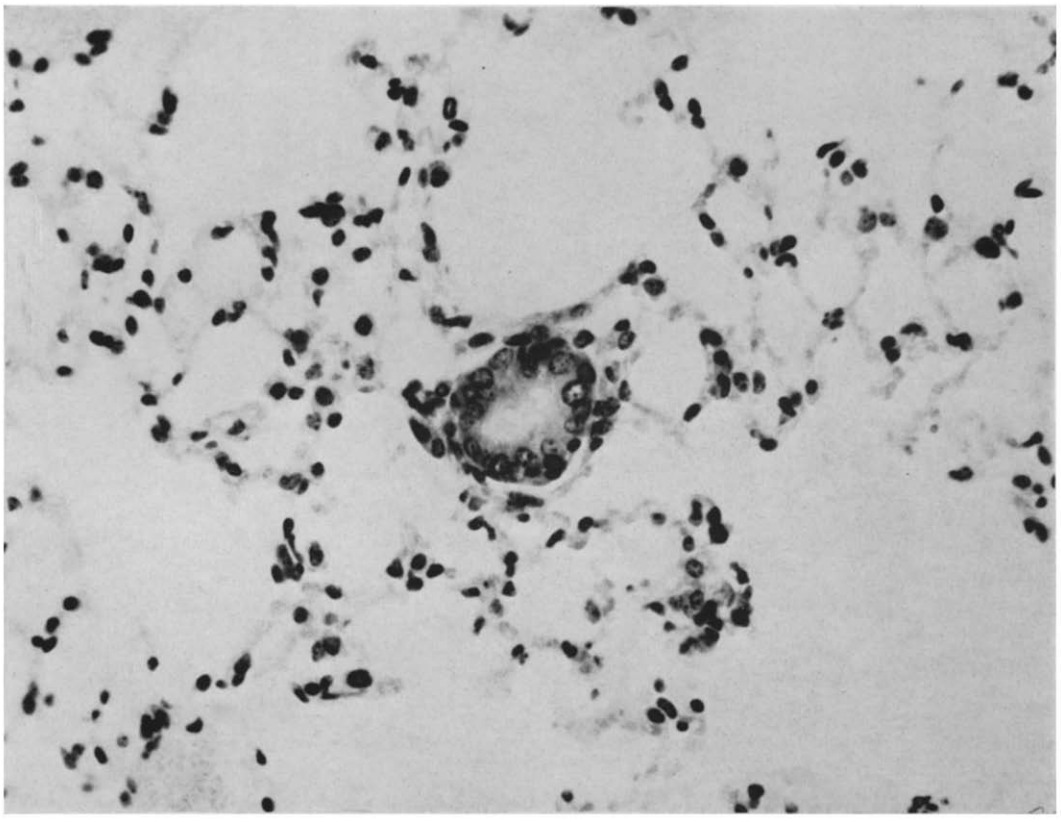


Fig. 3. *Lung section from MTU-treated mouse killed* 6 *weeks after i.v. injection of normal thyroid cells. A single thyroid follicle is present in this field, containing some colloid. The intra-alveolar nature of the thyroid follicle is evident. H, E* × 480.

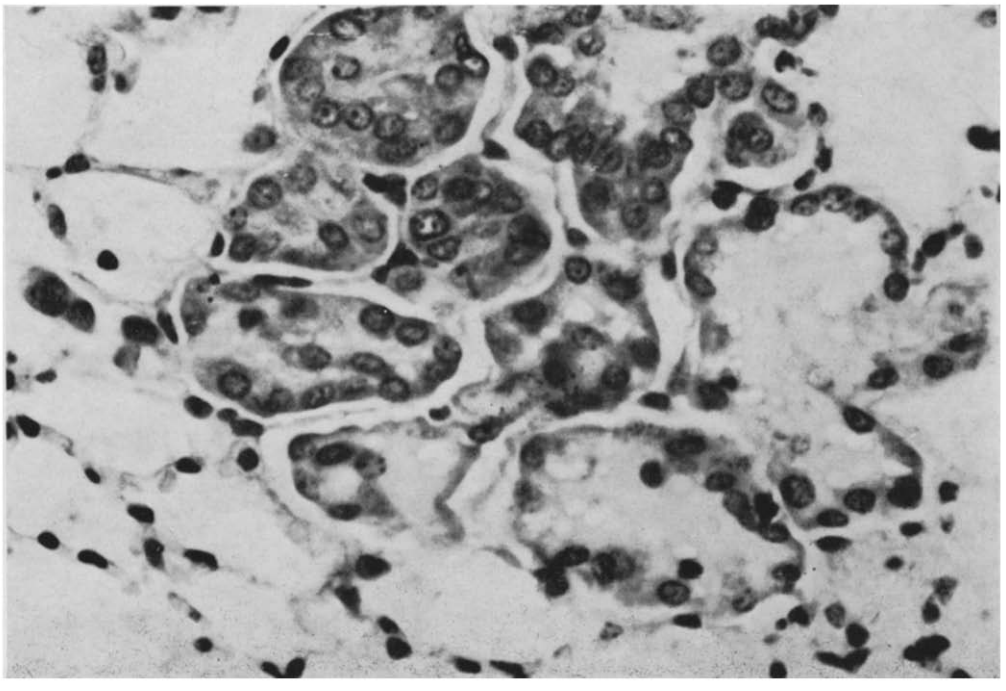


Fig. 4. *Lung section from MTU-treated mouse killed* 18 *weeks after i.v. injection of normal thyroid cells. There is simple hyperplastic thyroid tissue residing in the air sacs and containing PAS-positive colloid. PAS & Weigert's Haematoxylin.* × 800.

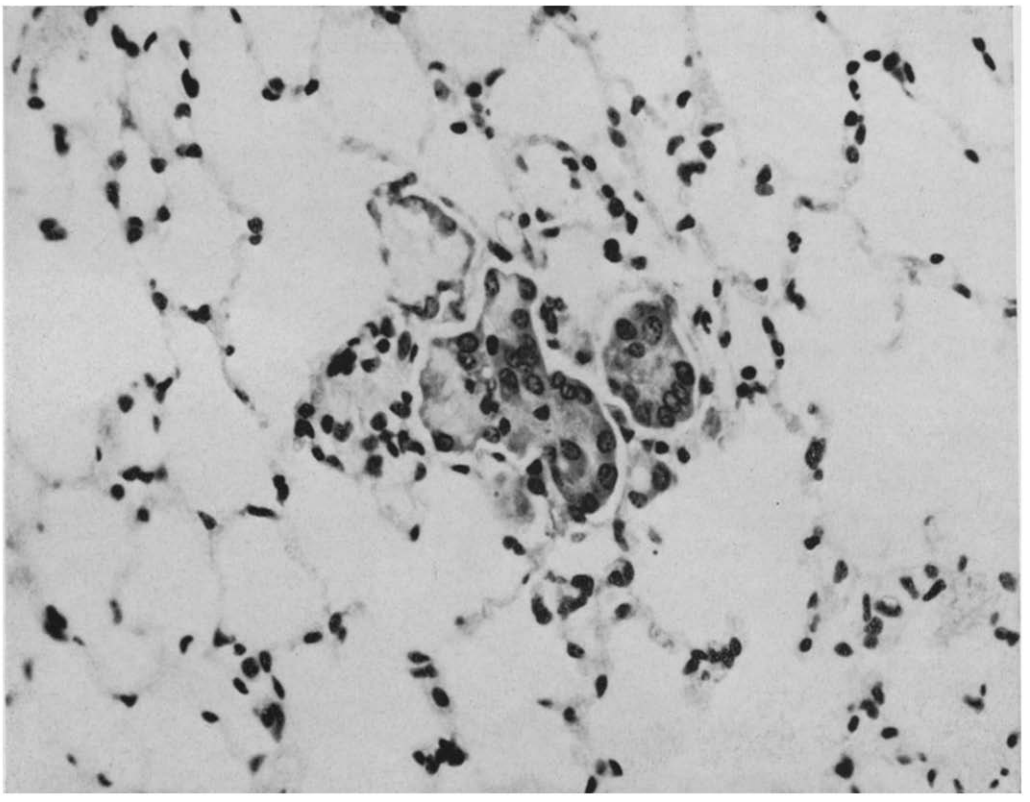


Fig. 5. *Lung section from similarly treated mouse as in Fig.* 3. *Small intra-alveolar aggregates of thyroid cells are present. Follicular structure is rudimentary. Consecutive sections of same lung revealed, in other areas, additional minute foci some consisting of one or two thyroid follicles. However, follicle formation by thyroid cells in the lung although frequent was not an invariable event. H, E* × 480.

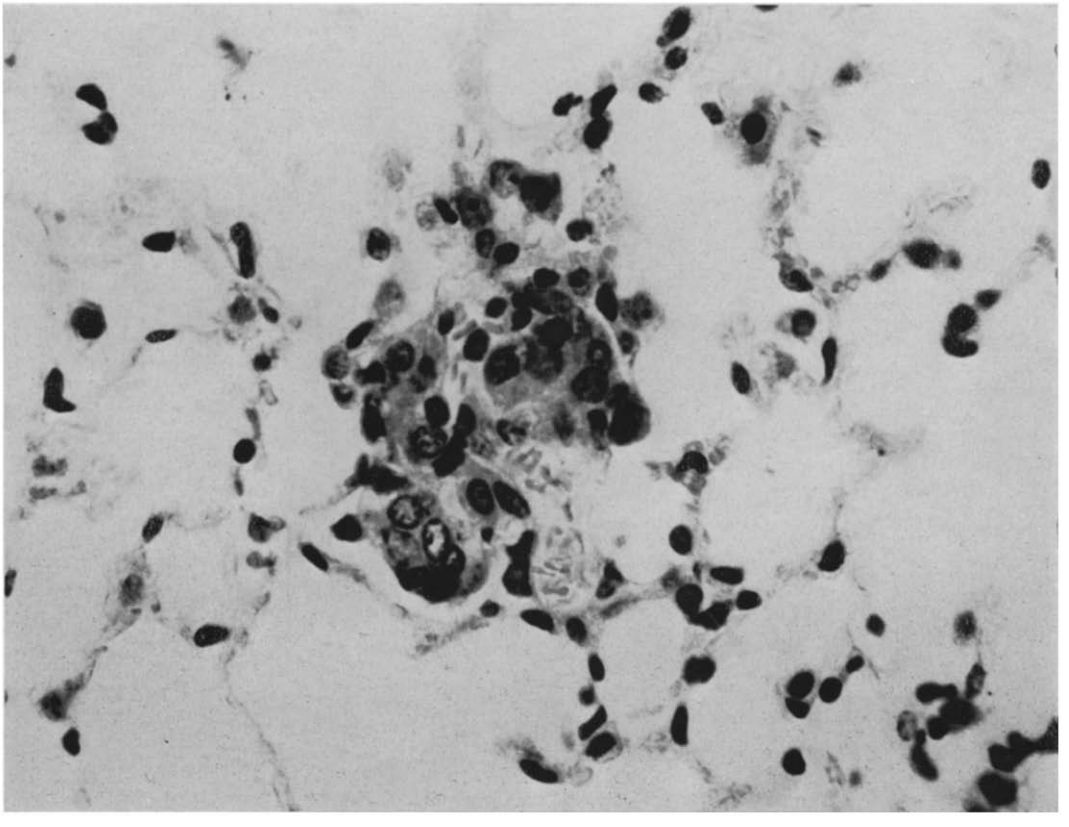


Fig. 6. *Lung section from a small pulmonary thyroid growth following i.v. injection of normal thyroid cells. The animal was killed* 6 *weeks after injection. Note perivascular proliferation of thyroid cells (from serial sections of the growth). H, E* × 750.

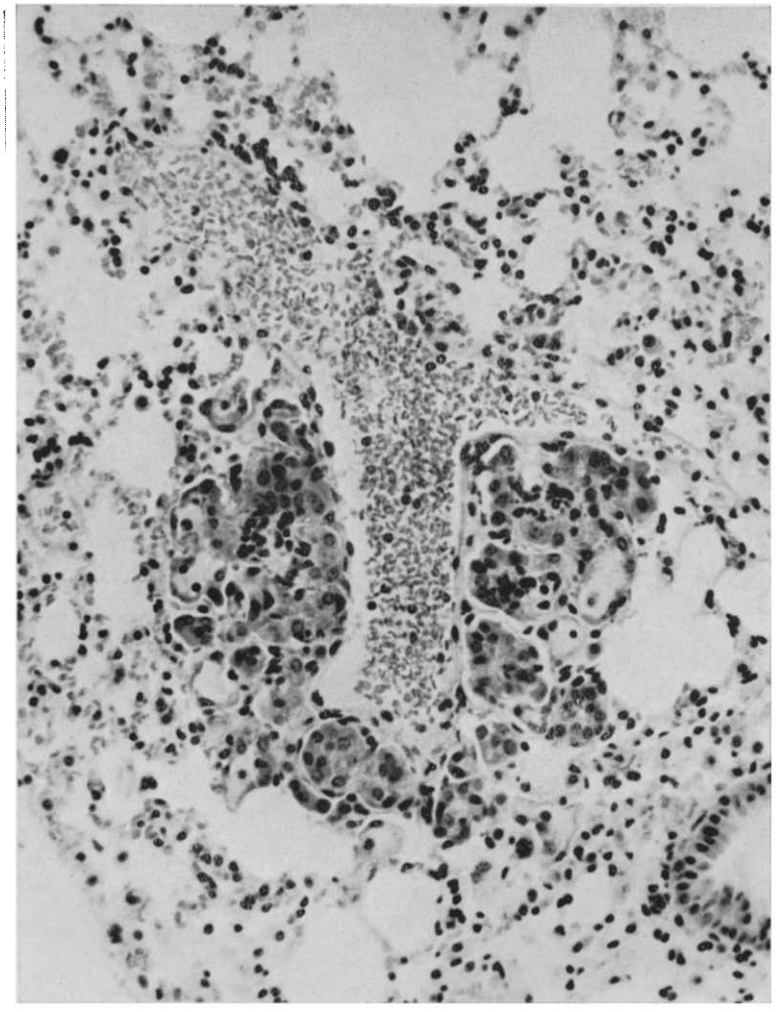


Fig. 7. Lung section from MTU-treated mouse killed 12 weeks after i.v. injection of hyperplastic thyroid cells. There is perivascular proliferation of thyroid tissue with no evidence of intravascular growth (from serial sections). H, E × 300.

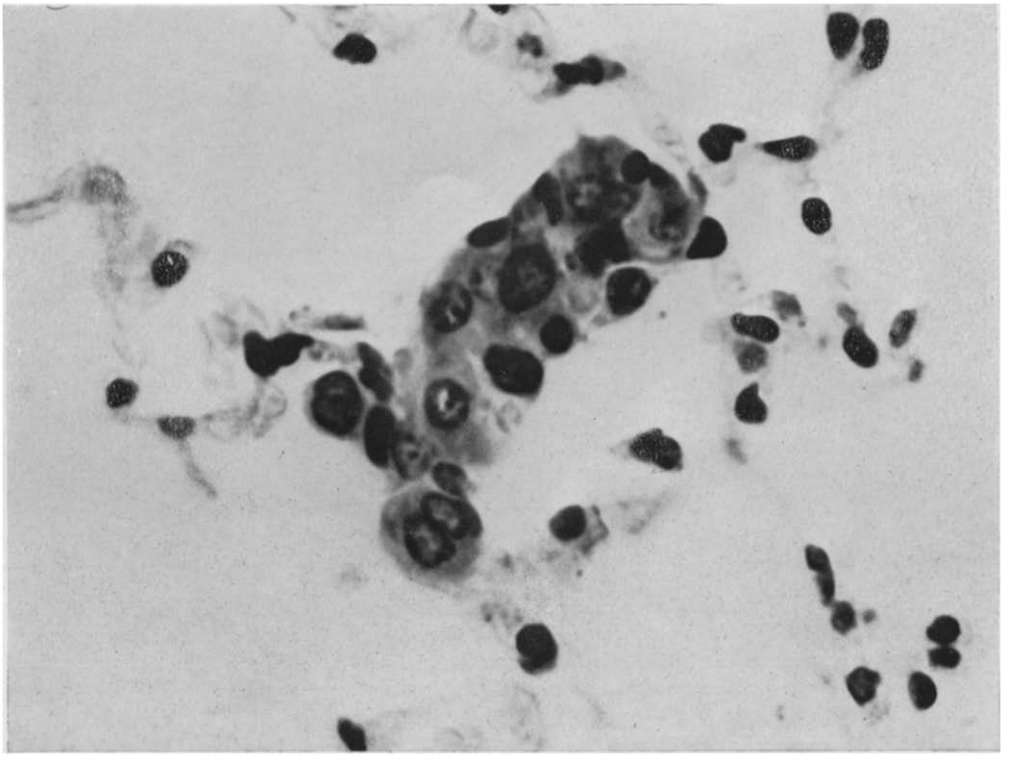


Fig. 8. Pulmonary capillary showing intravascular growth of thyroid cells. From mouse injected with normal thyroid cells and killed 6 weeks after injection. H, E × 1200.

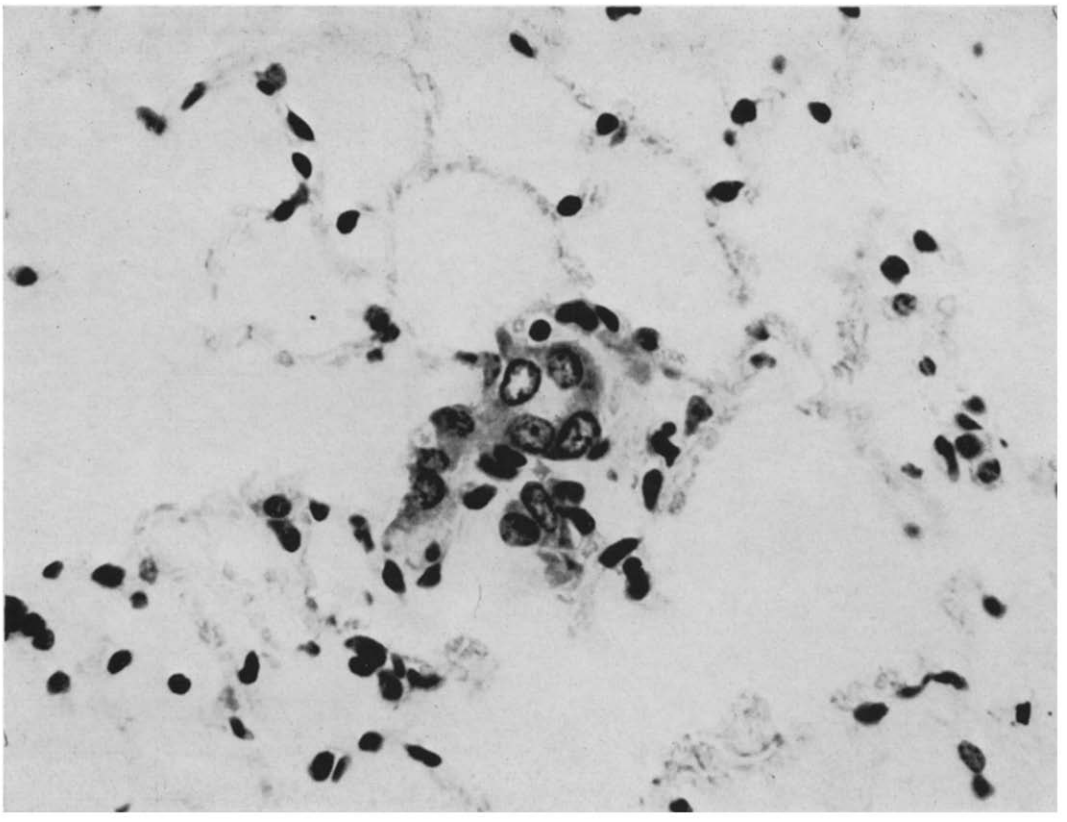
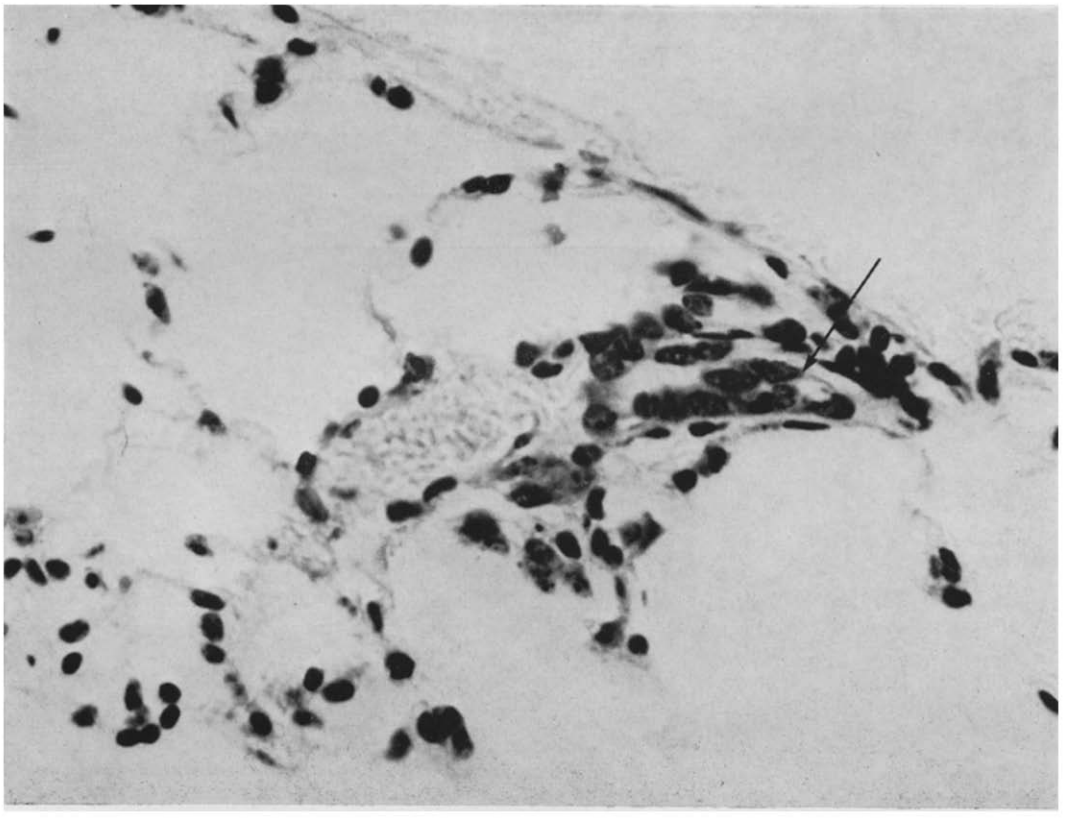


Fig. 9. *Intracapillary thyroid growth from another area of the lung of same mouse as in Fig.* 8. *Note early follicular structure extending into an air sac. H, E* × 900.



(*Figs.* 10–13 *are from serial sections of a small thyroid growth originating in a terminal arteriole, beneath the pleura, and induced by i.v. injection of hyperplastic thyroid cells. The figures demonstrate successive stages of: intravascular growth, penetration of the vascular wall and extension into a pulmonary alveolus. The recipient animal was killed* 6 *weeks after injection.*)

Fig. 10. *An intravascular thyroid growth is present* (*arrow*). *The vascular wall surrounding the growth appears to be continuous and is clearly seen. H, E* × 750.

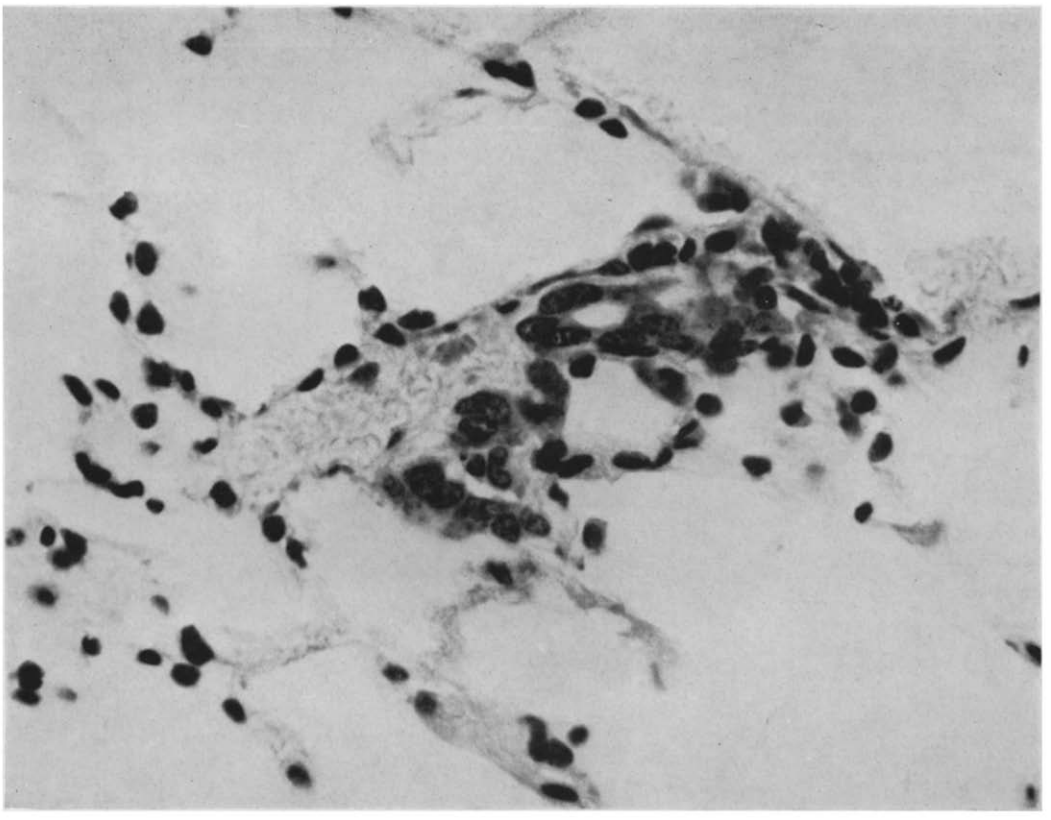


Fig. 11. *The endothelial wall above the intravscular growth is still evident. Below the growth the endothelial wall is obscure. To the left the arteriolar lumen is filled with blood. Its vascular diameter is similar to that part containing the intravascular growth. H, E* × 750.

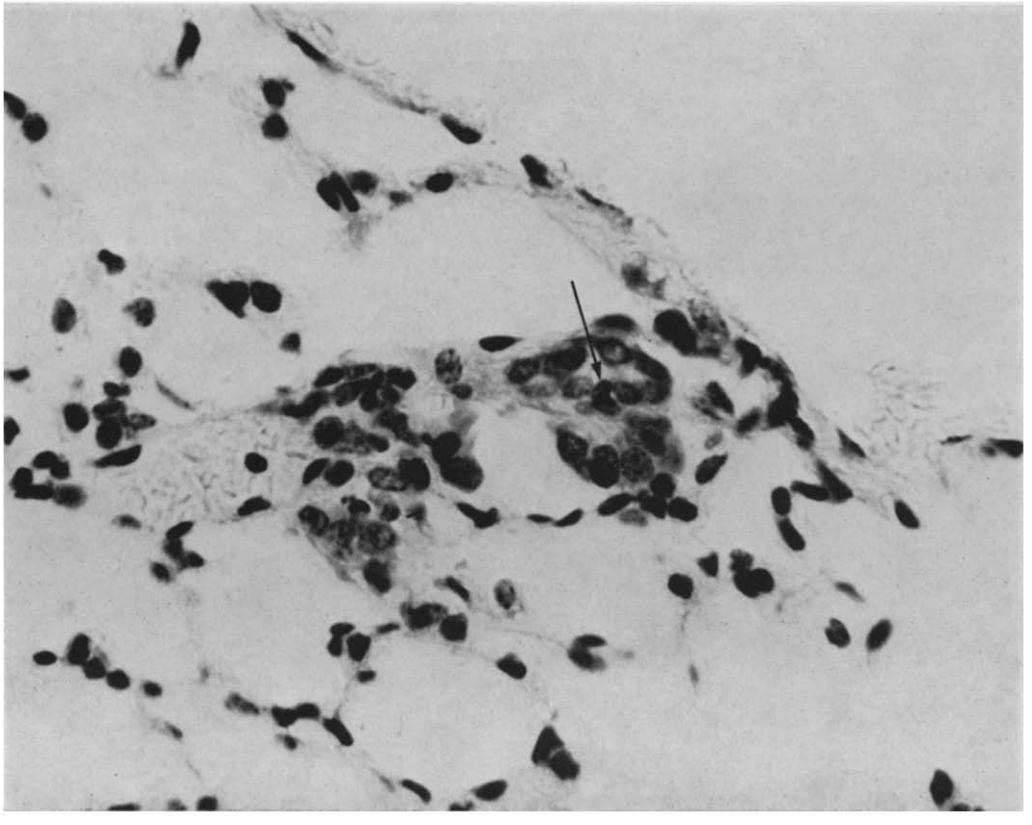


Fig. 12. *The intravascular growth has penetrated the vascular endothelium and has extended into an adjacent alveolus. Note formation of a waist by the growth at the region of endothelial penetration. At this region, one cell-nucleus of the growth (arrow) is kidney shaped probably as the result of the penetration. H, E* × 750.

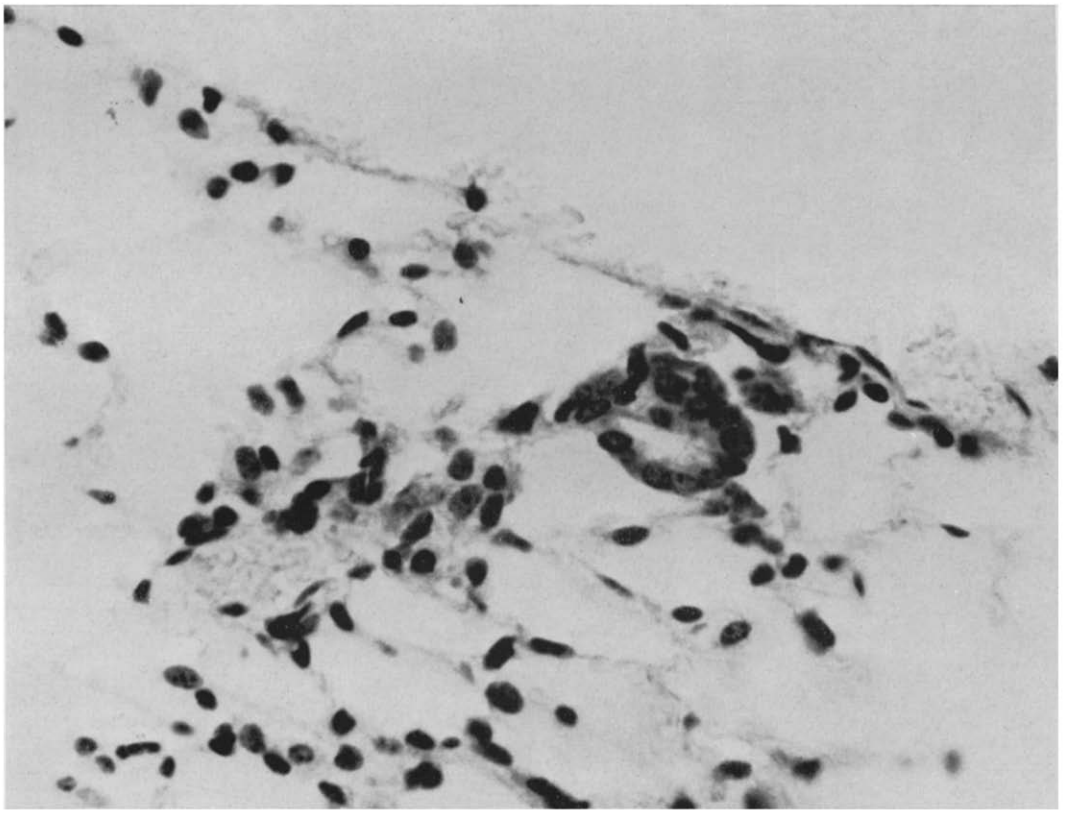


Fig. 13. *The sectioning has almost exhausted the arteriole containing the growth and mostly the extravascular part of the growth remains. H, E* × 750.

Fig. 14. *Lung section demonstrating invasion of a bronchus by neoplastic thyroid tissue, as the result of i.v. injection of neoplastic thyroid cells into an MTU-treated mouse. The animal was killed* 12 *weeks after injection. H, E* × 200.

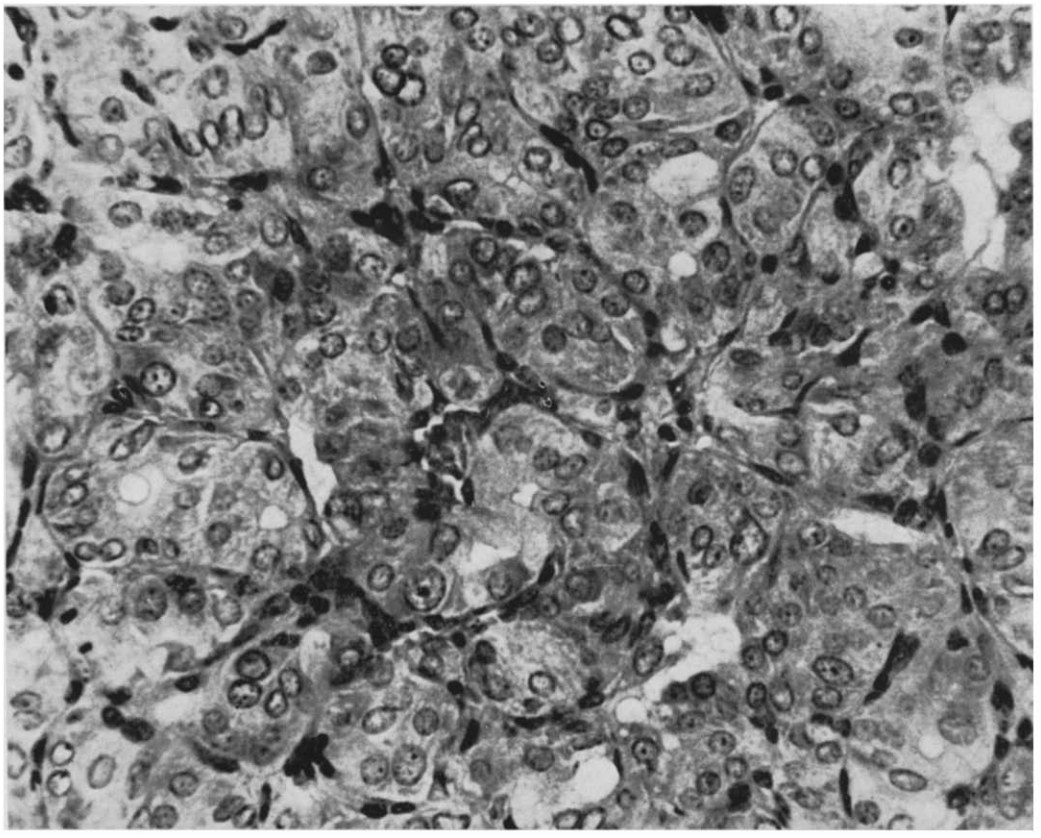


Fig. 15. *Cellular details from neoplastic thyroid growth shown in Fig.* 14. *There is a marked proliferation of thyroid cells with loss of polarity and almost complete obliteration of acinar spaces. H, E* × 512.

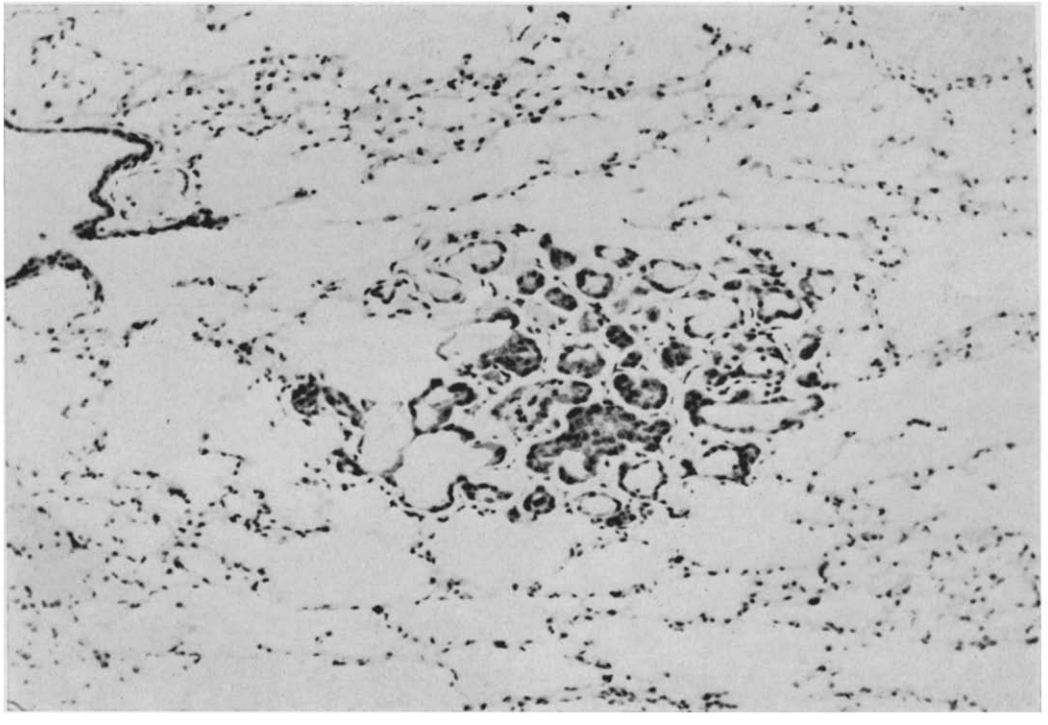


Fig. 16. *Lung section from* ^{131}I*-treated mouse injected i.v. with hyperplastic thyroid cells. The animal was killed* 13 *weeks after injection. It shows the intra-alveolar proliferation of follicular hyperplastic thyroid tissue. H, E* × 200.

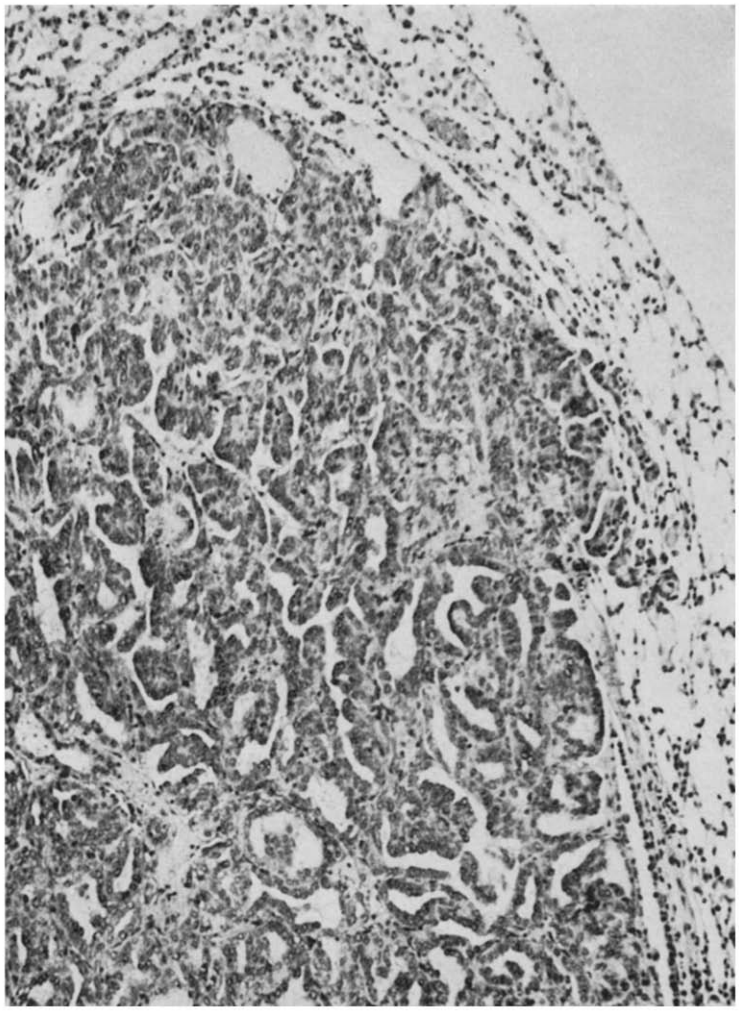


Fig. 17. *Lung section from* ^{131}I*-treated mouse killed* 26 *weeks after injection of hyperplastic thyroid cells. The animal's pituitary gland was grossly enlarged, weighing* 88·5 *mg. A large thyroid growth (papillary adenocarcinoma) is present in the lung.* $H, E \times 135$.

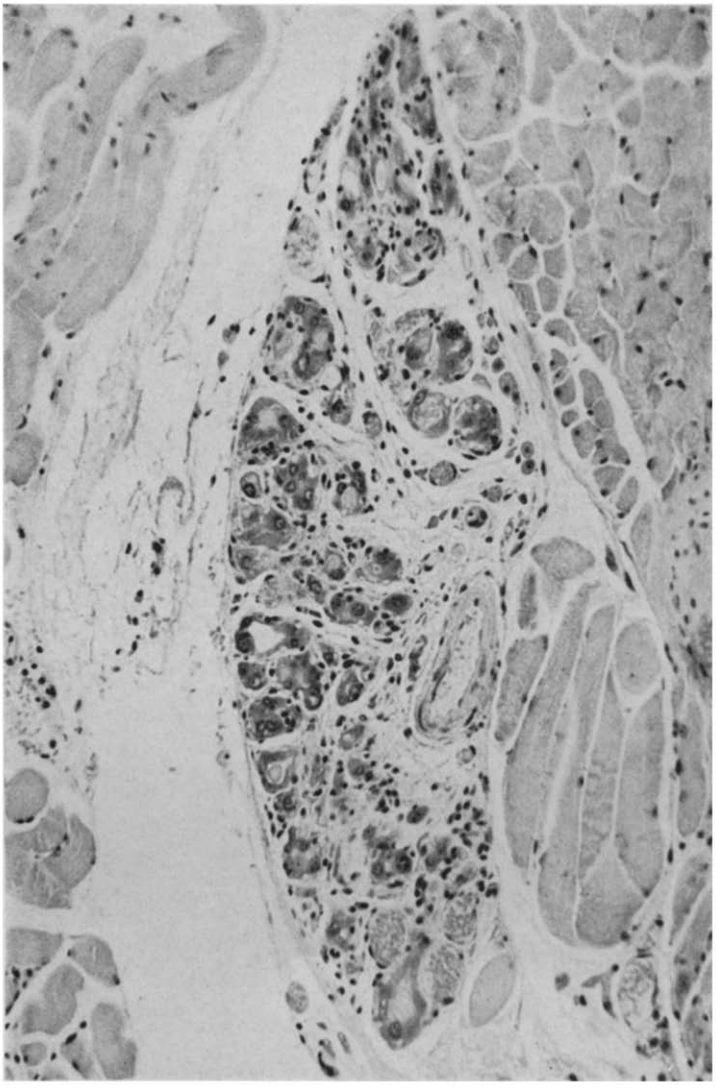


Fig. 18. *Thyroid gland remnants of same mouse as in Fig.* 17 *following irradiation with* 42μ*C of* ^{131}I. *The mouse was killed* 68 *weeks after subcutaneous administration of* ^{131}I. *The section shows some scanty and poorly formed follicular structures with some fibrosis and absence of stimulation of the gland. H, E* × 364.

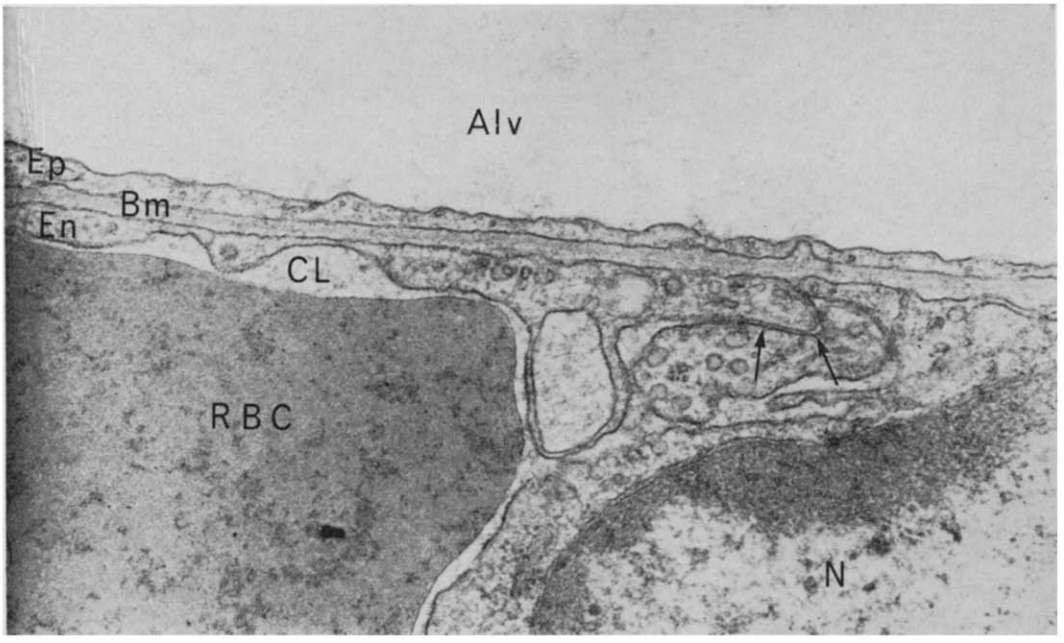


Fig. 19. *Section from mouse lung following* 23 *weeks of MTU treatment. Electron micrograph of an alveolar septum showing a continuous alveolar epithelium (Ep), basement membrane (Bm) and capillary endothelium (En). Arrows indicate an endothelial-cell junction and N represents a portion of an endothelial-cell nucleus. Part of an erythrocyte (RBC) is seen within the alveolar capillary lumen (CL). Alv indicates alveolar space.* ×32·000.

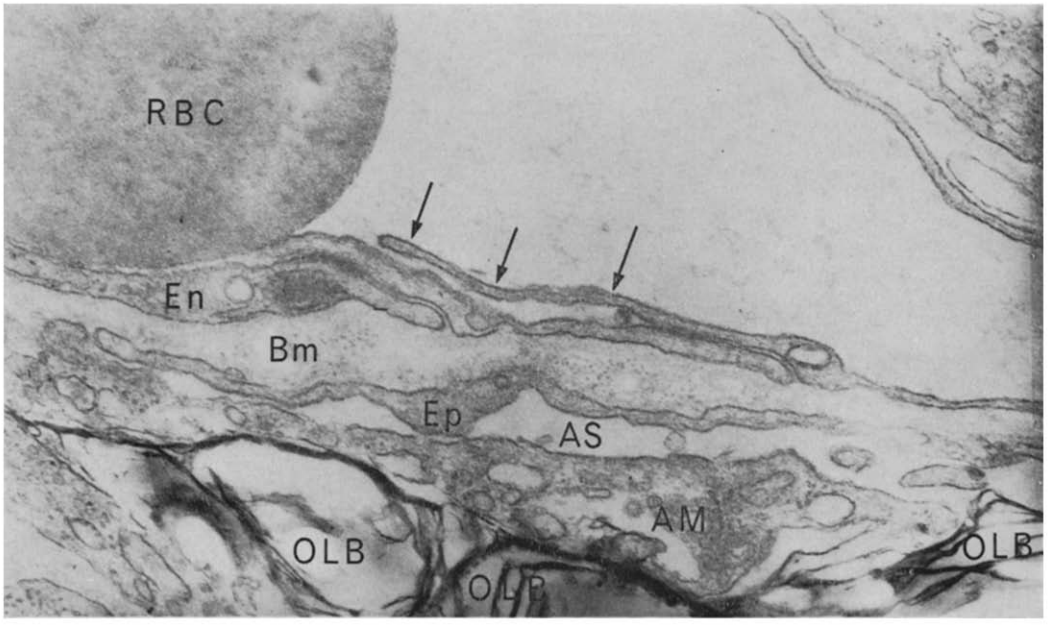


Fig. 20. *Section from mouse lung following* 57 *weeks of MTU treatment. Electron micrograph of an alveolar capillary containing an endothelial flap (arrows) with its tip pointing towards an erythrocyte (RBC). The capillary endothelium (En) and basement membrane (Bm) are continuous. The alveolar epithelium (Ep) is separated by a narrow air-space (AS) from an alveolar macrophage (AM) which contains osmiophilic lamellated bodies (OLB).* ×32·600.

papillary acinar and solid, structures with evidence of bronchial invasion. The presenting picture was that of a papillary adenocarcinoma (Fig. 17).

Thyroid gland remnants. The changes in the thyroid gland of mice and rats by ^{131}I administration have been fully described by Gorbman [15] and others [16, 17].

In the present experiments, thyroid tissue remnants were identified, microscopically, in all but one of ^{131}I-treated mice. Stenosing tracheitis was not seen. Although it is presumed, at least in those mice bearing enlarged pituitaries that their blood contained large amounts of thyrotrophic hormone [18], the remnants of tracheal thyroid tissue examined microscopically exhibited no evidence of proliferation. These remnants, therefore, were not capable of responding to stimulation by thyrotrophic hormone as non-irradiated thyroid cells do. Histologically (Fig. 18), the thyroid remnants contained, in some areas, a few irregularly shaped follicles, whereas in other areas the follicular pattern was absent. Some follicles contained a little colloid and others contained none. There was great variation in the size and shape of thyroid cells. The cytoplasmic–nuclear ratio was decreased. Some very large nuclei were observed containing fine chromatin granules but the presence of dispersed nuclear vacuoles gave an appearance of nuclear pallor. Some of the nuclei contained a slightly purple inclusion body. Mitotic figures were absent. Neighbouring muscle tissue and non-thyroid structures looked normal. The dissimilarity between the tracheal thyroid remnants and the intrapulmonary foci of thyroid tissue was evident.

C. *Injected controls*

Histologically, all lung sections of 6 mice, injected i.v. with PBS solution devoid of cells and treated with MTU for 6 or 26 weeks, looked normal. Their thyroid-gland sections revealed a diffuse follicular hyperplasia with no evidence of invasive growth.

D. *Non-injected controls* (*Table* 6).

Lungs. Whole lungs were sectioned serially. In all sections examined no evidence of thyroid tissue was found.

Thyroid glands. All sections showed the classical effect of MTU treatment as described above. There was no evidence of invasive growth in the gland, capsule or adjacent muscle tissue.

E. *Ultrastructure of pulmonary alveolar walls of MTU-treated mice*

The electron microscopic morphology of the human and mouse lung has been described by various investigators [13, 19, 20]. It has been shown that the capillary endothelium and alveolar epithelium are continuous without pores and both contain a full complement of intracellular organelles. A continuous basement membrane has also been noted.

The above observations are also confirmed in the present work involving the lungs of two MTU-treated NZO mice. The alveolar epithelium, basement membranes and capillary endothelium were continuous (Fig. 19) and appeared similar to those described by Karrer [13]. In over 400 sections examined, from several blocks, no true discontinuities at either the endothelial-cell junctions or of the endothelial cytoplasm were observed in capillaries, or other thin-walled vessels.

However, some elongated endothelial flaps in the lumen of the lung capillaries were noted (Fig. 20). Such endothelial flaps, arising from the endothelial surface of capillaries, are not uncommon and have been observed by others in various organs and in a number of mammals —see review by Majno [21]. The presence of endothelial flaps in the capillary endothelium of the mouse lung has not previously been reported. These and other electron microscopic observations, related to the presence of thyroid nodules in the lungs of NZO mice, have been more fully reported elsewhere [9].

DISCUSSION

The absence of thyroid tissue in the lungs of *normal* recipient mice after injection of neoplastic, hyperplastic, or normal thyroid cells was interpreted as follows: (1) inability of the injected cells to survive and proliferate in the lung in the absence of an excess of TSH; (2) survival in the lung in a state of mitotic arrest. The second hypothesis was tested by further experiments with normal thyroid cells and the results have been discussed elsewhere [10].

However, the development of thyroid tissue in the lungs of *stimulated* recipient mice after i.v. injection, indicates that *dissociated* thyroid cells are capable of extra-thyroidal proliferation when there is an excess of TSH secretion. In addition, the inability of the neoplastic thyroid cells to grow in the lungs of normal mice and their proliferation in the lungs of stimulated mice demonstrates that such tumour cells were TSH dependent. This is in conformity with the hormone dependence of goitrogen-induced tumours in rodents [7, 26].

Apart from the TSH-dependent thyroid tumours, there are other types of non-neoplastic thyroid growth some dependent and others independent of TSH secretion. These have been discussed by Doniach [22] and include the type known as reparative thyroid growth. This term implies the reconstitution of a thyroid autograft (in about 10 days in the rat) from surviving peripheral follicles, following ischaemic necrosis of most of the graft. Loeb [25] has demonstrated that this type of thyroid growth is capable of invading blood vessels and adjacent fat and muscle tissue but in contrast to carcinomatous growth, it is self limited. Reparative thyroid growth is not inhibited or prevented by thyroxine administration [23] or hypophysectomy [22] and appears to be independent of deficiency of circulating thyroid hormone [24]. The stimulus for reparative growth is thought to be connected with the ischaemic necrosis of the graft. In the present experiments the intravenous injection of dissociated thyroid cells into highly inbred animals is, in a way, a type of thyroid autograft but with some differences: the graft consisted of single cells with no stroma of their own and ischaemic necrosis could not occur; and whereas the stimulus for reparative growth is independent of TSH, the stimulus for proliferation of the injected cells was excess of thyrotrophic hormone. Therefore, the invasive growth (penetration and extension beyond the vascular endothelium) observed in the present experiments, by injection of non-neoplastic thyroid cells, cannot be attributed to invasive growth by regenerating thyroid tissue.

Furth [18] was first in demonstrating the development of nodules of thyroid tissue in the lungs of some prepared recipient mice after i.v. injection of minute thyroid *fragments*. The thyroid fragments were obtained from TSH-induced thyroid adenomas. The recipient mice were prepared by intramuscular implantation of autonomous, TSH-secreting pituitary tumours. The aim was to test the possibility of malignant transformation of the pulmonary thyroid-nodules in recipients secreting an excess of TSH. Furth reported the same degree of stimulation in the thyroid nodules in the lung and in the animal's own thyroid gland but observed no malignant transformation.

In the present experiments with normal or hyperplastic thyroid cells, the degree of stimulation of the pulmonary deposits was, histologically, similar to the recipient's own thyroid gland. That is, both the thyroid gland and the induced pulmonary deposits exhibited a simple hyperplasia. However, there was a striking dissimilarity between pulmonary deposits and thyroid gland remnants in the case of ^{131}I-treated mice and also in stimulated animals injected with neoplastic thyroid cells. In the ^{131}I-treated recipients, the thyroid gland remnants exhibited the typical radiation effect with no evidence of stimulation (Fig. 18). In contrast, their pulmonary deposits consisted of follicular hyperplastic thyroid tissue (Fig. 16). But in one mouse with a grossly enlarged pituitary gland (wt. 88·5 mg) the pulmonary deposits were, histologically, those of a thyroid papillary adenocarcinoma (Fig. 17).

The proliferative, rather solid pulmonary deposits occurring in stimulated mice, after injection of neoplastic thyroid cells, often contained morphological features indistinguishable from malignant growths (Fig. 14 and 15). This was in contrast to the simple follicular hyperplasia of their own thyroid gland. Such dissimilarity supports the belief that the origin of the pulmonary deposits was the injected cells. Furthermore, the absence of thyroid tissue in the lungs of control mice, indicates that the periods of MTU stimulation used in recipients of thyroid cells, were not likely to have produced metastases of their own thyroid tissue to the lung.

The mode of dissemination of proliferating thyroid tissue in the lungs of mice, following i.v. injection, appears to be the same with all kinds of dissociated thyroid cells used. Namely, intra-alveolar proliferation followed by extension from one alveolus to another through the antra or the pores of Kohn. Excitation of fibrous tissue was not observed. In the more solid structures of large pulmonary deposits (as seen after injection of neoplastic thyroid cells) the elimination of many of the alveolar septa was probably due to pressure atrophy. Whether such tumours were capable of elaborating lytic substances, as demonstrated in malignant tumours by Sylvén and Bois [27], is not known.

The presence of intrafollicular PAS-positive colloid in pulmonary lesions, derived from the injection of normal or hyperplastic cells (Fig. 4), was interpreted as a sign of follicular cell activity related to storage of thyroid hormone. However, in less differentiated pulmonary deposits (Fig. 15) derived from the injection of neoplastic thyroid cells, PAS-positive colloid was not present in all follicles. This is in agreement with the observations by Valenta and Jirasek [28] who found that in poorly-differentiated human thyroid tumours PAS-positive colloid was mostly absent.

TSH-dependent transplantable thyroid tu-

mours have shown varying degrees of impairment of thyroxine synthesis [8, 26]. It will be of interest to find out, by further experiments, to what extent iodine is bound organically in the various pulmonary deposits induced by the injection of dissociated thyroid cells.

One of the lysosomal enzymes is the non-specific esterase resistant to diethyl-*p*-nitrophenyl phosphate. The activity of this non-specific esterase (NE) is TSH dependent. In the rat it is accentuated after stimulation by thyrotrophic hormone and is diminished after administration of thyroxine [29]. Valenta and Jirasek [28] have found that NE activity is often present in the colloid of human thyroid carcinomas but not in benign thyroid growths. They also reported that in non-differentiated thyroid tumours NE was mostly absent. Whether NE activity was present in pulmonary deposits induced by the injection of *neoplastic* thyroid cells is unknown.

In experimental blood-borne metastases many investigators have used tumour material from rapidly growing, poorly differentiated tumours [5]. Such tumour material appears to be readily transplantable [1]. Experimental studies by others have further demonstrated that i.v. inoculation of tumour cells does not invariably produce metastases [2, 30]. For this, several explanations have been proposed. The various hypotheses advanced and some of the related experimental evidence have been discussed by a number of authors [6, 31, 32]. Experimental evidence suggests that only a small proportion of the i.v. injected tumour cells survive at the site of arrest and form metastases [1, 33, 34]. As to the site of arrest the capillaries of the lung are a frequent site of lodgement by tumour cells although arrest in arterioles may also occur [2, 3]. Capillary arrest appears to be independent of capillary diameter, vasomotor activity or rate of blood flow. The subsequent mode of endothelial penetration by tumour cells, *in vivo*, has been described by Wood [4].

In the present work, with i.v. injection of non-neoplastic thyroid cells, the results indicate that their lodgement in the lung occurs in capillaries and arterioles. Furthermore, their establishment in the lung is accomplished in two ways: (1) by production of an initial intravascular growth in capillaries and arterioles followed by penetration of the vascular wall; (2) by initial migration of thyroid cells to an extravascular position and subsequent growth. Both methods indicate that non-neoplastic thyroid cells are capable of penetrating the endothelial barrier in a manner somewhat similar to that described in tumour cells [5]. This is inferred from the presence of thyroid cells in the pulmonary alveolar spaces and also from the perivascular nature of some small pulmonary deposits. Serial sections of such deposits revealed absence of intravascular growth. In addition, a concomitant mode of endothelial penetration appears to be by initial intravascular proliferation, followed by extension of the growth beyond the endothelial wall as shown in Figs. 10–13. In terminal arterioles, the absence of distension of the arteriolar lumen by the intravascular growth, shows infiltration rather than mechanical stretching and breaking of the vascular wall. The mechanism involved in such infiltration is unknown.

That the penetration of the pulmonary vascular endothelium by non-neoplastic thyroid cells may not be exclusively related to stimulation by thyrotrophic hormone, is deduced from the dormancy of normal thyroid cells in the lungs of normal mice [10]. Furthermore, if TSH stimulation, which leads to cell proliferation, was solely responsible for endothelial penetration one should have expected penetration to occur mostly by initial intravascular growth. But even then, such benign intravascular growth (obtained by the use of normal or hyperplastic cells) seems capable of endothelial penetration.

Because of the slow progression of goitrogen-induced metastasizing tumours [26] and the necessity for keeping the present experiments uniform, mice injected with neoplastic thyroid cells were also sacrificed from 6 weeks onwards. However, in such mice, all pulmonary deposits were too advanced at 6 weeks. Therefore, the way by which these pulmonary growths were formed is not clear. Such animals should have been killed, at set periods, below 6 weeks. Since, however, all pulmonary deposits in these animals were *invariably* situated extravascularly, in the air sacs, it means that penetration of the vascular wall had occurred.

In experimental blood-borne metastases with other neoplastic cells (non-thyroid), administration of thyrotrophic hormone appears to have no effect on the number of pulmonary growths when compared to controls [5]. Similarly, thyroxine deficiency induced by the administration of propylthiouracil or thyroidectomy failed to alter the incidence, size, or extent of metastasis formation by Walker tumour cells [35].

As to the effect of MTU administration on the vascular endothelium of the lung of NZO mice, the results of the electron micro-

scopic investigation indicate that MTU treatment produced no structural defects. The pulmonary capillary endothelium and the endothelium of other thin-walled vessels was found to be continuous. In addition, the presence of extravascular thyroid tissue in the lungs of recipients treated with no MTU (^{131}I-treated), is further evidence that penetration was not due to any direct effect of the goitrogen on the vascular endothelium of the lung.

Various reports exist in the literature as to the effect of trypsin on normal cells with regard to the integrity of their plasma membrane [36]; breakage of their chromosomes with probable mutagenic action [37]; alteration of some of their surface properties [38, 39]; or the acquisition of ultrastructural changes similar to those of some malignant cells. Following electron microscopic studies of trypsinized human amnion cells, Edwards and Fogh [40] reported that such cells acquire micromorphological features like those seen in certain malignant cells. However, transplantation studies with trypsinized normal amnion cells have failed to produce tumour formation [41].

Active locomotion may be observed in most of the normal cells of vertebrates when cultured *in vitro* [42] and appears to be the result of the undulations of their cytoplasmic membrane [43]. Direct evidence of active locomotion *in vivo*, by dissociated non-neoplastic thyroid cells is lacking. Such evidence exists with V_2 carcinoma cells, in the rabbit ear chamber, and with normal lymphocytes and granular leucocytes [44]. However, the development of perivascular thyroid tissue with absence of intravascular growth (Fig. 7) indicate migration of non-neoplastic thyroid cells through the vascular wall. That such migration is not due to malignant transformation by trypsin has been discussed elsewhere [10].

Haematogenous autotransplantation of normal tissue *fragments* within blood vessels of the lung, after trauma, is known from human pathology. In endometriosis, viable benign endometrial tissue, consisting of both glands and stroma, has been reported in the lung and other organs [45]. In experimental studies, the i.v. injection of minced tissue from various normal organs has demonstrated the ability of minute tissue *fragments* to survive in blood vessels of the lung [46]. Embolism by such tissue is not metastasis. Equally, the production of benign thyroid growths in the lungs of mice by i.v. injection of non-neoplastic thyroid cells is not metastasis. For in spontaneous metastases, by blood-borne cancer cells, endogenous factors on the part of the host are thought to be also involved, in deciding the eventual character of biological behaviour of a given type of tumour cells.

However, the way by which these benign thyroid growths establish themselves in the lung bears such degree of similarity to the morphogenesis of blood-borne pulmonary metastases, as to raise the question: is the vascular endothelium container of and barrier to blood-borne cancer cells [5]; or, is a neoplastic state essentially the underlying cause of endothelial attachment and penetration?

It may be of some interest if further work indicates whether the mode of penetration shown by non-neoplastic thyroid cells is equally shared by dissociated epithelial cells from other normal tissues.

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RESUME

L'injection intraveineuse de cellules thyroidiennes dissociées, provenant de glandes neoplasiques, hyperplasiques et normales, chez la souris, met en évidence la capacité de ces divers types de cellules de pénétrer l'endothélium vasculaire du poumon. Une telle pénétration est démontrée chez les animaux receveurs qui secrètent un excès d'hormone thyréotrope.

Les cellules thyroïdiennes non néoplasiques semblent s'établir dans le poumon suivant des processus semblables à ceux qui sont observés dans les métastases expérimentales hématogènes de diverses cellules tumorales, c'est-à-dire: (a) par une prolifération intravasculaire initiale dans les capillaires ou les artérioles, prolifération suivie de la pénétration de la paroi endothéliale et de l'extension dans l'espace alvéolaire adjacent; (b) par la migration initiale des cellules thyroïdiennes vers l'espace extravasculaire, et leur croissance ultérieure.

Le fait que la pénétration de l'endothélium vasculaire du poumon, par des cellules thyroïdiennes normales, peut aussi s'observer en l'absence de stimulation par l'hormone thyréotrope est discuté.

A moins que le mode de pénétration, mis en évidence dans ce travail, ne soit particulier aux cellules thyroïdiennes, on peut mettre en doute l'hypothèse selon laquelle la pénétration de l'endothélium vasculaire du poumon, par des cellules cancéreuses circulantes, serait liée à des capacités invasives propres aux cellules cancéreuses.

SUMMARY

Parallel experiments with intravenous injection of dissociated thyroid cells derived from neoplastic, hyperplastic and normal thyroid glands of inbred mice, indicate common ability of penetration of the vascular endothelium in the recipient's lung. Such penetration is demonstrated in recipient mice secreting an excess of thyrotrophic hormone.

Non-neoplastic thyroid cells appear to establish themselves in the lung in some ways similar to those observed in experimental blood-borne metastases with various tumour cells. That is: (a) by initial intravascular proliferation, in capillaries or arterioles, followed by penetration of the endothelial wall and extension into adjacent alveolar space; and (b) by initial migration of thyroid cells to an extravascular position and subsequent growth.

That penetration of the pulmonary vascular endothelium by dissociated normal thyroid cells may also occur in the absence of stimulation by thyrotrophic hormone, has been discussed.

Unless the mode of penetration shown in the present work is peculiar to thyroid cells, it is questionable whether penetration of the pulmonary vascular endothelium, by blood-borne cancer cells, is related to invasive properties special to cancer cells.

ZUSAMMENFASSUNG

Parallel laufende Versuche mit intravenös injizierten dissozierten Thyreoideazellen von neoplastischen, hyperplastischen und normalen Schilddrüsen von Inzuchtmäusen zeigen die generelle Fähigkeit dieser Zellen durch das Gefässendothel in die Lunge des Empfängertieres einzudringen. Diese Penetration lässt sich durch einen Überschuss an thyreotropem Hormon im Sekret der Empfängermäuse nachweisen.

Die nicht-neoplastischen Thyreoideazellen scheinen sich in der Lunge in ähnlicher Weise festzusetzen wie es bei experimentellen intravenös gesetzten Metastasen verschiedener Tumorzellen der Fall ist: (a) durch anfänglich intravasculäres Wachstum in die Kapillaren oder Arteriolen dann Durchtritt durch das Endothel und Ausbreitung im anschliessenden Alveolarraum; (b) durch anfängliche Wanderung von Thyreoideazellen zu einem extravasculären Ort und von da ausgehendem Wachstum.

Die Tatsache, dass eine Durchwanderung der Lungengefässwände durch dissozierte normale Thyreoideazellen auch ohne die Stimulation durch thyreotrope Hormone erfolgen kann, wird diskutiert.

Wenn man annimmt, dass die in der vorliegenden Arbeit dargestellte Art des Penetrierens der Gefässwand nicht spezifisch für Thyreoideazellen ist, dann erscheint es fraglich, ob der Durchtritt durch das vasculäre Endothelium der Lunge bei Krebszellen aus der Blutbahn mit spezifischen Eigenschaften der Krebszellen verbunden ist.

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Chromosome Studies in a Case of Benzene-Induced Erythroleukaemia*

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INTRODUCTION

CHROMOSOME studies in some cases of acute erythroleukaemia (Di Guglielmo's syndrome) have been reported in the last few years [1–9]. As in acute leukaemias the chromosome patterns are quite variable, but in most cases one or more cell lines with abnormal karyotypes were found [2–9]. In the present paper a case of benzene-induced erythroleukaemia is reported in which chromosome studies were carried out.

CASE REPORT

S.M., female, age 37. Nothing important in the family history. One normal pregnancy at the age of 29, one spontaneous abortion at the age of 31. The patient had been working from 1959 to 1966 in a small factory of beauty cases; she usually worked at packing, but she was also often engaged in the mounting of the cases with a glue containing latex and para rubber, which was diluted with benzene and applied directly on the lining with a finger. The whole processing of the beauty cases was carried out by 4–5 persons in a small room. The patient reported that, especially during winter, there was a strong smell of benzene in the room.

Since 1965 the patient had suffered from frequent metrorrhagias. In July 1966 the patient started complaining of weakness, anorexia, pallor, irregular fever (up to 39°C). In August 1966, following a severe metrorrhagia, the patient was admitted to a gynaecologic department to have a currettage and, due to the findings of anaemia, she was transferred to the Clinica del Lavoro on 1 September 1966.

At admission the blood findings were: Hgb 8 g/100 ml, RBC 3,000,000/mm³, WBC 4500/mm³ with neutrophils 38%, eosinophils 2%, lymphocytes 14%, monocytes 6%, metamyelocytes 14%, myelocytes 26%, basophilic erythroblasts 10/100 WBC; platelets 125,000/mm³ with some giant, bizarre elements.

The bone marrow obtained by sternal puncture (9 September 1966) was very rich in elements of both series, especially of the more immature forms. Both erythroid and myeloid elements showed atypical features, like nucleo-cytoplasmic maturation asynchronism, bizarre nuclei; some giant mono- and binucleated elements were present. The myeloid–erythroid ratio was 1·1.

The patient was treated with transfusions and low doses of corticosteroids and was discharged improved on 24 September 1966.

A few days after (8 October 1966) the patient was readmitted in emergency due to severe metrorrhagia, thrombophlebitis of the left leg, high fever. The blood findings were: Hgb 6·2 g/100 ml, RBC 2,000,000/mm³, WBC 30,000/mm³ with neutrophils 69%, lymphocytes 4%, metamyelocytes 19%, myelocytes 2%, myeloblasts 1%, haemocytoblasts 5%; acidophilic erythroblasts 18, polichromatophilic erythroblasts 7, basophilic erythroblasts 1, proerythroblasts 1/100 WBC. Some of the immature elements of the erythroid series were PAS-positive. The leucocyte alkaline phosphatase score was high (262). At this time a direct chromosome preparation of peripheral blood was made.

From 22 October–1 November 1966 the patient was given a short course of 6-mercaptopurine (2·5 mg/kg/day), together with corticosteroids and transfusions. The patient had a partial clinical and haematological remission and was kept under check for a while.

On 23 December 1966 a new bone marrow sample was obtained, on which cytogenetic studies were performed. The pattern was similar to that previously described, but the bizarre and giant elements were more numerous. The myeloid–erythroid ratio was 0·46. Out of 1000 immature cells of both series, 2 mitoses of the myeloid and 6 of the erythroid precursors were found.

In the next months the patient became increasingly ill, had high irregular fever, complained of severe joint pains, and presented subcutaneous and submucous haemorrhages and liver and spleen enlargement.

The patient died on 16 February 1967. The autopsy findings were: bronchopneumonia, diffuse subcutaneous and submucous haemorrhages, liver and spleen enlarge-

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Table 1. *Chromosome counts in direct preparations of peripheral blood and bone marrow*

Date	Source	Chromosome counts							Total cells counted
		⩽43	44	45	46*	57	73	Near-tetraploid	
14 Oct. 1966	Blood		3	3	5			2	13
23 Dec. 1966	Bone marrow	6		2	14	1	1	5	29

*Pseudodiploid cells with 2 markers.

ment due to massive infiltration with erythroid and myeloid elements.

MATERIAL AND METHODS

The chromosome studies were performed on direct preparations of peripheral blood and bone marrow. The peripheral blood was treated with Colcemid CIBA for 2 hr, the bone marrow for 45 min at 37°C. Cells were washed twice with Hanks solutions, treated with diluted Hanks solution (Hanks 1 part: distilled water 4 parts), fixed in methanol–acetic acid (3:1), and chromosome preparations were made by the air-drying method, with slight flaming. No lymphocyte cultures were performed.

The leucocyte alkaline phosphatase was determined according to Kaplow's cytochemical score [10].

RESULTS

The direct preparation of peripheral blood was performed at a time when the patient had been treated only with corticosteroids and transfusions. The chromosome study of the bone marrow was made 1½ month after a short course of 6-mercaptopurine; unfortunately no chromosome studies had been performed at the time of the first bone marrow biopsy in September 1966.

The results of the chromosome counts are summarized in Table 1. Complete chromosome analysis was performed on 11 metaphases from the peripheral blood (Table 2) and on 12 metaphases from the bone marrow (Table 3). Both samples were characterized by a stem-line with a chromosome number of 46, but with a pseudodiploid pattern. In all pseudodiploid metaphases 2 chromosomes of the E group [17–18] were missing and 2 marker chromosomes were present (Figs. 1 and 2). The karyotype of the stem-line was therefore 46, XX, 2E−, mar 1+, mar 2+, according to the nomenclature of the Chicago conference [11].

Marker 1 was a small ring chromosome, appearing most of the times divided into the chromatids (Figs. 1 and 2). Marker 2 was a minute fragment, whose centromere was evident only in some metaphases. Both markers were quite recognizable and were observed also in some other metaphases which were not suitable for counting. The markers, and especially marker 1, occupied preferentially a peripheral position in the metaphase spreads (Figs. 1 and 2).

As shown in Table 1 quite a few metaphases were near-tetraploid; in these cells each marker was generally represented twice or more (Tables 2 and 3).

In some of the best metaphases a Bp+ chromosome was also somewhat evident; this chromosome was intermediate between chromosomes of pair 3 and chromosome 4 (Figs. 1 and 2). However, due to the quality of many metaphase spreads, it is difficult to state whether this chromosome abnormality was consistent.

Table 2. *Chromosome analysis of* 11 *metaphases from direct preparation of peripheral blood*

No. of cells	No. of chromosomes	A	B	C	D	E (17–18)	F	G	mar 1	mar 2
1	44			−1		−2			1	
1	44	−1	−2			−1			1	1
1	45			−1	−1	−1			1	1
1	45				−1	−2			1	1
1	45					−2				1
5	46					−2			1	1
1	86*		−1	−9	−1	−2			4	3

*In the near-tetraploid metaphases missing and extra-chromosomes are referred to the tetraploid karyotype.

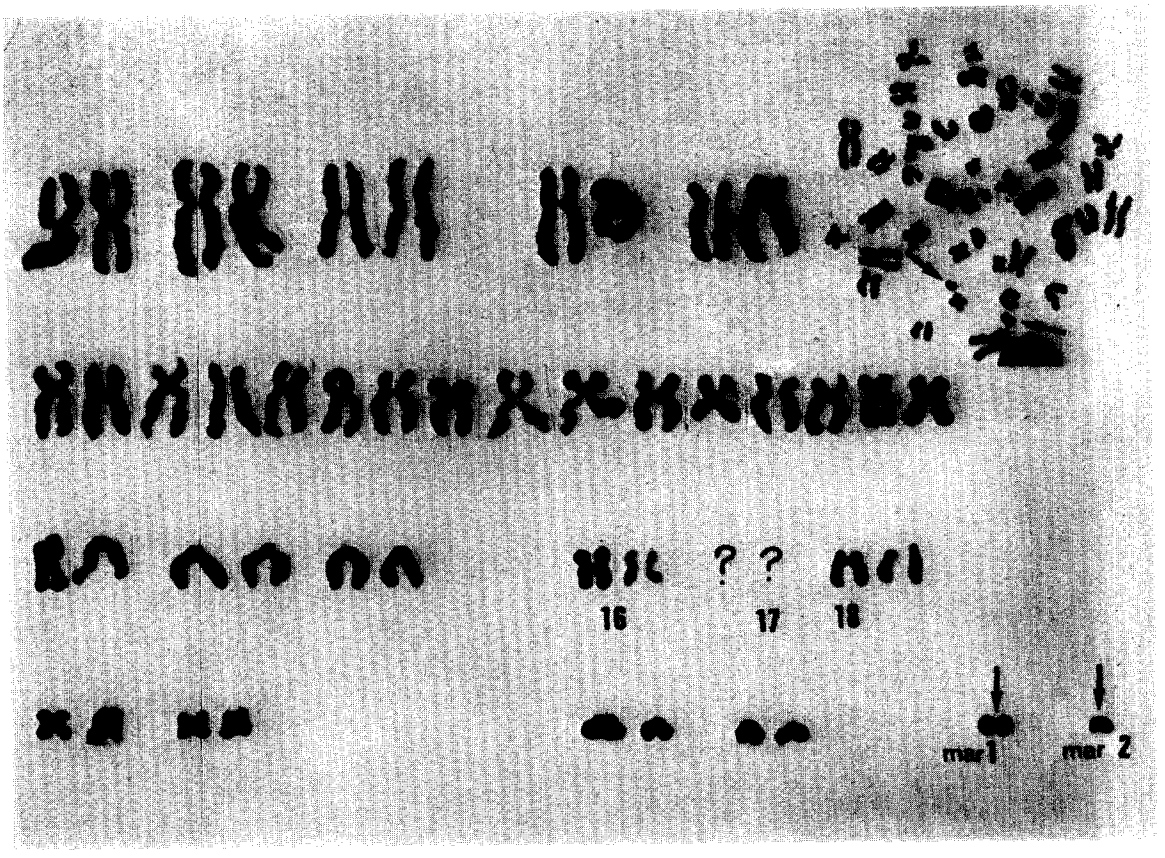

Fig. 1.

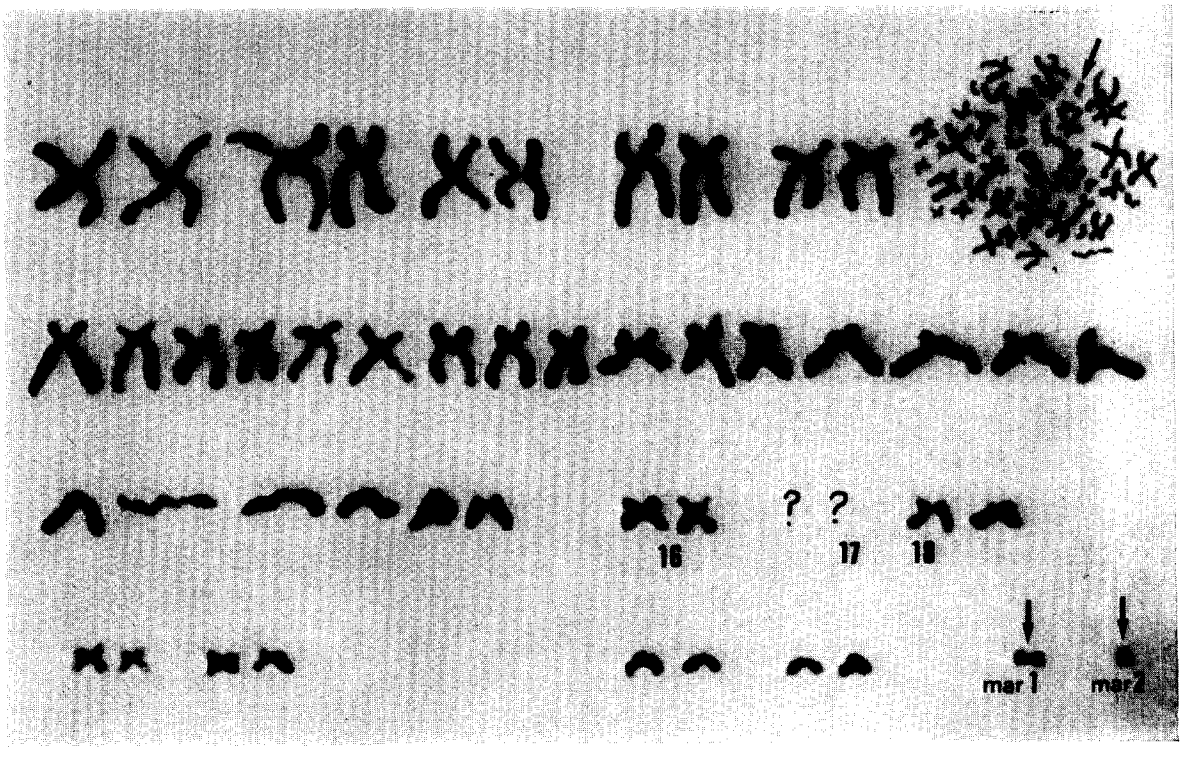

Fig. 2.

Figs. 1 *and* 2. *Pseudodiploid karyotypes from peripheral blood direct preparation. Note* 2 *missing chromosomes of the* E *group* (17–18) *and the* 2 *markers* (*arrowed*).

(to face p. 460)

Table 3. Chromosome analysis of 12 metaphases from direct preparation of bone marrow

No. of cells	No. of chromosomes	A	B	C	D	E 17–18	F	G	mar 1	mar 2
1	45			−1		−2			1	1
1	45					−2			1	
1	46					1q− −1 1q+				1
1	46			−1		−1 (1Eq−)			1	1
5	46					−2			1	1
1	57	+1		+4	+1		+1	+1	2	1
1	87*		−2			−5	−2		2	2
1	93*					−3			1	3

*See note below Table 2

DISCUSSION

The capacity of benzene to induce leukaemias is well known [12, 13]. Also cases of acute erythroleukaemia due to benzene are reported [14, 15]. In our case the illness started after 7 years of exposure to benzene in concentrations supposedly well above the threshold limit values. Although the patient had not had blood examinations, the frequent metrorrhagias in the year preceding the onset of leukaemia seem to indicate that disorders of the blood-forming organs were already present. For this reason we can assume that this case of erythroleukaemia was induced by benzene.

As far as the chromosome findings are concerned, from the comparison of our data with those existing in the literature some considerations can be made. The presence of one or more stem-lines with an abnormal karyotype seems to be a very frequent occurrence in acute erythroleukaemia [2–9]. As for the other acute leukaemias, the abnormal chromosome patterns are different from case to case. In the present case, consistent structural chromosome abnormalities were demonstrated in all metaphases, both in the sample of peripheral blood taken before antiblastic treatment and in the sample of bone marrow obtained at a time of relapse after treatment. The structural abnormalities seem therefore to belong to the neoplastic stem-line.

In two cases of acute erythroleukaemia reported in the literature a ring chromosome was observed [2, 6]. In the case of Di Grado *et al.* [2] the ring chromosome was the size of chromosome 2; in the case of Krogh Jensen [6] with 3 stem-lines, 2 stem-lines had a ring chromosome, one the size of a *C* chromosome, one the size of an *F* chromosome. The ring chromosome observed in our case was somewhat smaller than that of the second stem-line of the case of Krogh Jensen [6]. This author reports also in the same case the almost constant presence of a small acentric fragment in the metaphases with the marker ring chromosome. In our case the minute chromosome seemed actually to be centric.

A feature common to some reported cases of acute erythroleukaemia is a hypodiploid mode [1–3, 6, 7, 16]. In the case described the mode was pseudodiploid. The smaller size of the two marker chromosomes in comparison with the size of the two missing chromosomes of the *E* group suggests however a loss of genetic material, even though a translocation of part of this material on one *B* chromosome (?Bp+) cannot be ruled out. Loss of genetic material was also observed by Ricci [5] in his case of erythroleukaemia with a pseudodiploid pattern.

The involvement of chromosomes of the *E* group, particularly 17–18, in acute leukaemias and allied diseases, and in the acute transformation of chronic myeloid leukaemia, does not seem to be a rare occurrence [6, 7, 17–19]. Loss of 2 chromosomes [17–18] was a constant feature of the stem-line of the case described, even though in occasional hypodiploid cells only one *E* chromosome was missing together with chromosomes of other groups.

At variance with Castoldi *et al.* [9] no involvement of the *G* chromosomes was observed.

The tetraploid or near-tetraploid metaphases found in the direct preparation both of peripheral blood and of bone marrow represent probably mitoses of the abnormal giant elements, often binucleated, which were present in the smears.

The presence of the marker chromosomes in all the metaphases observed and analysed seems to indicate that all the dividing cells possessed an abnormal chromosome complement. As in the Giemsa-stained bone marrow smears made at the time of the chromosome prepara-

tion mitoses both of the erythroid and of the myeloid series were present, the abnormal metaphases observed seem to belong to both series. This fact, observed in acute leukaemias also by Krogh Jensen [6, 20, 21], seems to demonstrate that the abnormal cells derive from a common stem-cell with anomalous chromosome pattern.

Regarding the formation of the abnormal complement, it is noteworthy that some of the reported patients with acute leukaemia or erythroleukaemia had a history of exposure to X-rays [1, 6]. The patient with erythroleukaemia observed by us had been for some years exposed to benzene during her work. Benzene exposure is known to be able to produce chromosome changes similar to those induced by X-rays, both in lymphocytes and in bone marrow cells [22–26.] The hypothesis can therefore be suggested that in our case benzene exposure might have induced in a stem-cell chromosome changes (probably breakage of chromosomes of the *E* group), and that the consequent rearrangement might have resulted in a cell with a pseudodiploid complement, which gave rise to the abnormal clone. The leukaemic process might have been initiated by the proliferation of such clone and by its differentiation into cells both of the erythroid and of the myeloid series.

RESUME

On a étudié les chromosomes d'un cas d'érythroleucémie aiguë chez une malade qui avait été exposée au benzène. A l'examen direct tant du sang que de la moelle osseuse, il y avait une lignée cellulaire avec 46 *chromosomes, avec un caryotype pseudodiploïde par absence de deux chromosomes du groupe E et présence de deux chromosomes marqueurs (un chromosome en anneau et un petit fragment centrique). Le caryotype anormal paraissait être commun aux deux séries, puisque les chromosomes marqueurs étaient présents dans toutes les mitoses étudiées et appartenaient probablement tant à la série érythroblastique qu'à la série myéloïde. Les auteurs émettent l'hypothèse que l'exposition au benzène peut avoir induit des fragmentations et des réarrangements chromosomiques dans une cellule souche qui, en prolifération, produisait le clone érythroleucémique anormal.*

SUMMARY

Cytogenic studies were carried out in a case of acute erythroleukaemia, which occurred in a patient who had been exposed to benzene. In direct preparations both of peripheral blood and of bone marrow, a stem-line of 46 *chromosomes was present, with a pseudodiploid karyotype, due to the absence of* 2 *chromosomes of group E and to the presence of* 2 *small markers (a ring chromosome and a minute centric fragment). As the markers were present in all the mitoses observed, most probably belonging both to the erythroid and to the myeloid series, the abnormal chromosome complement seemed to characterize a stem-cell common to both series. The hypothesis is suggested that the exposure to benzene might have induced chromosome breakages and rearrangements in a stem-cell, and that proliferation of such abnormal element might have resulted in the erythroleukaemic clone.*

ZUSAMMENFASSUNG

Bei einem Patienten mit akuter Erythroleukämie, welcher der Einwirkung von Benzol ausgesetzt war, wurden die Chromosomen untersucht. Sowohl in der direkten Präparation des strömenden Blutes als auch in der des Knochenmarks war ein Zellstamm mit 46 *Chromosomen vorhanden. Durch die Abwesenheit von* 2 *Chromosomen der Gruppe E und das Vorhandensein von* 2 *kleinen Markerchromosomen (ein Ringchromosom und ein kleines zentrisches Fragment) entstand eine pseudodiploides Bild. Da die Markerchromosomen bei allen beobachteten Mitosen vorhanden waren, welche vermutlich sowohl der myeloischen als auch der erythroblastischen Serie angehören, scheint der pathologische Chromosomenbestand beiden Serien anzugehören.*

Die Autoren kommen zu der hypothetischen Folgerung, daß es durch die Wirkung des Benzols zu einem Bruch und einer Wiederanordnung der Chromosomen in einer Stammzelle gekommen war, wobei die Proliferation dieser Zelle zur Bildung eines anomalen Klons führte.

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New Observations on β-Glucuronidase in Human Cervical Cancer

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INTRODUCTION

β-GLUCURONIDASE (EC 3.2.1.31) is an enzyme which splits β-D-glucosiduronic acids. It is widely distributed in the human body, most abundantly in the liver, spleen and tissues of the reproductive and endocrine systems, and is confined in general to cells of epithelial origin [1, 2]. High activities have been found in many malignant tumours, particularly of breast, stomach, colon, lung and uterus [3]. This has been demonstrated both histochemically [4, 5] and by analyses of crude tissue homogenates [6, 7].

Interest has centred on β-glucuronidase in malignant and benign lesions of the female genital tract since the determination of the enzyme activity of vaginal fluid was claimed as a useful diagnostic aid by Odell and Burt [8] and by Kasdon, Fishman and Homburger [9]. β-Glucuronidase is found with increased activity in cervical carcinoma, as shown by histochemical procedures [10] and by analysis of homogenates [11].

In fractionation procedures on normal cells from rat and mouse tissues, 20% of the activity has been found in the nuclear fraction [12]. The remainder resides in the cytoplasm, mainly in mitochondria, microsomes and lysosomes with little free soluble enzyme in the cell sap [13, 14]. In contrast, over 50% of the cytoplasmic enzyme activity of mouse S37 and Ehrlich T2146 tumours was present in a soluble form [15, 16].

There have been no detailed reports on the intracellular distribution of the enzyme in cervical carcinoma, other than histochemical studies showing that the enzyme is confined to the cytoplasm of malignant cells [2–4, 17]. The present work was undertaken to examine this point in a search for the mechanism of raised β-glucuronidase in this tumour. Evidence was obtained which showed that the increased activity found in most carcinomas was mainly in the free soluble enzyme.

MATERIALS AND METHODS

Biopsy material was obtained from patients with carcinoma of the cervix uteri undergoing a course of radiotherapy. Nineteen specimens were obtained under anaesthesia just prior to insertion of the first radium implant. After removal, the specimens were placed immediately in iced water, washed free of blood and inflammatory exudates, dried on adsorbent paper, weighed and stored at −20°C till further processing. Histology showed two to be adenocarcinomas and the others squamous carcinomas of the cervix.

Twelve samples of non-malignant cervical tissue were obtained at operation on post-menopausal patients undergoing repair of uterine prolapse. The specimens were taken in such a way as to include as much of the epithelial layer as possible with minima amounts of underlying fibrous tissue which

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was never more than 50% of the entire sample. The specimens were classed as normal although microscopic evidence of varying degrees of chronic cervicitis was seen in some.

Preparations of cytoplasmic fractions

The specimens were cut on a freezing microtome into 10=40μ sections which were quantitatively added to a known volume of ice-cold 0·25M-sucrose in water. They were then homogenised for 3 min in a Waring Blendor. Cell debris and nuclei were then spun down at 500 **g** for 10 min and discarded as the material was unsuitable for precise measurement of enzyme activity and protein concentration. Cytoplasmic fractions were then obtained from the supernatant by the method of Goldberg and Pitts [18]. These were classified as (a) *Supernatant*, a clear solution containing predominantly cytoplasmic or soluble cell material, obtained after centrifugation at 35,000 **g** for 60 min (b) a *Mitochondrial* fraction spun down at 6000 **g** for 20 min; this contained predominantly mitochondria with some contamination by microsomes, and (c) a *Microsomal* fraction spun down at 35,000 **g** for 60 min; this contained mainly microsomes with occasional mitochondria and also lysosomes present. In some samples the particulate fractions were insufficient to be analysed separately and these were combined before analysis.

The particulate fractions were dispersed in a measured volume of ice-cold distilled water and the particles disrupted under ice by ultrasonic disintegration. β-Glucuronidase was measured in all fractions by a modification of the method of Talalay, Fishman and Huggins [19]. We added 0·1 ml of a suitable dilution of the tissue fraction to 0·8 ml of 0·1M-acetate buffer, pH 4·5; 0·1 ml of 0·01M-phenolphthalein-mono-glucuronic acid substrate (Sigma Chemical Co.) was added and the mixture incubated at 37°C for times varying from 1 to 4 hr depending on the enzyme activity. Four ml of 0·4M-glycine buffer, pH 10·4, were then added. A control was run consisting of the above reagents with the substrate added after the addition of the glycine buffer. The tubes were then spun to remove small amounts of precipitated protein and the test read against the control on a Unicam SP. 600 spectrophotometer at 550 mμ. The amount of phenolphthalein liberated was estimated from a calibration curve made from phenolphthalein standards. A unit of enzyme activity was defined as the formation of 1 μg phenolphthalein in 1 hr at 37°C under test conditions.

By this method duplicate enzyme analyses of the tissue fractions could be performed with a reproducibility of ± 2%. The enzyme reaction was found to be linear with time over at least a 6 hr period for all tissue fractions, and was also linear with respect to enzyme concentration over the range of dilutions used on the fractions [20].

The cytoplasmic enzyme activity of the tissue specimens was expressed as (a) units/g wet wt of tissue (g wet wt) and (b) specific activity, defined as units/mg protein as measured by the method of Lowry, Rosebrough, Farr and Randall [21].

RESULTS

Normal cervical tissue

Table 1 shows the results for the supernatant and particulate fractions of the normal specimens. An adequate yield of particles was obtained in only 7 of the 12 specimens, and separate mitochondrial and microsomal fractions obtained in only 4. In these, the β-glucuronidase activity on each fraction was added to give Particulate activity and divided by the sum of the protein content to give a combined Particulate specific activity. In the other 3 cases, the mitochondrial and microsomal fractions were combined together before analysis for enzyme activity and protein concentration. The total cytoplasmic activity/g wet wt was obtained by adding the supernatant and particulate results together and the percent of this total activity in each fraction calculated.

Table 1. *β-glucuronidase activity of cytoplasmic fractions of normal cervical tissue*

	Cytoplasmic fraction	
	Supernatant	Particulate
No. of samples	12	7
β-glucuronidase activity (units/g wet wt)	60±19	18±4
Specific activity (units/mg protein)	2·1±0·6	10·0±1·3
Per cent of total cytoplasmic activity/g wet wt	71±3	28±3
Protein (mg/g wet wt)	27·0±2·3	1·8±0·3

Results expressed as Mean±Standard Error.

The supernatant β-glucuronidase activity was low in the majority of these tissues, with a range of 10–240 units/g wet wt and specific activities of 0·6–8·0 units. Of the total

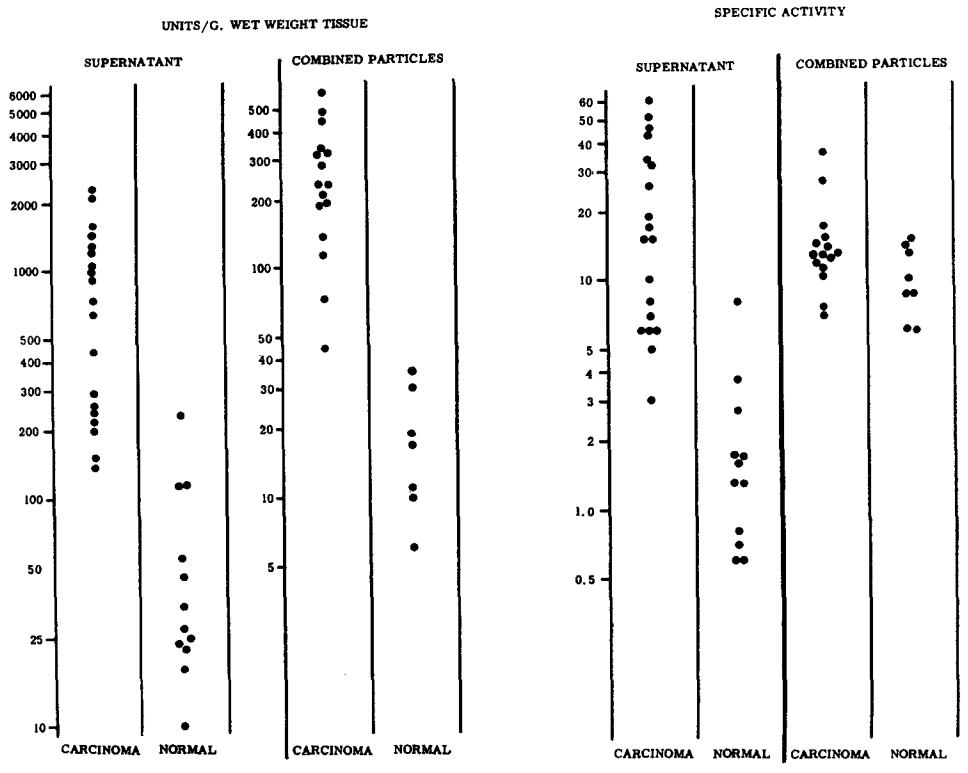

Fig. 1. *β-glucuronidase activity of the cytoplasmic fractions of normal and malignant cervical tissue.*

activity, 71% appeared in the supernatant fraction in those tissues in which particles were obtained. However, the particles had greater activity than the supernatant, relative to their protein content.

Cervical carcinoma tissue

Table 2 shows the results derived in a similar manner on the carcinoma specimens. Suitable particulate fractions were obtained in 15 of the 19 specimens. β-Glucuronidase activity was generally very much higher than in normal tissue, although the range was wide. The distribution of β-glucuronidase between supernatant and particles appeared to be very similar to that of normal tissue, the supernatant comprising 74% of the total cytoplasmic activity.

Table 2. *β-glucuronidase activity of cytoplasmic fractions of cervical carcinoma tissue*

	Cytoplasmic fraction	
	Supernatant	Particulate
No. of samples	19	15
β-glucuronidase activity (units/g wet wt)	855±153	242±33
Specific activity (units/mg protein)	22±4	15±2
Per cent of total cytoplasmic activity/g wet wt	74±3	26±2
Protein (mg/g wet wt)	43·0±9·9	17·0±1·8

Results expressed as Mean±Standard Error.

Comparison of carcinoma and normal tissue

Figure 1 shows a comparison of the β-glucuronidase activities of supernatant and particulate fractions in the normal and carcinoma specimens.

Although there was a wide range of activity in the supernatant of the carcinoma specimens, there was a highly significant difference between the two groups, with the results expressed as units/g wet wt ($t=4\cdot05$, $P<0\cdot001$) and as specific activities ($t=3\cdot80$, $P<0\cdot001$).

There was a higher yield of particulate β-glucuronidase/g wet wt from the carcinomas which again proved highly significant ($t=4\cdot67$, $P<0\cdot001$). However, when the results were expressed as specific activities, the difference was not significant ($t=1\cdot80$, $P>0\cdot05$), and the ranges were very similar (Fig. 1). This indicates that the differences in particulate β-glucuronidase/g wet wt were due to differences in particle yield.

β-Glucuronidase activity of the two particulate fractions

The mitochondrial and microsomal fractions

have so far been treated together for comparative purposes as few of the normal cervical tissues gave adequate particulate material for separate analysis.

However, in 4 specimens of normal tissue, both fractions were obtained. The β-glucuronidase activity of these was similar, a mean±s.e. of 6±0·8 units/g wet wt and 9±1·8 units specific activity for the mitochondrial fraction, and 8±1·7 units/g wet wt and 10±2 units specific activity for the microsomal fraction. The mean distribution of β-glucuronidase/g wet wt, in these 4 cases was 71±3% (Mean±s.e.) in the supernatant, 13±1% in the mitochondrial and 17±5% in the microsomal fraction.

Fourteen of the 19 carcinoma specimens had separate mitochondrial and microsomal fractions. The mitochondrial fractions showed a mean±s.e. β-glucuronidase of 114±15 units/g wet wt and specific activity of 14±2 units while the microsomal fraction had somewhat higher mean activities of 142±19 units/g wet wt and 16±2·4 units specific activity. The distribution of the total cytoplasmic activity was 74±3% in the supernatant, 12±1·8% in the mitochondrial and 14±2·2% in the microsomal fraction.

β-Glucuronidase and degree of malignancy

Because there was such a wide range of β-glucuronidase activities in the carcinoma series, it was felt necessary to see if this was associated with the severity of the disease. The specimens were classified into 3 groups on the basis of clinical staging according to the International Classification and histological criteria—

(*a*) *Severe* (9 cases)—these included specimens from Stage III cases, and those from Stage II cases which showed a poor differentiation, high mitotic rate and pronounced invasiveness.

(*b*) *Mild* (5 cases)—these included a specimen from a single Stage I case and from 4 stage II cases where the lesions were well-differentiated with a low mitotic rate and low invasiveness.

(c) *Intermediate* (5 cases)—these comprised specimens from Stage II cases which could not be placed with certainty in the other two groups.

The supernatant β-glucuronidase activity of the specimens in each group is shown in Table 3. There was a very obvious and significantly lower β-glucuronidase activity in the *Mild* cases compared with the *Severe* and *Intermediate* cases on an activity/g wet wt and specific activity basis. No difference was observed between the *Severe* and *Intermediate* groups. Particulate material was obtained from only 3 cases in the *Mild* group. The combined particle activity for these cases, 138, 73 and 48 units/g wet wt was very much lower than for the 9 cases in the *Severe* group—198–487 units/g wet wt. This difference was not apparent, however, with the results expressed as specific activity and was probably due to a small yield of particles in the *Mild* cases.

Table 3. Supernatant β-glucuronidase results on cases of cervical carcinoma classified by severity of the malignant lesion

	β-glucuronidase (Units/g wet wt) Mean±s.e.	*t**	*P*
Severe†	1044±225	2·74	<0·02
Intermediate†	1159±279	3·38	<0·01
Mild	212±20		
	Specific activity Mean±s.e.		
Severe†	24±5	2·47	<0·05
Intermediate†	33±10	2·51	<0·05
Mild	6±0·5		

*Value for Students' *t* test obtained by comparison with mild group.

†The severe and intermediate groups were not statistically different from each other.

There was no significant difference in the results on cancer specimens from patients who were pre-menopausal and those from patients who were post-menopausal.

Distribution of cytoplasmic β-glucuronidase in cervical carcinoma

On comparing the results of the 15 carcinoma tissues with supernatant and particulate fractions, those with high β-glucuronidase appeared to have a different distribution of the enzyme between the supernatant and particulate fractions from those with low enzyme activity. Consequently the specimens were split into 2 well separated groups—those with a total cytoplasmic activity over 1000 units/g wet wt—the *High Activity* group—and those with a total activity under 500 units/g wet wt—the *Low Activity* group. On this basis, only 1 sample with a total activity of 749 units/g wet wt. was not placed and this had a distribution of 71% in the supernatant and 29% in the particles. For the purpose of this comparison the mitochondrial and microsomal fractions on all the samples have been treated separately.

Table 4. Comparison of β-glucuronidase and protein results on cervical carcinoma of high and low β-glucuronidase activity

	High-activity group	Low-activity group	t*	P*
No. of cases	8	6		
Percentage of total cytoplasmic activity in each fraction				
Supernatant	82±1·9	61±6·0	4·04	<0·005
Mitochondrial	8±0·8	18±3·3	3·64	<0·005
Microsomal	10±1·2	21±4·7	2·81	<0·025
Protein content of each fraction (mg/g wet wt)				
Supernatant	56±4·6	36±3·6	4·15	<0·001
Mitochondrial	9±1·1	8±2·4	0·66	n.s.
Microsomal	11±1·3	7±0·9	2·14	n.s.
Specific activity of each fraction				
Supernatant	30±4·6	7±2·0	3·76	<0·005
Mitochondrial	17±2·9	10±0·9	1·86	n.s.
Microsomal	19±3·6	11±1·8	1·65	n.s.

Results expressed as Mean±Standard Error.
*Students' t-test and Probability values obtained by comparing the equivalent fractions of the two groups.
n.s. No significant difference.

The percentage activity occurring in the 3 fractions of both groups is shown in Table 4 and it can be seen that there is a highly significant difference in the distribution of the enzyme in the 2 groups. In the *High Activity* group, a greater proportion of the enzyme activity is found in the supernatant fraction than in the *Low Activity* group, indicating that most of the increase of β-glucuronidase in these tissues was mainly in the supernatant rather than in the particles.

There was no significant difference in the β-glucuronidase distribution between the two particulate fractions in the 2 groups.

DISCUSSION

It is clear that specimens of cervical cancer have a higher β-glucuronidase activity/g wet wt, than specimens of normal cervix. This is in accord with previous authors [8, 11, 22]. The cancer specimens, however, showed a wide range of β-glucuronidase activity (130–2300 units/g wet wt), a fact also noted by the above authors. It would appear that specimens from the less severe cases had a lower β-glucuronidase activity than from the more advanced cases (Table 3). Rauramo [22] found a higher incidence of low values in cases classified as Stage 0-I (38%) than in Stage II (13%) and Stages III and IV (7%). These results along with the present ones could be explained by the fact that normal cervical tissue would be removed more readily along with tumour tissue in the biopsy specimen from less widespread lesions.

The precise nature of the increased activity in the cervical cancers has not, as yet, been explained. Two possibilities present themselves, (a) an increased activity of β-glucuronidase in the malignant cells [4] or (b) the increased β-glucuronidase may be due to more cells with approximately the same β-glucuronidase activity as normal cells being found in the biopsy specimen of the tumour than in normal samples [17]. The results of this present study indicate that both mechanisms contribute to the increased enzyme activity.

The amount of soluble protein obtained in the supernatant fraction of the tissue homogenates is related to the cellularity of the specimen as this protein is derived mainly from the soluble intracellular protein and not from structural proteins of fibrous connective tissue. It can be seen from the results in Tables 1 and 2 that the carcinoma group had a higher supernatant protein content/g wet wt than the normal group, indicating that the malignant specimens had more epithelial tissue in the biopsy specimens. However, when the supernatant enzyme activity was expressed as specific activity, the carcinoma group was much more active than the normal group (Tables 1 and 2), thus upholding the view of Fishman *et al.* [4]. It should be emphasized, however, that there is not a pronounced or statistically significant difference in the specific activities of the Particulate fractions of the normal and malignant tissues (Fig. 1)

The data in the present study concern the cytoplasmic distribution as no account has been taken of any enzyme which may be present in the nuclei. It must be realized that the techniques of tissue preparation will influence the observed intracellular distribution of the enzyme as previously shown by Levvy and Conchie [16]. Conditions must be selected, however, which will give adequate breakdown of cellular material with a minimum of particle disruption. In a tissue as hard to disrupt as the cervix uteri, these conditions are difficult to achieve. The present conditions selected for cervical tissue were based on these considerations.

Two important factors must be considered when interpreting the distribution of β-glucuronidase in the normal and malignant cervical tissue. First, particles will be trapped and lost in the fibrous network formed from disrupted connective tissue of the specimen which will be centrifuged down with nuclei and cell debris. It is probable that this occurred extensively in the samples of normal cervix where fibrous tissue formed a high proportion of the total specimen and accounted for poor yield of particles. In the malignant tissue, however, this factor was very much less due to the higher ratio of epithelium to collagenous material.

Secondly, release of the contents of the particles into the supernatant fraction can occur during tissue fractionation due to mechanical disruption in the blendor and as a consequence of freezing and thawing during storage and slicing of the tissue. This factor would be common to both normal and malignant tissue preparations. It is unlikely that disruption of particles would invalidate the observed distribution to a notable degree for the following reasons. The specific activity of the supernatant in the carcinoma group was higher than that of the particles (Table 2), whereas that of the normal supernatant was only one-fifth that of the corresponding particles (Table 1). If the distribution were affected by factors common to both groups, one would not expect such a difference between the supernatant/particulate specific activity ratios. In addition, the protein content of the cancer supernatant was approximately 50% higher than that of the normals (Tables 1 and 2) while the specific activities of the particulate enzyme in the 2 groups were not widely different. It has previously been demonstrated that, during breakdown of subcellular particulate material by ultrasound, the release of β-glucuronidase and protein with time occurs in a relatively uniform manner [23]. If it is assumed that all the supernatant enzyme was derived from the particles in both tissues, it can be calculated from the mean values of specific activity and protein content of each fraction, that three-quarters of the normal particles, and four-fifths of the cancer particles would need to be ruptured to give the observed results. While the former possibility cannot be excluded in view of doubts about the recovery of the particles, the latter is quite untenable as this would require that 70 mg of particulate protein/g of tissue had escaped into the supernatant, the entire protein content of which was considerably lower than this (mean 43 mg/g wet wt).

Thus it seems likely that the supernatant enzyme is distinct from the particulate enzyme and that it constitutes approximately 75% of the cytoplasmic enzyme in the malignant tissue. Considerable variations in the cytoplasmic distribution of β-glucuronidase have been reported for different tissues ranging from values as low as 14% in the supernatant from mouse liver [16] to 70–90% in rat spleen [24].

It was not possible to assess quantitatively the degree of homogeneity of the 2 particulate fractions due to the inadequate amount of material obtained from the normal and carcinoma specimens. However, Ayre, Goudie and Goldberg [25] were able to make such an assessment of particle fractions obtained from thyroid tissues by the same fractionation procedure, by electron microscopic examination and analysis for RNA and succinate dehydrogenase in each fraction. They found the mitochondrial fraction to be 90%, and microsomal fraction to be 75–80% homogenous for the respective particles. In the particle fractions of the tumour cells, β-glucuronidase appears to be evenly divided between the mitochondrial and microsomal fractions. The enzyme has been referred to as 'lysosomal', although comparable activities were found in lysosomes and microsomes [14]. Levvy and Conchie [16] have shown it to be present in mitochondrial and microsomal fractions with approximately equal activity, as found in the present work.

In the tumour specimens with high β-glucuronidase activity, the increase appeared to be mainly in supernatant enzyme rather than in the particles. However, other explanations of this finding in terms of particle yield in the 2 groups might be considered:-

(a) Specimens in the *Low Activity* group could give a higher yield of particles than the *High Activity* group. However, the particulate protien/g wet wt can be taken as an index of the yield of particles from the tissues. From the protein contents in Table 4, there is not a significant difference in this respect between the 2 groups, and, if anything, there was a slightly smaller particle yield from the *Low Activity* group, the reverse of what would be required to explain the difference in β-glucuronidase distribution on this basis.

(b) The particles in the *Low Activity* group could have more β-glucuronidase than the *High Activity* group. However, from the specific activities in Table 4 it can be seen that there were no pronounced differences in β-glucuronidase activities of the particulate material in

both groups, and if anything the *High Activity* group was more active in this respect than the *Low Activity* group, again the opposite of that required to explain the different distribution.

(c) Fragility of the particles in the 2 groups might result in particulate contamination of the supernatant especially in the *High Activity* group. But it can be seen in Table 4 that the supernatant specific activity in the *High Activity* group was considerably higher than the particulate specific activities. It is not possible, therefore, to explain the increase in supernatant β-glucuronidase in terms of breakdown of particles during fractionation. There would have to be a selective release of β-glucuronidase-rich protein from the particles to give such large increases in supernatant specific activity in view of the fact that protein released from the particles would be greatly diluted due to the very large protein content of the supernatant relative to particles.

It would appear, therefore, that the increases in β-glucuronidase seen in the majority of specimens of cervical carcinoma are due to a rise in soluble cytoplasmic enzyme with only slight increase in particulate enzyme. This increase in the soluble enzyme is in contrast to the findings for ribonuclease and deoxyribonucleases I and II in normal and malignant cervical tissue processed in an identical way, where a higher percentage of the increased enzyme activities in the malignant tissue was in the particulate fractions [18]. Activities of phosphogluconate and NADP-isocitrate dehydrogenases were also raised in malignant cervical tissue but this was almost exclusively in the supernatant fraction [26]. The findings that in mouse and rat tumours a large proportion of β-glucuronidase was free in the cytoplasm of the tumour cells [15, 16] support the distribution described in this work. The estimation of β-glucuronidase activity in vaginal fluid has been widely applied in cancer diagnosis. High values have been seen in the majority of cases [8, 9, 22]. Fishman and Mitchell [27] stated that vaginal mucosa, uterine cervix or endometrium or cancer cells could be the source of the enzyme in vaginal fluid. It is feasible that in view of the intracellular distribution of the enzyme found in the present study, it could more readily escape from the malignant cells and enter vaginal secretions than if it were enclosed within cytoplasmic particles. The rapidity with which the enzyme of the uterine cervix, in contrast to that of vaginal epithelium, passes into an aqueous medium *in vitro* has already been demonstrated [23]. Again, radiation of cervical cancer has produced some fall in vaginal fluid β-glucuronidase activity [8, 28, 29] which has been accompanied by a parallel rise in serum and urine β-glucuronidase [30]. These facts would be more easily explained in terms of the enzyme being in a free soluble form in the cytoplasm of tumour cells if the mechanism involved leakage of the enzyme across a membrane whose permeability was altered by malignant change or radiation.

Acknowledgements—We should like to thank Dr. Mary Cowell and Dr. J. MacVicar for provision of specimens, Dr. E. B. Hendry for generous facilities and Professor D. F. Cappell for use of equipment within his department.

RESUME

On a mesuré l'activité de la β-glycuronidase dans des fractions cytoplasmiques provenant de cols utérins normaux et cancéreux.

L'activité enzymatique était beaucoup plus élevée dans la fraction surnageante de tissu malin que dans celle des spécimens normaux, en tenant compte du contenu en protéines et du poids tissulaire.

On n'a trouvé aucune différence significative dans l'activité de la β-glycuronidase, par mg de protéine du matériel microsomial et mitochondrial, entre les tissus malins et les tissus normaux.

Les tissus normaux ayant une très forte activité en β-glycuronidase ont montré une distribution cytoplasmique de l'enzyme, différente des spécimens ayant une activité plus basse en β-glycuronidase.

Il nous a semblé que les activités enzymatiques plus élevées étaient dues à l'augmentation de l'enzyme cytoplasmique libre soluble, et non à celle de l'enzyme liée à des particules.

SUMMARY

β-glucuronidase activity was measured in cytoplasmic fractions prepared from malignant and non-malignant human cervix uteri.

Enzyme activity in the supernatant fraction from malignant tissue was very much greater than that from non-malignant specimens, relative to protein content and tissue weight.

No significant difference was found in β-glucuronidase activity/mg protein of the mitochondrial and microsomal material from malignant and non-malignant tissues.

The cancer specimens with very high β-glucuronidase activities showed a different cytoplasmic distribution of the enzyme from those with lower β-glucuronidase activities. It appeared that the higher enzyme activities were due to increases in the free soluble cytoplasmic enzyme and not particle-bound enzyme.

ZUSAMMENFASSUNG

An Cytoplasmafraktionen maligner und nichtmaligner Cervix uteri wurde die β-glucuronidaseaktivität gemessen.

Bezogen auf Proteingehalt und Gewebegewicht war die Enzymaktivität beim malignen Gewebe sehr vie höher als beim nichtmalignen Gewebe.

Kein wesentlicher Unterschied in β-Glucuronidaseaktivität wurde zwischen den Mitochondrien- und Mikrosomen-Material maligner und nichtmaligner Gewebe gefunden.

Die Krebs-Gewebeproben mit sehr hohen β-glucuronidaseaktivitäten unterschieden sich von denen mit niederer β-Glucuronidaseaktivität durch ein anderes Verteilungsmuster des Enzyms im Cytoplasma. Es schien, als ob die höheren Enzymaktivitäten auf eine Vermehrung der frei löslichen Cytoplasmaenzyme und nicht der partikelgebundenen Enzyme zurückzuführen sei.

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Rat Hepatoma Cell Surface Antigens: Demonstration and Isolation of Membrane-Associated Isoantigens

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INTRODUCTION

NEOPLASTIC transformation of liver cells by aminoazo dye is accompanied by a series of modifications in the antigenic composition of the cell surface. There is a gain of new, tumour-specific antigens, which are demonstrable by transplantation methods [1] and also by immunofluorescence tests with sera from immunised syngeneic rats [2, 3]. A significant feature of these antigens is that they reflect the individuality of each tumour. Concomitant with antigenic gain, there is a loss, sometimes very marked, of normal liver antigens from the cell surface of these hepatomas which is also demonstrable by immunofluorescence methods [4]. Here, also, the pattern of normal liver antigen deletion varies between individual hepatomas. Such observations suggest that changes in plasma membrane composition as expressed by antigenic markers may be important in neoplastic transformation and tumour growth. For instance, it is now well established that tumour specific antigens evoke a host response similar in kind if not in degree, to that involved in homograft rejection, which influences growth of the hepatoma [1]. Likewise deletion of normal liver antigens from the hepatoma cell surface may be alternative expressions of those changes which lead to modified growth properties [5].

Studies were therefore carried out to establish methods for demonstrating normal isoantigenic components on hepatoma cells. A variety of methods including haemagglutination [6]; leukoagglutination [7]; immune cytolysis [8]; colony inhibition technique [9]; and immunofluorescence methods [10–12] have been used for demonstrating cell surface isoantigen on various cell types. Since, however, tumour specific antigen on hepatoma cells can only be detected *in vitro* by immunofluorescence methods [2, 3] these techniques have been used exclusively for isoantigen assay.

Cell membrane fractions have been isolated retaining a high percentage of the isoantigen content expressed on the intact tumour cell surface. These fractions are suitable material for a more detailed study of antigenic modification during aminoazo dye carcinogenesis and also provide an evaluation of fractionation techniques which are applicable to the isolation of cell surface expressed tumour specific antigen.

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MATERIALS AND METHODS

Rats. Three-month old inbred Slonaker and Wistar rats, maintained in this department since 1956 and 1952 respectively, were used for all experiments. The genetic identity of rats of each strain was routinely confirmed by exchange of skin grafts between rats of the same sex.

Tumour. A transplanted hepatoma (D23) originally induced by 4-dimethylaminoazobenzene (DMAB) in 1964 was maintained by

passage in syngeneic Wistar rats. Early generations of this tumour were stored at −70°C and withdrawn for re-implantation when the number of transfer generations reached 25. The tumour was highly strain specific and failed to grow in allogeneic Slonaker recipients.

Antisera. Isoantisera against tumour D23 were prepared by allografting the tumour subcutaneously by trocar into incompatible Slonaker rats. Immunisation was carried out at monthly intervals over a period from 2 to 8 months, after which time rats were bled out by heart puncture under ether anaesthesia, and serum collected in the usual manner.

Preparation of cells for absorption and immunofluorescence tests. Hepatoma D23 cells of high viability were prepared by trypsin treatment of finely minced tumour [1]. Single cell suspensions of Wistar spleen and lymph node were prepared by passing the freshly minced tissues through a stainless steel 60-mesh screen into medium 199, followed by a 120-mesh screen to remove clumps. Single cell suspensions of rat liver were prepared using a loose fitting silicone rubber plunger in a glass homogenizer tube (Glaves, unpublished technique). No attempt was made to separate parenchymal and reticuloendothelial cells.

Immunofluorescence studies. The indirect fluorescent antibody test described by Baldwin and Barker [2] was used. Briefly, viable hepatoma cells were incubated first with absorbed or in control tests, unabsorbed Slonaker anti-hepatoma D23 isoantiserum, then washed several times with medium 199 to remove excess serum and re-incubated with a $\frac{1}{3}$ dilution of fluorescein—conjugated rabbit anti-rat IgG (prepared in this laboratory) to "stain" bound isoantibody. Cells finally washed in medium 199 were resuspended and examined for membrane fluorescence staining. Cells exhibiting complete ring staining of the membrane or of isolated points on the membrane were scored as positive. Diffuse fluorescence of the cell indicated cell death and such stained cells were omitted from the test. In order to quantitate the results, fluorescence indices were calculated from the degree of membrane staining obtained with antiserum compared with that of normal rat serum. From these measurements, a fluorescence index (FI) was calculated as the proportion of unstained cells in the population exposed to normal rat serum minus the proportion of unstained cells in the population exposed to the test serum, divided by the former figure [2]. In 100 tests, the mean number of unstained cells following exposure to normal rat serum was $81 \cdot 1 \pm 6 \cdot 9\%$ and from this a fluorescence index of 0·3 or greater was considered to represent a significant reaction.

Absorption of antisera. The isoantigenicity of subcellular fractions of tumour D23 was assayed by the capacity to absorb isoantibody from iso-immune sera, compared with that of intact viable cells. For absorption, aliquots of antisera (0·1 ml of a $\frac{1}{3}$ dilution) were incubated with equal volumes of cell suspensions or subcellular fractions in phosphate-buffered saline (PBS), (a freshly prepared 1/10 dilution of a solution containing 76·5g NaCl, 7·25g Na_2HPO_4 and 2·12g KH_2PO_4 per liter), for 2 hr at 2°C. Cells were then centrifuged at 600 **g** for 10 min; large particles at 10,000 **g** for 20 min, and small particles and surface membrane fractions at 105,000 **g** for 60 min. Absorbed antisera, together with unabsorbed samples to serve as controls were then assayed against viable hepatoma D23 cells using the indirect fluorescent antibody test.

Isolation of subcellular fractions from hepatoma D23. Tumour cells were suspended in sucrose buffer (pH 7·4) as defined by Ozer and Wallach [13]. Homogenization was by nitrogen cavitation at 800–1000 lb per sq. inch for 30 min [14]. After cell rupture, the homogenate was made 0·001 M with respect to EDTA [13]. Nuclei were separated from the homogenate by centrifugation at 600 **g** for 10 min at 0°C. A second fraction, large particles, was sedimented from the 600 **g** supernatant at 10,000 **g** for 20 min, whilst a third fraction, small particles, was sedimented from the 10,000 **g** supernatant at 105,000 **g** for 60 min. Finally, the 105,000 **g** supernatant (soluble fraction) was re-centrifuged under the same conditions and concentrated.

A surface membrane preparation was isolated from tumour cells by adaptation of a method used for BP8 tumour cells [15, 16]. Pooled tumour cells in 0·9% NaCl (w/v) at a concentration of 2×10^7 cells/ml were gently agitated for 16 hr at 2°C with a silicone-covered magnetic stirrer. Under these conditions loss of viability did not exceed 20%. Cells were harvested by centrifugation (600 **g** for 15 min), extracted with distilled water to a point short of cell lysis, and the eluted cell-free membrane material separated from the aqueous extracts by centrifugation at 80,000 **g** for 30 min. Cytological examination revealed the presence of small fragments which stained with Oil Red O and Lissamine Green.

Chemical analyses. Protein was determined by the technique of Lowry *et al.* [17].

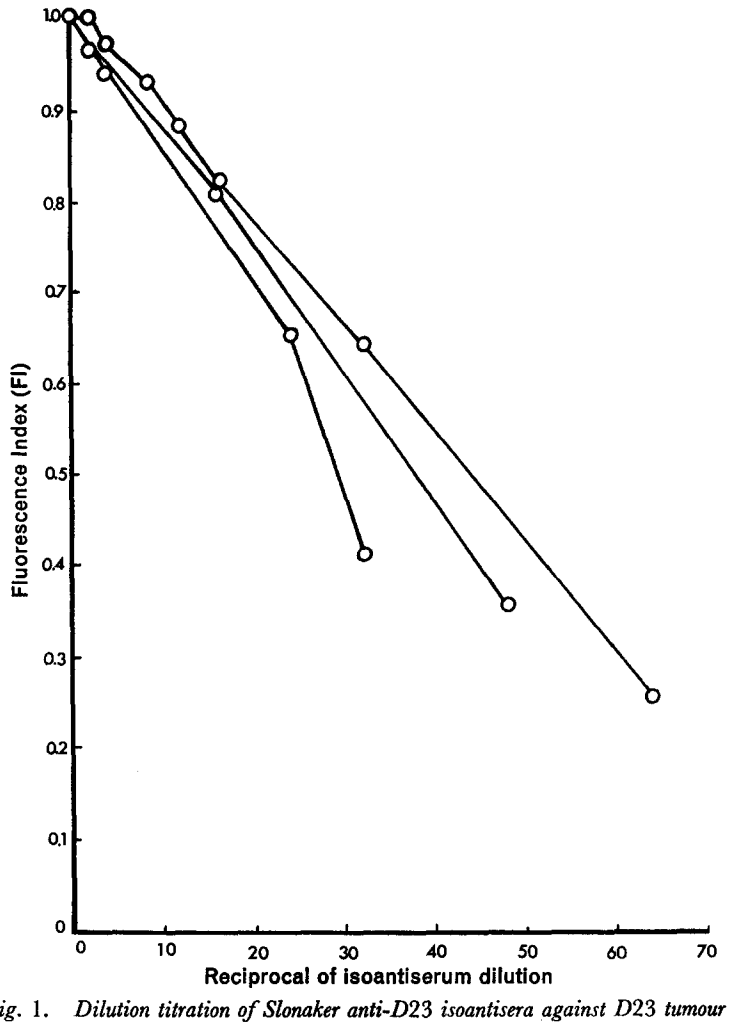

Fig. 1. Dilution titration of Slonaker anti-D23 isoantisera against D23 tumour cells.

RESULTS

Detection of isoantigen on the surface of hepatoma cells

In all immunofluorescence tests, undiluted Slonaker anti-hepatoma D23 isoantisera gave rise to staining of the vast proportion of D23 cells such that fluorescence indices (FI) were rarely below 0·95. Staining was characterised by complete ring reactions at the cell surface, although in a proportion of the cell population, dots or segments of fluorescence staining only were observed. In general, positive FIs against D23 cells were demonstrated at isoantiserum dilutions of 1/40, but at dilutions greater than this, the number of stained cells was not significant (Fig. 1).

Isoantisera prepared against tumour D23 also effectively stained other transplanted DMAB-induced hepatoma cells, although in many examples the reaction was far less intense than that obtained with the cells of hepatoma D23, observations which are consistent with the view that the surface antigen mosaic differs considerably for each hepatoma [4].

The reaction of anti-hepatoma D23 isoantiserum with D23 cells was completely inhibited by pre-absorption of the serum with D23 cells. Thus, after pretreatment of isoantiserum with approximately 2×10^8 cells/ml serum, the FI was below a significant value (Fig. 2). This end-point, in terms of the number of cells, varied according to the potency of the antiserum, but was reached at concentrations not usually less than 1×10^8 cells/ml serum (Table 1). The degree of fluorescence staining of D23 cells was effectively reduced by preabsorption of isoantiserum with comparable numbers of viable normal liver cells (Fig. 2), but the immunofluorescence reaction could not be completely abolished. Similarly, pre-absorption with relatively high concentrations (approximately 10^9 cells/ml antiserum) of spleen and lymph node cells failed to completely inhibit the reaction with D23 cells (Fig. 2). Thus, in all examples where isoantiserum prepared against grafts of hepatoma D23 was pre-absorbed with viable cells of normal Wistar tissues, a weak but positive immunofluorescence reaction was demonstrated against D23 cells (Fig. 2).

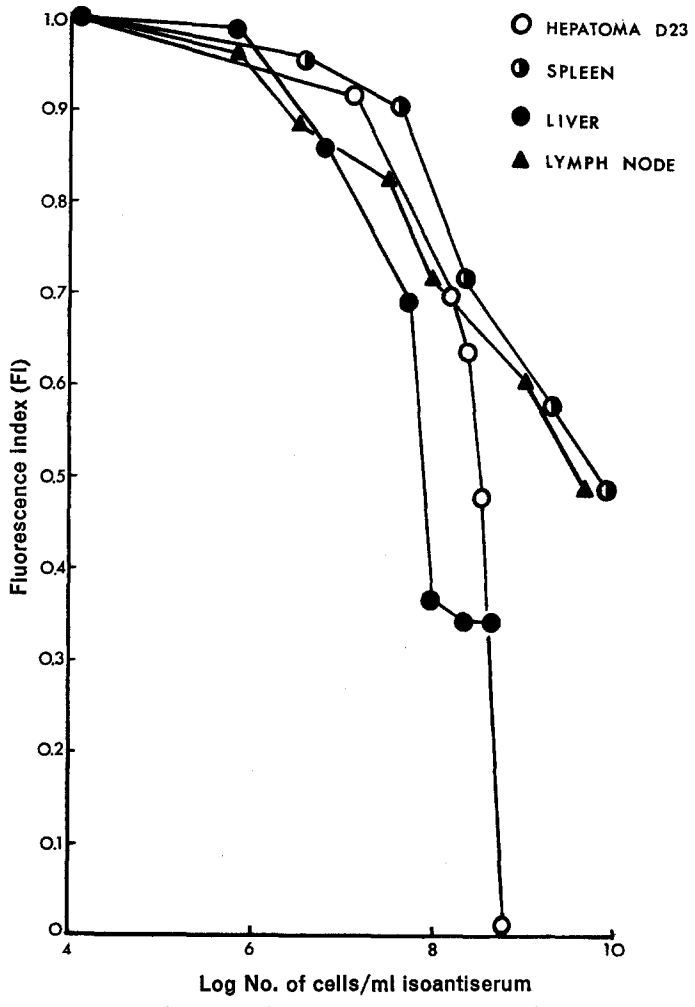

Fig. 2. Absorption of Slonaker anti-D23 isoantiserum by Wistar hepatoma D23 cells, and normal spleen, liver and lymph node cells.

Isolation of subcellular fractions retaining isoantigenic activity

The effectiveness and reproducibility with which isoantibody was absorbed by intact tumour cells under standard conditions were used as criteria for the evaluation of the antigenicity of subcellular preparations. In initial tests, an 'extra-nuclear membrane fraction' was isolated from homogenates of hepatoma D23 cells by centrifugation at 105,000 **g** following removal of nuclei at 600 **g**. Pre-absorption of isoantiserum with this fraction completely abolished the immunofluorescence reaction with D23 cells, the isoantigen recovery exceeding 63% of that on the surface of the intact cells (Table 1). Fractionation of this preparation into large and small particles produced two fractions of comparable isoantigenicity (Fig. 3). Thus, the specific activities of these fractions, expressed by the reciprocal of the smallest amount of protein (in mg) required in absorption tests to reduce the FI of isoantiserum to 0·3, were approximately the same (0·10 and 0·08 respectively; (Table 1). However, the recovery of isoantigen in the large particle preparation (60–65%) was greater than that in the small particle preparation (39–45%). Inhibition of the immunofluorescence reaction of Slonaker anti-hepatoma D23 antiserum was also achieved by pre-absorption with surface membrane preparations from the tumour (Fig. 3). Here, the specific activity (0·09) was comparable with the large and small particle preparations but the recovery of cell surface isoantigen retained in this fraction was more variable (34–68%). Whilst absorption of isoantibody was consistently demonstrated for particulate fractions of hepatoma D23 cells, little effect was achieved by the soluble fraction (105,000 **g** supernatant). Thus, the FIs for unabsorbed isoantiserum (0·99, 1·00) were marginally reduced (0·85, 0·86) after pre-absorption with soluble cytoplasmic protein at a concentration (45 mg/ml) four times greater than that at which the corresponding particulate fractions

Table 1. *Isoantigen content of hepatoma D23 cells and subcellular fractions estimated by absorption of Slonaker anti-D23 isoantibody*

Serum	Absorption conditions: Fraction	Protein (mg)	Cell equivalent ($\times 10^{-8}$)	Fluorescence index: Unabsorbed	Fluorescence index: Absorbed	Isoantigen content: Percentage recovery of surface antigen	Isoantigen content: Specific* activity
S1537	Whole cells	—	1·0	0·97	0·30	100	—
	Soluble fraction	45·0	—	0·99	0·86	—	<0·02
S1803	Whole cells	—	1·5	1·00	0·30	100	—
	Extra-nuclear membrane	18·0	2·4	0·96	0·05	>63	>0·06
	Large particles	9·6	2·3	0·97	0·27	65	0·14
	Small particles	10·9	3·3	0·97	0·24	45	0·09
	Surface membrane	11·5	4·4	0·98	0·30	34	0·09
S1989	Cells	—	1·5	1·00	0·30	100	—
	Large particles	10·0	2·5	1·00	0·30	60	0·10
	Small particles	12·5	3·8	1·00	0·30	39	0·08
S2028	Cells	—	3·0	1·00	0·30	100	—
	Soluble	38·3	—	1·00	0·85	—	<0·03
	Surface membrane	11·5	4·4	1·00	0·30	68	0·09

*Reciprocal of smallest amount of protein (mg) giving FI=0·3.

of the cell completely neutralised isoantibody reacting with D23 cells in the immunofluorescence test (Fig. 3; Table 1).

Immunogenicity of subcellular fractions

The immunogenic activity of subcellular preparations of hepatoma D23 was established by the induction of a humoral antibody response in allogeneic recipients. By this criterion, all the particulate fractions of the hepatoma which absorbed isoantibody prepared against intact tumour were immunogenic. Thus, sera from rats which had received two or more subcutaneous injections gave strong immunofluorescence reactions with hepatoma D23 cells approaching those obtained by grafting intact tumour (Table 2). In contrast, the humoral antibody response evoked against soluble fractions of hepatoma D23 (105,000 **g** supernatant) was much weaker and was demonstrated unequivocally only in the sera of repeatedly immunized recipients (Table 2).

DISCUSSION

These studies establish that rat isoantigens, like their H-2 counterparts in the mouse, are expressed on the cell surface and may be isolated in association with the particulate fractions of the hepatoma cell. Recovery of isoantigen in these fractions was considerable and retention of all isoantigenic specificities was confirmed by their ability to completely block the immunofluorescence reaction of isoantibodies directed against antigenic determinants expressed on the hepatoma cell surface. Evidence of retention of antigenicity was also furnished from *in vivo* experiments in which particulate fractions from the hepatoma were shown to evoke a humoral antibody response in allogeneic hosts comparable with that generated by grafting intact tumour.

In contrast, the soluble fraction of the hepatoma cell, in absorption tests, had only a marginal effect on the ability of isoantisera to interact with these cells. This fraction evoked a humoral antibody response in allogeneic hosts only after prolonged immunisation and

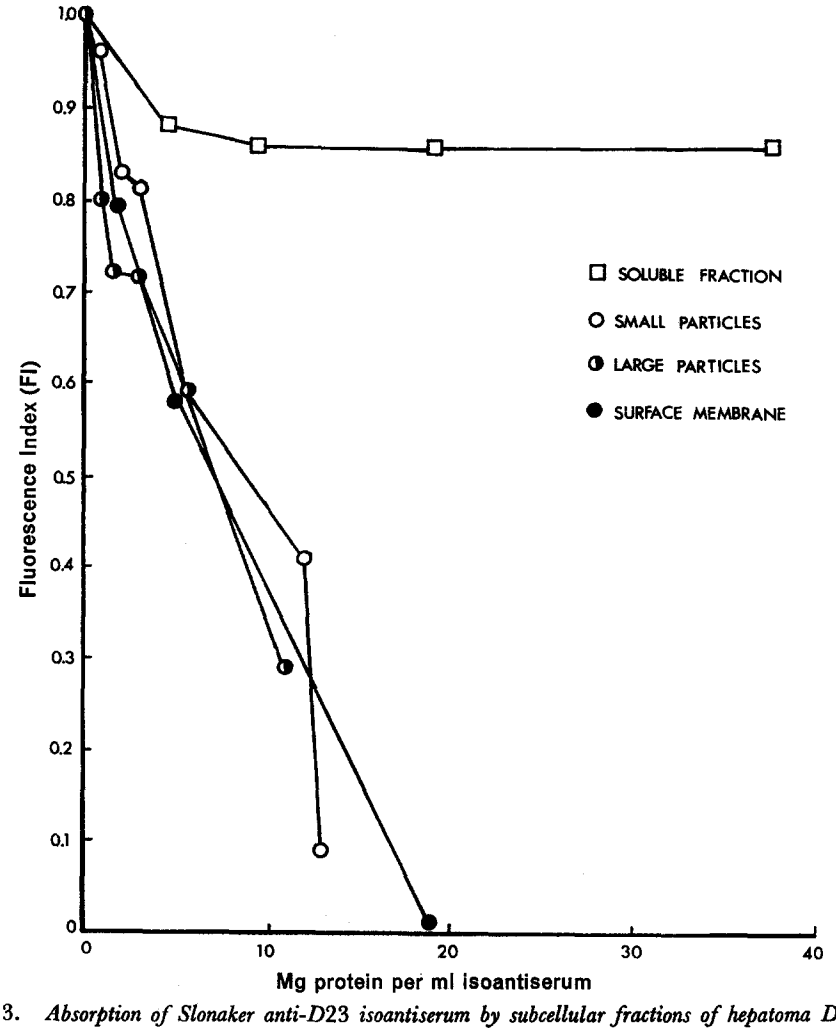

Fig. 3. Absorption of Slonaker anti-D23 isoantiserum by subcellular fractions of hepatoma D23.

this response was weak compared to that evoked by the particulate fractions. It is possible that, as shown for antigens of the mouse H-2 system [13, 18, 19], rat hepatoma isoantigens of one or more specificities find expression as soluble as well as particulate components. If such were the case, they would scarcely be detected in absorption studies using multispecific isoantisera, unless those determinants accounted for a large proportion of the hepatoma D23 cell surface. In common, therefore, with the H-2 isoantigens of the mouse and also human HL-A transplantation antigens [20, 21], rat isoantigens may be isolated from hepatoma cells principally in association with membranous components.

Table 2. Humoral antibody response evoked by hepatoma D23 cells and subcellular fractions in allogeneic recipients

Hepatoma fraction	No. of injections	Total immunizing dose mg protein	cell equivalent	Fluorescence index against D23 cells
Whole cells	7	—	Graft	0·93, 0·96, 0·84, 0·97
Whole Cells	8	—	Graft	1·00, 0·96, 1·00, 0.99
Large particles	4	16·4	$3·9\times10^8$	0·94, 0·97, 0·97
Small particles	4	13·3	$3·9\times10^8$	0·70, 0·73, 0·73
Surface membrane	2	1·9	7×10^7	0·71, 0·93, 0·79, 0·83
Surface membrane	3	4·8	$1·8\times10^8$	0·86, 0·89, 0·86, 0·80
Soluble fraction	3	78·6	—	0·22, 0·34
Soluble fraction	8	42·6	—	0·37, 0·51

Since graft rejection is based on reactions at the cell surface [22], isoantigens may serve as plasma membrane markers [13, 23]. Thus, the comparable activity of the large and small particle preparations is an indication that both these fractions are substantially contaminated with plasma membrane fragments. Clearly, when the cell is disrupted, the external membrane breaks up into particles of varying sizes depending upon cell type, the conditions of rupture and the fractionation scheme employed; thus several distinguishable classes of particles result from the breakage of Ehrlich ascites carcinoma cell membranes on which different H-2 antigenic specificities are expressed [13]. The present studies, however, imply that plasma membrane fragments isolated by differential centrifugation substantially overlap both small and large particles [24]. The other possibility, that antigenic determinants on intracellular membranes, identical to those expressed on the cell surface are exposed after cell rupture, is not supported by the finding that homogenisation did not demonstrably increase the ability of hepatoma D23 cells to inhibit the immunofluorescence reaction of isoantisera with intact D23 cells. However, single isoantigenic components on intracellular membranes would not be detectable using multispecific sera.

The failure, in absorption tests, of cells of normal rat tissues rich in isoantigens to completely neutralize the immunofluorescence reaction of Slonaker anti-hepatoma D23 isoantiserum with D23 cells, is consistent with the view that allografts of tumour D23 evoke the induction of antibody against D23 tumour-specific antigen [1–3], as well as against isoantigenic determinants. Furthermore, preabsorption of these isoantisera with subcellular particles completely abolished the immunofluorescence reaction with D23 cells, suggesting that membranous fractions retaining the activity of isoantigens also retain the activity of D23 tumour-specific antigen. This conclusion has been established in comparable absorption studies using tumour-specific immune sera from syngeneic hosts immunized with hepatoma D23 [3]. The preparation of subcellular fractions which retain the activity of tumour isoantigens is thus an important step in the isolation of tumour-specific antigens associated with carcinogen-induced transplanted hepatomas, and may be relevant to the isolation of neoantigens associated with other tumour types. Furthermore, these methods for isoantigen separation and assay could be applied to the fractionation of normal liver membrane components which are deleted from aminoazodye induced hepatomas [4]. These in turn will make possible immunochemical characterization of plasma membrane fractions, with regard to modified surface properties in the hepatoma cell.

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RESUME

Une technique d'immunofluorescence a été adaptée à l'étude de l'isoantigénicité de fractions subcellulaires d'un hépatome transplanté. La persistance de spécificités isoantigéniques, dans les fractions particulaires, fut confirmée (a) par leur capacité d'inhiber complètement la réaction immunofluorescente des isoanticorps dirigés contre des déterminants antigéniques de la surface cellulaire, (b) par l'apparition d'anticorps circulants à la suite de l'inoculation à des receveurs allogéniques.

Au contraire, la fraction soluble des cellules était relativement inactive dans ces deux systèmes.

Ces faits ont des conséquences importantes en ce qui concerne la séparation des composants de la membrane cellulaire du foie normal, composants qui peuvent être retirés des hépatomes provoqués par les colorants azoïques. Ils permettent aussi l'isolement d'antigènes tumoraux spécifiques des hépatomes et d'autres tumeurs.

SUMMARY

An immunufluorescence technique has been adapted for the assay of the isoantigenicity of subcellular fractions of a transplanted hepatoma. The retention, in particulate fractions, of isoantigenic specificities was confirmed by (a) their ability to completely inhibit the immunofluorescence reaction of isoantibodies directed against antigenic determinants expressed on the cell surface; and (b) by the generation of a humoral antibody response on

inoculation into allogeneic recipients. In contrast, the soluble fraction of the cell was relatively inactive in both respects. These findings have important implications for the separation of normal liver membrane components which are deleted from aminoazo-dye induced hepatomas; and also for the isolation of tumour specific antigens of hepatomas and other tumour types.

ZUSAMMENFASSUNG

Die Anwendung einer Immunofluoreszenzmethode wurde für die Prüfung der isoantigenen Eigenschaften der Fraktionen von Zellbestandteilen eines transplantierten Hepatoms ausgearbeitet. Das Weiterbestehen isoantigener Spezifität in bestimmten Fraktionen wurde festgestellt (a) durch ihre Fähigkeit, die Immunofluoreszenzreaktion solcher Isoantikörper zu verhindern, welche gegen antigene Determinanten der Zelloberfläche gerichtet waren; (b) durch die Erzeugung einer humoralen Antikörperreaktion bei Überimpfung auf artfremde Empfänger. Im Gegensatz hierzu erwies sich die lösliche Fraktion in beiden Prüfungen als relativ unwirksam. Aus diesen Befunden ergeben sich wichtige Folgerungen für die Isolierung bestimmter Komponenten der Membran normaler Leberzellen in Hepatomen, welche durch Aminoazofarbstoff induziert wurden, fehlen. Das gleiche gilt für die Isolierung von tumorspezifischen Antigenen bei Hepatomen und anderen Tumorarten.

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A Shift of Liver Aldolase Towards the Muscle Type in Hepatoma: Immunological Study

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SOME abnormalities of aldolase isozymes in human and rat hepatoma have been previously reported by Schapira *et al.* [1–3]. The activity ratio towards the two substrates fructose-1-6-diphosphate (F-D-P) and fructose-1-phosphate (F-1-P) was found to be increased (mean value 5·5 instead of 1 in normal liver). Adenosine triphosphate (ATP) (which inhibits muscle aldolase, as shown by Weinhouse *et al.* [5]), and antiserum anti muscle aldolase both strongly inhibited hepatoma aldolase: these results, strengthened by electrophoretic data, indicated a shift of liver aldolase toward a muscle pattern and also showed a similarity of hepatoma aldolase to foetal liver aldolase.

Muscle type aldolase (called aldolase A by Rutter [4]) and liver type aldolase (aldolase B) were reinvestigated in rat hepatoma and foetal liver, using the antibodies for crystalline rabbit liver and muscle aldolase: we measured and compared the amount of aldolase A and aldolase B present in these livers with that in normal adult liver.

MATERIALS AND METHODS

Rabbit muscle aldolase, prepared by the method of Taylor *et al.* [6] was recrystallized 4 times before use.

Rabbit liver aldolase was prepared by the Pogell's method [7] as modified by Morse and Horecker [8].

Rat hepatomas were obtained by feeding rats with 3'-methyl-4-dimethyl-aminoazobenzene‡.

The measurements of aldolase activity were made using the colorimetric method of Sibley and Lehninger [9] as modified by Schapira *et al.* [1].

Proteins were determined according to Lowry *et al.* [10].

Antiserum to rabbit liver aldolase (anti B) and to rabbit muscle aldolase (anti A) were prepared in chickens by repeated injections of crystalline rabbit liver or muscle aldolase, with or without complete Freund's adjuvant. Rabbit, rat and human aldolases are equally inhibited by chicken antisera: aldolases show no significant *species* specificities, but they display a strict *tissue* specificity (less than 2/100 of cross reacting material between muscle and liver aldolases).

The measurement of the proteins content of aldolases A or B was made by comparing the removal of neutralizing antibodies by hepatoma or foetal liver extracts with that by adult liver: standard curves were obtained by adding increasing amounts of normal liver extract to a constant amount of anti B or anti A. After

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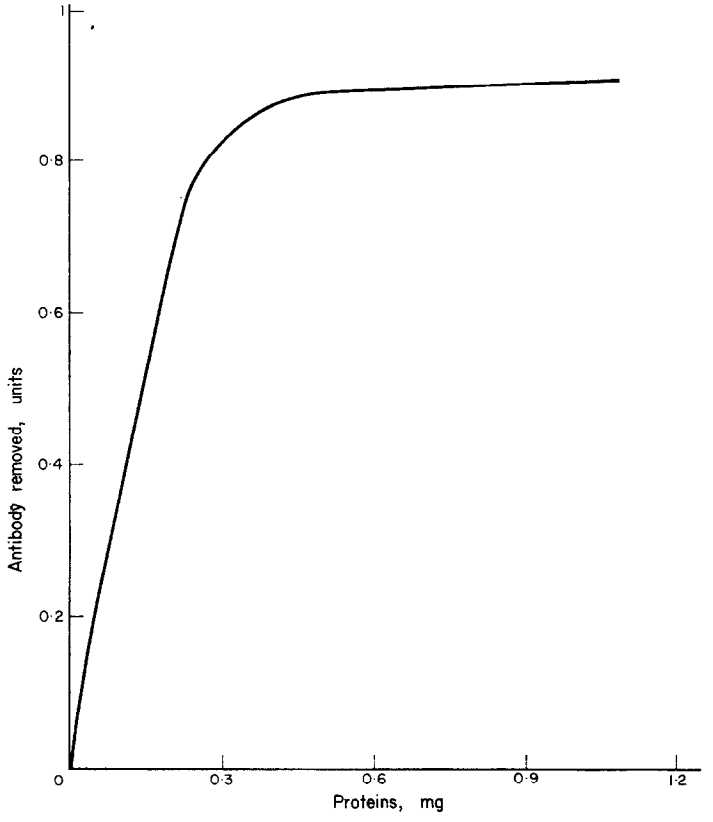

Fig. 1. *Removal of antibody anti B by normal adult rat liver. Increasing amounts of liver proteins were incubated with* 0·1 *ml* (1 *antibody unit*) *of anti B in a volume of* 0·25 *ml containing* 0·05 M *glycylglycine* (*pH* 7·5) *and* 2/100 *NaCl at* 37°*C for* 60 *min and at* 4°*C overnight. After centrifugation, supernatants were heated at* 56°*C for* 30 *min to destroy aldolase activity, and then centrifuged. The supernatants* (0·1 *ml*) *were analyzed for residual anti B by incubation with crystalline liver aldolase at* 37°*C for* 60 *min and at* 4°*C overnight and were then assayed for residual FDP cleavage activity. Controls included normal chicken serum.*

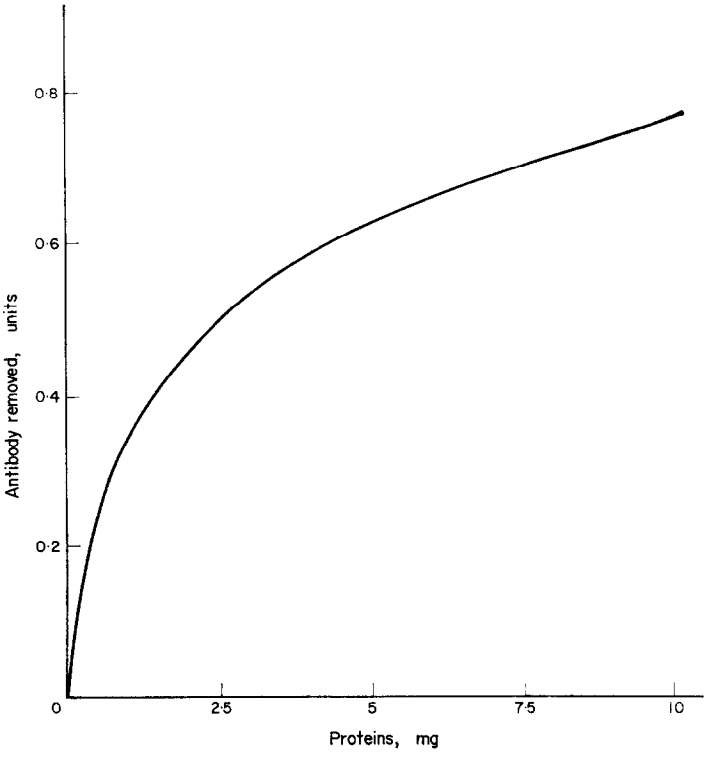

Fig. 2. *Removal of antibody anti A by normal adult rat liver. Same technique as described for Fig.* 1 *except that crystalline aldolase A is used.*

Table 1. Inhibition of F-D-P cleavage by antiserum anti aldolase A and/or anti aldolase B*

	F-D-P to F-1-P Activity ratio	% Inhibition by		
		Anti A	Anti B	Anti A + Anti B
Rat muscle	60	90	0	92
Rat liver	1·1	10	85	90
Foetal liver				
17	3·8	70	40·5	97
18	3·4	50	60	96
Hepatomas				
1	2·43	63	58	94
2	3	54	36	100
3	3·3	66	54	95
4†	16	83	0	

*The extracts were mixed with an equal volume of antiserum, incubated at 38° for 60 min then at 4°C for 24 hr and centrifuged. The supernatants were assayed for enzymatic activity. Controls included normal chicken serum.
†Reuber type hepatoma.

incubation and centrifugation, the supernatants were assayed for residual antibodies by determining the neutralization of the F-D-P cleavage activity of pure aldolase B or pure aldolase A.

The same type of experiments was then performed with hepatoma and foetal liver: referring to the standard curve (Figs. 1 and 2) we could determine the relative amount of each aldolase type; (other details of this method have been previously described in relation to liver aldolase in fructose intolerance [11]).

RESULTS

Table 1 shows the results of direct inhibition of the aldolase activity of rat hepatoma (4 cases) or foetal liver (2 litters) by antisera: in both types of livers the inhibition by anti A is much higher than in normal adult liver (cf. reference 3) and the reverse is true with respect to inhibition by anti B.

The Reuber type hepatoma is quite remarkable in that it appears to have only aldolase A.

Table 2 shows the removal of antibody anti aldolase A and anti aldolase B by rat hepatoma (6 cases) and foetal liver (2 litters).

Knowing the amount of normal liver proteins which remove the same quantity of antibody (cf. curves 1 and 2) it is easy to calculate the ratio of hepatoma (or foetal liver) aldolases to normal liver aldolases.

Table 2. Removal of antibody anti aldolase A and anti aldolase B by rat hepatoma or foetal liver

	F-D-P to F-1-P Activity ratio	Proteins (mg)	Removal of neutralizing antibody anti B (units*)	Removal of neutralizing antibody anti A (units†)	Ratio of hepatoma or foetal aldolases to normal adult quantities	
					A	B
Hepatomas						
1	2	0·95	0·66	0·48	1·77	0·22
2	2·01	0·87	0·19	0·56	3·1	0·28
3	2·43	2		0·52	2·5	
4	3·18	1·3		0·44	3	
5	3·3	1·5	0·52	0·49	3	0·22
6‡	16	0·5	0·06	0·75	17	0·16
Foetal liver						
17 days	3·8	0·29	0·02	0·18	1·2	0·15
18 days	3·4	0·45	0·03	0·24	1	0·17

*One unit: amount of antibody inhibiting 0·06 unit of F-D-P cleavage activity.
†One unit: amount of antibody inhibiting 0·23 unit of F-D-P cleavage activity.
‡Reuber type hepatoma.

In both these liver types the relative amount of aldolase B is lower than in normal liver (one sixth to one third).

Aldolase A is higher only in hepatoma (1·7 –17 times normal value).

In foetal liver at this stage, aldolase A is almost at normal values (0·9 –1·5).

DISCUSSION

Muscle type aldolase is present in *foetal liver* [3] as has been shown by electrophoretic pattern, inhibition by ATP and greater inhibition by anti A than in adult liver (Table 1).

The normal quantity of aldolase A that we found in 17–18 days foetal liver does not imply that in earlier stages aldolase A was not present in greater quantity. Nevertheless at this stage aldolase A still predominates, mainly because aldolase B is much lower than in adult liver (Table 2). Therefore the activity ratio F-D-P/F-1-P is higher in foetal than in adult liver because aldolase A has a higher affinity for F-D-P than aldolase B.

In *hepatoma* the results confirm previous data of our team [1–3] on the appearance (or reappearance) of a muscle type aldolase. Similar data have since been obtained with other hepatoma enzymes (pyruvate kinase [12], hexokinase [12], F-D-Pase [13] showing in each case a shift of liver enzyme towards a muscle pattern.

Using the antibody removal method we could directly measure the relative amount of proteins sharing the antigenic properties of aldolase A and B in hepatoma, as compared to normal liver (Table 2). The absolute increase of aldolase A and the decrease of aldolase B also gives a good explanation for the increase of activity ratio F-D-P/F-1-P. The most likely hypothesis for this absolute increase of aldolase A would be a "derepression" of the synthesis of this enzyme which was relatively predominant in foetal liver.

This hypothesis is strengthened by several reports of the reappearance of other foetal proteins in hepatoma in man [14], in the rat [15, 16] and in the mouse [17]. Drouet and Burtin [18] have also shown the reappearance of a foetal protein in kidney tumors. It therefore seems likely that the ability to synthesize foetal molecules has been preserved by adult tissues in a repressed form and that the neoplastic lesion leads to the "derepression" of the foetal protein synthesis.

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RESUME

Utilisant une méthode de mesure de consommation d'anticorps, nous avons pu doser (par rapport au foie adulte normal) les taux d'aldolase A et B présents dans le foie foetal et l'hépatome de rat. L'importante augmentation de l'aldolase A dans l'hépatome contraste avec la diminution de l'aldolase B.

Dans le foie foetal, l'aldolase A paraît proche du taux du foie adulte, mais l'aldolase B est également abaissée.

SUMMARY

Using the antibody removal method we could directly measure the relative amount of aldolase A and B in rat hepatoma and foetal liver, as compared to normal liver. We found a great increase of aldolase A in hepatoma, contrasting with an aldolase B much lower than normal values.

In foetal liver aldolase A is near the normal liver quantity, and aldolase B is lower.

ZUSAMMENFASSUNG

Unter Verwendung der Methode der Antikörperbindung konnte die relative Menge an Aldolase A und B in Hepatomen und foetaler Leber im Vergleich mit normaler Leber der Ratte direkt bestimmt werden. Es wurde eine starke Zunahme an Aldolase A im Hepatom gefunden, während Aldolase B im Gegensatz hierzu unter den normalen Werten lag. In foetaler Leber ist die Menge an Aldolase A nahe der in normaler Leber und die an Aldolase B geringer.

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Unterschiede in der Reifung des Kollagens zwischen Rücken- und Schwanzhaut von Mäusen im Hinblick auf die unterschiedliche Tumoranfälligkeit dieser Hautanteile

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EINLEITUNG

SPENCER [1] stellte fest, daß am Mäuseschwanz im Gegensatz zum Rücken mit Cancerogenen keine Carcinome induziert werden können. Diese entstehen erst, wenn die mit Cancerogen behandelte Schwanzhaut in den Rücken implantiert wird. Den Zellen der Schwanzepidermis ist also malignes Wachstum möglich, sie werden jedoch im Schwanzbereich durch die dort wirksame Kontrolle des Wachstums daran gehindert.

Wachstum und Differenzierung der Epidermis werden, wie aus der Embryologie und Untersuchungen über die Regeneration bekannt ist, durch die Dermis kontrolliert. Besondere Bedeutung scheint dabei dem Kollagen der Dermis zuzukommen. Möglicherweise ist deshalb malignes Wachstum der Epidermis auf eine Störung in der Dermis bzw. im Kollagen der Dermis zurückzuführen, wie an anderer Stelle [2] bereits ausführlicher dargelegt wurde. Im Einklang damit stellten wir in früheren Untersuchungen fest, daß Cancerogene eine starke Verminderung des Kollagengehaltes der Rückenhaut von Mäusen bewirkten [2]. Diese Verminderung ist in erster Linie auf eine kollagenolytische Wirkung zurückzuführen, welche von der Epidermis auszugehen scheint, da sie nicht beobachtet werden kann, wenn Cancerogen direkt auf die Dermis gebracht wird [3]. In der Schwanzhaut dagegen kann auch nach Behandlung mit größeren Dosen von Cancerogen kein Kollagenverlust festgestellt werden.

Schrumpfungsversuche [4] an Rücken- und Schwanzhaut von Mäusen haben bereits nahegelegt, daß der Vernetzungsgrad des Kollagens in diesen beiden Hautanteilen unterschiedlich ist. Es soll deshalb im folgenden der Verlauf der Kollagenvernetzung der Dermis bei Mäusen im Hinblick auf die angeführten Zusammenhänge mit dem Tumorwachstum der Epidermis in Rücken- und Schwanzhaut während der ersten Lebensmonate näher analysiert werden.

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MATERIAL UND METHODEN

Untersucht wurden Männchen von Swiss-Albino-Mäusen in vier Altersgruppen. Je fünf Tiere im Alter von 130 und 64 Tagen sowie je zehn Tiere im Alter von 34 und 19 Tagen wurden getötet. Von Rücken- und Schwanzhaut der getöteten Mäuse wurden subcutanes Fett- und Muskelgewebe sowie Haare bzw. Schuppen sorgfältig mechanisch entfernt. Die Häute wurden fein zerschnitten, 24 Stunden in Äther entfettet und im Exsikkator

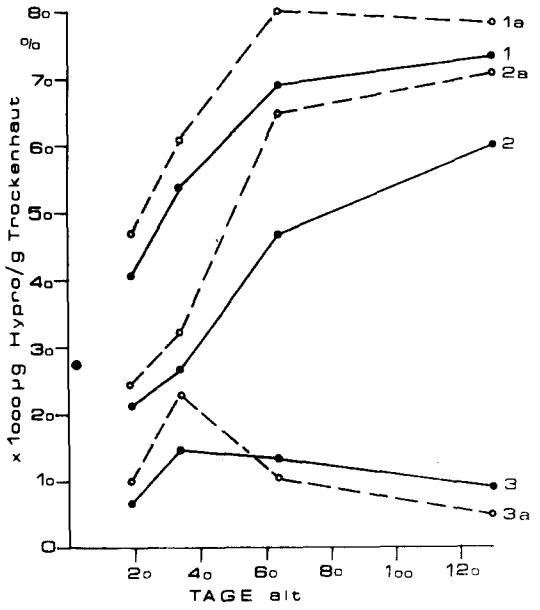

Abb. 1. *Hydroxyprolingehalt von Rücken* (1, 2, 3) *bzw. Schwanz* (1*a*, 2*a*, 3*a*) *verschieden alter Mäuse.*
1. *Gesamthyrdoxyprolingehalt.*
2. *Gehalt in der 'alten' (hochvernetzten) durch TCA extrahierbaren Kollagenfraktion*
3. *Gehalt der 'jungen' durch Cysteamin extrahierbaren Kollagenfraktion.*

———*Rücken.*
----- *Schwanz.*

bis zur Gewichtskonstanz getrocknet. Die entsprechenden Anteile (Rücken- und Schwanzhaut) innerhalb jeder Altersgruppe wurden vereint, 0,2 bis 0,5 g Trockenhaut zur Analyse verwendet.

Bei der stufenweisen Extraktion des Kollagens folgten wir im wesentlichen der Methode von Nimmi *et al.* [5], Abweichungen davon wurden bereits beschrieben [2]. Von den mit dieser Extraktionsreihe gewonnenen Fraktionen enthält der NaCl-Extrakt frisch synthetisiertes, noch nicht fest im Fibrillenverband befindliches Kollagen, durch Cysteamin wird ebenfalls junges, jedoch bereits fest aggregiertes Kollagen erfaßt, welches vermutlich noch nicht in jener Weise kovalent verknüpft ist, wie der verbleibende, durch heiße Trichloressigsäure extrahierbare Rest. Die Bedeutung der Citratfraktion ist nicht genau bekannt. Bei Applikation radioaktiv markierten Prolins an Ratten erschien die Aktivität zuerst in der NaCl-, dann gleichzeitig in der Cysteamin- und Citrat- und zuletzt in der TCA-Fraktion [5].

Die Hydrolyse der Proben wurde mit 6 n

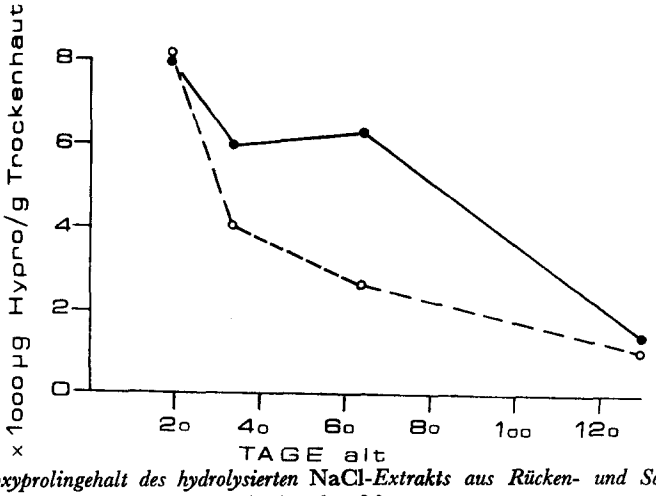

Abb. 2. *Hydroxyprolingehalt des hydrolysierten* NaCl-*Extrakts aus Rücken- und Schwanzhaut verschieden alter Mäuse.*

———*Rücken.*
----- *Schwanz.*

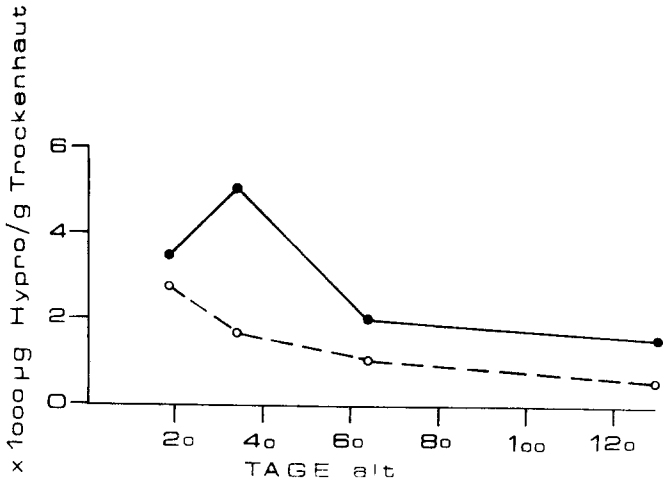

Abb. 3. Hydroxyprolingehalt des hydrolysierten Citratextrakts aus Rücken- und Schwanzhaut verschieden alter Mäuse.
——— *Rücken.*
- - - - - *Schwanz.*

HCL 18h bei 110°C durchgeführt. Die Hydroxyprolin-bestimmung lerfolgte nach der Methode von Woessner [6].

ERGEBNISSE

Die Abb. 1 zeigt, daß der Gehalt an Gesamtkollagen in jeder der untersuchten Altersstufen im Schwanz höher ist als im Rücken. Die größten Unterschiede ergeben sich um den 64. Tag, wenn die Schwanzhaut bereits den maximalen Kollagengehalt erreicht hat, während die Menge in der Rückenhaut noch langsam bis zum 130. Tag zunimmt. Der zu diesem Zeitpunkt festgestellte Wert von *ca.* 74.000 µg Hydroxyprolin/g Trockenhaut erhöht sich später nicht mehr.

Die stärkste Kollagensynthese (ersichtlich aus der Größe der NaCl-Fraktion) findet—etwa gleich stark in Rücken- und Schwanzhaut—um den 19. Tag oder vorher statt (Abb. 2). Zwischen 19. und 34. Tag, in der Zeit der Entwöhnung von der Mutter, sinkt die Synthese sehr stark ab, und zwar in der Schwanzhaut bedeutend stärker als im Rücken. Während die Synthese im Rücken zwischen 34. und 64. Tag wieder leicht ansteigt, um dann rasch zu sinken, wird in der Schwanzhaut lediglich ein Abfall der Synthese beobachtet.

Das weitere Schicksal des Kollagens aus dem starken Syntheseschub um den 19. Tag ist anhand der Abb. 1–3 gut zu verfolgen und in Rücken und Schwanz etwas unterschiedlich: während es in der Schwanzhaut größtenteils rasch in die durch Cysteamin extrahierbare Fraktion übernommen wird, was sich durch ein starkes Maximum in dieser Fraktion am 34. Tag ausdrückt (Abb. 1), ist im Rücken der Anstieg in der Cysteaminfraktion viel

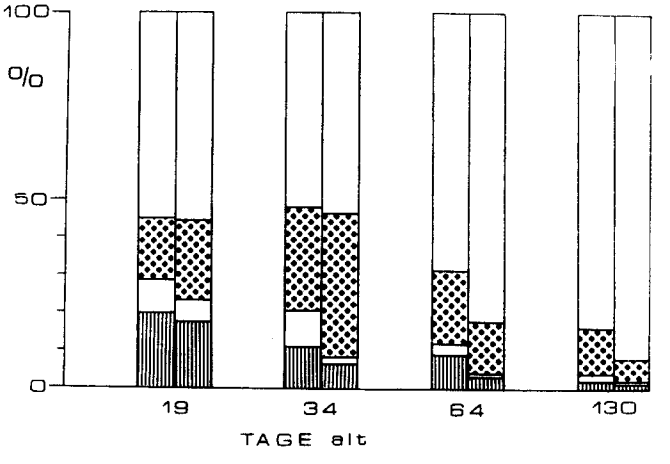

Abb. 4. Prozentuelle Zusammensetzung des Gesamtkollagens von Rücken- (linke Hälfte) und Schwanzhaut (rechte Hälfte der Doppelsäulen) verschieden alter Mäuse aus NaCl- (strichliert), Citrat- (darüber, leer), Cysteamin- (punktiert) und TCA- (darüber, leer) extrahierbarem Material.

schwächer, dafür ist ein ausgeprägtes Maximum im Citratextrakt zu beobachten (Abb. 3). Ein Teil der NaCl-Fraktion, welcher im Schwanz in die Cysteaminfraktion übergeht, ist im Rücken in den Citratextrakt gelangt. Prozentuell ist in Schwanz- und Rückenhaut am 34. Tag etwa gleich viel Kollagen in den "jungen" Fraktionen (NaCl-, Citrat- und Cysteaminfraktion) enthalten (Abb. 4), innerhalb dieser Fraktionen sind allerdings die Anteile zwischen Cysteamin und Citrat verschoben.

Zwischen 34. und 64. Tag sinkt der Kollagengehalt der Cysteaminextrakte im Schwanz stark ab, während jener der hochvernetzten TCA-Fraktion steil ansteigt, die Vernetzung schreitet also im Schwanz sehr rasch fort. Viel weniger steil ist dagegen der Anstieg der TCA-Fraktion im Rücken und parallel dazu sinkt der Cysteamin-extrahierbare Anteil langsamer. Zu dieser Zeit eilt die Kollagenvernetzung in der Schwanzhaut jener in der Rückenhaut deutlich voraus (Abb. 1). Auch später wird im Rücken der Grad der Schwanzvernetzung nicht mehr erreicht.

DISKUSSION

Ein wesentlicher Unterschied in der Kollagenreifung zwischen Schwanz- und Rückendermis besteht nach unseren Ergebnissen darin, daß in der Schwanzdermis frisch synthetisiertes Kollagen zu einem hohen Prozentsatz und rasch in einer Form aggregiert, welche das Fortschreiten der kovalenten Vernetzung (geschlossen aus der Verschiebung im Kollagengehalt von Cysteamin- und TCA-Extrakt) sehr begünstigt. In der Rückendermis erscheint ein viel kleinerer Prozentsatz in dieser Form (durch Cysteaminlösung extrahierbar), ein bemerkenswerter Anteil dagegen findet sich als säurelösliches (Citrat-) Kollagen, welches die weitere Vernetzung zu behindern scheint, da diese im Rücken langsamer vonstatten geht als in der Schwanzhaut. Säurelösliches Kollagen ergibt bei Denaturierung nach Untersuchungen mittels Diskelektrophorese einen so hohen Anteil an ß-Kollagen, daß der größte Teil der in dieser Fraktion enthaltenen Kollagenmoleküle eine intramolekulare kovalente Bindung enthalten muß. Nach Ansicht einiger Autoren [7, 8] ist das ein Seitenweg bzw. eine Fehlentwicklung, weil diese Möglichkeit der Bindung dann für die intermolekulare Vernetzung bereits blockiert sei. Damit könnten die beobachteten Unterschiede in der Kollagenreifung zwischen Rücken- und Schwanzdermis bei Mäusen folgendermaßen erklärt werden: frisch synthetisiertes Kollagen kann in der Rückendermis nicht so rasch fibrillär aggregieren wie in der Schwanzdermis. Dadurch bleibt ein längerer Zeitraum, in dem nur intramolekulare Bindungen geknüpft werden können, was sich in einer erhöhten Menge säurelöslichen Kollagens ausdrückt. Das säurelösliche Kollagen bildet Fehlstellen im Kollagennetzwerk, welche die Geschwindigkeit der weiteren Vernetzung beeinträchtigen und das Rückenkollagen insgesamt weniger vernetzt erscheinen lassen als das Kollagen der Schwanzdermis.

Eine kollagenolytische Wirkung, gerichtet auf die apolaren Bereiche der Kollagenkette, wie sie bei malignem Wachstum verstärkt beobachtet wird [9], wird dort rascheren Abbau bewirken, wo die Vernetzung geringer ist. Wird nun die Epidermis mit einem Cancerogen behandelt, fällt die Kontakthemmung der Epidermiszellen weg. Nach Strauch [9] ist aber der Wegfall der Kontakthemmung mit einer Aufnahme der Synthese eines kollagenolytischen Enzyms verknüpft. Im Rücken werden die Kollagenstrukturen rascher aufgelöst, die dagegen wirkende Aggregation erfolgt langsamer, sodaß die Kollagenolyse die Oberhand behält. Im Schwanz dagegen erfolgt die Auflösung langsamer, die Aggregation neuer Fibrillen rascher, sodaß die Kollagenolyse nicht fortschreiten kann. Dies würde erklären, daß bei Bepinselung mit Cancerogen nur im Rücken, nicht aber in der Schwanzhaut von Mäusen ein Kollagenverlust auftritt.

RESUME

1. *Après le traitement de la peau de la souris avec une substance cancérogène, on a observé la formation de cancers dans la région dorsale, et non sur la queue. Considérant le rôle du collagène dermique sur la croissance et sur la différenciation de l'épiderme, on a étudié, chez la souris, les différences entre la maturation du collagène de la peau de la queue et celle de la région dorsale.*

2. *Dans la queue, la plus grande partie du collagène récemment synthétisé s'agrège sous une forme extractible par la cystéamine. Cette forme favorise la formation du réseau covalent; celle-ci se passe plus rapidement et atteint un degré plus élevé que dans la peau dorsale du même animal. Dans la région dorsale par contre, un pourcentage relativement*

élevé du collagène récemment synthétisé a pu être retrouvé plus tard sous une forme acido-soluble.
3. *Les résultats soutiennent l'hypothèse que le collagène acido-soluble représente une voie à part dans le processus de la maturation du collagène.*
4. *L'impossibilité d'induire des cancers dans la peau de la queue pourrait s'expliquer d'un côté par une plus grande vitesse d'agrégation et de formation de fibrilles, et de l'autre côté par un ralentissement de l'agression par les collagénases. Ces deux facteurs empêchent la perte de collagène qui a été observée à la suite du badigeonnage du dos par le méthylcholanthrène.*

SUMMARY

1. *Painting with carcinogenic hydrocarbons produces carcinomas in the back skin of mice, but is ineffective in tail skin. Since dermal collagen is thought to be involved in the control of epidermal proliferation and differentiation, it was investigated whether there are differences between back and tail skin with respect to the maturation of collagen.*
2. *Newly synthesized collagen of tail skin is composed predominantly of a cysteamin-extractable form which appears to be favourable for further cross linkage. Cross linkage in tail skin collagen proceeds faster and to a greater extent than in collagen of the back skin of the same animal. By contrast, a relatively high percentage of newly synthesized collagen of back skin is later found in an acid-soluble form.*
3. *The results support the hypothesis that the acid-soluble form of collagen is not a precursor of the insoluble collagen.*
4. *The faster rate of collagen aggregation and fibril formation in tail skin is thought to result in a highly cross linked form of collagen more resistant to degradation by collagenases. This process obviously prevents a loss of collagen analogous to that observed in back skin after methylcholanthrene application. This might possibly be the reason for the inability to induce carcinomas in tail skin.*

ZUSAMMENFASSUNG

1. *Durch Behandlung mit Cancerogen können in der Rücken-, nicht aber in der Schwanzhaut von Mäusen Carcinome erzielt werden. Im Hinblick auf die Rolle des Kollagens der Dermis bei der Steuerung von Wachstum und Differenzierung der Epidermis wurden die Unterschiede in der Kollagenreifung zwischen Schwanz- und Rückenhaut von Mäusen untersucht.*
2. *Frisch synthetisiertes Kollagen aggregiert in der Schwanzhaut zum Großteil in einer mit Cysteaminlösung extrahierbaren Form, welche die weitere kovalente Vernetzung sehr zu begünstigen scheint, da diese sowohl rascher vonstatten geht als auch einen höheren Grad erreicht als in der Rückenhaut der gleichen Tiere. Demgegenüber wird in der Rückenhaut ein verhältnismäßig großer Prozentsatz der frisch synthetisierten Kollagens später als säurelösliches Kollagen gefunden.*
3. *Die Ergebnisse stützen die Ansicht, daß das säurelösliche Kollagen einen Seitenweg in der Kollagenreifung darstellt.*
4. *Die Nichtinduzierbarkeit von Carcinomen in der Schwanzhaut könnte damit zusammenhängen, daß dort mit einer rascheren Kollagenaggregation und Fibrillenbildung eine durch die höhere Vernetzung bedingte langsamere Auflösung durch Kollagenasen einhergeht; beide Faktoren verhindern einen Kollagenverlust, wie er in der Rückenhaut bei Einwirkung von Methylcholanthren zu beobachten ist.*

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Solubilization and Partial Fractionation of the Sulphur-Containing Nuclear Proteins of Hepatoma 223 Ascites Cells

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LITTLE IS known about the synthesis, metabolism and function of the non-histone proteins of the cancer cell nucleus. Considerable quantities of this type of protein are present in somatic cell nuclei [1, 2]. Debov [3, 4] found that the concentration of these proteins was higher in tumours and regenerating liver than in normal tissues. Nevertheless, no satisfactory separation nor classification of this group of proteins has been reported (see references 5 and 6 for reviews). This is because their relative insolubility in normal aqueous solvents makes them a difficult problem from the analytical point of view and is a serious impediment to further characterisation. However, their chemical composition has been studied after rather drastic extraction procedures, which may have broken labile covalent links [7]. Thus although studies of NH_2 terminal amino-acids indicate great heterogeneity [8], the only distinguishing feature (apart from insolubility) of these non-histone proteins is the preponderance of aspartic and glutamic acids over the basic amino-acids, lysine, arginine and histidine.

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Recently further attempts at subfractionation and classification of rat liver chromatin and residual proteins based on the solubility of some of their components in deoxycholate and detergent mixtures have been reported [9, 10]. Little progress has been made, so far, with the acidic proteins from tumour nuclei, probably as a result of problems involved in obtaining clean nuclei from tumour tissues [11].

The acidic proteins have been known for some time to contain most of the sulphur present in the nucleus [1]. However, although the histones were formerly considered by some workers to contain little cysteine or methionine [12], the F3, or arginine rich, fraction has been shown to contain appreciable levels of sulphydryl (-SH) groups [13–15] which might be important in the control of transcription [16, 17].

Marsh, Ord and Stocken [18], in their studies on the -SH and -S-S- content of nuclei, noted that a doubling of nuclear -SH values occurred on treatment with 4M urea. Further investigations using rat liver nuclei (Gronow, unpublished observation) showed that 88% of the -SH material could be solubilized in 8M urea 0·05M sodium phosphate pH 7·6. This technique was successfully applied to nuclei isolated from a rat ascites hepatoma and details are given of results described in preliminary communication [19].

METHODS AND RESULTS

Tumour

Hepatoma 223 (a 4-dimethylaminoazobenzene-derived tumour) was maintained in an ascitic form by weekly serial transplant in the peritoneal cavity of 200 g albino male Wistar rats (Scientific Products, Ash, Canterbury, England). The animals were used for experiment 7 days after the initial transplant of approximately 1 ml of undiluted ascitic fluid.

Isolation of nuclei

The use of osmotic shock for preparing nuclei from ascites cells is well known [20, 21]. For the proposed preparations it was thought that the addition of divalent cations in the hypotonic medium might help to bind protein–nucleic acid complexes in a tighter fashion and thus prevent leakage during isolation. Also, in order to remove cytoplasmic contamination, homogenization in all-glass ball-type homogenizers [22] with a tight gap was attempted. Homogenizers with a 0·5, 1·0, 1·5, and 2·0 thousandths of an inch gap were used. The most successful was with a one-thousandth gap, as judged by the number of clean nuclei seen on phase microscopy.

The pooled tumour from a minimum of five rats was centrifuged at 350 **g** for 3 min and this was sufficient to sediment all the cells. The fluid was discarded and the cells resuspended in an equal volume of 0·005M calcium chloride (in distilled water) at 4°C. The cells were spun down as before and the wash repeated. The erythrocyte contamination was removed by these washes. After resuspending the cells for a third time they were homogenized in an all-glass homogenizer (with a one-thousandth inch gap) using 8–10 complete up-and-down strokes. The mixture of nuclei and whole cells was spun down at 750 **g** for 3 min. The homogenization was repeated once more; all procedures being carried out at 0–4°C. Finally the pellet was resuspended in 2·4M sucrose 0·005 Ca^{2+} using a teflon glass motor-driven homogenizer and made up to a final volume of approximately three times the volume of ascitic fluid initially taken. Nuclei were separated from whole cells by centrifugation at 40,000 **g** for 70 min [23]. In order to check the purity of these nuclei, samples were examined in the electron microscope.

Samples of nuclei, extracted nuclei and whole cells were fixed for 2 hr (4°C) in a solution containing 4% (w/v) paraformaldehyde, 1% (w/v) glutaraldehyde in 0·05M sodium phosphate buffer. They were then washed with 0·2M sodium phosphate pH 7·3–7·4 containing 5% sucrose, stained with 1% osmium tetroxide in this buffer and finally embedded in Durcupan (Fluka Chemische Fabrik). Sections were stained with lead acetate.

Extraction of nuclear proteins.

(a) *Extraction of urea soluble proteins.* Nuclei were extracted twice with 8M urea 0·05M sodium phosphate buffer pH 7·6 containing a two-fold excess of 1-^{14}C *N*-ethylmaleimide (NEM) (Schwarz BioResearch, Orangeburg, N.Y. diluted with unlabelled NEM to a specific activity of 0·125–0·25 μC/μ mole) in a hand-operated all-glass homogenizer with a 10 thousandth of an inch gap at 5–10°C.

A small portion of the homogenate was tested with Ellman's [24] reagent to ensure that all the -SH groups had reacted with the NEM. This was usually complete within minutes of the first extraction. Unreacted ^{14}C-NEM was quantitatively removed by gently extracting with ether. -SH measurements using the Ellman method always agreed with those obtained using ^{14}C-NEM.

The nuclei, which were essentially intact after this extraction, were spun down at 3000 **g** for 15 min. They were then re-extracted with half the initial volume of buffered 8M urea without ^{14}C-NEM.

(b) *Extraction of histones.* After centrifuging as before the nuclei were homogenized with 0·25M HCl (at 4°C) until the gel had been broken up. The mixture was spun at 3500 **g** for 25 min and the insoluble residue re-extracted with half the initial volume of 0·25M HCl.

The residue was insoluble in HCl and was extracted with 1M NaOH at room temperature overnight.

Protein, DNA and -SH assays

Diluted samples of suspensions of nuclei or whole cells were counted in a Neubauer chamber. Aliquots of these suspensions were subjected to the Schmidt–Thannhauser procedure. The protein content was determined by the method of Lowry, Rosebrough, Farr and Randall [25] using bovine plasma albumin (Armour Chemical Co.) as a standard.

DNA was estimated by the Burton modification of the diphenylamine procedure [26] using highly polymerized calf thymus DNA (Sigma) as a standard.

Suitable volumes of each of the nuclear fractions were taken and trichloracetic acid (TCA) added at 4°C to give a concentration of 30% (w/v). The precipitate was centrifuged (3000 **g** for 20 min), washed with cold 20%

(w/v) TCA and finally dissolved in 1M NaOH at room temperature. Aliquots of this solution were taken, neutralized and the protein and DNA content determined as previously described.

The -SH content of the nuclear extracts was determined, either directly or after neutralization, from the amount of ^{14}C-NEM present. For this, suitable aliquots of up to 100 µl of the labelled mixture were diluted to 1 ml with water and 10 ml of scintillator fluid added. The latter consisted of 8 g butyl PBD (Ciba Products Ltd.) and 80 g naphthalene in 1 litre dioxan. The resultant clear fluid was counted in a Beckman automatic liquid scintillation counter to 5% error at about 70% efficiency.

Chromatography of nuclear protein fractions

A. 8M *Urea* 0·05M *phosphate extract*

It was found that any attempt to remove urea by dialysis against distilled water or even against salt solutions or buffers led to copious precipitation of protein material. Therefore it was necessary to carry out subsequent chromatography in strong urea solutions. Also metal ions were found to interfere with ion exchange chromatography causing irreversible aggregation of the proteins on column material (e.g. DEAE). To remove these ions and to achieve an initial separation of different molecular weight thiols, chromatography on Sephadex G-100 was attempted.

For this an 18 × 4·3 cm column of G-100 was made up in, and eluted with, 8M urea (deionized using a Deeminac Deionizer, Baird and Tatlock) containing 0·005M EDTA 0·005M tris pH 8·0; 12 ml ^{14}C-NEM labelled nuclear urea extract containing 50–60 mg protein were put on this column after making 0·01M with respect to EDTA. Next 5·2 ml fractions were collected at an elution rate of about 1 ml/2 min.

The absorbancy of the column eluant was read at 260 and 280 mµ (after dilution when necessary) in a Hilger–Watts spectrophotometer; 50 or 100 µl were directly assayed for radioactivity as previously described.

Chromatography on DEAE cellulose

Whatman microgranular DEAE cellulose (DE 52) was washed, as recommended, with acid and base and suspended in 8M urea (deionized as previously described) containing 0·005M EDTA 0·005M tris pH 8·4. The excluded protein and RNA peak from the G-100 column was applied to a column of this ion-exchange cellulose.

A solution of the ^{14}C-NEM labelled G-100 protein peak containing about 25 mg of protein was applied to a 10 × 1 cm column of the DEAE cellulose. Fractions were eluted using discontinuous sodium chloride gradients made up in 8M urea, 0·005M EDTA, 0·005M tris pH 8·0.

Attempts to fractionate material not absorbed on DEAE cellulose

The protein solution passing straight through the DEAE was dialysed exhaustively at 4°C against distilled water pH 7·0. The protein was quantitatively precipitated, centrifuged down and dissolved in 1M acetic acid. Attempts were made to fractionate this mixture by four different methods:

(a) *On CM-cellulose.* Fifteen ml of M acetic acid containing 15–20 mg of ^{14}C-NEM labelled protein were passed through a 10 × 2 cm column of Whatman CM-cellulose (CM11) previously equilibrated with this solvent. After a wash with M acetic acid the column was eluted with 0·01M, 0·02M, 0·10M and 1M HCl.

(b) *On G-200 Sephadex.* To a 95 × 3 cm column in M acetic acid (the void volume of which was 160 ml) was applied 3 ml of M acetic acid containing 15 mg of ^{14}C-NEM labelled DEAE "breakthrough" protein. The protein was eluted with M acetic acid at the rate of approximately 12 ml/hr. Fractions of 4 ml were collected and these were assayed for radioactivity as previously described.

(c) *By isoelectric focussing.* The technique of Svennson [27] was applied to these acetic acid soluble proteins. Using an apparatus supplied by LKB Instruments the mixture was subjected to electrophoresis for 40 hr in a pH gradient of 3–10; the carrier ampolytes being dissolved in 6M urea (deionized).

(d) *By gel electrophoresis.* Attempts were made to obtain electrophoretic separations of all the protein fractions previously described. The polyacrylamide gel systems of Ornstein [28] were used with acrylamide monomer concentrations varying from 3 to 15% at pH values of 4·4, 7·6 and 8·6, with and without 8M urea.

Also electrophoresis was attempted on 1·5% agrose gels (supplied by Seravac Laboratories).

B. *Chromatography of* 0·25M *HCl soluble extract*

This was dialysed at 4°C against three changes of 0·1M sodium acetate buffer pH 4·2 and an equal volume of 11M urea (deionized) added.

Fifty ml of this solution containing approximately 32 mg of the ^{14}C-NEM labelled protein were applied to a 15 × 2 cm column of Whatman microgranular CM cellulose (CM52

—precycled with alkali and acid and equilibrated with 0·1M sodium acetate buffer pH 4·2).

Essentially the elution system employed by Johns, Phillips, Simson and Butler [29] was used. The flow rate was 12 ml/hr and 4 ml fractions were collected. The absorbancy at 275 mμ and the radioactivity of each fraction was determined.

Also a sample of the 8M urea 0·05M phosphate nuclear extract was exhaustively dialysed (at 4°C) against 0·25M HCl. After centrifuging the dialysed material at 100,000 **g** for 1 hr the supernatant was applied to a CM-cellulose column and fractionated as described above.

Amino-acid analysis

Pooled samples were dialysed exhaustively against distilled water, passed through a 0·45 μ Millipore filter and finally freeze dried. One to two mg of solid were taken, constant boiling point HCl added (200 μl per mg) and the tubes sealed under reduced pressure. Samples were hydrolysed at 108°C for 24 hr. Amino-acid analyses were made with a Spinco 120c automatic analyser (Beckman Instruments) [30].

RESULTS

Purity of nuclear preparation

Nuclei prepared by the method described appear to be free of cytoplasmic contamination when viewed in a phase contrast microscope. This was confirmed by electron microscopy. Figure 1a shows an example of the original ascites cells and Fig. 1b the nuclei isolated from these cells. The double membrane can be seen to be intact in these nuclei with large prominent nucleoli characteristic of tumour cells. An electron micrograph of urea extracted nuclei (Fig. 1c) show clearly the loss of density in the nucleolar region. These "holes" can also been seen under the phase microscope.

Isolated nuclei contained 6·7 ± 0·2 pg of DNA per nucleus and had a protein to DNA ratio of 8·0 ± 1·0 (whole cells, 30·7 ± 1·0). As a comparison nuclei were isolated in a 2 or 4% citric acid medium [21]. These had a more transparent appearance and the protein to DNA ratio was about half of that given above, showing considerable loss of protein during the isolation procedure.

Extraction of proteins from isolated nuclei

Of the total nuclear protein material 93% can be solubilized by these two extraction procedures and of the -SH, 95%; i.e. initially with buffered 8M urea, then with 0·25M HCl. These results are given in Table 1. Figures in parentheses show that similar results were obtained with rat liver nuclei.

No DNA could be detected in either the 8M urea or 0·25M HCl nuclear extracts.

Chromatography of protein fractions

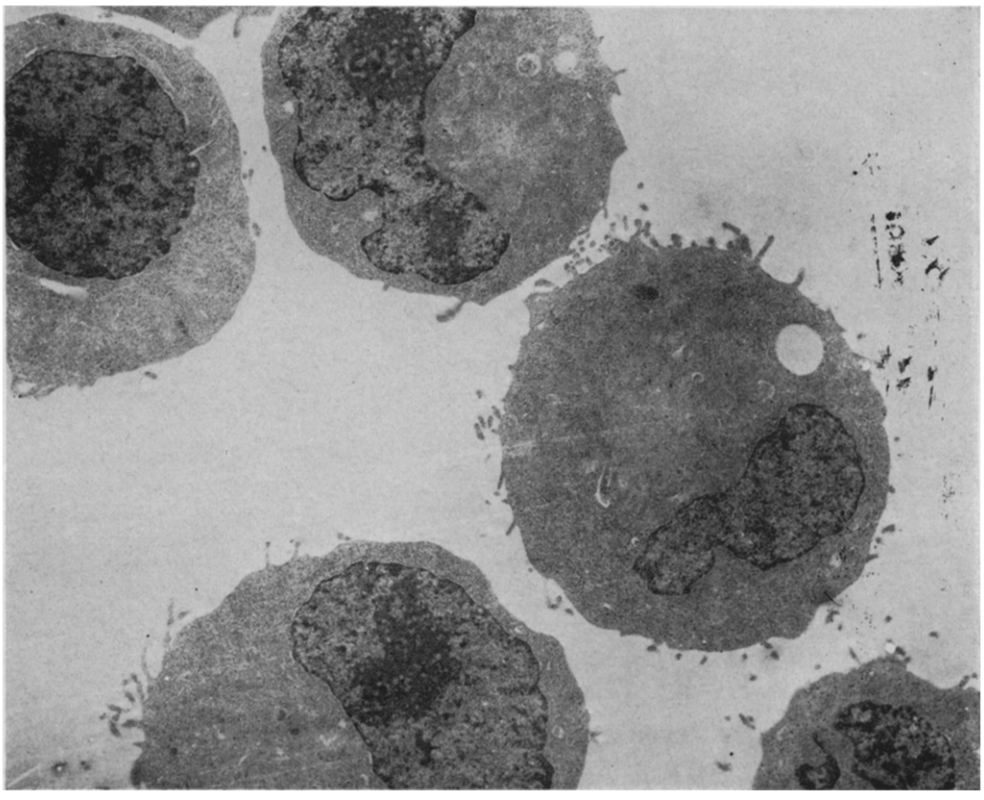
A. Figure 2 shows the elution profile obtained from chromatography of the 8M urea extract on a G-100 column. Blue Dextran when applied to this column under these conditions was eluted after 94 ml and collected in tubes 1–11. It can be seen that the only protein peak obtained was eluted, along with some RNA, in the void volume. Some RNA and a discrete peak of low molecular weight -SH compounds (23% of total -SH applied) were retained in the gel. Attempts to absorb the latter on cation or anion exchange resins, Norit A charcoal, or to extract into ether were unsuccessful; 85–90% recovery of applied radioactivity was attained.

A histogram of the results obtained from chromatography of the G-100 void volume material on microgranular DEAE cellulose is given in Fig. 3; 75–80% recovery of applied ^{14}C-NEM was achieved.

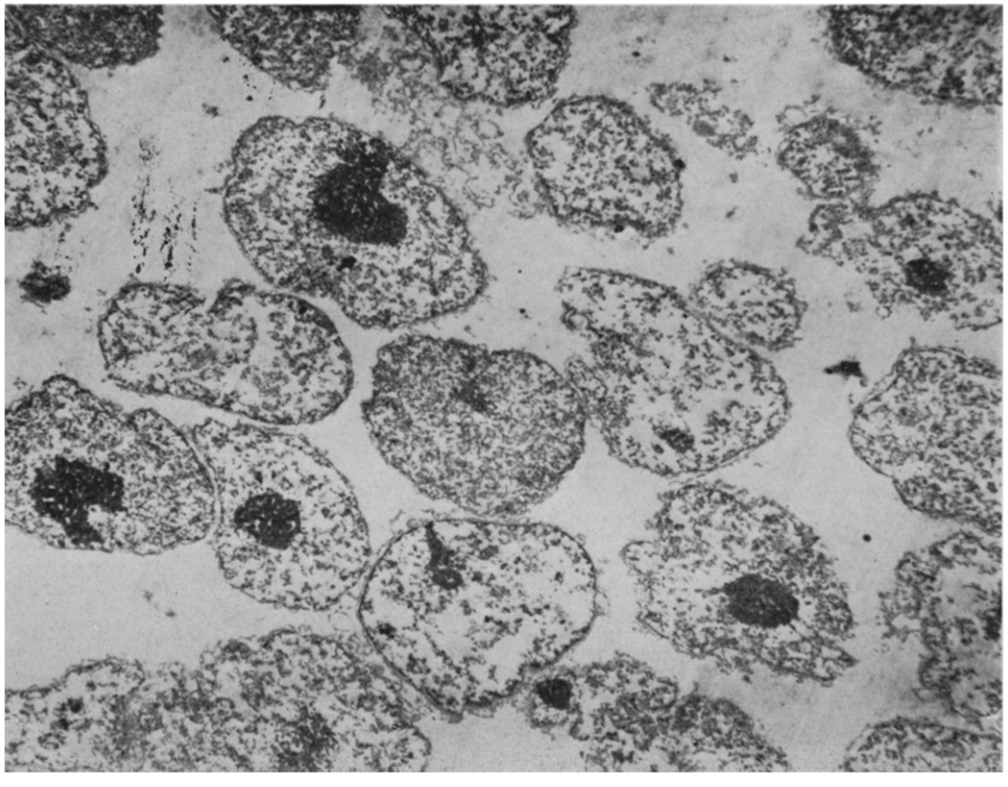
Table 1. *Extraction of -SH containing nuclear proteins from isolated Hepatoma* 223 *nuclei.*

Extraction procedure	Percentage of total nuclear protein extracted	Percentage of *total nuclear -SH	Specific activity *total n moles -SH/mg protein
1st Extraction 8M urea 0·05M Sodium phosphate pH 7·6	69·8±2·3 (70·3±5·0)	88·9±0·2 (88·2±1·4)	83·4±3·0 (70·0±3·0)
2nd Extraction 0·25N HCl	23·2±2·1 (27·3±3·0)	6·0±0·3 (6·2±1·5)	15·0±1·2 (20·0±2·2)
Residue (Soluble in N NaOH)	7·0±0·2 (2·4±3·1)	5·0±0·3 (5·6±1·7)	5·3±1·0 (75–400)

Figures based on average of 5–7 different preparations (Mean±S.D.).
Figures in brackets are those obtained from isolated rat liver nuclei.
*In 8M urea.



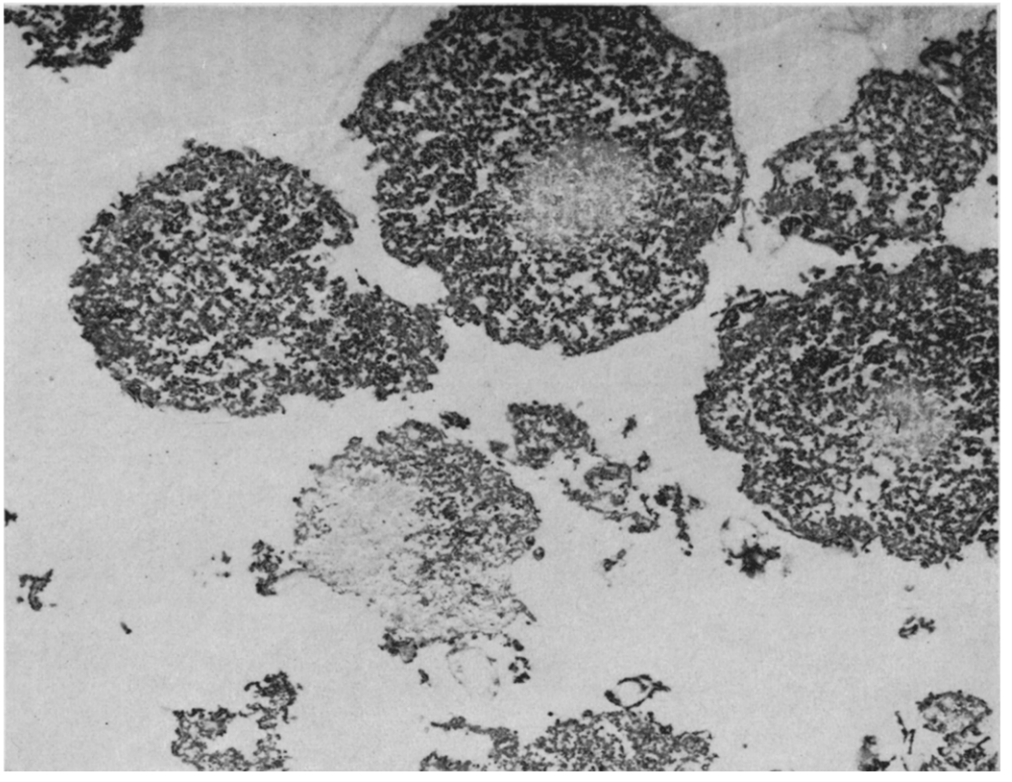
(*a*)



(*b*)

Fig. 1. *Electron micrographs of* (*a*) *rat Hepatoma* 223 *ascites cells;* (*b*) *nuclei isolated from these cells;* (*c*) *isolated nuclei after extraction with buffered* 8M *urea.*

(to face p. 500)



(*c*)

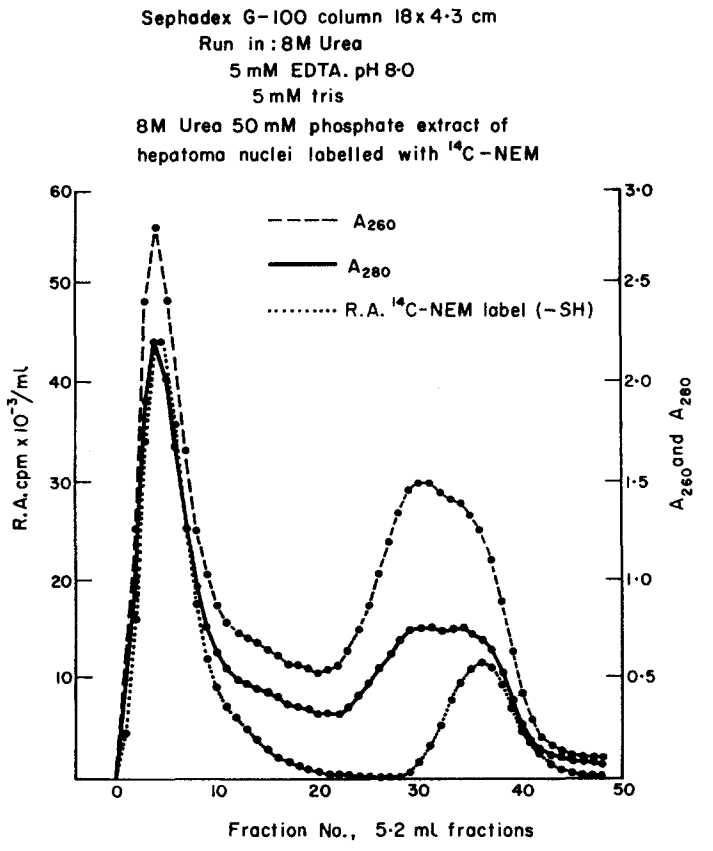


Fig. 2. Pattern obtained from chromatography on Sephadex G-100 of ^{14}C-NEM labelled 8M *urea* 50 *mM phosphate extract of hepatoma nuclei. The* 18×4·3 *cm column was eluted with* 8M *urea* 0·005M *EDTA* 0·005M *tris buffer pH* 8·0 *at the rate of* 1 *ml/2 min.*

Folin–Lowry estimations showed that nearly half (45% ± 5%, average of three runs) of the protein material passed straight through the column unabsorbed. Doubling the column volume did not affect this result. Most of the remaining absorbed protein could be removed with sodium chloride solutions (in 8M urea 0·005M tris, 0·005M EDTA) up to 0·20M. A little more radioactivity could be removed with 1M NaOH. Nearly all the RNA was removed with 0·3–1·0M NaCl and very little protein was present. The RNA amounted to

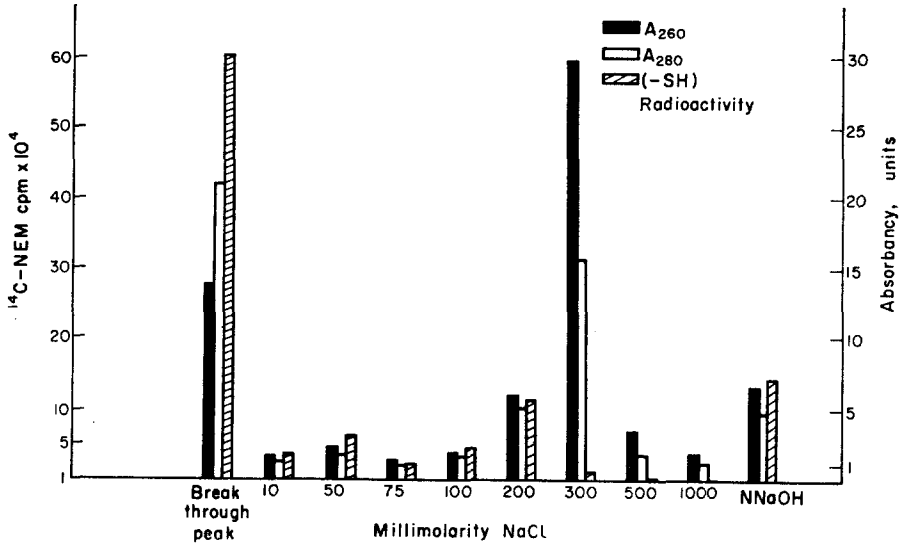


Fig. 3. Chromatography of void volume material from G-100 (Fig. 2) on microgranular DEAE cellulose (Whatman). A 10×1 *cm column was used and eluted with a discontinuous sodium chloride gradient in* 8M *urea* 0·005M *EDTA* 0·005M *tris pH* 8·0.

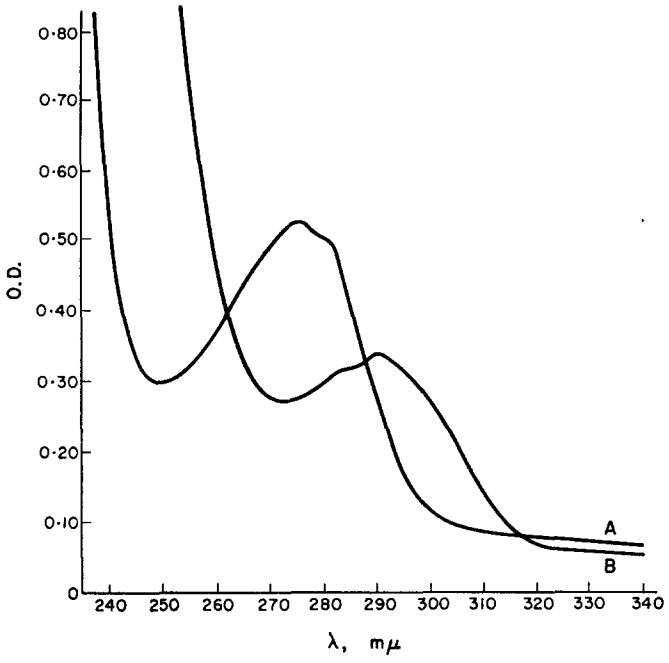


Fig. 4. u.v. spectra of acetic acid soluble protein. A. 0·3 *mg protein/ml N acetic acid (in a* 1 *cm cell). B. Half this concentration at pH* 12·0.

6–7% of the protein by weight as estimated by the extinction at 260 mμ.

The material not absorbed on the DEAE did not contain RNA. On dialysis against distilled water pH 7·0 it was quantitatively precipitated. Attempts to dissolve this protein (with ^{14}C-NEM label) in strong or dilute sodium chloride solutions, buffers at pH 4·4, 7·5 and 8·6 or 0·25M HCl failed. Apart from 8M urea solutions the labelled material was found to dissolve readily in 1M acetic acid, leaving a small amount of insoluble unlabelled amorphous material. That the protein was truly in solution was checked by spinning the

Table 2. Values are percentages of total moles of amino-acid recovered.

	DEAE fractions (mM NaCl)					CMC fractions (mM MCl)	
	10	50	75	100	200	10	20
Lysine	5·98	6·35	9·44	7·28	8·10	9·45	5·30
Histidine	1·48	1·07	1·98	1·20	1·97	3·34	1·64
Arginine	5·71	5·20	6·80	6·36	6·90	6·33	5·22
Aspartic Acid	11·00	10·50	10·30	12·30	11·90	9·60	10·60
Glutamic Acid	14·60	16·30	14·00	14·20	15·50	11·50	12·90
Ratio Acidic/Basic	1·95	2·20	1·34	1·78	1·61	1·10	1·94
*Cysteine	1·10	0·98	0·98	1·00	0·94	0·46	0·93
Methionine	1·98	1·87	1·52	1·49	2·14	2·12	1·19
Threonine	4·43	4·95	4·29	4·90	3·92	4·52	4·89
Serine	8·36	8·25	7·72	8·83	9·07	7·42	7·82
Proline	5·98	5·51	6·68	8·25	6·28	5·06	5·42
Glycine	8·75	7·52	7·75	8·46	7·45	11·20	14·40
Alanine	7·75	8·54	8·11	7·20	7·05	7·36	6·10
Valine	6·25	5·80	5·61	5·00	5·37	5·83	5·68
Isoleucine	3·89	4·08	3·33	3·14	3·04	3·66	3·69
Leucine	8·86	8·93	8·17	6·92	6·95	6·65	6·62
Tyrosine	2·18	2·08	2·05	2·08	1·65	2·35	3·18
Phenylalanine	3·00	3·01	2·84	2·54	2·67	3·61	3·93

*Calculated from specific activity of ^{14}C-NEM (assuming all the -SH is in the form of cysteine).

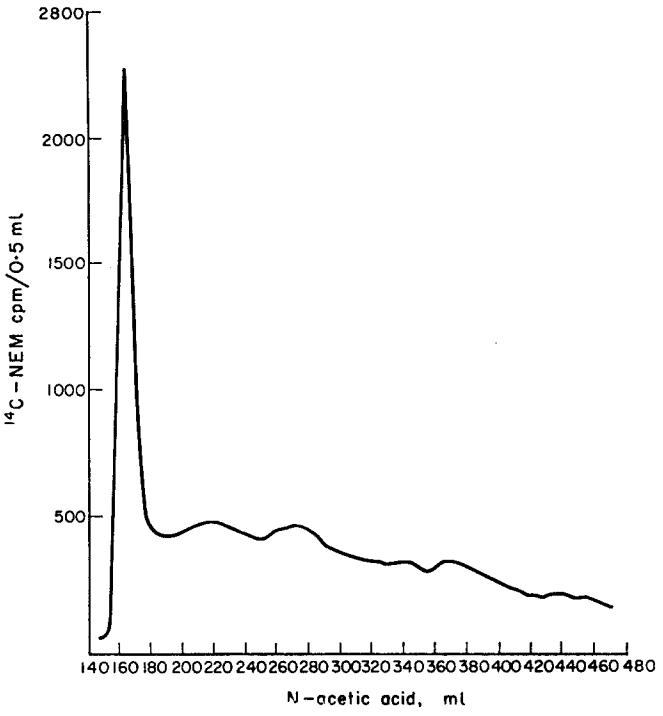


Fig. 5. Chromatography of ^{14}C-NEM labelled acetic acid soluble nuclear proteins of Hepatoma 223 *ascites cells on Sephadex G-*200. *A* 95 × 3 *cm column was used which was eluted with N acetic at* 4 *ml/fraction/*20 *min. Blue Dextran eluted after* 160 *ml in this system.*

solution at 100,000 **g** for 1 hr; no change in the radioactivity of the supernatant could be detected after this centrifugation. The u.v. spectrum of this solution (0·3 mg protein/ml) is given in Fig. 4A (4B contains 0·15 mg protein/ml at pH 12·0). Little change in spectrum occurred at pH 7·5, it was almost identical to that in M acetic acid.

When this protein (in M acetic acid) was applied to a CM-cellulose column practically 100% absorption occurred as ascertained by ^{14}C-NEM measurement. Of the applied radioactivity, 11·7% could be eluted in 0·01M HCl and this material contained 38·0 n moles of -SH/mg of protein; 0·02M HCl removed 77·5% of the protein containing 77·0 n moles of -SH/mg; 0·10M HCl removed a further 1·5% and 0·10M NaOH the remaining 9·3% of radioactivity.

Amino-acid analyses on these protein fractions (Table 2) were corrected for the destruction of threonine (8·2%), serine (12·5%) and tyrosine (2·5%) during hydrolysis. Tryptophan was not estimated. One feature of these analyses is the high glycine content of the 0·02M HCl protein from the CM-cellulose.

One-sixth of the applied ^{14}C-NEM came through in the void volume on gel filtration of the acetic acid soluble protein on G-200 Sephadex (Fig. 5). When the pooled contents of each peak were freeze dried (after passage through a Millipore filter) it was found that only 4–6% of the solid obtained consisted of protein.

Isoelectric focussing of the DEAE "breakthrough" protein did not give a satisfactory result as most of the protein material precipitated at or about its isoelectric point. However the main bulk of precipitated material appeared to be in the 6·5–6·7 pH region.

Attempts to obtain electrophoretic separations by the methods described were unsuccessful when applied to any of the protein fractions (c.f. reference 43). However, the 0·02M HCl protein from the CM-cellulose preparation did give a rather diffuse pattern when run on a 15% polyacrylamide gel in glycine-HCl buffer (pH 2·6, $\mu = 0{\cdot}1$) in 8M urea at 4 mA tube for 2½ hr. Four major, slow moving bands (relative to histones) and 4 faster moving, minor bands could be clearly seen in the gel (stained with naphthalene black).

B. The pattern obtained from the chromatography of the 0·25M HCl soluble nuclear proteins on microgranular CM-cellulose is shown in Fig. 6. About 9% of the ^{14}C-label passed through the column unabsorbed and 16% was recovered in 0·42M NaCl 0·17M sodium acetate; 0·01, 0·0125 and 0·015M HCl failed to remove any protein material from this column. Fifty-one per cent of the counts were recovered in 0·02M HCl.

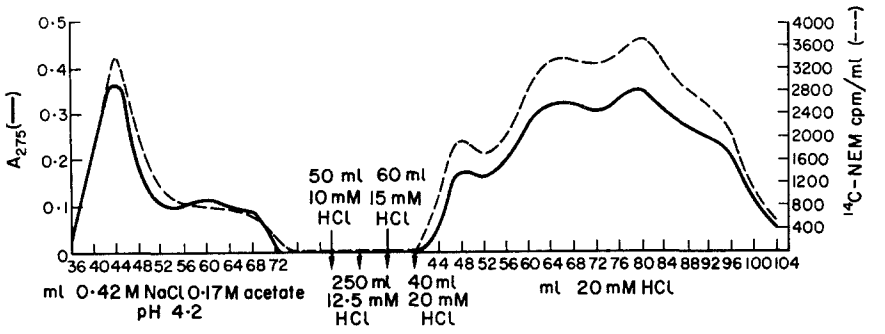


Fig. 6. Chromatography of nuclear 250 mM HCl soluble proteins (obtained after extraction of nuclei with buffered 8M urea). 15×2 cm column of Whatman microgranular CM-cellulose used which was eluted as shown.

Twenty-five per cent of the total 8M urea soluble protein was found to be soluble in 0·25M HCl. A preliminary examination of this material by polyacrylamide gel electrophoresis at pH 4·4 showed one major fast moving band to be present with a mobility similar to that of the F2b fraction. Chromatography, using CM-cellulose system described, produced two clearly separated protein peaks eluted from the column with 0·02M HCl amino acid analysis of which gave almost identical results.

The results of amino-acid analysis of the CM-cellulose peaks is given in Table 3.

DISCUSSION

The solubilization of the non-histone protein achieved by this method represents a quicker and perhaps superior alternative to the conventional saline extraction procedures. One distinct advantage is that none of the DNA is solubilized. The usual initial 0·14M saline wash to remove the so-called nuclear "sap" was not applied to these nuclei. Samarina [44] has shown that this fraction accounts for only 17·8% of the total dry weight of nuclear protein in ascites nuclei (prepared by osmotic shock). It is possible that the exposure of the

Table 3. Amino-acid compositions of protein peaks obtained by chromatography on microgranular CM-52 cellulose.

	Urea insoluble, acid soluble nuclear proteins*						Acid soluble protein present in urea extract	
	Protein eluted in 0·42M NaCl 0·17M acetate pH 4·2		Protein eluted in 20mM HCl				Protein eluted in 20mM HCl	
	A	B	C	D	E	F	A	B
Lysine	19·82	11·72	10·58	10·12	10·48	9·46	9·31	10·83
Histidine	1·17	2·24	2·15	1·83	2·20	1·62	2·02	2·10
Arginine	5·80	10·02	8·76	10·47	9·08	8·13	7·61	7·39
Aspartic acid	4·99	5·84	7·37	6·61	6·68	7·15	10·20	10·10
Glutamic acid	8·14	8·92	12·02	10·75	11·22	12·27	12·80	12·70
Ratio Acidic/Basic	0·49	0·615	0·90	0·77	0·82	0·98	1·21	1·12
Threonine	5·72	6·08	5·96	5·62	5·72	6·14	4·50	4·50
Serine	9·11	6·04	7·36	6·70	6·55	7·32	7·41	7·31
Proline	8·10	4·84	4·51	5·70	6·27	5·71	7·75	7·65
Glycine	7·82	9·30	7·57	7·06	9·22	7·26	8·60	8·04
Alanine	15·13	10·92	9·76	9·94	9·68	9·94	7·04	7·00
Valine	5·02	6·13	5·94	5·35	5·74	5·87	5·06	5·32
Methionine	0·68	0·96	1·28	1·32	1·05	1·31	2·06	2·00
Isoleucine	2·07	4·37	3·68	3·91	4·10	3·93	3·18	2·80
Leucine	4·65	8·27	7·83	7·96	8·08	8·17	6·61	6·75
Tyrosine	0·75	2·39	2·12	2·09	1·31	1·88	2·17	2·13
Phenylalanine	0·96	1·92	3·06	2·91	2·56	2·91	3·13	3·04

*Combined Cysteine and Cystine contents were less than 1%.

nucleo-protein Ca^{2+} – complex to high concentrations of monovalent cations may alter the chromatin macromolecular structure sufficiently to trap, or even change the solubility properties, of some nuclear proteins.

The "acid" residual fraction representing nucleolar proteins has been solubilized judging by the completeness of the nucleolar extraction shown in the electron micrographs. The major part of the dry weight of isolated nucleoli has been shown to consist of acidic type protein [31]. They showed that the nucleoli of tumour contained nearly twice as much of this component as liver. This type of protein is notorious for its insolubility in aqueous solvents. The problems involved have been discussed at length by Busch [5] and Busch and Steele [7]. These workers obtained some fractionation by salt and phenol extraction procedures but failed to sub-fractionate the acidic proteins on DEAE columns. Zbarskii and Samarina [32] and later Wang [9] used 1% sodium deoxycholate to solubilize some of the protein material left after the extraction of rat liver nuclei with 1M NaCl. Wang obtained four fractions on the basis of pH and ammonium sulphate precipitation. These proteins interacted strongly with histones to give insoluble complexes suggesting a possible function of derepression. A similar technique [33] was applied to rat liver chromatin acidic proteins. However, few of the proteins reported had as high a ratio of acidic to basic amino-acids as those isolated in these studies (Table 2).

The observations presented here also indicate that the acidic nuclear proteins are not simple proteins. Thus 6% of the total solids of the void volume peak from a G-200 Sephadex column (Fig. 5) consisted of protein material. About 20% consisted of carbohydrate material and no phosphate could be detected in the solid (Gronow unpublished observations). After acid hydrolysis (prior to amino-acid analysis) of the DEAE fractionated proteins (Fig. 3) a considerable amount of insoluble residue was left which did not dissolve in lipid solvents. Problems encountered in the gel electrophoresis of these proteins [43] could be due to the presence of covalently linked unknown high molecular weight macromolecules. That gel electrophoresis could be performed on protein eluted from CM cellulose (Fig. 6) in 0·02M HCl seems to indicate that an acid-labile link may be present. This is being investigated further. Also this observation indicates that 'contamination' of histone fractionations extracted from tumour tissue by acidic protein of this type is possible.

The -SH content of the 0·25M HCl soluble (histone) fraction as shown by the ^{14}C-NEM labelling was only about 20% of that of the urea soluble proteins. It is interesting that this label is nearly evenly distributed amongst the CMC fractions. However, as the amino-acid composition of these peaks indicates (Table 3), some contamination by non-histone protein is likely. Polyacrylamide gel electrophoresis of these fractions gave characteristic histone bands of different mobilities, each peak giving approximately three bands of differing stain intensity. In contrast the 0·25M HCl soluble protein present in the urea extract gave only one band (fast moving). However, amino-acid analysis of the CMC fractions showed an overall acidic composition.

The specificity of *N*-ethylmaleimide for reaction with -SH has been questioned [35]. However, the short exposure times, pH, and concentration of reagent (two fold excess) used in these experiments makes reaction with groups other than -SH unlikely. Also direct -SH measurements using the Saville method [36] on the histone fractions confirmed the ^{14}C-NEM result.

The relatively high -SH content (83 $\pm$ 3 n moles -SH/mg protein) of the urea soluble proteins is of interest especially in view of the involvement of -SH in the process of cell division [37], carcinogenesis [38, 39] and irradiation damage to the nucleus [40]. Since the oxidation of -SH and -S-S- can bring about a large change in macromolecular configuration, these molecules could be important in the masking and unmasking of the DNA to allow transcription to occur [16]. The acidic proteins themselves represent a substantial part of the nuclear protein content. Their association with RNA [9], DNA [41], and rapid rate of turnover (see 6 for review) points to their importance in the regulation of genetic information. Holoubek, Fanshier, Crocker and Hnilica [42] showed that the rapid incorporation of labelled amino-acids into the DNA associated protein parallels the rate of RNA synthesis in the nuclei of Ehrlich ascites tumour, indicating the possible importance of these proteins as a mechanism of controlling the rate of RNA synthesis in response to physiological requirements. Also the insolubility of the acidic nuclear proteins may be an important factor in the transport of messenger or ribosomal RNA, its function being prevention of degradation by the ubiquitous RNase.

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RESUME

Une préparation de noyaux, exempte de contamination cytoplasmique, a été obtenue à partir des cellules ascitiques de l'Hépatome 223, *chez le rat, par la technique du choc hypo-osmotique. Deux traitements successifs de ces noyaux, par une solution d'urée* 8*M et de phosphate de sodium* 0,05M *à pH* 7,6, *permettent l'extraction de* 89% *du total du thiol nucléaire et de* 70% *des protéines. Deux extractions supplémentaires avec l'acide chlorhydrique* 0,25M *permettent de retirer un surplus de* 6% *du thiol et de* 23% *des protéines. L'acide désoxiribonucléique n'est pas solubilisé par ces procédés.*

*Le fractionnement du matériel soluble dans l'urée sur le Sephadex G-*100, *le DEAE, et les celluloses CM, permet la séparation de l'acide ribonucléique et de sept fractions protéiques, dont la composition en acides aminés est décrite. On a aussi essayé de pousser plus loin la séparation d'une fraction acide, riche en glycine, par passage sur le Sephadex G-*200, *par électrophorèse sur gel et par concentration isoélectrique.*

On a pu réaliser la séparation de certains composés thiol de la fraction insoluble dans l'urée et soluble dans l'acide chlorhydrique 0,25M, *c'est-à-dire les protéines nucléaires (histones).*

SUMMARY

Nuclei, free from cytoplasmic contamination, have been prepared from rat hepatoma 223 *ascites cells disrupted by hypo-osmotic shock. Two extractions of these nuclei with* 8M *urea* 0·05M *sodium phosphate pH* 7·6 *removed* 89% *of the total nuclear thiol material and* 70% *of the protein. A further two extractions with* 0·25M *HCl removed a further* 6% *of the thiol material and* 23% *of the protein. No DNA was solubilized by these procedures.*

*Fractionation of the urea soluble material on G-*100 *Sephadex, DEAE and CM-celluloses separated RNA from seven protein components, the amino-acid composition of which is reported. Further attempts at fractionation of an acidic, glycine-rich fraction on G-*200 *Sephadex, by gel electrophoresis and isoelectric focussing are also described.*

Separation of some of the thiol components of the urea insoluble, 0·25M *HCl soluble nuclear proteins (histones) on microgranular CM-cellulose has been achieved.*

ZUSAMMENFASSUNG

Von Asciteszellen des Hepatoms 223 *der Ratte wurden, nach Auflösung durch hypo-osmotischen Schock, Kernpräparationen angefertigt, die frei waren von cytoplasmatischen Verunreinigungen. Zwei Extraktionen dieser Kerne mit* 8M *Harnstoff in* 0,05M *Natriumphosphat bei pH* 7,6 *entfernten* 89% *des gesamten SH-Gruppen-haltigen Kernmaterials und* 70% *des Proteins. Zwei weitere Extraktionen mit* 0,25M *Salzsäure entfernten weitere* 6% *des SH-Gruppen-haltigen Materials und* 23% *des Proteins. Durch dieses Vorgehen wurde keine DNS gelöst.*

*Die Fraktionierung des mit Harnstoff löslichen Materials mit G-*100 *Sephadex, DEAE und CM-Zellulose trennte die RNS von sieben Proteinkomponenten. Über deren Aminosäurezusammensetzung wird berichtet. Weitere Versuche zur Fraktionierung einer sauren glycinreichen Fraktion auf G-*200 *Sephadex durch Gel-Elektrophorese und isoelektrische Fokussierung werden beschrieben.*

Die Isolierung einiger der SH-Gruppen-haltigen Komponenten der harnstoffunlöslichen, in 0,25M *HCl löslichen Nuklearproteine (Histone) auf mikrogranulärer CM-Cellulose ist erreicht worden.*

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The Formation of Diethylstilbestrol Monoglucuronide in Hamster Liver Homogenates

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INTRODUCTION

VASQUEZ-LOPEZ [1] observed for the first time the presence of renal tumours in the male golden hamster treated with diethylstilbestrol (DES). Later studies confirmed the appearance of renal tumours in male [2–5] and ovariectomized female hamsters [6] but not in other animals of experimentation after administration of natural estrogens or DES. These tumours are hormone-dependent and transplantable [7]; in the first transfers they keep their hormonal dependency but after successive generations become developed without external estrogen contribution [8, 9].

This phenomenon 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. Horning [10] has formulated the hypothesis that hamster liver had a smaller DES conjugation capacity than other rodents; this fact would cause non-conjugated high DES levels to be eliminated becoming an irritative stimulus to the hamster kidney, eventually leading to tumour formation.

In view of Horning's hypothesis and the little known about DES metabolism we thought it worthwhile to do a comparative study of UDP glucuronyl transferase activity with DES as substrate in rat and hamster livers. Due to the fact that estrogens cause a decrease in liver glucuronyl transferase activity [11], we have considered of interest to also study the *in vivo* effect of DES upon hamster liver.

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MATERIAL AND METHODS

Chemicals. DES, uridin diphosphate glucuronic acid (UDPGA) and saccharic acid were provided by Sigma Chemical Co.; tris(hydroxymethyl)aminomethane "Tris", propylene glycol and perchloric acid, by Schuchardt; β-glucuronidase (Ketodase) 5000 units/ml by Warner Chilcott and 25 mg DES pellets (Cyren A), by Bayer. The other reagents were of analytical grade, the solvents were redistilled and diethyl ether was maintained free from peroxides by storage over ferrous sulfate and was freshly distilled before use. Saccharolactone approximately 0·016 M was obtained starting from a 0·05 M saccharic acid solution following Dutton's method [12]. Diethylstilbestrol monoglucuronide (DESGA) was obtained from the urine of rabbits fed DES at a daily rate of 500 mg for 5 days following the procedures given by Dodgson *et al.* [13].

Animals. The following types of animals were used: (1) three months old male hamsters; (2) three months old female hamsters; (3) eleven months old male hamsters, castrated at three months; (4) hamsters of types 1, 2 and 3 seven days after a 25 mg DES subcutaneous pellet implantation; (5) three months old male Wistar rats.

The animals were sacrificed by cervical dislocation and the livers immediately removed and washed with 0·15 M KCl-$3 \cdot 2 \times 10^{-4}$ M $KHCO_3$ solution at 0°C.

Determination of UDP glucuronyl transferase activity. It has been done by measuring the DES consumed after an incubation period with the liver homogenate, following the technique

Table. 1. *DES consumption by hamster and rat liver homogenates**†*

	Without UDPGA	With UDPGA
Castrated ♂ hamster [10]‡	−0·2±7·5	64·4±9·2
Castrated and implanted ♂ hamster [10]	2·1±6·7	74·4±9·3
♂ hamster [10]	9·7±18·2	77·6±8·2
Implanted ♂ hamster [8]	2·1±6·3	64·7±16·6
♀ hamster [9]	15·0±10·0	67·5±13·8
Implanted ♀ hamster [11]	6·3±7·4	50·8±15·7
♂ rat [10]	3·1±5·7	12·3±8·6

*The composition of the incubation mixture was as follows: tris, 35 mM; $MgCl_2 \cdot 6H_2O$, 10·5 mM; UDPGA, 0·67 mM; DES, 0·149 mM; 3% v/v propylene glycol; 15%homogenate, 1 ml; final volume, 5 ml; pH, 7·4.

†DES consumption is expressed as mcg/150 mg of fresh liver/90 min at 37°C. Results are given as the mean ± S.D.

‡Numbers in parentheses indicate number of animals used.

described by Gabaldón *et al.* [14]. The amount of DES consumed was calculated by difference with a blank incubated without substrate and with DES added after protein precipitation. Assays were done in triplicate. The composition of the incubation mixture is described in Table 1.

DESGA identification. The volume and time of incubation used to identify DESGA, were twice that of the quantitative assays. The reaction was stopped with 2 ml of 12·5% perchloric acid and 20 min later, 8 ml of ethanol was added. After 20 min the mixture was filtered; 0·5g of $KHCO_3$ was added to the filtrate in order to eliminate excess $HClO_4$, $KClO_4$ being separated by cold filtration. The filtrate was extracted four times with an equal volume of diethyl ether. From here on, methods given by Gabaldón *et al.* [14] were followed. The final residue obtained was dissolved in 0·5 ml of 80% ethanol. An aliquot was applied to two silicagel plates; one plate was developed in ethyl acetate:2-propanol: water (6:2·5:1·5) and the other in *n*-butanol: acetic acid: water (6:2:2); both plates were sprayed with 50% H_2SO_4 and heated to 100°C for 3 min. Another aliquot was subjected to hydrolysis with β-glucuronidase. The composition of the incubation mixture was as follows: extract, 0·15 ml; β-glucuronidase, 1500 units; saccharolactone, approximately 0·5 mM; acetate buffer pH 4·5, 85 mM; final volume, 3 ml. It was incubated at 37°C for 90 min, evaporated to dryness and the residue dissolved in 0·5 ml of 80% ethanol. An aliquot was applied to a silicagel plate, developed in benzene: ethanol (9:1) and DES detected by spraying with 0·5 N Folin–Ciocalteu's reagent and exposure to ammonia vapor.

RESULTS

Table 1 shows DES consumption in absence and presence of UDPGA by liver homogenates of male rat, male, castrated male and female hamsters and by liver homogenates of the same type of hamsters a week after a 25 mg DES pellet subcutaneous implantation. DES consumption is expressed in mcg/150 mg of fresh liver/90 min at 37°C. In absence of UDPGA, DES consumption is negligible and variable and can be due to residual UDPGA levels or to other unknown pathways of utilization.

The statistical significance levels of differences in DES consumption by rat and hamster liver homogenates have been determined. Each one of the variables has been studied: sex, DES pellet implantation, castration and species. Statistical significance levels have been calculated according to the Student *t*-test. Differences are considered significant when $P<0·01$.

DES consumption differences in presence and absence of UDPGA are highly significant in all cases. In absence of UDPGA none of the variables studied determines statistically significant differences of DES consumption. In the presence of UDPGA, only species and castration determine values of $P<0·01$. In this last case considering the differences of age of castrated animals with respect to the intact, differences found could be due to age instead of castration.

When the ethanolic extract obtained from the incubation mixture (see experimental) was subjected to chromatography development in ethyl acetate: 2-propanol: water (6:2·5:1·5) and *n*-butanol: acetic acid: water (6:2:2), a spot appeared having the same R_f (0·3 and 0·82 respectively) as standard DESGA obtained from rabbits urine treated with DES. The incubation of the ethanolic extract with

β-glucuronidase liberated DES which was detected by thin layer chromatography. DES formation was inhibited in presence of saccharolactone. DESGA has not been detected in ethanolic extracts obtained from a DES-free incubated mixture. Detection of DESGA has been accomplished with positive results in all series, being the only metabolite detected under conditions utilized.

DISCUSSION

In liver of animals treated with DES a decrease of glucuronyl transferase activity could be expected due to an estrogen depressor effect upon that activity. Nevertheless in implanted animals no differences have been observed in DES consumption in relation to non-treated animals even if the estrogen is absorbed during this period as shown by the decrease in pellet weight observed seven days after implantation.

Liver UDP-glucuronyl transferase levels are usually higher in males than females [15]. Even so, it has been known that in hamster the conjugation capacity of progesterone with glucuronic acid is higher in female than in male. In our case sex has not determined statistically significant differences of DES consumption neither in control nor in implanted animals.

Horning suggested that the renal tumour in hamster produced by DES could be due to a diminished liver conjugation capacity as compared to other rodents that do not exhibit the phenomenon. Later reports by Fishman and Sie [16] and Rao and Taylor [17] sustained this point of view by showing that the production of glucuronides from testosterone and progesterone was lower in hamster liver than in rat liver and at the same time smaller in male hamster than in female.

If Horning's hypothesis were true, glucuronyl transferase activity with DES as substrate should be higher in rat than in hamster and in this last animal greater activity in female than in male. Our results do not agree with this hypothesis as we have found activity in hamster to be from 5 to 6 times higher than in rat and the differences between the sexes have not been statistically significant.

It must be taken into consideration that in our experiences the UDPGA concentration does not constitute a limiting factor, while *in vivo* the levels of the same can be decisive for the conjugation reaction. Wong and Sourkes [18] found that liver UDPG levels are considerably lower in hamster as compared to other experimental animals, the hamster level being one third that of rat. UDPGA level is identical in both animals. This attainability deficiencies for UDPG could, in heavy requirement cases, determine an *in vivo* precarious conjugation reaction.

Our results obtained *in vitro* together with Kirkman's experiences [8] who failed in the attempt to produce renal tumours by DES pellet implantation in the spleen or in the path of the hepatic portal drainage suggest that the liver is not involved directly in the renal tumour phenomenon produced by DES in hamster.

Ghaleb [19] while working with labelled DES found that the tracer hormone was taken up and bound to renal cellular proteins, being the amount of labelled DES concentrated in renal epithelium double in male hamster than in female. In view of this it seems more reasonable to explain the mechanism of renal tumour by DES as a hormone balance alteration in which cellular receptors would have a predominant role.

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RESUME

On a démontré, in vitro, *la réaction de conjugaison du diéthylstilboestrol (DES) avec l'acide glycuronique sur des homogénats de foie du hamster doré et du rat. La mesure de l'activité de UDP-glycuronyl transférase a été réalisée par la détermination photocolorimétrique du DES consommé, après une période d'incubation avec l'homogénat. Le diéthylstilboestrol monoglycuronide (DESGA) a été identifié comme un produit de réaction par la chromatographie sur couche mince, et par l'hydrolyse enzymatique par la β-glycuronidase. On a fait une étude comparative des activités de l'UDP-glycuronyl transférase chez le rat mâle, le hamster mâle, le mâle castré et la femelle, et sur les mêmes groupes d'animaux, une semaine après l'implantation sous-cutanée d'une pilule de 25 mg de DES.*

En l'absence de UDPGA, il n'y a pour ainsi dire aucune consommation de DES dans aucun des cas. Des variables étudiées, sexe, castration, implantation et espèce, seule

cette dernière détermine des différences statistiquement significatives de consommation de DES. Le foie du hamster consomme une quantité de DES six fois supérieure à celui du rat. On discute le rôle du foie dans le processus de la genèse de la tumeur du rein chez le hamster par le DES.

SUMMARY

The in vitro *DES conjugation reaction with glucuronic acid in golden hamster and rat liver homogenates has been made evident. The measurement of UDP-glucuronyl transferase activity has been done by photocolorimetric determination of DES consumed after an incubation period with the homogenate.*

DESGA has been chromatographically and enzymatically identified as a reaction product. A comparative study of activity in male rat, male, castrated male and female hamsters and in the same type of hamsters a week after a 25 *mg DES pellet implantation, has been done.*

In absence of UDPGA there is practically no DES consumption in any one of the cases. Of all variables under study: sex, castration, implantation and species, only this last one determines DES consumption differences of high statistical significance. Hamster liver consumes six times as much DES as that consumed by rat liver. The role of liver in renal tumour processes in hamster by DES is discussed.

ZUSAMMENFASSUNG

Es wurde in vitro *die Konjugation von Diaethylstilboestrol (DAS) mit Glukuronsäure in Leberhomogenaten von Goldhamstern und Ratten nachgewiesen. Die UDP-Glucuronyltransferaseaktivität wurde durch photocolorimetrische Messung des nach einer Inkubationszeit mit dem Homogenat verbrauchten Diaethystilboestrols bestimmt. DASG ist chromatographisch und enzymatisch als Reaktionsprodukt identifiziert worden. Es wurde vergleichend eine Aktivitätsbestimmung bei männlichen Ratten, männlichen kastrierten Ratten und weiblichen Hamstern, sowie im gleichen Hamstertyp, eine Woche nach der Implantation von Pillen aus* 25 *mg DAS, durchgeführt. In Abwesenheit von UDPGA besteht praktisch in keinem Fall ein DAS-Verbrauch. Von allen experimentellen Variablen wie Geschlecht, Kastration, Implantation und Spezies hat nur die letztere Einfluß auf Unterschiede im DAS-Verbrauch, die von hoher statistischer Signifikanz sind. Die Leber des Hamsters verbraucht sechsmal soviel DAS wie die Leber der Ratte. Die Rolle der Leber bei renalen Tumorentwicklungen im Hamster durch DAS wird diskutiert.*

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Untersuchungen zur Mobilisierung von vitalen Zellen aus Transplantationstumoren durch mechanische Faktoren

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Bei experimentellen Tiertumoren kommt es im Verlauf des Tumorwachstums, ebenso wie bei malignen Geschwülsten des Menschen, zur Ausschwemmung von Tumorzellen in die Blutbahn, wo sie dann—vor allem in den vom Tumor abführenden Venen—cytologisch nachgewiesen werden können [1–8]. Die Bedeutung dieser abgelösten Zellen in Bezug auf eine Verschlechterung der Prognose der Erkrankung wird untershchiedlich beurteilt [9–18].

Mechanische Irritationen des Tumors führen zu vermehrtem Auftreten von pathologischen Zellem im Blut [7, 12, 15, 19–21]. Als Ursache solcher "Tumorzellschauer" kommen beim Menschen verschiedentlich banale Traumen während des täglichen Lebens in Frage, wie auch die "Massage" der Geschwulst durch die umgebende Muskulatur. Neben diesen unvermeidbaren Reizungen können im Verlauf des diagnostischen und therapeutischen Vorgehens auch iatrogene Maßnahmen zur Tumorzellausschwemmung führen; so z.B. wiederholte Palpationen der Geschwulst zwecks Feststellung von Größe, Konsistenz und Verschieblichkeit, Probepunktion oder Probeexcision oder Manipulationen bei der operativen Entfernung des Tumors. Inwiefern durch solche Ereignisse eine Metastasierung begünstigt und damit die Prognose getrübt wird, läßt sich auf Grund der bisherigen Literaturangaben nicht eindeutig beurteilen. Manche Autoren lehnen eine Gefahrdung des Patienten durch Eingriffe am Primärtumor ab [22–32], während andere Untersucher einen Zusammenhang zwischen mechanischer Irritation und erhöhter Metastatenbildung für möglich halten [33–40].

Beweisende Untersuchungen am Menschen lassen sich zur Beantwortung dieser Frage begreiflicherweise nicht durchführen. Bei retrospektiven Krankengeschichtsanalysen läßt sich die Bedeutung solcher "Traumatisierungen" des Primärtumors nicht absächtzen, da exakte Vergleichsbedingungen infolge des unterschiedlichen biologischen Verhaltens der menschlichen Malignome, der wechselnden Krankheitsdauer, des verschiedenen therapeutischen Vorgehens usw. nie gegeben sind. Es muß daher eine Antwort mit Hilfe des Tierexperimentes gesucht werden, wobei Rückschlüsse auf den Menschen erst nach Auswertung umfassender experimenteller Ergebnisse gezogen werden dürfen.

Die folgenden Versuche sollen einen Beitrag zu der Frage leisten, ob durch Massage eines Transplantationstumors vitale Geschwulstzellen vermehrt in die Blutbahn augeschwemmt werden.

MATERIAL UND METHODE

Die Untersuchungen erfolgten an ingsesamt 427 Mäusen der Stämme GP, BDF und C_3H, mit einem durchschnittlichen Körpergewicht von 20 g. Die Tiere wurden in Gruppen von 5–10 in. Drahtkäfigen gehalten und erhielten standardisiertes Pellettrockenfutter und Wasser *ad libitum*.

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Tumorimpfung

1. *Lewis-Lung-Tumor.* Dieser wird seit 1952 durch 14 tägige Überimpfung am Institut gehalten. Von durchschnittlich 5 g schweren Tumoren wird unter sterilen Cautelen aus den makroskopisch vitalen Randabschnitten Material entnommen, 1:10 mit isotoner NaCl-Lösung+Suprakombinzusatz (zur Infektionsprophylaxe) verdünnt und durch Behandlung mit dem Potter'schen Homogenisator ein Tumorhomogenisat hergestellt. Von demselben erhielten 211 weibliche BDF-Mäuse 0, 1 ml in den linken hinteren Unterschenkel intramuskulär injiziert.

2. *Sarkom* 180*. Das sarkom 180 erhielten wir in Form einer Eisampulle. Nach einigen Passagen des Tumors auf weibliche GP-Mäusen erfolgte die Überimpfung auf insgesamt 188 weibleiche GP-Mäuse: Verdünnung der steril entnommenen Tumorstücke 1:1 mit NaCl-Suprakombinlösung, mechanische Homogenisierung, Impfung von 0, 05 ml pro Tier intramuskulär in den linken hinteren Unterschenkel.

3. *Das Osteosarkom der Maus*† (siehe später). Dieses wurde uns in Form von 5 beimpften C_3H-Mäusen am 3.5.1968 übersandt. Nach Beobachtung des Tumors bei zwei Generationen C_3H-Mäusen wurden die 58 verwendeten Testtiere (weibliche C_3H Mäuse) geimpft: Verdünnung der homogenisierten Tumorstücke 1:4 mit NaCl-Suprakombinlösung, Impfung von 0, 1 ml intramuskulär in den linken hinteren Unterschenkel.

Tumorzellennachweis

Der Nachweis vitaler Tumorzellen in den Lungen des tumortragenden Tieres wird durch einen biologischen Test geführt, im folgenden als "Lungenprobe" bezeichnet (41): nach Tötung der Tiere sterile Entnahme der Lunge, Einfüllen derselben in eine 1 ccm Tuberkulinspritze nach Wegnahme des Stempels. Subkutane Applikation der Lunge mittels dünner Nadel in die Inguinalgegend eines Testtieres (im folgenden "Lungenempfänger"). Die linke Lunge wird in der beschriebenen Weise links inguinal, die rechte Lunge rechts inguinal injiziert. Bei Vorhandensein vitaler Tumorzellen entwickelt sich aus der implantierten Lunge ein Tumor. Das Zeitintervall zwischen Injektion und klinisch sicher nachweisbarem Tumor schwankt je nach Transplantationstumor und Anzahl der vitalen Tumorzellen in der transplantierten Lunge zwischen 8 und 70 Tagen. Nach diesem verschieden langen Intervall kommt es zu einem raschen Tumorwachstum und zum Tode der Tiere an Tumorkachexie. Der Tod des "Lungenempfängers" an dem sich aus der Lunge entwickelnden Tumor gilt als Beweis für das Vorhandensein vitaler Tumorzellen in der Lunge der primär beimpften Testtieres.

Tumormassage

Ab dem Inokulationstag wird die beimpfte hintere Extremität bei der Hälfte der verwendeten Mäuse zweimal täglich während einer Dauer von jeweils 45 Sekunden kräftig massiert. Durch diese Traumatisierung kommt es zu einer lokalen Propagation der Geschwulst. Dies zeigt sich bei Amputationsversuchen, wobei die massierten Tiere eine wesentlich höhere Rate an Lokalrezidiven aufweisen, da der Tumor infolge der weiten Ausdehnung nach proximal durch die Operation nicht mehr radikal entfernt werden kann. Das Tuorvolumen und das Gewicht der massierten und nicht massierten Geschwülste bleibt jedoch gleich.

RESULTATE

1. *Lewis-Lung-Tumor*

Beim Lewis-Lung-Tumor [32, 43] treten zwischen dem 10. und 14. Tag nach Inokulation regelmäßig Lungenmetastasen auf. Zur gleichen Zeit wird auch die "Lungenprobe" positiv.

In einer ersten Versuchsanordnung (Tab. 1) erfolget die "Lungenprobe" am 4., 6., 8. und 10. Tag nach Tumorimpfung. Es zeigt sich, daß beim ersten Versuch (Tab. 1, Versuch 1) weder bei den massierten noch bei den nicht massierten Tieren vitale Tumorzellen in den Lungen vorhanden waren. Alle Empfänger der Lungen überlebten. Bei längerem Zuwarten mit der "Lungenprobe" stirbt eine zunehmende Zahl von "Lungenempfängern", an den sich aus den überimpften Lungen entwickelnden Tumoren (Tab. 1, Versuche 2–4). Der zahlenmäßige Unterschied zwischen den toten "Lungenempfängern" massierter und nicht massierter Mäusegruppen ist gering, doch ist die Absterberate bei der massierten Gruppe jeweils höher.

In einer zweiten Versuchsserie sollte geprüft werden, inwieweit die "Massage" der Geschwulst durch die umgebende Muskulatur bei den normalen Bewegungen der Tiere die

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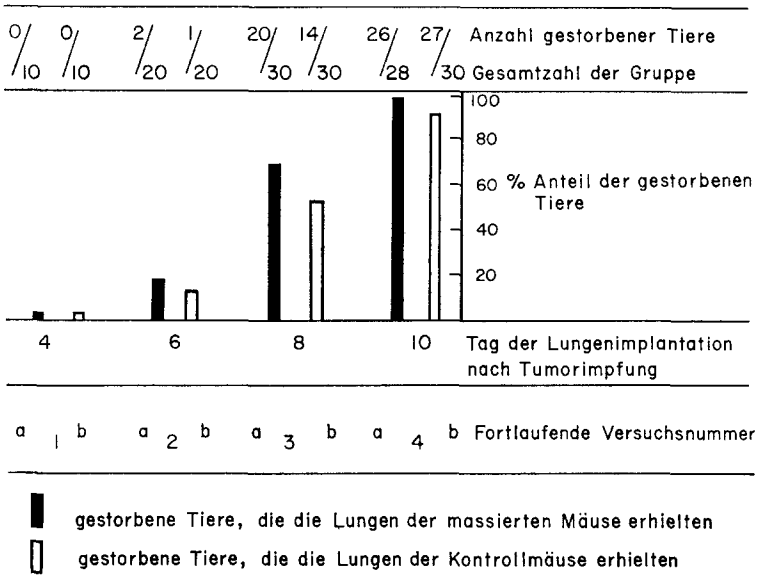
Tabelle 1. *Lewis-Lung-Tumor: Verteilung der an den implantierten Lungen gestorbenen Tiere bei* 4 *Versuchspaaren*



Tumorzellausschwemmung begünstigt. Um die Muskeltätigkeit der beimpften Extremität einzuschränken, wurde zunächst eine mechanische Ruhigstellung versucht. Diese scheiterte jedoch daran, daß die Tiere durch die Fixationsvorrichtung irritiert, andauernde Befreiungsversuche mit gesteigerten Muskelaktionen unternahmen. Aus diesem Grunde erfolgte eine medikamentöse Dämpfung der Tiere mit zweimal täglich 25 mg Valium pro kg Maus ab dem Tag der Überimpfung des Tumors. Die Hälfte dieser Mäuse wurde in der üblichen Weise massiert, um den Einfluß der "äußeren" Massage bei Hemmung der "inneren" Massage auf die Zellverschleppung zu untersuchen. Während dieser medikamentösen Ruhigstellung kam es zu einer verminderten Nahrungsaufnahme. Dies kann durch den reduzierten Energiebedarf infolge der fehlenden Bewegungen erklärt werden. Einflüsse auf andere Stoffwechselvorgänge und damit auch auf solche des Tumors künnen jedoch nicht ausgeschlossen werden. Aus diesen Gründen lassen sich mit dieser Methode noch keine sicheren Rückschlüsse auf die Frage des Einflusses der "Automassage" auf die Tumorzellverschleppung ziehen. Allerdings sind die Absterbequoten bei den "Lungenempfängern" nicht massierter Tiere der Versuche 3 und 4 (Tab. 1, Versuche 3b und 4b) und diejenigen der "Lungenempfänger" massierter Mäuse der Versuche 5 und 6 (Tab. 2, Versuche 5a und 6a)

Tabelle 2. *Lewis-Lung-Tumor: Verteilung der an den implantierten Lungen gestorbenen Tiere bei* 2 *Versuchspaaren. Die massierten bzw. nicht massierten tumortragenden Mäuse erhielten ab Tumorinokulation eine allgemein dämpfende Behandlung mit Valium.*

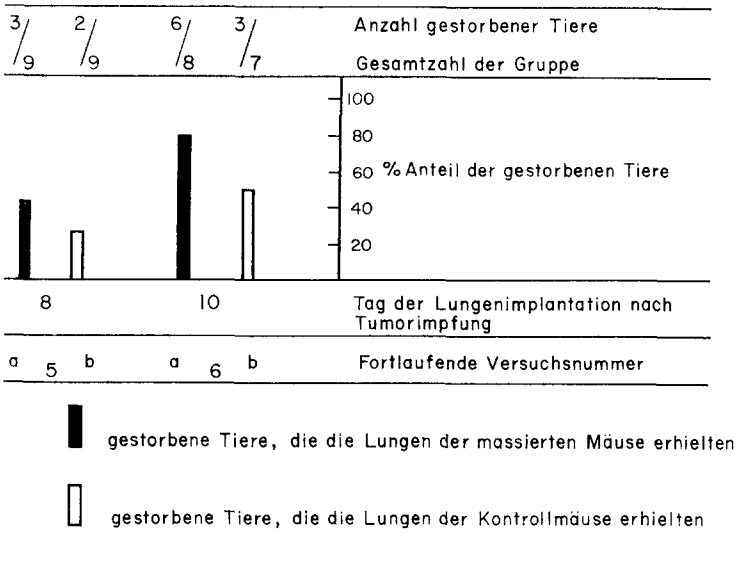
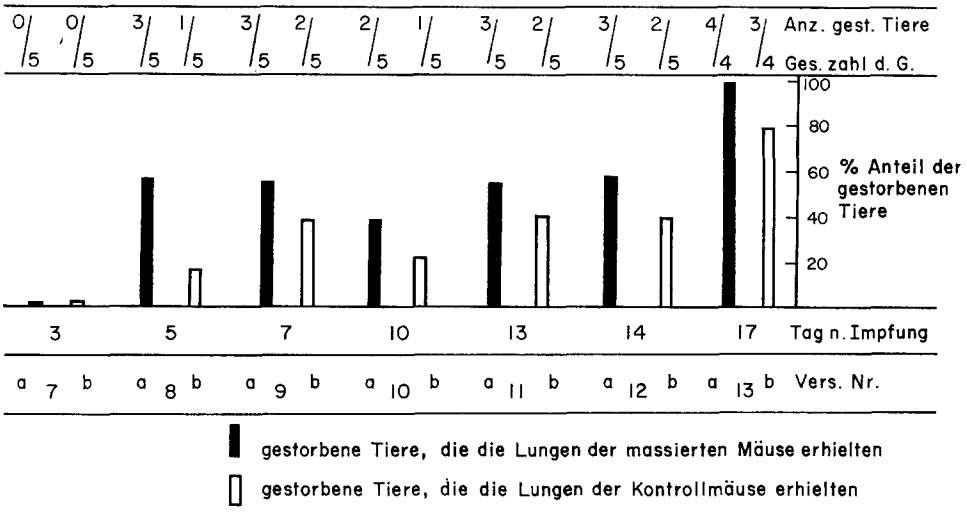


Tabelle 3. *Sarkom* 180: *Verteilung der an den implantierten Lungen gestorbenen Tiere bei* 7 *Versuchspaaren*



fast gleich. (Die "Lungenprobe" erfolgte bei den Versuchen 3 und 5 jeweils am 8. Tag, bei den Versuchen 4 und 6 jeweils am 10. Tag nach der Tumorimpfung).

Bei den Versuchen mit medikamentöser Dämpfung (Tab. 2) waren die Bedingungen für die massierten bzw. nicht massierten Tiere gleich, sodaß sich die Resultate bezüglich des Einflusses der Massage verwerten lassen. Bei beiden Versuchen zeigt eine grössere Zahl von massierten Mäusen vitale Tumorzellen der Lunge, dargestellt durch eine größere Zahl positiver "Lungenproben".

2. *Sarkom* 180

Bei Durchführung der "Lungenprobe" am 3. Tag nach Tumorimpfung lassen sich mit dieser Methode beim Sarkom 180 keine vitalen Tumorzellen nachweisen, alle Tiere überlebten (Tab. 3, Versuch 7). Die "Lungenprobe" vom 5–17. Tag nach der Impfung führt zu einer wechselnden Absterberate der "Lungenempfänger", wobei diejenige der massierten Versuchsgruppen durchwegs höher war (Tab. 3, Versuche 8–13). Auf Grund dieser Versuchsreihe mit kleinen Einzelgruppen wurde ein Test mit größerer Tierzahl durchgeführt, bei welchem am 5. und 7. Tag die "Lungenproben" erfolgten. Der Impftumor wuchs bei diesem Test deutlich langsamer, sodaß scheinbar später Tumorzellen ins Blut abgegeben wurden und somit an den gewählten Testtagen nur wenige implantierte Lungen zu einer Tumorbildung Anlaß gaben (Tab. 4). Auch hier zeigen die Empfänger der massierten Tiere etwas häufiger positive "Lungenproben".

3. *Osteosarkom der Maus*

Das verwendete Osteosarkom gelangte 1955 am National Cancer Institute Bethesda, Md.,

Tabelle 4. *Sarkom* 180: *Verteilung der an den implantierten Lungen gestorbenen Tiere bei* 2 *Versuchspaaren*

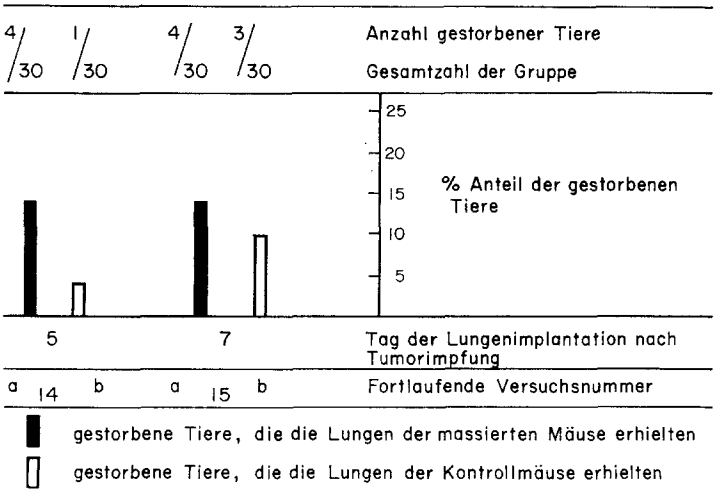
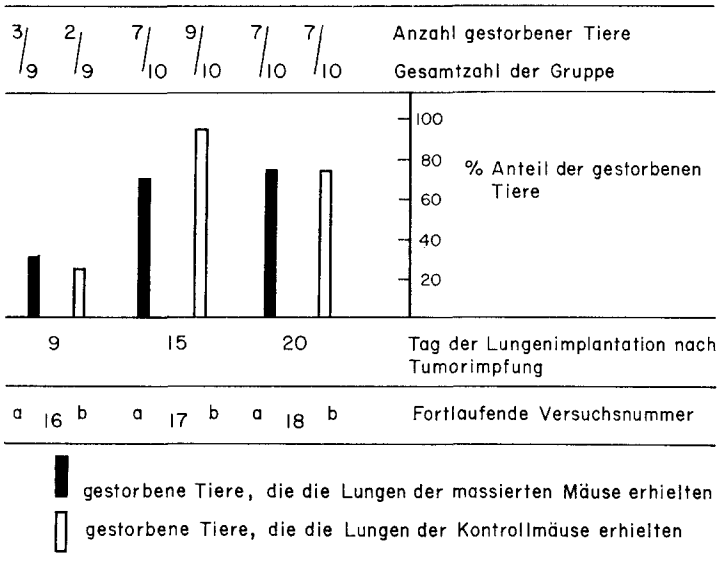


Tabelle 5. *Osteosarkom der Maus: Verteilung der an den implantierten Lungen gestorbenen Tiere bei* 3 *Versuchspaaren*



USA, zur Beobachtung. Bei der routinemäßigen Sektion einer weiblichen C3H-Maus fand sich an der Nierenkapsel ein linsengroßer weißlicher Tumor. Die Röntgenuntersuchung des Skelettes deckte ein primäres Osteosarkom in einem lumbalen Wirbelkörper auf (44). Seither wird diese Geschwulst an C3H Mäusen am National Cancer Institute weiter überimpft.

Bei subkutaner, intramuskulärer und intraossärer Impfung mit Mengen ab 0, 01 ml eines 1:4 verdünnten Tumorhomogenates kommt es immer zur Tumorbildung. Spontanregressionen werden nie beobachtet. Histologisch findet sich das Bild eines polymorphzelligen Osteosarkoms mit gitterförmiger Osteoid- und Knochenbildung und Neigung zu ausgedehnten zentralen Nekrosen. Das Wachstum der Geschwulst ist wesentlich langsamer als das der verwendeten übrigen Mäusetumoren. Der Spontanexitus tritt nach durchschnittlich 68 Tagen auf. Ungefähr 80% der Tiere zeigen bei der Obduktion Lungenmetastasen. Der Zeitpunkt der Metastasierung und des Exitus schwankt mehr als bei den rascher wachsenden Impfgeschwülsten.

Die Massageversuche wurden bei der noch geringen Erfahrung mit dem Tumor nur probeweise vorgenommen und zeigten uneinheitliche Resultate (Tab. 5). Bei Durchführung der "Lungenprobe" am 9. Tag nach Tumorimpfung starb wohl ein Tier mehr als aus der massierten Gruppe, am 15. Tag fanden sich jedoch umgekehrte Verhältnisse. Am 20. Tag starben in beiden Gruppen 7 von 10 Mäusen. Das klinische Verhalten und das histologische Bild des verwendeten Osteosarkoms der Maus sind menschlichen Knochensarkom sehr ähnlich. Es werden deshalb die Untersuchungen mit diesem Modell fortgesetzt.

STATISTISCHE AUSWERTUNG*

Die statistische Auswertung der Resultate der einzelnen Versuche erfolgte zunächst in der Form, daß unabhängig vom verwendeten Tiertumor, unabhängig von der Anzahl der Versuchstiere und unabhängig vom prozentuellen Ausfall des Einzeltests, die positiven Versuche (höhere Mortalität bei der massierten Gruppe), den negativen Tests (höhere Absterbequote bei nicht massierten Tieren bzw. gleiche Resultate bei beiden Gruppen) gegenübergestellt wurden.

Insgesamt sind 16 verwertbare Gruppen vorhanden, da die Versuche 1 und 7 infolge zu früh angesetzter "Lungenprobe" bei beiden Gruppen kein Angehen der überimpften Lungen zeigten und daher ausgeschieden wurden. Bei 14 Versuchen lag die Absterbequote der "Lungenempfänger" der massierten Tiere höher als die der nicht massierten Kontrollmäuse und nur zweimal mußte der Test als negativ bezeichnet werden (Versuche Nr. 17 und 18). Nach dem Vorzeichentest ergibt sich mit p kleiner als 0, 01 eine hohe Signifikanz zugunsten der positiven Tests.

Bei dieser Art der statistischen Prüfung der Differenz zwischen positiven und negativen Tests, wird der prozentuelle Unterschied zwischen den gestorbenen "Lungenempfängern" der massierten bzw, nicht massierten Tiere

*Für die statistische Beratung bin ich Herrn Prof. Dr. F. X. Wohlzogen, (Vorstand der Lehrkanzel für medizinische Statistik und Dokumentation der Universität Wien) zu großen Dank verpflichtet.

nicht berücksichtigt. Bei Betrachtung der Tabellen scheint dieser Unterschied meist gering. Es erfolgte deshalb die Signifikanzprüfung aller 92 gestorbenen "Lungenempfänger" der massierten Mäuse, gegen die 70 gestorbenen der nicht massierten Tiere bei einer Gesamtzahl von 457 untersuchten Tieren. Nach dem 4-Felder-Test ergibt sich auch hier mit p kleiner als 0, 05 eine signifikant höhere Todesrate bei den Tieren, die die Lungen der massierten Mäuse überimpft bekamen.

DISKUSSION

Aus obigen Versuchen geht hervor, daß bei den drei verwendeten transplantablen Mäusetumoren durch die Massage des Primärtumors vermehrt vitale Tumorzellen in die Blutbahn und somit auch in die Lunge gelangen.

Zum Nachweis der ausgeschwemmten Tumorzellen fand die Methode der "Lungenprobe" Anwendung. Bei der gegebenen Fragestellung scheint dieselbe günstiger als die Suche nach Tumorzellen im Blut, denn erstens ist die Unterscheidung isolierter Geschwulstzellen von anderen großen Zellen (Megacaryocyten oder Endothelzellen) schwierig oder auch unmöglich [15, 18, 45, 46] und zweitens sagt das Vorhandensein vom Tumorzellen im Blut noch nichts aus über die Lebens- und Vermehrungsfähigkeit derselben. Auch gegenüber dem Nachweis der Lungenmetastasen oder des früheren Exitus des massierten Tieres selbst, erweist sich die "Lungenprobe" als genaueres Modell, weil sich bei den verwendeten Impftumoren bei Vorversuchen stets mehr positive Lungenproben: als makroskopisch nachweisbare Lungenmetastasen fanden. Dies liegt einerseits darin, daß es technisch leichter gelingt die subkutan gelegenen, aus den implantierten Lungen gewachsenen Tumoren zu beurteilen als kleinste Lungenmetastasen nachzuweisen. Andererseits dürfte durch körpereigene Abwehrmechanismen ein Teil der Tumorzellen soweit geschädigt werden, daß sie nicht zur Metastasenblidung führen [23, 35, 38, 47–50], während dieser Faktor bei der "Lungenprobe" wegfällt.

Einige Autoren, die an einem großeren Material experiementelle Untersuchungen anstellten, gelangten ebenfalls zu dem Resultat, daß sich mechanische Irritationen des Primärtumors prognostisch ungünstig auswirken: so fanden Knox [35], Marsh [36] und Tyzzer [39] nach Massage des Implantationstumors eine erhöhte Rate von Lungenmetastasen. Kaae [34] und Riggins [38] erhielten ein ähnliches Resultat nach Probeexzisionen aus der Impfgeschwulst, Peyton [37] konnte sogar nach intratumoraler Injektion mehr Lungenmetastasen beobachten und Agostini [33] fand erhöht Lebermetastasen nach Manipulation während der operativen Entfernung experimenteller Coloncarcinome bei Mäusen.

Alle diese Untersucher verwendeten transplantable Ratten- oder Mäusetumoren und werteten das Auftreten von Lungen- oder Lebermetastasen. Es liegen allerdings auch Arbeiten vor, wo sich bei ähnlichen Versuchsbedingungen keine erhöhte Metastasenrate nach mechanischen Irritationen zeigte. [23, 24, 30, 32].

Das Auftreten von Lungenmetastasen scheint (aus den oben erwähnten Gründen) kein sicheres Kriterium für die Ausschwemmung vitaler Tumorzellen zu sein. Diese Tatsache ist für Modellversuche von entscheidender Bedeutung, da es für eventuelle Rückschlüsse auf den Menschen darauf ankommt, ob vitale Geschwulstelemente durch Traumatisierung des Tumors verschleppt werden können, und nicht darauf, ob im Experiment diese Zellen zur Metastasenbildung Anlass geben. Aus diesem Grunde dürfte der gewählte Weg zum Nachweis der Vitalität der Zellen durch den biologischen Test der Implantation der Lungen günstiger sein als die Beobachtung der Lungenmetastasen.

Unsere Versuchsergebnisse und andere experimentelle Beobachtungen (siehe oben) zeigen somit die Möglichkeit auf, daß es auch beim Menschen durch Manipulationen am Primärtumor zu einer Ausschwemmung vitaler Tumorzellen kommen kann. Solange diese Annahme widerlegt worden ist, ergibt sich für alle diagnostischen und therapeutischen Maßnahmen die Forderung strengstens darauf zu achten, jede überflüssige mechanische Irritation der Geschwulst zu vermeiden.

RESUME

L'effet du massage sur la dissémination des cellules tumorales a été étudié sur trois types de tumeurs transplantables chez la souris. Le moyen utilisé pour mettre en évidence la dissémination des cellules tumorales vivantes était l'inoculation, par injection sous-cutanée, des poumons des souris ainsi traitées, à d'autres souris, à differents intervalles. La présence de cellules cancéreuses vivantes, dans les poumons des souris testées, était considérée comme étabile lorsque les animaux receveurs mouraient avec les tumeurs.

En comparant 228 *souris, dont les tumeurs avaient été massées, à* 229 *souris qui n'avaient pas subi ce traitement, on constata une mortalité nettement plus élevée dans le premier groupe. Ces résultats montrent que la dissémination des cellules tumorales viables est nettement augmentée par le massage des tumeurs.*

SUMMARY

Solid tumours from three different strains of transplantable cells were treated by massage. In order to assay dissemination of living tumour cells, lungs of the tested animals were subcutaneously inoculated into host animals at different periods of time. Death of the tumour-bearing host animals indicated the presence of living tumour cells in the lungs of the tested animals.

Mortality was much higher in 228 *mice, the tumours of which had been treated by massage than in* 229 *control mice. These results show that dissemination of living tumour cells is significantly enhanced by massage of the tumours.*

ZUSAMMENFASSUNG

An 3 *transplantablen Mäusetumoren werden Massageversuche unternommen. Zum Nachweis vitaler Tumorzellen werden die Lungen der Testtiere nach verschiedenen Zeiträumen Empfängertieren* subkutan *überimpft. Das Vorhandensein vitaler Geschwulstzellen scheint dann bewiesen, wenn die Empfängertiere an den sich aus den überimpften Lungen entwickelnden Tumoren sterben. Bei einem Vergleich von* 228 *massierten Mäusen mit* 229 *Kontrolltieren, die in jeweils* 18 *Versuchsgruppen aufgeteilt werden, ergibt sich eine signifikant höhere Todesrate bei den Empfängern der Lungen des massierten Tiere.*

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Enzymhistochemische Untersuchungen an experimentellen Glioblastomen des Kaninchens

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EINLEITUNG

Über die Induktion von Hirngeschwülsten bei Kaninchen durch *N*-Methyl-*N*-nitrosoharnstoff (MNH) haben Jänisch und Mitarb. [1, 2] erstmalig berichtet. Der Befund ist von besonderem Interesse, da diese Tierart gegenüber kleineren Laboratoriumstieren wesentlich günstigere Voraussetzungen für zukünftige pathophysiologische und therapeutische Versuche bietet. Nachdem die Morphologie dieser Neubildungen bereits ausführlich beschrieben ist [3], sollen an dieser Stelle die enzymhistochemischen Untersuchungsergebnisse an 14 intracerebralen Glioblastomen des Kaninchens mitgeteilt werden.

MATERIAL UND METHODE

Mischrassigen Kaninchen beiderlei Geschlechts (Anfangsgewicht 1120–4260 g) wurde MNH in steriler physiologischer Kochsalzlösung unter Zusatz von 1% NaH_2PO_4 (pH 4, 2–4, 4) in eine Ohrrandvene gespritzt. 14 Kaninchen wurden nach Auftreten neurologischer Symptome in Äthernarkose ausgeblutet. Innerhalb von 10 Minuten nach dem Tode wurden Teile der Hirntumoren in Pentan bei −80°C eingefroren. Die wichtigsten Versuchsdaten sind aus Tab. 1 ersichtlich.

Von den Geschwülsten wurden in jedem Falle auch Paraffinschnitte angefertigt und mit Hämatoxylin-Eosin, gelegentlich auch mit Kresylechtviolett sowie nach Gomori gefärbt. Die Färbungen mit Sudan und nach van Gieson haben wir, ebenso wie die histochemischen Fermentnachweise, an 12–16 µm dicken, bei −20°C angefertigten und auf Objektträger aufgezogenen Kryostatschnitten ausgeführt. Zum Nachweis hydrolytischer Enzyme wurden die Schnitte bei 0–4°C 10–15 Minuten in neutralem, 4%-igem Formalin mit Zusatz von 1% $CaCl_2$ fixiert. Die übrigen Nachweise erfolgten an unfixierten Kryostatschnitten.

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Folgende Methoden wurden benutzt

1. Alkalische Phosphatase:
   (a) Calcium–Kobalt-Methode nach Gomori [4],
   (b) Kupplungsmethode, Naphthol-AS BS-phosphat-Na [5]
2. Saure Phosphatase:
   Naphthol-AS BS-phosphat-Na [6]. Ein Teil der Schnitte wurde vor dem Enzymnachweis 20–30 Minuten in eine 10^{-3} m NaF-Lösung bzw. in eine 1%-ige wäßrige Lösung von Triton X 100 (Octylphenoldecaäthylenglykoläther) gestellt.
3. Adenosinmonophosphatase [7].
4. Glucose-6-phosphatase [7].
5. Arylesterase:
   α-Naphthylacetat [8]. Beim Nachweis dieses Enzyms wurden folgende Inhibitoren verwendet:
   (a) 10^{-5} m Eserinsalicylat,
   (b) 10^{-4} m E 600 (*p*-Nitrophenyl-diäthylphosphat),
   (c) 10^{-4} m *p*-Chlormercuribenzoat-Na,
   (d) 10^{-3} m Monojodessigsäure.
   Zur Fermenthemmung mit Eserinsalicylat, *p*-Chlormercuribenzoat-Na und Monojodessigsäure wurden die Schnitte in den einzelnen Hemmstofflösungen 30 Minuten bei Raumtemperatur präinkubiert und anschließend in Inkubationslösungen gebracht, die die entsprechenden Inhibitoren in den genannten Konzentrationen enthielten. Mit E 600 wurden die Schnitte nur 30 Minuten präinkubiert.
6. β-D-Glucosidase:
   Postkupplungsmethode, 6-Brom-2-naphthyl-β-D-glucopyranosid [9].
7. β-D-Galactosidase:
   Postkupplungsmethode, 6-Brom-2-naphthyl-β-D-galactopyranosid [9].
8. $NADH_2$-Tetrazoliumsalzoxydoreduktase, red. NAD [10], Nitro-BT.
9. Succinatdehydrogenase [11], Nitro-BT.
10. Lactatdehydrogenase [12], Nitro-BT.
11. Cytochromoxydase [13], Kontrolle: KCN als Inhibitor.

Bei den Nachweisen wurden Kontrollschnitte, wenn nicht anders angegeben, ohne Substrat inkubiert.

Tabelle 1. *Enzymhistochemisch untersuchte, durch* N-*Methyl*-N-*nitrosoharnstoff* (*MNH*) *induzierte Glioblastome des Kaninchens. Versuchsgruppe A:* 10 mg *MNH*/kg *Körpergewicht in zweiwöchigem Abstand injiziert. Versuchsgruppe B:* 20 mg *MNH*/kg *Körpergewicht in vierwöchigem Abstand injiziert*

Vers.-gruppe	Vers. Nr.	Sitz des Tumors	Zahl d. Injektionen	Gesamtdosis MHN in mg	Latenzzeit in Tagen
A	89	rechte Hemisphäre	21	544	290
A	112	rechte Hemisphäre	19	452	255
A	129	parietal rechts	22	480	299
A	136	parietal rechts	20	538	273
A	153	frontoparietal rechts	18	509	234
B	97	Thalamus rechts	13	648	340
B	101	Großhirn rechts	12	548	328
B	119	frontoparietal rechts	11	526	322
B	124	parieto-occipital links	14	655	381
B	134	frontoparietal links	12	533	331
B	135	occipital links	12	653	314
B	144	frontoparietal links	11	501	288
B	156	parieto-occipital rechts	10	488	285
B	159	parietal links	10	600	281

ERGEBNISSE

1. *Histologische Befunde*

Die Glioblastome zeigen eine hochgradige Zell- und Kernpolymorphie sowie herdförmige Nekrosen und Blutungen. Astrozytenähnliche Zellen sind nur stellenweise vorhanden: meist herrschen Zellatypien vor. Mitosen sind häufig zu beobachten. In der Regel enthalten die Geschwülste massenhaft vielkernige Riesenzellen. In einem Teil der Fälle sind ihre Kerne nahe der Zellmembran lokalisiert und umschließen ringförmig den ausgedehnten, meist runden oder ovalen eosinophilen Zelleib. Die Glioblastome lassen oft eine multizentrische Geschwulstentstehung erkennen; infolgedessen sind unterschiedlich große, isolierte oder konfluierende Tumorherde in wechselnder Zahl anzutreffen. In den meisten Glioblastomen fallen nekrobiotische Tumorriesenzellen auf, die unregelmäßig im Tumorgewebe verstreut liegen und durch wabenförmig aufgelockertes Zytoplasma gekennzeichnet sind. Nur bei einem Teil dieser Zellen können mit der Sudanfärbung kleine Fetttropfen nachgewiesen werden.

2. *Enzymhistochemische Befunde*

In 7 der 14 untersuchten Hirntumoren kann *alkalische Phosphatase* erfaßt werden, wobei die Ergebnisse beider Nachweismethoden übereinstimmen. In 6 Glioblastomen ist die Enzymaktivität sehr gering und auf wenige verstreut liegende Tumorzellen und gelegentlich auf kleine Wandanteile einzelner Gefäße beschränkt. Lediglich eine Neubildung zeigt lebhafte Phosphatasereaktion. Hier ist das Ferment in einer Reihe von Kapillarwänden zu erkennen. Weiterhin können besonders in einigen Tumorbezirken zahlreiche Geschwulstzellen, darunter auch mehrkernige Riesenzellen, mit enzymaktivem Zytoplasma beobachtet werden.

Der Nachweis der *sauren Phosphatase* ist in allen untersuchten Tumoren positiv. Mäßige bis starke Fermentaktivität wird in der Mehrzahl der Tumorzellen beobachtet. Das Verteilungsmuster des Enzyms in den Geschwulstzellen ist unterschiedlich. In den kleineren Zellen findet man meistens einen kleinen Zytoplasmabezirk mit hoher Aktivität, der oft in unmittelbarer Umgebung des Zellkerns lokalisiert ist. In den Riesenzellen ist die Phosphataseaktivität gleichmäßig über das gesamte Zytoplasma verteilt. Besonders hohe Fermentaktivität enthalten die mehrkernigen Riesenzellen, deren Zytoplasma von den Zellkernen kranzförmig umschlossen wird (Abb. 1). In nekrobiotischen Tumorriesenzellen sind die Farbstoffniederschläge in dem schaumig anmutenden Zytoplasma auffallend grobgranulär. Allerdings ist die Zahl der nekrobiotischen Riesenzellen, die beim Nachweis der sauren Phosphatase positiv reagieren, geringer als beim Arylesterase-Nachweis (s.u.). Tumorareale im Stadium beginnender Nekrose sowie die Ränder weniger kleiner nekrotischer Bezirke sind ebenfalls phosphataseaktiv. Das hier lokalisierte Ferment wird wiederum in Form grober Farbstoffgranula angezeigt.

In allen untersuchten Hirntumoren kann die saure Phosphatase durch Natriumfluorid gehemmt werden. Lediglich bei einigen Riesenzellen tritt noch eine schwache Farbstoffbildung auf. Durch das Detergens Triton X 100

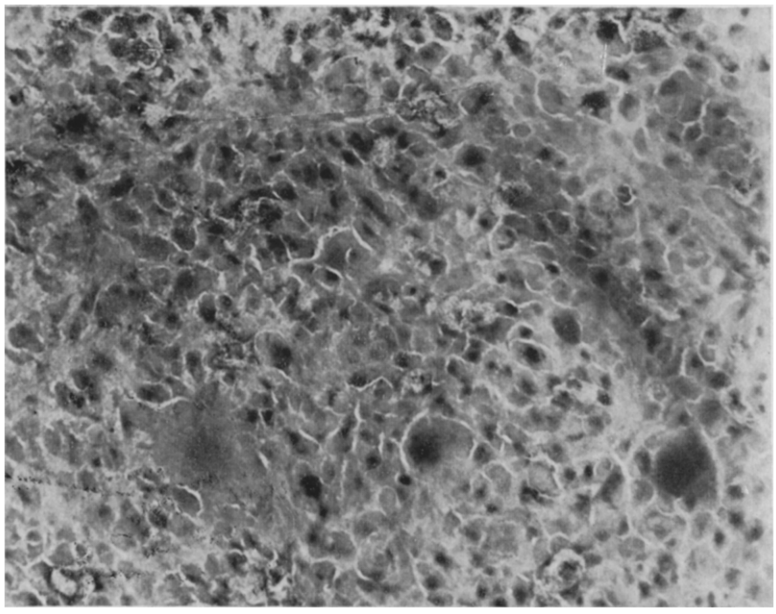

Abb. 1. *Glioblastom Vers. Nr.* 124. *Saure Phosphatase im gesamten Zytoplasma der Riesenzellen sowie in kernnahen Bezirken der übrigen Tumorzellen.* 300-*fach.*

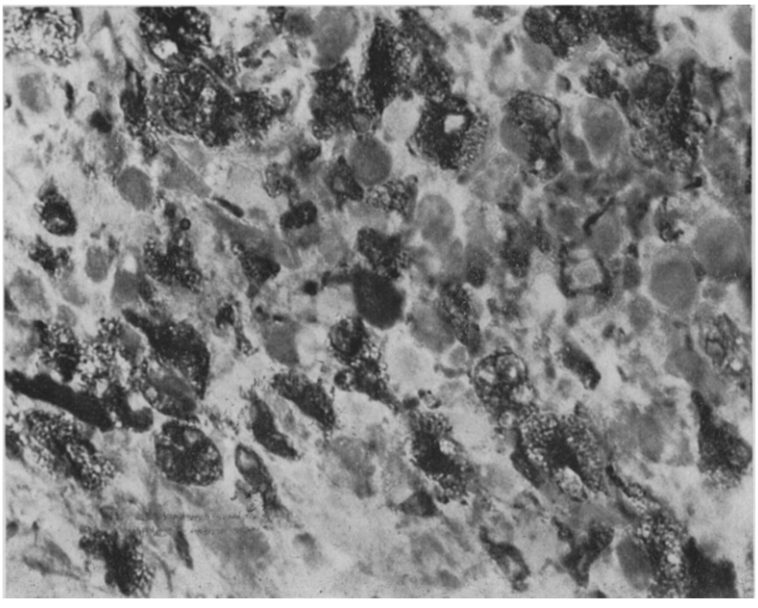

Abb. 2. *Glioblastom Vers. Nr.* 144. *Hohe granuläre Aktivität der Arylesterase im Zytoplasma der nekrobiotischen Riesenzellen.* 450-*fach.*

(to face p. 526)

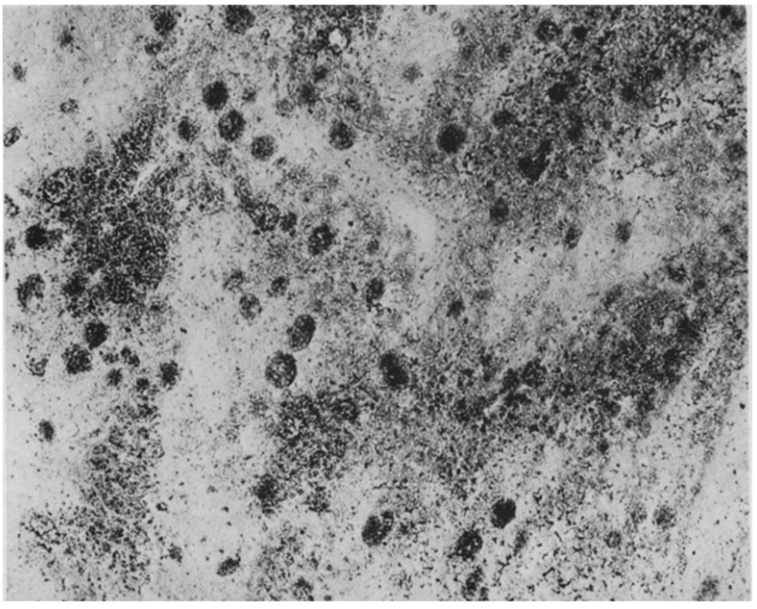

Abb. 3. *Glioblastom Vers. Nr.* 129. *Starke* ß-D-*Galactosidase-Aktivität in zahlreichen kleinen Zellgruppen. Schwächerer Reaktionsausfall in ausgedehnten nekrobiotischen Gebieten.* 85-*fach.*

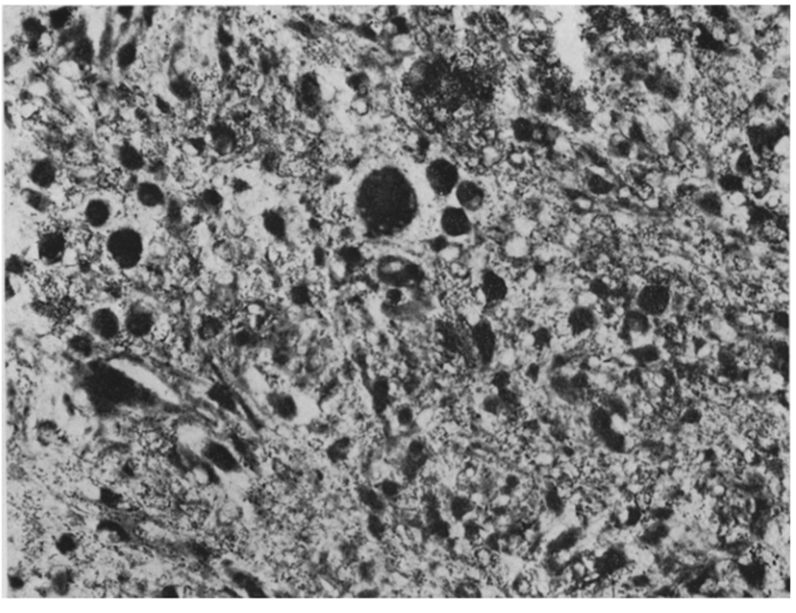

Abb. 4. *Glioblastom Vers. Nr.* 144. *$NADH_2$-Tetrazoliumsalzoxydoreduktase. Hohe Aktivität im Zytoplasma der Riesenzellen.* 190-*fach.*

wird die Nachweisbarkeit des Ferments unterschiedlich beeinflußt. In 5 Geschwülsten ist der Reaktionsausfall völlig negativ. Dagegen wird im angrenzenden normalen Hirngewebe durch diese Behandlung die Fermentaktivität gesteigert. In den übrigen Neubildungen ist nach Einwirkung des Detergens das Enzym hauptsächlich noch im Perikaryon der kleineren Tumorzellen nachweisbar. So zeigt beispielsweise das kleinzellige Glioblastom (Vers. Nr. 156) eine ungeschwächte Aktivität. Frei von saurer Phosphatase sind nach Einwirkung von Triton X 100 alle nekrobiotischen sowie die Mehrzahl der erhaltenen Riesenzellen.

Die Untersuchungsergebnisse beim Nachweis der spezifischen Phosphatasen *Glucose-6-phosphatase* und *Adenosinmonophosphatase* weichen stark von den oben beschriebenen Befunden der alkalischen und sauren Phosphatase ab. Während Glucose-6-phosphatase in keiner Geschwulste nachgewiesen werden kann, tritt Adenosinmonophosphatase (AMP-ase) in 8 Hirntumoren auf. Neben einer mäßig starken AMP-ase-Aktivität in wenigen Kapillar- und Gefäßwänden wird regelmäßig starker Reaktionsausfall in nekrotischen Tumorbezirken beobachtet. Im Zytoplasma mehrkerniger Riesenzellen ist ebenfalls Fermentaktivität anzutreffen.

Weiterhin zeigen unterschiedlich große Zellgruppen eine zytoplasmatische Aktivität mittlerer Intensität. Diese Bezirke des Tumorparenchyms lassen sich auch mit Sudan schwach anfärben, wobei zum Teil sehr kleine Lipidtröpfchen dargestellt werden. Die nekrobiotischen Riesenzellen mit aufgelockertem Zytoplasma sind inaktiv.

Arylesterase ist mit einer Ausnahme (Vers. Nr. 129) in allen Hirngeschwülsten nachweisbar. In der Regel ist der Hauptanteil des Tumorparenchyms nur schwach bis mäßig aktiv. Das Ferment ist im Zytoplasma dieser Zellen in feingranulärer Form enthalten. Der überwiegende Teil der Arylesteraseaktivität ist in Nekroserändern und in bestimmten Zellarten konzentriert, deren Vorkommen und wechselnde Häufigkeit das histochemische Bild bei diesem Fermentnachweis bestimmen. Auffallend ist zunächst, ebenso wie beim Nachweis der sauren Phosphatase, der starke Reaktionsausfall in den meisten Riesenzellen. In einem Teil derselben, besonders in den mehrkernigen Formen, ist das gesamte Zytoplasma gleichmäßig intensiv angefärbt. Daneben kann in dem aufgelockerten Zytoplasma der nekrobiotischen Riesenzellen hohe grobgranuläre Aktivität nachgewiesen werden. Abb. 2 zeigt eine Häufung derartiger Zellen, an denen die einzelnen Stadien der Zytoplasmaauflockerung zu erkennen sind. Weiterhin findet man im Tumorgewebe kleine Zellen, die im Zytoplasma und in ihren Fortsätzen lebhafte Arylesterase-Aktivität aufweisen. Darüberhinaus kommen in den einzelnen Neubildungen aktive Tumorzellen in wechselnder Zahl und unterschiedlicher Größe vor. In einem Glioblastoma multiforme (Vers. Nr. 101) sind die Endothelproliferationen einer Reihe von Gefäßen reich an Arylesterase. Die Nekroseränder in den Geschwülsten sind bei diesem Fermentnachweis, im Gegensatz zur sauren Phosphatase, fast ausnahmslos dargestellt. In dem oft mehrere Zellschichten breiten Saum enthält das zerfallende Gewebe zahlreiche große Farbstoffgranula. An den Randbezirken der Tumoren kommen häufig ortsständige Ganglienzellen vor. Ihre Unterscheidung von den ebenfalls fermentaktiven Riesenzellen ist oft schwierig.

Die in der Hirntumoren nachweisbare Arylesterase wird durch Jodessigsäure, E 600 und Eserin völlig gehemmt. Werden die Schnitte mit Eserin allerdings nur präinkubiert, ist das Enzym noch in Nekroserändern und nekrobiotischen Zellen aktiv. Nach Verwendung von *p*-Chlormercuribenzoat ist in 3 Glioblastomen noch eine positive Reaktion zu beobachten. In 2 Fällen sind nur kleine Tumorzellen und ihre Fortsätze bzw. die nekrobiotischen Riesenzellen selektiv dargestellt, während beim Tumor der Vers. Nr. 124 keine Fermenthemmung eintritt.

Die lysosomalen Enzyme β-D-*Glucosidase* und β-D-*Galactosidase*, die mit Ausnahme von 2 Fällen (Vers. Nr. 153, 156) in allen untersuchten Kaninchentumoren nachzuweisen sind, besitzen beide die gleiche Lokalisation. Dabei ist der Reaktionsausfall beim Nachweis der β-D-Galactosidase stets stärker. Von beiden Fermenten werden sowohl nekrotische als auch solche Tumorabschnitte markiert, die sich möglicherweise in einem pränekrotischen Zustand befinden, denn neben Nekroseherden zeigen auch Tumorareale eine erhebliche granuläre Farbstoffbildung, die noch keine morphologischen Zeichen des Zelluntergangs aufweisen. Im übrigen Tumorparenchym, das nur schwache zytoplasmatische Aktivität besitzt, liegen unregelmäßig verstreut enzymaktive Herde, die oft nur aus 3 bis 4 Zellen bestehen (Abb. 3).

$NADH_2$-Tetrazoliumsalzoxydoreduktase ist in allen Neubildungen enthalten. Das beobachtete Enzymmuster erinnert an ähnliche Befunde beim Nachweis der Arylesterase. Relativ isomorphe Tumorabschnitte zeigen zytoplas-

matische Fermentaktivität mittlerer Stärke, die den polymorphen Kern etwa gleichmäßig umgibt (Abb. 4). In dem kleinzelligen Glioblastom (Vers. Nr.156) ist dagegen der Hauptanteil des Enzyms an umschriebener Stelle in Kernnähe konzentriert. In polymorphen Abschnitten der übrigen Tumoren treten demgegenüber Riesenzellen stärker hervor. Besonders auffallend ist der intensive Reaktionsausfall im ausgedehnten Zytoplasma jener Riesenzellen, deren zahlreiche Kerne an der Zellmembran angeordnet sind (Abb. 4). In gelegentlich vorkommenden runden Zellen mittlerer Größe wird das gesamte Protoplasma von Formazan ausgefüllt. Weiterhin enthalten einige Tumorzellen gesteigerte Fermentaktivität im Zytoplasma und in ihren Fortsätzen. Fermentaktiv sind außerdem Gefäßwände sowie Gefäßproliferationen. In den meisten Fällen ist die Formazanbildung in den mesenchymalen Strukturen und im Tumorparenchym etwa gleich stark. Dagegen treten in einigen Geschwülsten die Gefäßwände stärker hervor. In den Nekroserändern kommt es zur Bildung von Formazangranula unterschiedlicher Intensität. Ebenso wie beim Nachweis der Arylesterase werden auch die im Tumorrandgebiet gelegentlich vorkommenden Ganglienzellen dargestellt.

Succinatdehydrogenase ist im Gegensatz zur $NADH_2$-Tetrazoliumsalzoxydoreduktase schwach aktiv und kann nur in 10 von 14 untersuchten Geschwülsten histochemisch erfaßt werden. Die Lokalisation beider Fermente zeigt dagegen weitgehende Übereinstimmung.

Lactatdehydrogenase mittlerer bis starker Aktivität findet man in allen Hirngeschwülsten des Kaninchens. Im überwiegenden Teil der Tumorzellen ist die Fermentaktivität weitgehend gleich und wird sowohl durch homogene Farbstoffbildung als auch durch einzelne Formazangranula im Zytoplasma angezeigt. Riesenzellen weisen einen gesteigerten Reaktionsausfall auf. Die Aktivitätsunterschiede zwischen Riesenzellen sind jedoch nicht so groß wie beim Nachweis der $NADH_2$-Tetrazoliumsalzoxydoreduktase. In nekrobiotischen Riesenzellen, die auch bein anderen Enzymnachweisen auffallen, können nur tiefblaue Granula beobachtet werden. Nekrosegebiete, in denen bereits ein vollständiger Verlust der übrigen untersuchten Dehydrogenasen eingetreten ist, sind ebenfalls noch beträchtlich aktiv.

Cytochromoxydase ist weder in den Tumoren noch in dem die Geschwülste unmittelbar begrenzenden normalen Hirngewebe nachweisbar. Erst mit wachsender Entfernung vom Tumorrand steigt die Fermentaktivität allmählich an.

DISKUSSION

Nach bisherigen Befunden sind Kaninchen neben Ratten die zweite Tierart, bei der MNH regelmäßig und unter vergleichbaren Bedingungen Geschwülste im Nervensystem induziert [3]. So ist ein Vergleich der fermenthistochemischen Befunde von Geschwülsten des Zentralnervensystems bei Ratten [14–16] und Kaninchen von großem Interesse.

Die Hirngeschwülste von Kaninchen und Ratten zeigen beim Nachweis der oxydativen Fermente gleiches Verhalten bezüglich der Reaktionsintensität, jedoch Unterschiede im Verteilungsmuster. Die Abnahme des Reaktionsausfalls in den Geschwülsten beider Tierarten entspricht folgender Reihe: $NADH_2$-Tetrazoliumsalzoxydoreduktase, Lactat-, Succinatdehydrogenase und Cytochromoxydase. Die unterschiedliche Verteilung, besonders der beiden erstgenannten Dehydrogenasen, in den neurogenen Geschwülsten beider Spezies wird vorwiegend von den morphologischen Besonderheiten bestimmt, wie beispielsweise durch das gehäufte Vorkommen von Riesenzellen in den Hirntumoren der Kaninchen. Ähnliches gilt auch für das Verteilungsmuster der sauren Phosphatase. Allerdings ist die höhere Aktivität dieses Ferments in den Geschwülsten des Kaninchens nicht nur durch die Riesenzellen bedingt, sondern das übrige Parenchym zeigt regelmäßigeren und intensiveren Reaktionsausfall als das der Neubildungen der Ratte.

Die auffallendsten fermenthistochemischen Unterschiede, die nicht durch die histologische Struktur der Geschwülste bedingt sind, können beim Nachweis der alkalischen Phosphatase und der β-D-Glycosidasen beobachtet werden. Dem regelmäßigen Vorkommen alkalischer Phosphatase in den Gefäßwänden der Hirn- und Nervenwurzeltumoren der Ratte stehen die fast negativen Befunde bei den Hirngeschwülsten der Kaninchen gegenüber. Die fehlende Fermentaktivität in den mesenchymalen Anteilen dieser Geschwülste ist sicher nicht durch das Vorliegen von Gefäßneubildungen bedingt, wie das bei schnell wachsenden menschlichen Gliomen gefunden wurde [17]. Vielmehr muß hier berücksichtigt werden, daß auch die Kapillar- und Gefäßwände des normalen Kaninchengehirns nur einen sehr geringen Enzymgehalt aufweisen (vgl. 18).

Im Gegensatz zu entsprechenden Befunden bei Kaninchen konnten in primären Hirntumoren der Ratte weder β-D-Galactosidase noch

β-D-Glucosidase festgestellt werden. Bei denjenigen Geschwulstzellen, die beim Nachweis dieser Fermente in den Kaninchentumoren in Form kleiner, verstreuter Herde hervortreten, handelt es sich vermutlich um die Riesenzellen, die auch durch ihren Gehalt an Arylesterase, saurer Phosphatase und Dehydrogenasen auffallen und von denen einige mit Sudan angefärbt werden. Während jedoch bei diesen Reaktionen stets nur jeweils eine Zelle mit Farbstoffgranula ausgefüllt ist, werden die β-D-Glycosidasen auch in einigen umliegenden Zellen beobachtet. Dieser Befund deutet darauf hin, daß die gesteigerte Aktivität beider Fermente in den Nachbarzellen durch Stoffwechselprodukte der nekrobiotischen Zellen induziert wurde. Wie der intensive Reaktionsausfall an den Nekroserändern zeigt, sind die Enzyme in den vorliegenden Geschwülsten an Phagocytose- und Pinocytosevorgängen beteiligt. Während das Vorkommen der β-D-Glycosidasen in den Hirntumoren der Kaninchen auf nekrotische und nekrobiotische Gewebsabschnitte beschränkt ist, konnte Hanefeld [19] bei menschlichen Gliomen den Hauptanteil dieser Fermente in zahlreichen Tumorzellen, insbesondere in großen Astrozytomzellen und in mehrkernigen Riesenzellen, sowie in zerfallenden Markscheiden nachweisen.

Weitere Unterschiede zwischen den Hirntumoren des Kaninchens und denen der Ratte ergeben sich auch bei der Untersuchung des Arylesterasevorkommens. Intensive Reaktion an den Randbezirken von Nekrosen ist nur bei den Kaninchengeschwülsten zu beobachten. Arylesteraseaktivität, die nur in etwa der Hälfte der intracerebralen Neubildungen der Ratte nachweisbar war [15], konnte weder durch Jodessigsäure noch durch *p*-Chlormercuribenzoat-Na gehemmt werden. Dagegen wird das Enzym in den Hirntumoren der Kaninchen von allen verwendeten Hemmstoffen weitgehend inhibiert.

Das fermenthistochemische Verhalten von Hirngeschwülsten ist somit auch speziesbedingt, wie besonders die Untersuchungsergebnisse solcher Tumoren zeigen, die durch das gleiche Cancerogen induziert wurden und die die gleiche Histogenese besitzen. Es bestehen somit deutliche biochemische Unterschiede zwischen den Glioblastomen der Kaninchen und Ratten sowie offenbar auch des Menschen. Das ist bei künftigen chemotherapeutischen Versuchen an experimentellen Hirngeschwülsten zu beachten.

Anerkennung—Frau Ch. Lehnguth möchten wir für die technische Mitarbeit danken.

RESUME

L'activité enzymatique de 14 *glioblastomes du lapin, provoqués par l'injection intraveineuse de N-Méthyl-N nitrosurée (MNU), a été étudiée par des méthodes histochimiques. L'existence et la distribution de* 11 *enzymes ont été examinées dans les tumeurs cérébrales. Une forte activité de phosphatase acide, d'arylestérase, de $NADH_2$-tétrazolium-oxydoréductase et de déshydrogénase lactique a été retrouvée dans toutes les tumeurs; elle était surtout localisée dans le cytoplasme des cellules géantes. La β-D-galactosidase et la β-D-glucosidase étaient présentes dans les foyers nécrotiques et en dégénérescence. La glucose-6-phosphatase et la cytochrome-oxydase n'étaient pas décelables, mais l'adénosine monophosphatase et la déshydrogénase succinique furent mises en évidence dans certaines tumeurs. L'absence presque totale de la phosphatase alcaline dans les glioblastomes du lapin est digne d'être notée. La distribution des enzymes, particulièrement des enzymes hydrolytiques, était nettement différente de celle observée dans les tumeurs du système nerveux du rat, produites également par le MNU.*

SUMMARY

The results of enzyme histochemical investigations of 14 *glioblastomas induced in rabbits by the intravenous injections of N-methyl-N-nitrosourea (MNU) are reported. The occurrence and the distribution of* 11 *enzymes were studied. High activity of acid phosphatase, arylesterase, $NADH_2$-tetrazoliumoxydoreductase and lactate dehydrogenase was found in all samples and was especially localized in the ctyoplasm of the giant cells. β-D-galactosidase and β-D-glucosidase were present in necrotic and degenerative foci. Glucose-6-phosphatase and cytochrome oxidase were not detected, but adenosine monophosphatase and succinic dehydrogenase were found in some tumours. It was noteworthy that the glioblastomas of the rabbit exhibited almost no activity of the alkaline phosphatase. The topic pattern, particularly of the hydrolytic enzymes, was distinctly different from the corresponding findings in tumours of the nervous system of the rat also induced by MNU.*

ZUSAMMENFASSUNG

Es werden enzymhistochemische Befunde an 14 Glioblastomen des Kaninchens mitgeteilt, die durch intravenöse Injektion von N-Methyl-N-nitrosoharnstoff (MNH) induziert wurden. Die Hirntumoren werden auf das Vorkommen und die Verteilung von 11 Fermenten untersucht. Hohe Aktivität von saurer Phosphatase, Arylesterase, $NADH_2$-Tetrazoliumsalzoxydoreduktase und Lactatdehydrogenase ist in allen Neubildungen vorhanden und bevorzugt im Zytoplasma der Riesenzellen lokalisiert. Nekrosegebiete und nekrobiotische Gewebsanteile werden beim Nachweis der β-D-Galactosidase und der ß-D-Glucosidase dargestellt. Während Glucose-6-phosphatase und Cytochromoxydase in keinem Fall nachweisbar sind, ist Adenosinmonophosphatase- und Succinatdehydrogenase-Aktivität nur in einem Teil der Geschwülste zu finden. Bemerkenswert sind die weitgehend negativen Ergebnisse beim Nachweis der alkalischen Phosphatase. Vorkommen und Lokalisation, besonders der hydrolytischen Fermente, unterscheiden sich deutlich von entsprechenden Befunden an mit MNH induzierten Tumoren des Nervensystems der Ratte.

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Announcements

National Cancer Institute Monograph 30 May 1969

Human Tumor Cell Kinetics *Edited by:* SEYMOUR PERRY. *For sale by the Superintendent of Documents, U.S. Government Printing Office, Washington, D.C.* 20402. *Price* $4.00

THIS monograph includes papers given at a series of seminars held at the National Institutes of Health between 1 November 1967 and 17 April 1968.

The monograph gives data on cell kinetics and cell cycle characteristics of both normal and neoplastic cells in man which were discussed by 14 leading investigators. Their findings relate to clinical problems that should be of value to the cancer chemotherapist, radiotherapist, pharmacologist, toxicologist, and others concerned in the effort to improve the control of cancer through the use of antitumor agents.

Les Problèmes Therapeutiques Suscités par l'Utilisation des Progestatifs de Synthèse dans le Traitement du Cancer

Mardi 9 *décembre* 1969

UN COLLOQUE International se tiendra à Lyon (Palais des Congrès) sous la présidence de Monsieur le Professeur Marcel DARGENT, Directeur du Centre Léon Bérard.

Des exposés introductifs sur les problèmes biologiques et cliniques seront suivis de discussions en table ronde.

Pour tous renseignements s'addresser à

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Fibrinogen Metabolism in Experimental Tumours

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INTRODUCTION

EXTRAVASCULAR deposition of fibrin in human carcinomas was found related to an increased thromboplastic activity in the tumour tissue by O'Meara [1–4]. Similar observations were made in studies on both spontaneous and transplanted experimental tumours [5–13]. O'Meara suggested that fibrin in the periphery of a growing malignant tumour was a suitable stroma for tumour cell invasion and thus *favoured* the growth and spread of malignant tumours. Antifibrinolytic therapy was found to decrease the tumour growth rate in experimental tumours [14], which suggested that fibrin might instead be part of a reaction *against* the growing tumour. Such a mechanism might be influenced by the thromboplastic and fibrinolytic properties of the tumours. Thus, it was found that antifibrinolytic therapy decreased the growth of a spontaneous transplantable mouse mammary carcinoma which had high thromboplastic activity and low spontaneous fibrinolytic activity. The growth of a 20-methylcholanthrene induced mouse sarcoma, with comparatively low thromboplastic activity and high spontaneous fibrinolytic activity, was not significantly influenced by the antifibrinolytic agent. It was also found that the high fibrinolytic activity in this tumour was only partially inhibited by antifibrinolytic therapy when administered in highest tolerated dose.

After intravenous administration of ^{125}I- and ^{131}I-labelled fibrinogen to rats with experimental tumours a maximum uptake of fibrinogen in tumour tissue was found to occur within 6–12 hr [7, 9, 15–18]. The registered uptake of labelled fibrinogen was about 40 per cent of the dose given in some tumours, but large variations between different tumours were found. There was a relatively rapid decay of labelled fibrinogen in tumour tissue. This decay was delayed by administration of an antifibrinolytic drug. It was therefore suggested that the fibrinolytic activity of a tumour is responsible for the disappearance rate of fibrinogen or fibrin from tumour tissue [15].

The large variations in uptake of injected fibrinogen in different experimental tumours might be due to differences in their thromboplastic and fibrinolytic activity. The aim of the present study was to investigate the uptake and disappearance rate of injected labelled fibrinogen in relation to the thromboplastic and fibrinolytic properties of well defined experimental tumours.

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MATERIAL AND METHODS

Animals. C$_3$H hybrid mice, 3–4 months old, were studied in the first experiment, while syngeneic C$_3$H mice of the same age were studied in the second experiment. The animals were allowed free access of water and rat pellets.

Tumours. 1. A mouse mammary carcinoma, which developed in a female mouse of the C$_3$H strain in 1963 and which was later continuously transplanted intramuscularly in animals of the same strain. Tumour transfer generations 97 and 113 were studied.

2. A 20-methylcholanthrene induced sarcoma, which developed in February 1967 in a C$_3$H mouse injected subcutaneously 3 months earlier with 1 mg of 20-methylcholanthrene suspended in sesame oil. This tumour was also continuously transplanted intramuscularly in animals of the same strain. Tumour transfer generations 27 and 46 were studied. Both tumours were little differentiated and metasta-

sized spontaneously to the lungs of the animals. The mouse mammary carcinoma was found to be highly thromboplastic but had a low fibrinolytic activity, while the mouse sarcoma had a comparatively low thromboplastic activity but a high spontaneous fibrinolytic activity bound to single tumour cells. Tumours and their properties were described in detail by Peterson [14].

All animals were transplanted with a solid piece of tumour tissue intramuscularly into one hindleg. The experiments were performed 12 days after tumour transplantation with a mean tumour diameter of about 1 cm. Female mice were transplanted with the mammary carcinoma while male mice were transplanted with the sarcoma.

Fibrinogen. In the first experiment homologous mouse fibrinogen was used. This fibrinogen was prepared from pooled mouse plasma according to Blombäck and Blombäck [19]. Since it was desirable to include a larger number of animals in the second experiment and necessary to have a larger volume of fibrinogen, the obtainable volumes of homologous mouse fibrinogen were not sufficient. No species differences between rat and mouse fibrinogen have been reported. Therefore, rat fibrinogen was prepared from pooled rat plasma and accepted for use in this experiment.*

Labelling of fibrinogen. Labelling of fibrinogen with ^{125}I was done with the capillary technique of McFarlane [20] as modified by Blombäck *et al.* [21], but with the use of iodine monochloride instead of molecular iodine [22]. Two ml of a mouse fibrinogen solution in 0·3 M NaCl to a protein concentration of 1·17% was labelled with 1mC ^{125}I to a final volume of 8 ml. Each animal was injected intravenously with 0·2 ml of this solution in a tail vein. The rat fibrinogen was labelled in the same way. Twelve ml of a rat fibrinogen solution with a protein concentration of 2·93% was labelled with 0·5 mC ^{125}I. The fibrinogen concentration in the final solution was 0·6 g per cent. Each animal was injected intravenously in a tail vein with 0·3 ml of this solution.

Radioactivity measurements. These measurements were made in a well scintillation counter with a one channel pulse analyzer. In the first experiment the activity was calculated to cpm/ml whole blood or cpm/g tissue. In the second experiment the activity of whole blood was measured and the activity per ml plasma was calculated. The activity in solid tissue and plasma was corrected for the animal weight, since the same volume of labelled fibrinogen was given to all animals independent of their weight. The per cent retained activity of the given dose in plasma and tissue was calculated and transformed to a mean animal weight of 30 g.

Antifibrinolysis. Antifibrinolysis was induced in animals by administration of a 3% concentration of a potent antifibrinolytic agent, tranexamic acid (Cyclokapron)* in the drinking water of the animals continuously from 2 days before the experiment. A high antifibrinolytic activity in blood could be induced in this way [14].

Fibrinolysis. Fibrinolysis was induced by injecting the animals intraperitoneally twice daily during the experiment with 0·3 casein units (Sgouris) of human plasminogen* activated with 1000 units of streptokinase (Kabikinas)* in a volume of 0·1 ml/10 g body weight. A prolonged fibrinolytic activity in blood was obtained as earlier described [14].

EXPERIMENTS

1. *Uptake of labelled homologous mouse fibrinogen in tumour tissue*

Procedure. Fifteen female mice transplanted with the mouse mammary carcinoma and fifteen male mice transplanted with the mouse sarcoma were studied. All animals were injected intravenously with 0·2 ml of the labelled mouse fibrinogen solution in a tail vein. Five animals from each tumour group were sacrificed by exsanguination under ether anaesthesia after 2, 12 and 24 hr respectively. One tenth ml blood was drawn from each animal. Biopsies of macroscopically vital tumour tissue, liver and normal leg muscle were taken from each animal and weighed to the nearest 0·001 g. The activity in blood and solid tissue was registered and calculated to cpm/ml blood and cpm/g tissue.

Results. Since all animals were injected with the same volume of fibrinogen independent of their weight and since only biopsies were taken

*Mouse and rat fibrinogen was prepared by courtesy of Laborator Birger Blombäck, M.D., Karolinska Sjukhuset, Stockholm, Sweden.

*Kindly supplied by AB Kabi, Stockholm, Sweden.

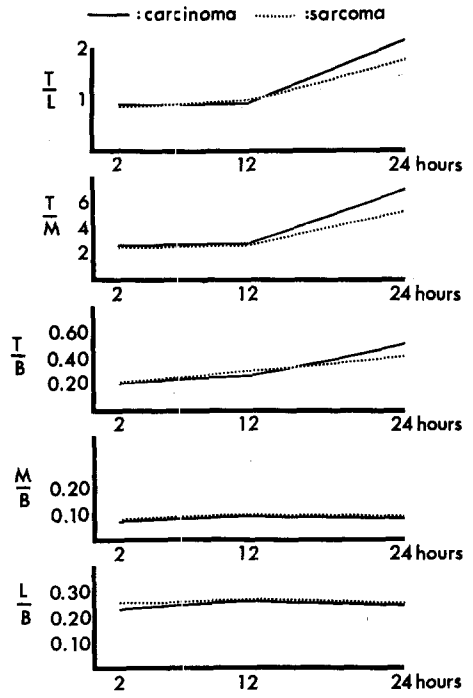

Fig. 1. *Ratios between registered activity* (cpm/g *or* ml) *in tumour, muscle, liver and blood. Note increased activity in tumour* 24 *hr after fibrinogen injection, more pronounced for the mammary carcinoma.*
T=tumour, M=muscle, L=liver and B=blood.

from the different tissues, the presentation of the results are based on ratios between the activity in tumour tissue and liver, muscle and blood. The activity in liver and muscle was compared to blood (Fig. 1). There was an increased activity ratio after 24 hr in tumours compared to normal tissues and to blood. This increase was most marked in the mammary carcinoma. The ratio between liver, muscle and blood was practically unchanged during the obervation period. These data indicate an increased uptake of labelled fibrinogen in tumour tissue. $t_{\frac{1}{2}}$ for labelled mouse fibrinogen in blood was 17 hr in both tumour groups.

2. *Uptake of labelled rat fibrinogen in tumour tissue*

Procedure. Seventy-five female mice transplanted with the mouse mammary carcinoma and seventy-five male mice transplanted with the mouse sarcoma were studied. All animals were injected intravenously with 0·3 ml of the labelled rat fibrinogen solution in a tail vein. From each tumour group 25 animals were used as controls, while antifibrinolysis was induced in 25 animals and fibrinolysis was induced in 25 animals. An increased activity in tumour tissue compared to normal tissue was found in the first experiment 24 hr after the injection of labelled fibrinogen. Therefore, in this second experiment 10 control animals, 10 animals with induced antifibrinolysis and 10 animals with induced fibrinolysis were sacrificed at this time, while only 5 control or treated animals were sacrificed 2, 10 and 48 hr respectively after the fibrinogen injection. Animal weights were registered. From each animal 0·1 ml blood was drawn. During the growth of the tumours there was a development of central necroses, which was most marked in the sarcoma. Central macroscopically necrotic tumour tissue was removed and the tumours were thereafter weighed to the nearest 0·001 g. In the animals transplanted with the sarcoma and sacrificed 48 hr after injection, the removed necroses were also weighed and submitted to registration of activity. Normal muscle from the contralateral leg of all animals was removed and weighed. The activity in blood, whole tumour, necrotic tumour tissue and muscle was registered and corrected to a mean animal weight of 30 g. Student's *t*-test was used for statistical analyses on the larger number of animals sacrificed 24 hr after the fibrinogen injection. The statistical analyses were carried out at a 5 per cent level of significance, i.e. there was a statistically significant difference when $P<0·05$.

During the experiment the animals were kept in clean plastic cages. At the end of the experiment each cage was thoroughly rinsed with 100 ml saline and the activity in this solution was analysed in order to measure the excreted activity as loss in urine and faeces.

Results. The mean per cent retained activity of the dose given per ml plasma (calculated to a mean hematocrit of 40) and the mean per cent retained activity per g tissue at different intervals after fibrinogen injection are presented in Table 1 and Fig. 2. The activity in plasma decreased in a similar way in both tumour groups as did the activity in normal muscle tissue. $t_{\frac{1}{2}}$ of the labelled rat fibrinogen was about 10 hr. Induced antifibrinolysis significantly delayed the decay of the plasma activity, while induced fibrinolysis had no significant effect on this parameter. Similar observations were made in normal muscle tissue.

The activity in both tumours was markedly increased compared to normal muscle. Antifibrinolysis delayed the decreasing activity in the mouse mammary carcinoma, but did not influence the activity in the sarcoma. No effect of the induced fibrinolysis on tumour activity was found until 48 hr after the fibrinogen injection. At this time a lower activity was registered in animals with induced fibrinolysis compared to control animals in both tumour

Table 1. *Per cent retained activity of injected dose per* ml *plasma or per* g *tissue given with standard deviations, in mice transplanted with the mammary carcinoma and with the* 20-*methylcholanthrene induced sarcoma. Control animals and animals with induced antifibrinolysis or induced fibrinolysis*

Hours after injection	Plasma			Muscle			Tumour		
	Control	Antifibrinolysis	Fibrinolysis	Control	Antifibrinolysis	Fibrinolysis	Control	Antifibrinolysis	Fibrinolysis
					Carcinoma				
2	30·9 (2·1)	37·5 (7·1)	39·1 (5·2)	0·81 (0·13)	0·78 (0·08)	0·72 (0·10)	2·4 (0·4)	2·1 (0·8)	2·5 (0·7)
10	11·3 (5·3)	19·8 (3·7)	12·6 (3·0)	0·48 (0·08)	0·67 (0·17)	0·64 (0·10)	2·5 (0·9)	3·1 (0·7)	2·4 (0·4)
24	4·1 (1·9)	7·2[s] (2·8)	4·6 (2·0)	0·35 (0·09)	0·57[s] (0·15)	0·49 (0·11)	1·8 (0·6)	2·5[s] (0·6)	1·9 (0·2)
48	0	0	0	0·07 (0·02)	0·27 (0·07)	0·06 (0·07)	1·3 (0·5)	1·4 (0·1)	0·9 (0·3)
					Sarcoma				
2	35·3 (2·0)	38·8 (4·0)	39·5 (4·9)	0·88 (0·13)	0·92 (0·08)	0·80 (0·11)	2·4 (0·8)	2·4 (0·1)	2·4 (0·2)
10	16·0 (1·6)	25·2 (2·0)	18·3 (4·4)	0·51 (0·08)	0·97 (0·18)	0·68 (0·16)	3·3 (0·4)	3·1 (0·2)	3·5 (0·1)
24	2·9 (2·3)	8·0[s] (1·9)	3·8 (2·0)	0·40 (0·08)	0·56[s] (0·13)	0·48 (0·12)	3·2 (0·8)	2·9 (1·2)	2·8 (0·5)
48	0	0	0	0·07 (0·03)	0·16 (0·06)	0·08 (0·02)	2·4 (0·7)	2·2 (0·9)	1·6 (0·2)

Mean activity in necrotic sarcoma tissue at 48 hr after injection: 5·74 (2·17). s=significant difference ($P<0·05$) compared to control animals sacrificed at the same time.

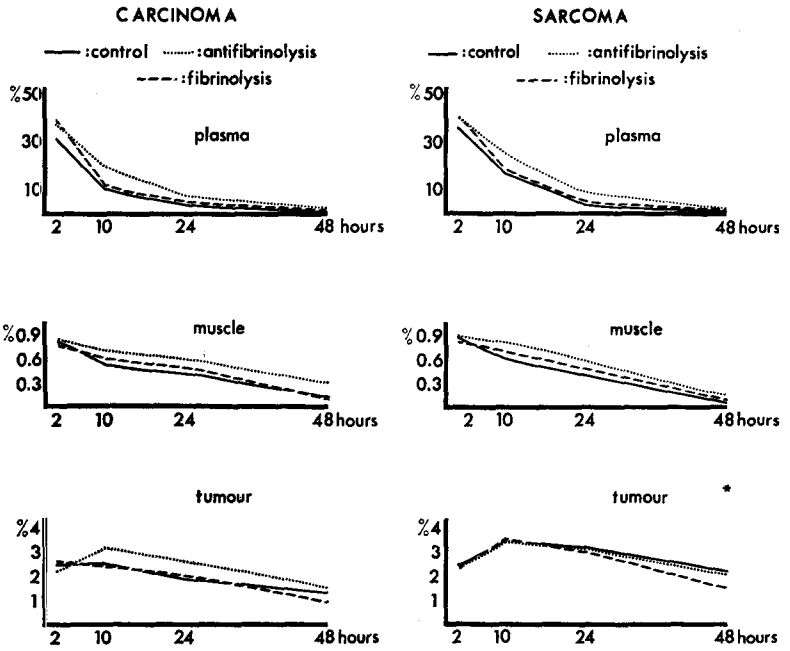

Fig. 2. Per cent retained activity of given dose in plasma, muscle and tumour. Note the delayed decrease of activity in both tumours compared to plasma and muscle. Antifibrinolysis delays the decreasing activity in the mammary carcinoma, but does not influence the activity in the sarcoma. A low activity in tumours is found in animals with induced fibrinolysis 48 hr after the fibrinogen injection.
**Mean activity in necrotic sarcoma tissue.*

groups. A markedly high activity was found in necrotic sarcoma tissue compared to macroscopically vital tumour tissue.

The excreted activity calculated to animal and hour is presented in Fig. 3. This activity was higher in control animals transplanted with the carcinoma compared to animals transplanted with the sarcoma. Induced antifibrinolysis decreased the excreted activity in carcinoma animals but did not influence this activity in sarcoma animals. Induced fibrinolysis did not influence the excreted activity in carcinoma animals, while the activity in sarcoma animals was increased.

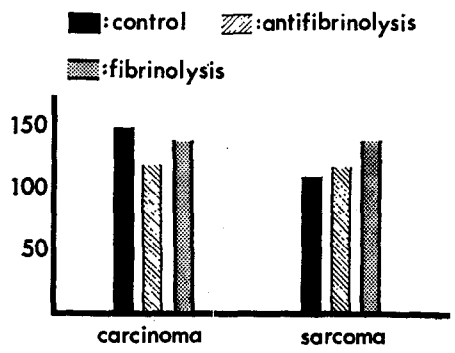

Fig. 3. Excreted activity calculated to cpm/animal/hr. Note the lower activity in sarcoma control animals compared to carcinoma animals. Antifibrinolysis decreases the excreted activity in carcinoma animals but does not influence the activity in sarcoma animals. Fibrinolysis increases the excretion in sarcoma animals.

DISCUSSION

After intravenous injection of labelled homologous mouse fibrinogen an increased activity was found in tumour tissue compared to normal muscle and liver. The activity in muscle and liver was unchanged compared to blood. These observations therefore indicate an increased uptake of labelled fibrinogen in tumour tissue, which is in accordance with earlier studies on experimental tumours. The increased uptake was more pronounced in the mammary carcinoma than in the sarcoma.

In the second experiment rat fibrinogen was used, since mouse fibrinogen was not obtainable in sufficient volumes for the larger number of animals included in this experiment. An increased uptake in tumour tissue was confirmed to occur also for rat fibrinogen. However, in this experiment, the increased uptake of fibrinogen was more pronounced in the sarcoma. This difference between the two experiments might be explained by varying experimental technique. In the first experiment the activity in small biopsies of macroscopically vital tumour tissue was analysed, while in the second experiment whole tumour activity was analysed after removal of central necroses. From earlier studies on these tumours it is known that the sarcoma has a greater tendency for development of central necroses during growth and has a lower capillary transport function compared

to the carcinoma [23]. Though central macroscopically necrotic tumour tissue was removed before registration in the second experiment, there is still a greater risk of including necrotic tumour material compared to the technique used in the first experiment with small biopsies of vital tumour tissue.

The observed increased uptake of mouse fibrinogen in biopsies of vital carcinoma tissue compared to the sarcoma might be fully explained by the high thromboplastic activity of this tumour combined with a low spontaneous fibrinolytic activity. On the other hand the sarcoma had a comparatively low thromboplastic activity and a high spontaneous fibrinolytic activity, which might limit the uptake and enhance the removal of fibrinogen or fibrin in the vital sarcoma tissue. However, a high retention of injected labelled fibrinogen in necrotic tumour tissue, which was also found in the second experiment, might overshadow a decreased uptake in vital sarcoma tissue and explain the results in the second experiment. The retention of injected fibrinogen in necrotic sarcoma tissue is not due to a high thromboplastic activity in such necroses, since in earlier studies a significantly lower thromboplastic activity was found in necrotic tumour tissue compared to vital tumour tissue [14].

The unchanged ratio between muscle and blood in the first experiment suggests that the activity registered in muscle is bound to the blood activity in this tissue. The induced antifibrinolysis delayed the decay of the activity registered in plasma and normal muscle in both tumour groups, probably due to a decreased fibrinolytic activity in blood. Induced fibrinolysis did not influence the activity in blood and muscle. The $t_{\frac{1}{2}}$ for the labelled rat fibrinogen was only about 10 hr which might be due to a lower quality compared to the mouse fibrinogen. This rapid decay in control animals might explain the failing effect of the induced fibrinolysis to further promote the breaking down of circulating fibrinogen.

The induced antifibrinolysis significantly delayed the decay of activity in the mammary carcinoma, while no influence on the activity in the sarcoma was found. This difference might be explained by different properties of the two tumours. The high spontaneous fibrinolytic activity of the sarcoma was only partially inhibited by antifibrinolytic therapy given in the highest tolerated dose [14]. Thus, there might be a remaining fibrinolysis sufficient for removal of labelled fibrinogen from this tumour, while a weak spontaneous fibrinolytic activity in the carcinoma is inhibited by the induced antifibrinolysis, resulting in a decreased elimination of fibrinogen bound to the carcinoma. Consequently, in animals with induced fibrinolysis a decreased activity compared to control animals was found 48 hr after the fibrinogen injection in both tumour groups.

In the second experiment an attempt was made to register the breaking down of labelled fibrinogen by measuring the excreted activity. A decreased excretion was found in animals transplanted with the sarcoma compared to animals transplated with the carcinoma. This difference might be explained by the large volumes of labelled fibrinogen retained in necrotic sarcoma tissue. Antifibrinolysis decreased the excretion in carcinoma animals in which there was also a delayed activity in tumour tissue. Neither the tumour activity nor the excretion was influenced by antifibrinolysis in animals transplanted with the sarcoma. Induced fibrinolysis increased the excretion from sarcoma animals but did not influence the same parameter in carcinoma animals.

Thus, an increased uptake of intravenously injected, labelled homologous or heterologous fibrinogen occurred in the studied experimental tumours as compared to normal tissues. Induced antifibrinolysis, when sufficient to inhibit spontaneous fibrinolytic activity in tumour tissue, decreased the disappearance of labelled fibrinogen from the tumours. Induced fibrinolysis, on the other hand, enhanced the disappearance of labelled fibrinogen from the tumours.

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RESUME

On a étudié la captation, dans deux tumeurs transplantées de la souris, du fibrinogène marqué de souris et de rat. Une forte concentration a été atteinte dans ces tumeurs. Cette concentration accrue est en relation avec les propriétés thromboplastiques et fibrinolytiques des tumeurs. On a constaté qu'une antifibrinolyse provoquée, lorsque celle-ci était suffisante pour inhiber l'activité fibrinolytique spontanée dans le tissu tumoral, ralentissait la disparition du fibrinogène dans les tumeurs. Au contraire, une fibrinolyse provoquée a accéléré cette disparition.

SUMMARY

The uptake of intravenously injected labelled mouse and rat fibrinogen in two transplantable mouse tumours was studied. A high uptake occurred in tumours compared to normal tissues. This increased uptake was related to the thromboplastic and fibrinolytic properties of the tumours. Induced antifibrinolysis, when sufficient to inhibit spontaneous fibrinolytic activity in tumour tissue, decreased the disappearance of fibrinogen from tumours. Induced fibrinolysis, on the contrary, enhanced the disappearance of fibrinogen from tumour tissue.

ZUSAMMENFASSUNG

Die Speicherung von intravenös verabreichtem radioaktiven Mäuse- und Rattenfibrinogen in transplantierten Mäusetumoren wurde untersucht. Im Vergleich zu normalem Gewebe konnte im Tumorgewebe ein hoher Fibrinogengehalt nachgewiesen werden. Der Grad der Speicherung war von den thromboplastischen und fibrinolytischen Eigenschaften des Tumors abhängig. Induzierte Antifibrinolyse konnte unter der Voraussetzung, daß sie jede spontane fibrinolytische Aktivität des Tumorgewebes unterband die Abnahme des Fibrinogengehaltes der Tumoren vermindern. Induzierte Fibrinolyse hingegen beschleunigt die Beseitigung des Fibrinogens aus dem Tumorgewebe.

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Erratum

JOACHIM MARK, Rous sarcomas in mice: the chromosomal progression in primary tumours. *Europ. J. Cancer* **5,** 307 (1969).

It is regretted that Table 2 of the above article was omitted; the article is now published again with apologies to the Author and readers.

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Sur l'Origine et le Comportement des Tumeurs à Cellules Claires de la Thyroïde du Rat

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ON PROVOQUE des néoplasmes de la thyroïde du rat lorsqu'on inhibe la synthèse de ses hormones en employant soit un régime carencé en iode, soit la thyroïdectomie partielle, soit des antithyroïdiens, soit enfin des radiations ionisantes, en particulier celles du radioiode.

L'efficacité de ces méthodes varie avec la souche d'animaux employés et les conditions expérimentales. Les résultats les plus marqués ont été obtenus en faisant suivre l'application du radioiode (30 μc) de l'administration d'un antithyroïdien, en l'occurrence le propyl-thiouracil, poursuivie, durant la vie de l'animal, jusqu'au moment où les épithéliomas apparaissent [1, 2].

Les tumeurs obtenues comprennent les adénomes β, de structure plus ou moins papillaire, et les adénomes γ, tumeurs compactes constituées de cellules claires, ou cellules parafolliculaires; ces deux types de néoplasmes se rencontrant chez tous les mammifères.

Ceux-ci peuvent se cancériser ultérieurement, et, selon l'interprétation classique, la formation de ces néoplasmes bénins et malins est due à l'action directe de la TSH hypophysaire élaborée en excès par réaction à la carence permanente en iodothyronines que les traitements tumorigènes engendrent.

Cette interprétation appelle des réserves en ce qui concerne les tumeurs à cellules claires. En effet, on sait que la thyroïde normale du Rat, comme celle des autres mammifères, contient des cellules claires analogues à celles qui forment les adénomes γ, et qu'elles sont capables de se multiplier sous l'influence d'une administration expérimentale de G.H. [3]. On sait aussi que ces adénomes se développent avec une grande fréquence après le rétablissement d'un régime normal chez le Rat, dont on a initialement arrêté l'élaboration des iodothyronines et stimulé ainsi la production de TSH grâce à une carence alimentaire en iode [4]. Enfin, nous avons montré que ces adénomes se développent plusieurs mois après l'arrêt de tout traitement par les antithyroïdiens, à un moment où il semble peu vraisemblable que la TSH soit encore sécrétée en excès [5].

Dans le présent travail, nous avons tout d'abord eu pour but de déterminer l'origine histologique des cellules claires constitutives des adénomes γ, afin de voir s'ils se forment à partir des cellules claires de la thyroïde normale ou s'ils naissent directement par transformation des cellules vésiculaires elles-mêmes. En second lieu, nous avons recherché si la production de ces adénomes se présente avec des particularités différentes suivant que le traitement qui leur donne naissance est cancérigène ou non. En troisième lieu, nous avons étudié l'aspect du tissu thyroïdien normal entourant les adénomes, afin de voir si les critères histophysiologiques qu'il présente sont en faveur d'un excès de TSH expliquant la formatoin de ces tumeurs, selon l'opinion classique.

Enfin, dans le même ordre d'idées, nous avons administré de fortes doses de TSH à des rats porteurs d'adénomes γ, afin de voir si ces néoplasmes réagissent ou non à l'action de cette hormone.

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MATERIEL ET METHODES

Pour obtenir des néoplasmes à cellules claires, nous avons repris ici les protocoles expérimentaux utilisés dans notre travail antérieur [5]. Des rats mâles de souche Wistar, issus d'un élevage en 'in breeding', sont nourris de façon standard (Feedine), et reçoivent, deux fois par semaine, de la verdure. L'eau de boisson, qui est donnée *ad libitum*, contient du propylthiouracil (PTU: 0,60/00), dont l'administration est réglée de manière que les rats arrivés à l'âge de 9 mois aient été traités par le PTU pendant 1 ou 3 mois.

Au terme de cette opération, une partie des animaux reçoit alors 30 µc d'^{131}I par la voie sous-cutanée, et tout traitement est ensuite définitivement suspendu. Des lots de rats, traités seulement par l'antithyroïdien ou le radioiode, sont pris comme témoins, ainsi que des rats normaux.

Le sacrifice des animaux a été réalisé dans les 4 à 17 mois qui ont suivi l'arrêt du traitement. Le prélèvement des thyroïdes est fait 24 h. après une injection sous-cutanée de colchicine (0, 5 mg), qui permet de dénombrer les mitoses thyroïdiennes bloquées en métaphase.

Les coupes de thyroïde, colorées par la méthode de MacManus–Hémalun, nous ont permis d'étudier un certain nombre de critères histo-physiologiques de la fonction thyroïdienne, tels que les dimensions de l'épithélium vésiculaire et la teneur en colloïde de la glande. Ces deux dernières données sont établies par la méthode classique du poids de carton, suivant laquelle de grandes surfaces de thyroïde, projetées à l'aide d'un microscope horizontal sur un papier bristol d'épaisseur constante, sont ensuite dessinées, puis découpées suivant le dessin. Les constituants recueillis sont ensuite pesés et les poids respectifs enregistrés permettent d'en calculer la proportion relative.

Enfin, pour tester la sensibilité éventuelle des adénomes γ à la TSH, nous avons traité des rats susceptibles d'être porteurs de telles tumeurs, pendant 3 jours de suite, par une dose journalière de 8 unités R.P. de cette hormone. La dernière injection, réalisée 24 h. avant le sacrifice, est associée à l'administration de 0.5 mg de colchicine destinée comme précé-

Tableau 1.

Durée de traitement	Age du rat au sacrifice (mois)	Nombre de rats	Poids de la thyroïde (mg)	Pourcentage de colloïde	Mitoses pour 10.000 cellules	Hauteur de l'épithélium	Sujets porteurs d'adénome
PTU 3 mois	9	5	70	15	12,5	6	0
	13	2	48	17	4,4	3	0
	16	2	46	29	2,7	—	0
	20	3	54	28,5	1,6	1,6	2
	26	3	54	36	0,5	2,7	1
PTU 3 mois	13	2	46	16,5	4,1	2,3	0
+30 µc ^{131}I	16	2	37,5	28	3,4	1,3	2
	20	3	49	28	1,2	2	3
	26	2	56	38	1,1	2,3	2
PTU 1 mois	13	2	42,5	28	2,5	2,4	0
+30 µc ^{131}I	16	2	40	32	2,1	1,5	0
	20	2	42	29	1,2	2,1	1
	26	3	41	—	2,3	2,3	2
^{131}I 30 µc	11	2	35	22	7	4,4	0
	13	2	27	19	3,9	4,1	0
	20	2	19	19	5,9	3,8	0
	24	2	15,5	—	—	—	0
Normal	11	3	21	20	4,5	4	0
	13	4	19	21,5	3,9	3,7	0
	20	5	19,5	17	0,6	3	0
	26	1	—	—	0,5	2,5	0

Caractéristiques thyroïdiennes de rats soumis à des traitements générateurs ou non d'adénomes à cellules claires. On notera l'action tumorigène précoce du traitement susceptible d'entraîner la cancérisation des adénomes (PTU 3 mois+^{131}I) et on remarquera que les caractéristiques histophysiologiques des thyroïdes ne sont pas en faveur de la présence de TSH en excès lors de l'apparition des néoplasmes.

Le dénombrement des mitoses a été effectué après blocage colchicinique.

demment à apprécier la fréquence des mitoses.

Dans ce travail, nous avons limité nos investigations au cas des néoplasmes γ à cellules claires, tumeurs les plus fréquemment obtenues dans nos conditions expérimentales.

RESULTATS

Origine histologique des adénomes à cellules claires

Nous ne croyons pas que ces néoplasmes proviennent de la prolifération de cellules claires préexistantes que l'on trouve dans la thyroïde normale du rat, sans que, pour autant, on puisse éliminer totalement une telle filiation.

Les images observées nous donnent plutôt à penser que les adénomes dérivent en fait de la transformation d'une partie plus ou moins étendue de la paroi vésiculaire dont les cellules prolifèrent en prenant l'aspect clair.

Les divers aspects de cette évolution se voient bien chez les rats traités par le PTU et le radioiode. Dans certains cas, l'adénome procède de la transformation totale d'une microvésicule pratiquement dépourvue de colloïde, fréquente chez les sujets ainsi traités, et dont les cellules constitutives donnent d'emblée un microadénome.

Le plus souvent, le néoplasme résulte de la transformation d'un groupe de cellules vésiculaires qui prennent simultanément l'aspect caractéristique tout en proliférant. La tumeur reste alors solidaire de la vésicule sous une capsule conjonctive commune, image distincte de celle que donne l'inclusion d'une vésicule déformée à petits noyaux et que l'on trouve assez fréquemment dans ce type d'adénome. Plusieurs vésicules voisines peuvent présenter simultanément cette transformation.

Les adénomes à cellules claires dans les traitements cancérigènes ou non

Nos expériences nous ont montré que le traitement qui s'est révélé cancérigène dans nos expériences antérieures (PTU 3 mois + ^{131}I) se distingue des autres par le fait qu'il amène une production relativement précoce et générale d'adénomes γ dont l'activité mitotique est supérieure à celle des vésicules thyroïdiennes ambiantes. Chez les sujets plus âgés, ces adénomes présentent un volume accru, et leur activité mitotique est nettement plus forte qu'elle ne l'est initialement. Nous n'avons pas poursuivi nos observations jusqu'au moment où ces adénomes présentent une transformation épithéliomateuse avec le maximum de fréquence, mais nous avons cependant observé un tel néoplasme qui présentait des signes de transformation maligne à l'exception de l'envahissement vasculaire.

Le traitement mixte non cancérigène (PTU 1 mois + ^{131}I) donne lui aussi naissance à des adénomes γ qui sont alors d'apparition moins précoce et de fréquence plus réduite que dans le cas précédent. A égalité de taille, ils présentent une activité mitotique comparable à celle des adénomes induits par le traitement cancérigène. Du fait de leur apparition plus tardive, leur taille est généralement plus faible que celle qui est le propre des adénomes observés chez les sujets du même âge, soumis au traitement cancérigène.

Chez les rats soumis au traitement par le PTU seul, les adénomes apparaissent tard et plus sporadiquement. Ils sont de très petite taille et ne manifestent pas d'activité mitotique appréciable, comme c'est généralement le cas lorsqu'on est en présence d'adénomes γ aussi réduits.

Enfin, nous n'avons vu apparaître aucun adénome chez les rats traités par le radioiode seul, alors que nous en avions eu quelques-uns dans nos expériences antérieures.

En somme, une des caractéristiques fondamentales qui sépare le traitement cancérigène de ceux qui ne le sont pas nous paraît représentée par la précocité d'apparition des néoplasmes qui aboutissent à de volumineuses tumeurs richement mitotiques chez les sujets les plus âgés, par opposition aux traitements non cancérigènes qui donnent des adénomes plus tardivement. De ce fait, et à égalité d'âge, ces tumeurs sont nettement plus petites et moins mitotiques qu'elles ne le sont chez des sujets soumis au traitement cancérigène.

Le structure thyroïdienne lors de la genèse des adénomes à cellules claires.

Les adénomes à cellules claires apparaissent et évoluent dans des thyroïdes dont les caractéristiques histophysiologiques ne sont pas en faveur de la présence de TSH en excès, à ce moment.

En effet, après l'arrêt des traitements tumorigènes, la thyroïde, jusqu'alors hypertrophiée et hyperplasiée, retourne vers sa structure habituelle. Elle prend ensuite un aspect de repos manifeste qui se traduit par une hauteur d'épithélium vésiculaire inférieure à la normale et par une proportion de colloïde anormalement élevée. Si le poids des thyroïdes reste supérieur à la normale, malgré l'arrêt du traitement, le fait est imputable à l'hypertrophie résiduelle qui persiste toujours comme séquelle après l'interruption d'une stimulation thyroïdienne intense et prolongée par l'hormone thyréo-

trope. C'est au moment où la thyroïde présente, depuis plusieurs mois, ces signes de repos fonctionnel que se développent les premiers adénomes à cellules claires (Tableau 1).

L'aspect morphologique que présente la thyroïde n'est donc pas en faveur d'un excès de TSH comme cause directe de cette tumorigénèse. Cependant, sur ce critère, on ne peut pas exclure la possibilité que le traitement subi rende le tissu vésiculaire peu sensible à la TSH, alors que les cellules claires conserveraient une sensibilité normale à cette hormone, ce qui expliquerait les images observées.

Pour cette raison, il nous a paru indispensable de tester la sensibilité du parenchyme thyroïdien et celle des adénomes γ en administrant directement la TSH à des rats porteurs de telles tumeurs.

Sensibilité à la TSH des adénomes à cellules claires

Les expériences que nous avons conduites en traitant ou non par la TSH des Rats porteurs d'adénomes γ, permettent de répondre à l'objection précédente, et mènent aux conclusions suivantes.

Chez les rats non traités par l'hormone thyréotrope, le taux des mitoses, dans les adénomes à cellules claires, oscille dans d'assez larges limites. En effet, les adénomes les plus petits, de taille vésiculaire, sont pauvres en mitoses, alors que les néoplasmes les plus gros sont les plus riches en divisions cellulaires. En revanche, l'index mitotique de l'épithélium vésiculaire entourant les tumeurs reste très faible, comme c'est le cas dans la thyroïde, ainsi qu'on le sait classiquement.

Chez les rats soumis au traitement par la TSH, le taux des mitoses dans les adénomes oscille dans les mêmes limites que chez les sujets non traités, à condition de comparer des tumeurs de même volume. Par contre, les cellules des vésicules thyroïdiennes ambiantes présentent une augmentation marquée de la fréquence mitotique, phénomène qui traduit la sensibilité bien connue de ces cellules à cette hormone. Les faits observés montrent donc que les tumeurs à cellules claires sont pratiquement insensibles à l'action de la TSH qui conserve cependant son influence sur les cellules vésiculaires ambiantes (Tableau 2).

Les images morphologiques présentées par la thyroïde lors de la formation des adénomes à cellules claires et le fait que ces néoplasmes ne répondent pas à l'action de la TSH montrent que cette hormone hypophysaire n'est pas la cause directe de la prolifération des cellules claires qui les constituent, bien que ces néoplasmes apparaissent dans des thyroïdes stimulées d'une façon intensive par cette hormone, longtemps avant leur apparition.

DISCUSSION DES RESULTATS ET CONCLUSIONS

Les résultats que nous avons apportés et que nous allons discuter concernent en premier lieu l'origine histologique des tumeurs à cellules claires; en second lieu, les différences

Tableau 2.

	Sans TSH		Avec TSH	
Rats	Adénome	Vésicule	Adénome	Vésicule
No.	1	0,03	0	0,6
	7	0,05	2	0,6
			5	18
			10	60
PTU et	2,5	0,3	0	1
PTU+^{131}I	5	0	1	2
	8	0.08	6	11
	8	0,3	36	38
	12,5	0,1		
	13	0,08		
	14	0,1		
	17	0,2		
	17	0,17		
	18	0,2		
	25	0,1		

Ce tableau donne la fréquence des mitoses pour 1.000 noyaux après blocage colchicinique, tant dans les adénomes à cellules claires que dans le parenchyme vésiculaire de la thyroïde de rats âges de 20 à 30 mois, traités ou non par la TSH. Certains de ces sujets n'ont subi aucun traitement préalable (No) et leurs tumeurs sont donc spontanées. Les autres ont absorbé entre 6 et 9 mois d'âge, du propylthiouracil associé ou non à 30 μc de radioiode (PTU et PTU+^{131}I).

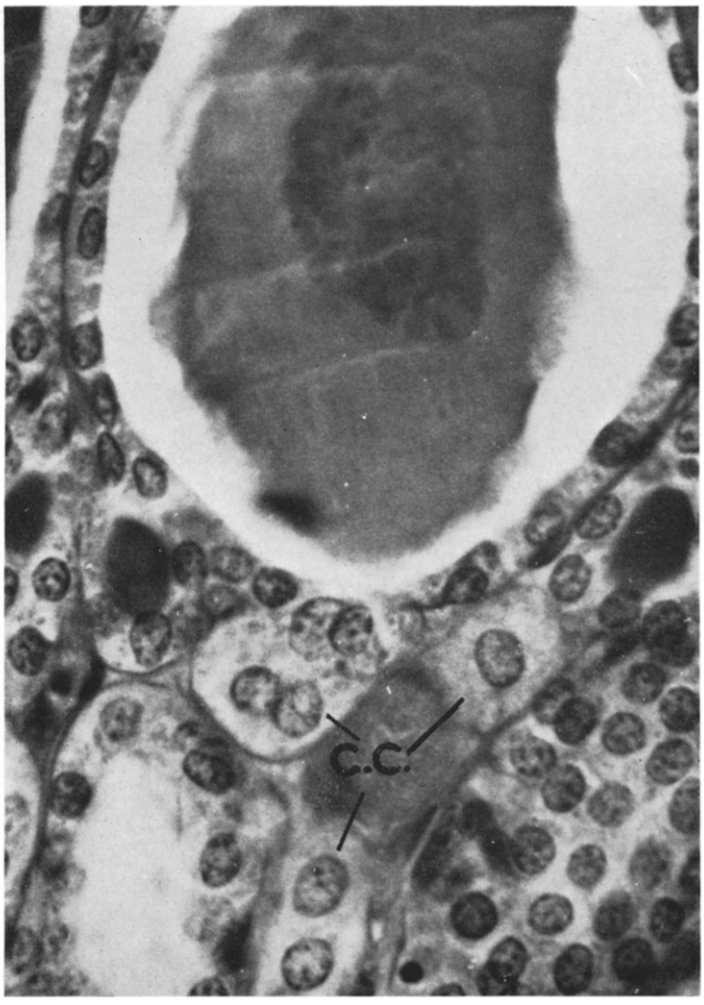

Fig. 1. *Rat de* 20 *mois* (*PTU* 3 *mois*+ ^{131}I)
Groupe de cellules claires s'isolant de la paroi vésiculaire. Elles sont aisément reconnaissables à leur noyau volumineux et à leur cytoplasme transparent.
La thyroïde de ce sujet présente par ailleurs toutes les transitions entre cette prolifération localisée de cellules claires et les adénomes eux-mêmes (*cf. Fig.* 2).

(to face p. 546)

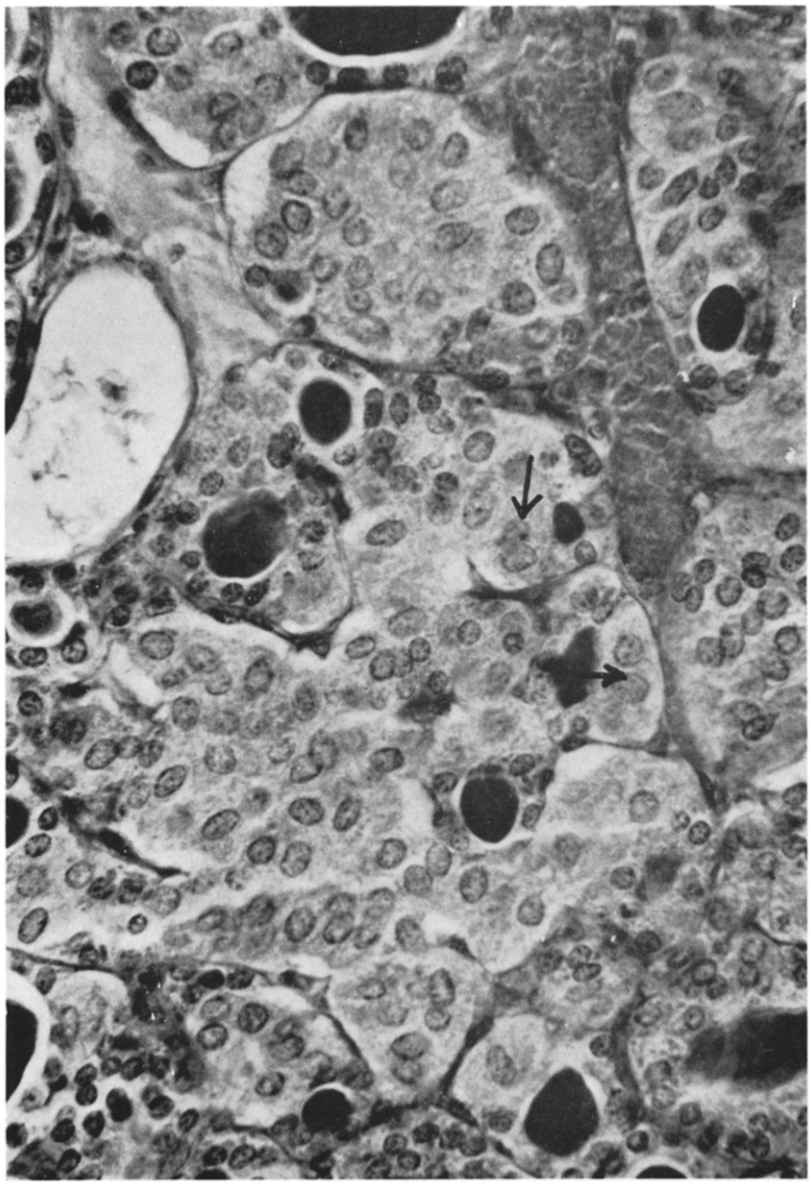

Fig. 2. *Même rat que pour la Fig.* 1.
Amas pluricentriques de petits adénomes γ à cellules claires. Dans certains d'entre eux, on voit la paroi vésiculaire se poursuivre par le zone adénomateuse, dont les cellules claires n'élaborent plus de colloïde.

d'apparition de ces tumeurs suivant que le traitement est cancérigène ou non, et enfin, en troisième lieu, le rôle de la TSH dans leur genèse.

Origine histologique des adénomes à cellules claires

On sait que la nature des cellules claires appelées initialement cellules parafolliculaires par Nonidez [6], et qui existent en petite quantité dans la thyroïde de tous les mammifères, fait l'objet de controverses. On trouvera un exposé succinct des différentes interprétations proposées et la bibliographie correspondante, en particulier dans les articles de Ponse [7] et de Thompson, Isler et Sarkar [3].

Certains auteurs considèrent ces éléments tantôt comme des phagocytes, tantôt comme des cellules parathyroïdiennes, tantôt comme des restes du canal thyréoglosse, tantôt comme des cellules vésiculaires en cours de mitose, tantôt comme des éléments issus du corps ultimo-branchial. Les travaux récents sur l'origine cellulaire de la calcitonine adoptent généralement cette dernière interprétation [8].

Selon Sarkar et Isler [9], qui ont étudié le comportement de ces cellules chez le rat, "Possibly light cells are follicular cells which have lost their contact with the lumen of the follicle . . .". Par ailleurs, elles présentent la même teneur en ADN que les cellules vésiculaires, ce qui exclut l'éventualité d'un aspect préparatoire à la mitose [10].

Les observations que nous avons faites montrent que les adénomes dérivent directement des cellules de la paroi d'une ou plusieurs vésicules qui prolifèrent sur une étendue plus ou moins grande, prenant l'aspect caractéristique des cellules claires à gros noyaux et dont le cytoplasme est dépourvu de tout grain de sécrétion MacManus positif. Les adénomes ainsi obtenus possèdent une vitrée commune à la tumeur et à la vésicule qui lui a donné naissance, signe même de leur communauté d'origine.

Bien que nous ne puissions pas exclure totalement l'éventualité de la naissance d'un adénome à partir de cellules claires isolées et préexistantes, nous n'avons pas observé d'image en faveur d'un tel processus.

L'ensemble de nos observations va donc dans la même sens que celles de Sarkar et Isler [9], et on peut en conclure que les cellules claires de la thyroïde normale du Rat ainsi que les cellules claires des adénomes γ sont des éléments épithéliaux dérivés des cellules vésiculaires. Par voie de conséquence, les épithéliomas qui résultent de la transformation de ces adénomes présentent la même origine.

Les cellules claires représentent donc une différenciation morphologique et physiologique des cellules vésiculaires puisqu'elles acquièrent une sensibilité aux stimuli hormonaux, différente de celle que présentent les cellules vésiculaires, ainsi que nous le verrons plus loin.

Apparition des adénomes à cellules claires dans les traitements cancérigènes ou non

Le traitement, qui s'est montré cancérigène dans nos expériences antérieures, et que nous avons repris ici (PTU 3 mois+^{131}I), se traduit en particulier par le fait qu'il entraîne une production précoce d'adénomes γ chez presque tous les rats, tumeurs qui apparaissent plus tard et plus sporadiquement chez les animaux soumis aux traitements non cancérigènes.

A égalité d'âge, les sujets traités par le régime cancérigène présentent alors des adénomes plus volumineux que ceux qui s'observent chez les autres sujets. Par ailleurs, le taux des mitoses allant en augmentant avec el volume des néoplasmes, les rats soumis au traitement cancérigène présentent de ce fait des tumeurs dont le taux mitotique est plus élevé que celui qui existe dans les adénomes de sujets de même âge, non soumis à ce type de traitement.

Nous pensons qu'il y a là un des facteurs importants qui séparent le traitement cancérigène de ceux qui ne le sont pas, même si ces derniers sont générateurs d'adénomes à cellules claires. En effet, et compte tenu de la durée de vie du rat, l'apparition tardive des adénomes dans le traitement non cancérigène empêche ces néoplasmes d'atteindre le temps de latence qu'exige la cancérisation et qui est peut-être lié à l'acquisition d'un certain taux d'hyperplasie adénomateuse que seuls les adénomes obtenus par le traitement cancérigène sont susceptibles d'atteindre du fait de leur apparition précoce.

Le rôle de la TSH dans l'apparition des adénomes à cellules claires

Selon l'opinion classique, la production des néoplasmes bénins ou malins de la thyroïde par les procédés classiques tels que les antithyroïdiens, le radioiode, la carence alimentaire en iode, la thyroïdectomie partielle, est imputable à la carence hormonale thyroïdienne que déclenchent ces procédés. La privation hormonale amène, par réaction, une production excessive de TSH qui entraîne l'hyperplasie de la thyroïde, puis la production des divers types de néoplasmes bénins ou malins. L'hormone

thyréotrope, ainsi sollicitée, deviendrait à la longue carcinogène, opinion que Purves et Griesbach [11] ont en particulier défendue.

En ce qui concerne les tumeurs à cellules claires, cette interprétation soulève des objections que nous avons déjà signalées dans l'introduction. On sait en effet que les adénomes à cellules claires se forment plus ou moins longtemps après suppression des régimes générateurs de la carence hormonale, qu'il s'agisse d'un régime carencé en iode [4] ou de l'emploi des antithyroïdiens [5] à un moment où la sécrétion excessive de TSH devrait avoir cessé.

En premier lieu, nos résultats apportent des preuves qu'un excès de TSH n'est pas la source directe de la genèse des adénomes à cellules claires (Tableau 1). L'examen histophysiologique de la fonction thyroïdienne montre que ces néoplasmes apparaissent à un moment ou la glande présente, depuis plusieurs mois, un aspect de repos, signé par l'augmentation du taux de colloïde et l'existence d'un épithélium vésiculaire moins haut qu'à l'état normal, aspect qui s'oppose à l'hypothèse d'un excès de TSH.

Goldberg et Chaikoff [12] avaient déjà enregistré une constation analogue mais l'importance des doses de radioiode utilisées dans leurs expériences (400 μc) ne permet pas d'assimiler les deux conclusions, l'état de repos thyroïdien observé dans leaur travail étant peut-être lié à l'irradiation thyroïdienne intense.

En second lieu, les données apportées ici permettent également d'exclure une autre hypothèse selon laquelle les adénomes résulteraient d'une sensibilité exagérée de leurs cellules à la TSH présente sous un taux normal dans l'organisme.

En effet, l'injection de fortes doses de TSH, en un temps suffisamment court pour ne pas créer d'anticorps antihormonaux, n'amène aucune hausse du taux des mitoses dans les adénomes des sujets ainsi traités. Par contre, l'activité mitotique augmente notablement dans les cellules vésiculaires de ces sujets, observations qui montrent la conservation de la sensibilité de ces éléments à l'hormone hypophysaire et l'insensibilité des cellules claires adénomateuses à l'action mitotique de cette même hormone.

L'ensemble de ces arguments montre de maniére évidente que les adénomes γ à cellules claires ne prennent directement leur source ni dans une sécrétion excessive de TSH, ni dans une sensibilité anormale des cellules claires qui les constituent à l'hormone thyréotrope.

On voit ainsi que les adénomes à cellules claires et les épithéliomas qui en dérivent, apparaissent dans les suites lointaines d'une stimulation initiale de la thyroïde par la TSH élaborée en excès sous l'influence de traitements qui inhibent la production des iodothyronines bien qu'au moment où les néoplasmes apparaissent l'excès de TSH ait pratiquement cessé depuis longtemps. Cette stimulation initiale des cellules vésiculaires par la TSH a pour conséquence ultérieure leur transformation en cellules claires néoplasiques, privées de réactivité à la TSH. Elles obéissent alors à un déterminisme hormonal nouveau et présentent probablement des propriétés sécrétoires différentes de celles que possèdent les cellules vésiculaires dont elles procèdent.

Les recherches effectuées sur les cellules claires de la thyroïde normale du rat donnent des indications sur les facteurs endocriniens susceptibles d'entrer en jeu dans le contrôle de ces adénomes.

En effet, les cellules claires de la thyroïde normale se multiplient sous l'influence de l'hormone somatotrope et, selon Sarkar et Isler [9], l'hypophyse contiendrait une substance inhibitrice à l'égard de leur multiplication, ce qui expliquerait l'augmentation de leur nombre après hypophysectomie [9, 13].

Peut-être s'agit-il de la TSH puisque l'administration de thyroxine augmente leur nombre, tandis que l'injection de thyréostimuline aux rats hypophysectomisés diminue leur nombre, propriété qui ne paraît pas se manifester dans le cas des adénomes à cellules claires une fois constitués.

Ce déterminisme endocrinien, particulier aux cellules claires normales, s'accompagne de propriétés sécrétoires autonomes dont on peut se demander si elles s'étendent aux adénomes.

On sait en effet que ces cellules contiennent des granulations qui répondraient à la calcitonine, substance hypocalcémiante d'origine thyroïdienne [8, 14–18].

Elles présentent également une activité cholinestérasique [19] et selon Larson, Owman et Sundler [20], interviendraient aussie dans le mécanisme régulateur de la production de diverses amines biogènes.

Considérations sur les tumeurs à cellules claires de l'Homme

Les considérations précédentes montrent que les tumeurs à cellules claires ainsi que les cellules claires normales représentent des entités morphologiquement et physiologiquement différentes des éléments proprement vésiculaires

responsables de l'élaboration de la thyroglobuline et des iodothyronines.

Dans le cas des tumeurs thyroïdiennes de l'homme, il existe encore des ambiguïtés en ce qui concerne l'appartenance de certaines d'entre elles à la catégorie des néoplasmes à cellules dites claires ou parafolliculaires. En effet, les histopathologistes considèrent parfois que les néoplasmes à cellules de Hürthle relèvent de cette rubrique. D'autres auteurs estiment que les épithéliomas médullaires à stroma amyloïde, qui contiennent de la calcitonine, font également partie de ce type de tumeurs.

Depuis plusieurs années, on administre parfois des hormones thyroïdiennes aux malades atteints d'épithéliomas thyroïdiens dans le but d'arrêter la sécrétion de TSH hypophysaire et, par cette voie, de ralentir l'évolution du néoplasme en se fondant sur son hormono-dépendance éventuelle [21].

Si cette méthode a des bases rationnelles en ce qui concerne les épithéliomas thyroïdiens dérivés des cellules vésiculaires élaboratrices de thyroglobuline, il n'en est apparemment plus de même en ce qui concerne les tumeurs malignes à cellules claires puisque ces dernières, qui ne sécrètent pas de thyroglobuline, se révèlent insensibles à la TSH chez le rat et qu'il n'y a pas de raisons actuelles de penser que le conditionnement hormonal de ces cancers est différent chez l'homme.

Dans la mesure où les tumeurs citées plus haut sont constituées de cellules claires, on peut même craindre que l'administration d'hormones thyroïdiennes, loin d'avoir un effet bénéfique, ait au contraire un effet néfaste puisque l'on sait que la thyroxine exerce une action stimulante sur les cellules parafolliculaires de la thyroïde normale du Rat [9, 13].

RESUME

1. *Les auteurs étudient le comportement des adénomes thyroïdiens à cellules claires, déclenchés par un traitement associant le propylthiouracil et le radio-iode, et qui, selon les modalités de ce traitement, évoluent ou non en tumeurs malignes.*
2. *Les adénomes ainsi obtenus procèdent de la transformation de cellules vésiculaires en cellules claires dont la prolifération aboutit à la tumeur. Parmi les divers traitements générateurs d'adénomes, le traitement qui aboutit à la cancérisation se caractérise par le déclenchement précoce des adénomes qui atteignent alors une grande taille chez les sujets âgés. Par contre, les traitements non cancérigènes donnent des adénomes plus tardivement.*
3. *Quel que soit le type de traitement, l'activité mitotique des tumeurs ainsi obtenues va en croissant avec leur dimension. L'administration de TSH n'amène pas de modification significative du taux des mitoses dans les adénomes à cellules claires, contrairement à ce qui se passe au niveau des cellules vésiculaires où ce taux est fortement augmenté. Ces tumeurs ne résultent donc directement ni d'une sécrétion excessive de TSH, ni d'une sensibilité anormale des cellules claires à cette hormone.*
4. *Les faits expérimentaux donnent donc à penser que les tumeurs thyroïdiennes à cellules claires représentent une entité distincte des autres types de tumeurs thyroïdiennes.*

SUMMARY

1. *The authors study the behaviour of light cell thyroid adenomata induced by an association of propylthiouracil and radio-iodine, and which, depending on the method used for this treatment, either develop cancerous tumours or do not.*
2. *The adenomata obtained in this way are the result of the transformation of vesicular cells into light cells, the proliferation of which leads to the tumour. Among the various treatments of adenomata, the one resulting in cancerization is characterized by the early production of adenomata reaching large size in old subjects. On the other hand, non cancer-inducing treatments produce adenomata at a later stage.*
3. *Whatever type of treatment is used, the mitotic activity of the tumours so obtained increases in proportion to their size. Treatment with TSH does not bring about any modification in the rate of the mitoses in light cell adenomata, in contrast to what happens at the level of vesicular cells where this rate is significantly increased. These tumours do not result from an excessive secretion of TSH, nor from an abnormal sensitivity of the light cells to this hormone,*
4. *Experimental facts would therefore tend to show that light cell thyroid tumours are a distinct entity as compared to other type of thyroid tumours.*

ZUSAMMENFASSUNG

1. *Die Autoren untersuchten das Verhalten von 'hellen Zellen' bei Schilddrüsenadenomen, die durch eine Kombination von Propylthiouracil und Radioiod ausgelöst wurden und die sich, je nach Behandlungsart, in bösartige Geschwülte verwandeln können.*
2. *Die so erhaltenen Adenome enstanden durch Unwandlung der vesikulären Zellen in 'helle Zellen' deren Wucherung zum Tumor führt.*

Die krebserzeugende Adenombehandlung ist charakterisiert durch eine frühzeitige Entwicklung von Adenomen, welche bei alten Tieren eine beträchtliche Größe erreichen. Nach Behandlungen, die nicht krebserzeugend sind, erscheinen dagegen die Adenome später.
3. *Welche Behandlung auch angewendet wird, die mitotische Tätigkeit der so erhaltenen Geschwülste nimmt mit der Größe der Tumoren zu. TSH-Zufuhr bringt keine wesentliche Veränderung der Mitoserate bei den 'Hellzellenadenomen, im Gegensatz zu den vesikulären Zellen, bei welchen der Prozentsatz erhöht ist. Diese Tumoren entstehen also nicht unmittelbar durch eine übermäßige TSH-Sekretion oder einer abnormen Empfindlichkeit der hellen Zellen gegen dieses Hormonen.*
4. *Die experimentellen Ergebnisse zeigen, daß die 'hellen Zellen' der Schilddrüsentumoren wahrscheinlich einen speziellen Typ darstellen, der sich von dem anderer Schilddrüsentumoren unterscheidet.*

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The Role of the Carrying Molecule in the Cross-Resistance against Sugar Alcohol Derivatives

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INTRODUCTION

EXPERIMENTAL tumours rendered resistant to one of the alkylating compounds, e.g., mustards, ethyleneimines or alkylsulphonic esters, have been often found to be cross-resistant to all other types of alkylating agents [1, 2]. Detection of elevated levels of non-protein sulphhydryl groups in some resistant tumour tissues suggested that a deactivation of the alkylating agent by interaction with sulphhydryl groups may be involved in the mechanism of the cross-resistance [3–5].

On the other hand, in our previous investigations NK lymphomas and Yoshida sarcomas rendered resistant to the sugar alcohol derivative mannitol mustard (Degranol) or dibromodulcitol showed resistance to all the hexitol derivatives: mannitol mustard, dibromodulcitol, dibromomannitol and mannitolmyleran, but remained sensitive to some other types of alkylating agents, namely to TEM, trenimon and Myleran [6, 7]. It seemed to be possible that in the case of alkylating sugar alcohol derivatives resistance might be developed against both the carrying molecule itself and the alkylating function of the agent.

In order to approach this problem the possible role of the length of the carbon chain was studied by means of two dibromotetritols, 1,4-dibromo-1,4-dideoxy-DL-threitol and 1,4-dibromo-1,4-dideoxy-meso-erythritol containing a four-carbon chain like Myleran. The role of the hydroxyl groups of the carrying molecule was studied on the 1,6-dibromohexane, the hydroxyl-free analogue of dibromohexitols (Fig. 1).

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MATERIAL AND METHODS

Mannitol mustard (Degranol), dibromodulcitol and dibromomannitol (Myelobromol) and mannitolmyleran (Mannogranol) were kindly supplied by L. Institóris from Pharmaceutical Factory, Chinoin; dibromothreitol and dibromo-*meso*-erythritol by Dr. T. Horváth from Pharmaceutical Research Institute. Dibromohexane and Myleran were commercial reagents.

Four sublines of Yoshida ascitic tumours, resistant to mannitol mustard (Degranol) (Y-DR), to dibromodulcitol (Y-DBDR), dibromohexane (Y-DBHR) and dibromothreitol (Y-DBTR), were developed by a previously reported procedure [8].

The treatment started with the administration of insufficient doses and was continued with gradually increasing doses during several passages. As a result of this treatment we observed a prolongation of the survival time of the animals and a decrease of the total number of tumour cells. After the 20–25th passages, however, in spite of high range therapeutic doses the total number of tumour cells and the survival time proved to be the same as in the case of the untreated animals, showing that a resistance manifested itself to the compound.

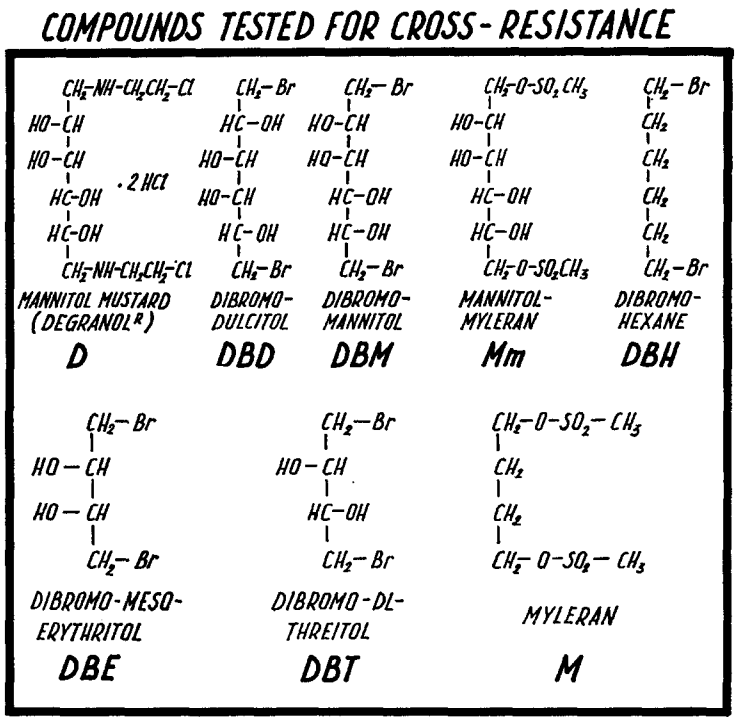

FIG. 1.

Daily doses of the compounds were administered i.p. between the following limits:

Mannitol mustard	1–10 mg/kg
Dibromo-dulcitol	10–200 mg/kg
Dibromohexane	5–50 mg/kg
Dibromo-DL-threitol	8–50 mg/kg

A dose of 5×10^6 cells derived from the resistant ascites tumours was transplanted in the left dorsal region of Wistar rats weighing 150 g. Cells derived from the sensitive ascites tumour line were inoculated on the right side.

One day following transplantation daily treatment of the animals with a multiple of the therapeutic dose of the compounds was initiated. Drugs were administered intraperitoneally as aqueous solutions or suspensions with Tween 80.

On the 8th day following transplantation the bilaterally growing tumours were extirpated and weighed. The tumour inhibitory effect of each compound was tested on thirty animals.

RESULTS

Comparison of the growth inhibitory effect of the compounds on bilaterally growing sensitive and resistant Yoshida tumours is presented on Table 1. The tumours of sensitive Yoshida ascites tumour line were particularly responsive to DBD, DBT and Degranol. DBM, MM and Myleran also exerted an inhibitory effect of about 50%. In experiments reported by Institóris *et al.* [9] DBH (6×15 mg/kg) did not exert any antineoplastic effect on Yoshida tumours, and the chronic treatment with DBE (6×20 mg/kg) induced a tumour growth inhibition of only 39%. Increasing the dose of DBH to 5×50 mg/kg we could observe a slight tumour growth inhibition, whereas in the case of DBE an augmentation of the dose to 5×50 mg/kg did not lead to an increase of the inhibitory effect. DBH and DBE which exert a slight effect on the growth of sensitive Yoshida tumours did not show any effect on the corresponding resistant lines.

Table 2 presents the ratio of the wet weight of bilaterally growing sensitive and resistant tumours treated with the compounds tested. The symbol '++' indicates the cases where the resistant tumours weighed more than twice the sensitive ones, and '+' where only half as much again of the sensitive ones. The symbol '—' presents cases where the drugs inhibited the growth of the resistant tumours to the same degree as that of sensitive tumours.

The Yoshida tumour lines Y-DBDR and Y-DR were found to be cross-resistant to Degranol, DBD, DBM and MM but remained sensitive to DBE, DBT and to Myleran. On the other hand, the tumour line (Y-DBTR) retained full sensitivity to DBD, DBM, Degranol or MM, and proved to be cross-resistant to Myleran and DBE.

An incomplete cross-resistance, marked by '+' on Table 2, was found between DBH and the hexitol derivatives. The R/S ratios leading

Table 1. *Comparison of the growth-inhibitory effect of the compounds on bilaterally growing Yoshida tumours sensitive and resistant*

Compound (mg/kg)	Sensitive* tumours	Y-DBDR†	DY-DR† wet weight in g	Y-DBHR‡	Y-DBTR†
Without treatment	11·9±0·23	12·6±0·81	12·3±0·35	12·5±0·13	12·2±0·81
DBD 5×200	2·3±0·13	10·9±0·23	10·2±0·11	8·1 ±0·27	2·1±1·12
DBH 5×200	5·6±0·59	12·1±0·41	12·2±0·07	8·52±0·23	5·7±1·04
DBM 5×50	6·8±0·39	9·8±1·02	9·7±0·72	11·9 ±1·03	5·9±1·01
D 5×10	4·3±0·37	11·4±0·12	10·7±1·13	7·9 ±0·61	4·1±0·07
Mm 5×300	5·7±0·79	12·2±0·92	11·6±1·03	10·62±0·22	5·6±0·83
DBT 5×50	3·8±0·13	3·1±0·24	3·9±0·218	4·1 ±0·3	12·9±0·08
DBE 5×50	7·1±0·72	5·9±0·83	7·1±0·19	6·9 ±0·71	13·9±0·042
M 5×10	5·8±0·56	6·3±0·41	6·1±0·31	6·2 ±0·01	12·2±0·03

*Average value from 80 tumours.
†Average value from 20 tumours.
‡Average value from 40 tumours.

Standard deviation $\varepsilon x - \frac{(\varepsilon x)^2}{N} = ss$

$$s^2 = \frac{ss}{N-1} \quad s\bar{x} = \frac{s^2}{N} \times 2{\cdot}26$$

where x=individual value, εx=sum of individual values, N=number of data, s^2=square of deviations, $s\bar{x}$ = mean deviation.

to '+' call for some explanation. Comparing the R/S ratios with the wet weight data of Table 1, it is evident that DBH treatment was without any effect on the DBH-resistant tumour and the ratio 1,7 is a result of the slight effect of the compound on the sensitive tumour. On the other hand, in the cases of DBM, Degranol and MM treatment of the DBH resistant tumour and in the case of DBH-treatment of DBDR or Degranol-resistant tumours the ratios 1,4–1,8 are the result of a moderate inhibitory effect of these compounds on the resistant tumours.

The DBH-resistant tumour showed no cross-resistance to Myleran and to dibromotetritols.

DISCUSSION

Our results point to the decisive role of the carrying molecule in the mechanism of resistance to alkylating sugar alcohol derivatives. Tumours rendered resistant to a compound containing a six-carbon chain showed cross-resistance to other related six-carbon compounds carrying various functional groups.

The tumours with acquired resistance to dibromo-DL-threitol proved to be cross-resistant

Table 2. *Cross-resistance between cytostatic sugar alcohol derivatives*

Compounds	Y-DBDR R/S	Y-DR R/S	Y-DBHR R/S	Y-DBTR R/S
DBD	++ (4·7)	++ (4·3)	++ (3·5)	−− (0·9)
DBM	++ 2·1	++ 2·1	+ 1·5	−− 1·0
DBH	+ 1·4	+ 1·4	+ 1·7	−− 0·8
D	++ 2·6	++ 2·2	+ 1·8	−− 0·9
Mm	++ 2·0	++ 2·0	+ 1·6	−− 0·9
DBT	−− 0·8	−− 1·0	−− 1·0	++ 3·3
DBE	−− 0·8	−− 0·9	−− 0·9	++ 1·9
M	−− 1·0	−− 1·0	−− 1·0	++ 2·1

R/S=ratio of the wet weight of the bilaterally growing resistant and sensitive tumours.

to the other compounds of the four-carbon series. No cross-resistance could be demonstrated between the six-carbon and four-carbon series.

Inactivation of the agent by removal of the functional groups may be generally involved in the resistance to alkylating agents. Our results suggest that the removal of the functional groups of the four-carbon compounds may occur on another pathway than those in the case of six-carbon compounds.

The problem of cross-resitance to sugar alcohol derivatives becomes complicated by the fact that these compounds are present in biological systems both in cyclized and in intact forms.

Formation of epoxides from mannitolmyleran, dibromomannitol and dibromothreitol *in vitro*, was reported by Davis and Ross [10]. The 1,2-5,6-diepoxides of mannitol and dulcitol were isolated by Jarman and Ross [11]. In animals treated with mannitolmyleran or DBM Elson and co-workers demonstrated a complex blood response pattern showing both Myleran-like and epoxide-like features. These complex response patterns could be reproduced by treatment with a combination of Myleran and 1,2-5,6-dianhydro-D-mannitol. Myleran-like effects seems to be derived from the intact form of mannitolmyleran and DBM [12].

In the course of our recent investigations the presence of 1,2-5,6-dianhydrohexitol and other alkylating metabolites could be detected in the serum and ascitic fluid of rats treated with DBD, DBM or mannitolmyleran [13].

It is possible that resistance has to develop both to the cyclized and to the intact forms of the drugs.

One of the possible mechanisms of resistance to the intact form of the halogen-containing derivatives is the hydrolytic cleavage of the C–halogen bonds. In our previous experiments the C–halogen bond splitting capacity of sensitive and resistant (Y-DBDR and Y-DR) tumours was investigated. Using DBM as substrate, the extent of degradation of C–Br bond was measured by the bromide formation. Tumours resistant to Degranol and DBD showed a larger capacity to split the C–Br bond of DBM than the sensitive ones [14]. These tests were carried out in Krebs–Ringer phosphate buffer where no conversion of DBM to diepoxide occurs.

In the case of the hydroxyl-free compound dibromohexane no epoxide formation can occur. The incomplete cross-resistance of the Y-DBHR tumour line to the hexitol derivatives may be due to an inactivating mechanism which can attack the C–halogen bound but inactive against the cyclized forms of these agents.

A particular mechanism for inactivation of four-carbon compounds may be given by the cycloalkylation demonstrated by Roberts and Warwick [15]. In the case of the 1,4-dimesyloxy-butane (Myleran) both ends of the four-carbon chain of the compound react with the same sulphydryl group forming a cyclic compound. A similar mechanism may be supposed in the case of the four-carbon containing sugar alcohol derivatives DBT, DBE and their epoxide derivatives.

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RESUME

Les auteurs étudient les rapports entre la structure chimique et le mécanisme de résistance croisée envers des dérivés cytostatiques de polyalcools. Le rôle de la molécule porteuse a été étudié sur les tumeurs de Yoshida rendues résistantes aux dérivés hexitol, Dégranol et dibromodulcitol, au dérivé tétritol, dibromothréitol, ainsi qu'au dibromohexane.

L'effet antitumoral des divers dérivés de polyalcools a été examiné sur les tumeurs dérivées de la lignée sensible, ainsi que des lignées résistantes du sarcome de Yoshida, et inoculées bilatéralement chez l'animal.

Les tumeurs résistant au Dégranol ou au dibromodulcitol ont manifesté une résistance croisée aux autres dérivés hexitol. Les tumeurs résistant au dibromothréitol ont montré une résistance croisée envers le dibromoérythritol et le Myleran. Une résistance croisée incomplète a été trouvée entre le dibromohexane et les dérivés hexitol. On ne peut démontrer aucune résistance croisée entres les composés à six et à quatre atomes de carbone.

SUMMARY

The role of the carrying molecule in the mechanism of cross-resistance against cytostatic sugar alcohol derivatives was studied on Yoshida ascitic tumours which were rendered resistant to the hexitol derivatives Degranol, dibromodulcitol, to the tetritol-compound

1,4-*dibromo*-1,4-*dideoxy*-DL-*threitol as well as to the hydroxyl-free analogue of dibromo-hexitols, dibromohexane. The growth inhibitory effects of various sugar alcohol derivatives were investigated on bilaterally growing solid tumours derived from the sensitive as well as from the resistant lines. The Degranol and DBD resistant tumours proved to be cross-resistant to other alkylating hexitol derivatives. The Yoshida tumours resistant to dibromo-threitol were cross-resistant to dibromo-erythritol and to Myleran. An imcomplete cross-resistance was found between dibromohexane and the hexitol derivatives.*

No cross-resistance could be detected between the hexitol and tetritol derivatives.

ZUSAMMENFASSUNG

Die Rolle des entscheidenden Moleküls in dem Resistenzmechanismus gegen alkylierende Zuckeralkoholabkömmlinge wurde gegen Degranol, Dibromodulcitol, Dibromothreitol und Dibromhexan an resistenten Yoshida Tumoren untersucht. Die antineoplastische Wirksamkeit der Substanzen wurde an bilateral gewachsenen sensitiven und resistenten Tumoren geprüft.

Die degranol- und dibromodulcitolresistenten Tumoren erwiesen sich mit anderen alkylierenden Hexitolverbindungen als kreuzresistent. Die dibromothreitolresistenten Tumoren waren kreuzresistent mit Dibromomeso-erythritol und Myleran. Eine unvollständige Kreuzresistenz wurde zwischen Dibromhexan und den Hexitolabkömmlingen gefunden. Zwischen den Hexitol- und Tetritolreihen konnte keine Kreuzresistenz nachgewiesen werden.

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Radioimmunoassay of Rat Prolactin

Comparison of Rat Prolactin Preparations Isolated from the 'Granular Fraction' of Pituitary Tumour Transplants and from Normal Pituitary Glands

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INTRODUCTION

THE ISOLATION of rat prolactin from the 'granular fraction' of transplants of pituitary tumours and some properties of a rabbit antiserum raised against RPT* have been described [1, 2] and a radioimmunoassay based on ^{125}I-labelled RPT has been reported briefly [3]. Parallel competitive inhibition curves indicated antigenic identity as far as radioimmunoassay is concerned between RPT, native prolactin present in crude homogenates of rat pituitary tissue and in rat plasma samples. The assay is sensitive enough to detect physiological fluctuations during the oestrous cycle [4] and to detect the release of prolactin into the circulation as a response to a vigorous suckling stimulus in lactating rats ([5] 1969).

However, since RPT has been isolated from 'storage granules' it may represent a prolactin precursor rather than prolactin itself (viz. pancreas storage granules). Moreover a degraded prolactin molecule may have resulted from either faulty replication or faulty transcription, or faulty reading of the code in prolactin synthesis by pituitary tumour tissue. Great discrepancies in biological activity estimates were reported when this preparation was tested in various *in vitro* or *in vivo* assay systems (e.g. 15·6 i.u./mg by mouse mammary gland gland culture assay compared to 2 to 2 i.u./mg by the local crop as well as by the intra-ductal rabbit mammary gland assay [5].

Recently the isolation of rat prolactin from whole, frozen normal pituitary glands was reported [6], followed by a report of an isolation procedure starting from 20 fresh rat pituitary glands ([7], 1968). The first group reported a yield of 1·1 mg/g of wet glands and a biological activity of 25 i.u./mg assayed by the local crop test in pigeons; the biological activity being 17 i.u./mg when assayed in the systemic pigeon crop method. More recent data (Dr. Ellis, personal communication) indicated in the systemic assay a potency of 10·9 i.u. (95% confidence limits 8·18–14·6) and in the local assay using 6-week-old pigeons 30 i.u./mg (95% confidence limits 16–53·4).

The present study compares the performance of RPT in radioimmunoassay with RPN*; whereby both preparations were labelled with ^{125}I, antisera raised against either of the two preparations were compared and both preparations were employed unlabelled as respective standards.

*RPT=Rat prolactin derived from tumours.

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*RPN=Rat prolactin derived from pooled frozen normal pituitary glands.

MATERIAL AND METHODS

Rat prolactin preparations: RPT, isolated as described earlier [2], except that only first passage tumour transplants were used as starting material. Biological activity was estimated to be ±10% (i.e. 2–3 i.u./mg) of that of the ovine reference preparation by the local crop test in pigeons, but estimated to be ± equipotent with the same reference preparation as judged by the lactogenic effect on mouse mammary gland tissue *in vitro* (both assays kindly performed by Dr. I. A. Forsyth at the National Institute for Research in Dairying, England). RPN, preparation H9-6B, kindly made available by Dr. S. Ellis (Ames Research Center, NASA, Moffett Field, U.S.). Biological activity 28 i.u./mg by the local crop test in pigeons.

Heterologous prolactin preparations: mouse prolactin, the same preparation employed as reference preparation in radio-immunoassays of mouse plasma and pituitary homogenate in earlier experiments [8] and bovine prolactin isolated as described earlier [9] were used for inhibition experiments. The bovine preparation had a biological activity of 14 i.u. (95% fiducial limits 11·9–16·4) estimated by the intra-ductal rabbit mammary gland assay (kindly performed by Dr. I. A. Forsyth) and has threonine as the sole NH_2-terminal amino acid (personal communication Dr. W. S. Bont).

Rabbit antisera: Antiserum no. 359 raised to RPT and antiserum no. 405 raised to RPT coupled to bovine γ-globulins in an effort to raise its antigenicity; immunization procedure as described earlier [2]. Antiserum V-20-B30 raised to RPN and made kindly available to us in lyophilized form by Dr. R. E. Grindeland.

Preparation and storage of rat prolactin solutions for radio-immunoassay: A small amount (0·2–0·4 mg) of rat prolactin is weighed on a Cahn (gram) Electrobalance to better than 1% accuracy and is dissolved in 0·125 M borate/HCl buffer, pH 7·65, to exactly 1 mg/ml. Six to twelve Microcaps (100 µl size, Drummond Scientific Company, Broomall, PA, U.S.) are calibrated and 0·025 ml rat prolactin solution is aspirated into each of the capillaries in such a way that the solution occupies the middle of the capillary; both ends of the capillaries are plugged with small amounts of wax and the capillaries are stored at −20°C. (personal communication Dr. F. C. Greenwood).

Iodination procedure: The chloramine-T method of Greenwood, Hunter and Glover [10] is slightly modified and the mixing of the reagents is carried out essentially as described by Reith and Brown [11] for tyrosine labelling. The modifications are: (1) the use of 0·125 M borate/HCl buffer, pH 7·65 to dissolve all reagents; (2) care is taken to avoid over-iodination of the rat prolactin preparations by standardizing to 25 µg of rat prolactin and 1 mC of ^{125}I when the specific activity of the IMS-3 (Amersham) preparation is stated to be 10 mC/µg of iodine or better. When the specific activity of the IMS-3 preparation is less than 10 mC/µg of iodine, proportionally less than 1 mC is used for labelling of the standardized amount of hormone.

To ensure rapid mixing of the reagents and to minimize oxidation damage of the hormone during the labelling procedure the following modifications were employed: (1) the free end of the Teflon tubing is heated in a hot stream of air; the end is drawn out and cut at the smallest diameter to obtain a fine nozzle; (2) instead of air, N_2 is used for spacing the small volumes of reagents in the Teflon tubing.

Calculations of iodination efficiency and of yield of labelled prolactin in peak fraction: essentially as described by Greenwood *et al.* [10]. However, since eluated radioactivity is measured in polystyrene tubes and the amount of Na ^{125}I employed for the reaction is calculated from measurements of radioactivity in the glass ampoule, a conversion factor has to be used, which takes care of the difference in geometry (radioactivity is concentrated in less than 0·1 ml in the ampoule tip, whereas it is evenly distributed over 10 drops volume in the polystyrene tubes) and in absorption of the gamma radiation by respectively the (varying) thick glass ampoule tip and the polystyrene tube wall.

Antiserum titration curves: six series of 7 polystyrene tubes covered the 6 possible combinations, i.e. (1) either labelled RPT or labelled RPN (both in 12 ng/ml working solution) and (2) seven dilutions of each of the 3 antisera (dilutions used were $3^0 \times 10^{-2}$ down to $3^{-6} \times 10^{-2}$). Incubation time 20–24 hr at 4°C.

Comparison of standard curves: eight series of 7 polystyrene tubes covered the 8 possible combinations of: (1) either unlabelled RPT or unlabelled RPN in 7 dilutions (from $3^0 \times 1200$ ng/ml down to $3^{-6} \times 1200$ ng/ml); (2) a standardized dilution of antiserum 405 (1:900) or of antiserum V-20-B30 (1:2700) and after 'preincubation' of 40 hr at 4°C; (3) either labelled RPT or labelled RPN (both in 12 ng/ml working solution) is added. The reaction mixtures are left for a further 24 hr at 4°C.

Collection of plasma samples: heparinized

capillary tubes (length 75 mm; i.d. 1·1–1·2 mm, Propper manufacturing Co., Long Island City, N.Y., U.S.) are broken into three parts of approximately equal length. A rat is brought under light ether anaesthesia. Light pressure applied by thumb and fore finger of the left hand on both sides behind the jaw bones causes the eye ball to protrude and increases the venous pressure. A piece of capillary is inserted with its sharp edge into the eye socket, laterally and caudally of the right eye ball. By rolling the capillary gently between thumb and fore finger of the right hand, the sharp edge cuts into the retrobulbar venous plexus and blood starts to drip from the capillary into a polystyrene tube held under the free part of the capillary. The capillary is withdrawn at the tenth drop. At the same time engorgement is stopped, the eye lid is closed and gently pressed in order to stop bleeding by pressing the eye ball on the wound in the retrobulbar venous plexus wall. The polystyrene tube is centrifuged for 10 min at 500 **g**; the plasma is pipetted off into another tube and either used for radioimmunoassay immediately or stored for up to a few months at −20°C. For radioimmunoassay the samples are used in at least three serial (1:3 steps) dilutions, usually starting at the dilution 1:6, 1:18, 1:54, etc.

Pituitary tissue homogenates: at least three rats are decapitated under light ether anaesthesia; the cranial vaults are opened and the pituitary glands are taken out, weighed on a torsion balance and homogenized in a small glass tube cooled on ice by applying ten strokes with a loosely fitting Teflon pestle at 900–1100 rev/min, suspended in physiological saline (10 mg wet wt/ml). After centrifuging the homogenate for 10 min at 350 **g** the supernatant is pipetted into another tube and diluted 1:10 with diluent buffer. Starting from this seven serial (1:3 steps) dilutions are made for radioimmunoassay.

Chromato-electrophoretic separation of 'bound' and 'free' hormone: DEAE-c paper (Whatman Paper Chromedia DE 81; cat. No. 24813) is cut into strips of 28 × 2½ cm. Pencil lines are drawn to mark the middle of the strip and at 6½ cm and at 4½ cm from one end. The strips are wetted in buffer (0·025 M borate/HCl buffer, pH 8·6 and 0·14 M NaCl). Two glass or Perspex plates are put on the table with their edges parallel to each other and ±2 cm apart. Blot the wetted strips between two sheets of blotting paper and put the strips in such a way on the two plates that the 4½ cm pencil markings are suspended loosely between the two plates, but are still free from the surface of the table. Apply 0·1 ml of a 1:3 dilution of IAA-horse serum (1 part of a 5% iodo-acetamide solution (w/v) + 9 parts of horse serum) on the 4½ cm markings. When this has been entirely absorbed by the paper, 0·1 ml amounts of the reaction mixtures are applied also on the 4½ cm markings. The strips are then put on an electrophoretic apparatus (platinum electrodes; electrode compartments containing each at least 400 ml 0·025 M borate/HCl buffer, pH 8·6 and 0·14 M NaCl) in such a way that the application sites hang free and are at least 2½ cm above the anode buffer surface. Apply 300 V over the electrodes (constant voltage) during 15–20 min (depending on the relative humidity of the room). Dry the strips horizontally in a stove (100°C). Cut each strip into three pieces: (1) a short (anode) piece of 6½ cm, which incorporates the application site; (2) a middle piece of ± 8 cm length and (3) a long cathode piece, which is discarded.

Fold or roll the two pieces of each strip of DEAE-c paper in such a way that they can be accommodated in the *plastic* test tubes of the Autogamma Spectrometer—Packard, Tri-Carb model 3003 with a 2 in. crystal, settings: gain 100%; window at 10 keV (lower) and 90 keV (upper). The counts registered for the middle piece represent the labelled hormone bound by antibody and the anode piece the free labelled hormone for each reaction mixture;

$$\frac{\text{cpm 'bound'}}{\text{cpm 'bound'} + \text{cpm 'free'}} \times 100\%$$

gives the '%-bound' of a particular reaction mixture. Plotting the %-bound linearly on the vertical axis and either the ng amounts of unlabelled prolactin, or the dilutions of either standard preparations or plasma samples or pituitary homogenates in a log scale horizontally, will give the respective regression lines. These can be checked for parallelity and used for calculating the relative potency of two preparations or for expressing the prolactin level of a plasma sample or pituitary homogenate in ng equivalents of a reference preparation per ml.

RESULTS

Comparison of ^{125}I-labelling of RPT and RPN

The first iodinations of RPT and of RPN performed on the same day were carried out with an IMS-3 batch, stated to have a specific activity of 10 mC/μg of iodine. The eluation patterns from the Sephadex columns are represented in Fig. 1 (a) and (b). Iodination efficiency of the labelling procedure for RPT was 70·7% and that for RPN was 58·0%. The slightly higher iodination efficiency in the

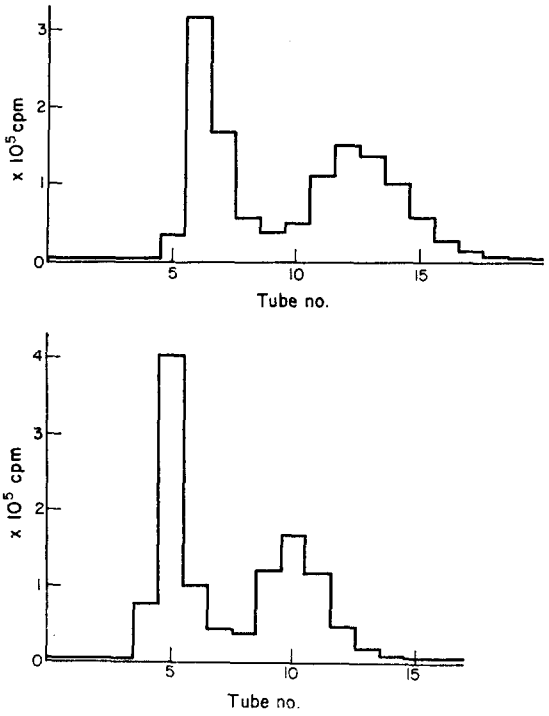

Fig. 1. *Elution patterns showing distribution of radioactivity of labelled rat prolactin separated on Sephadex G-50 (coarse grade) from unreacted iodide fractions.*
(a) *upper part; results of labelling* 25 μg *RPT with* ±1 *mC of* $Na^{125}I$. *Iodination efficiency* 70·7% (±28 μC/μg *RPT*). *Column size* ±10 *cm*×0·9 *cm; tube no.* 5 *only is used; volume is brought to* 1 *ml and an aliquot is further diluted* (1:660) *with diluent buffer to* 12 *ng labelled RPT/mm.*
(*b*) *lower part; results of labelling* 25 μg *RPN with* ±1 *mC of* $Na^{125}I$. *Iodination efficiency* 58·0%(±23 μC/μg *RPN*). *Column size* ±11 *cm*×0·9 *cm; tube no.* 6 *only is used; volume is brought to* 1 *ml and an aliquot is further diluted* (1: 625) *with diluent buffer to* 12 *ng labelled RPN/ml.*

labelling of RPT may have caused more 'damage' to the protein, since the column retained 13·6 μg (54·4%) of the labelled RPT compared to 11 μg (44%) of the labelled RPN.

The second iodinations carried out 3 weeks later with an IMS-3 batch (specific activity 6 mC/μg iodine) resulted in iodination efficiencies for RPT and for RPN of respectively 76% and 66%. Both labelled preparations remained for a great part adsorbed to the Sephadex columns; respectively 90% of the labelled RPT and 87% of the labelled RPN.

Comparison of antiserum titration curves

Figure 2 shows the antiserum titration curves obtained with the two labelled rat prolactin preparations of the first iodinations. The percentages of the labelled rat prolactin preparations (100 μl of a 12 ng/ml dilution of labelled prolactin per tube) bound by the seven serial (1:3 steps) dilutions of the three antisera employed. Both antisera raised against RPT showed their maximal binding of labelled RPT at dilutions $3^0\times10^{-2}$ and $3^{-1}\times10^{-2}$ and start to fall off from maximal binding at $3^{-2}\times10^{-2}$. The antiserum raised against RPN has a higher titre of specific antibodies; maximum binding of labelled RPT occurred at the dilution $3^{-2}\times10^{-2}$ and starts to fall off at $3^{-3}\times10^{-2}$. Apparently some precipitation of antigen–antibody complex occurred with this antiserum at the dilutions $3^{-0}\times10^{-2}$ and $3^{-1}\times10^{-2}$.

Maximal binding of labelled RPN and distinct fall off from maximal binding by the three antisera in general followed the same pattern, except that: (1) presumably precipitation of antigen–antibody complex occurred with all three antisera at the dilution $3^0\times10^{-2}$ resulting in a smaller percentage of the bound labelled preparation moving from the application site; and (2) maximal binding percentages for labelled RPN are 10–15 15%-binding higher than for labelled RPT (i.e. for labelled RPN it is approximately 70%, which migrated from the application site, compared to 55 to 60% for labelled RPT).

Maximal binding percentages shown by the three antisera with the two labelled preparations obtained at the second iodination series, however, were 42·3%, 50·9% and 63·8% of the labelled RPT, compared to 37·5%, 47·8% and 54·9% of the labelled RPN. The lower binding percentages shown by all three antisera suggest greater 'damage' at the antigenically active sites of the labelled RPN in the second iodination series.

Comparison of competitive inhibition curves

The 8 standard curves covering the eight possible combinations of: (1) unlabelled prolactin (either RPT or RPN); (2) antiserum in the reaction mixture (either antiserum 405 against RPT, or antiserum V-20-B30 raised to RPN) and (3) labelled prolactin (either RPT or RPN), are represented in Fig. 3. The four regression lines represented in the upper half of Fig. 3 show the results obtained with the reaction mixtures in which labelled RPT is employed. The four regression lines in the lower half of Fig. 3 represent the combinations in which labelled RPN was used. The four standard curves shown in the left half of the figure represent the results with antiserum V-20-B30 raised against RPN, while the four standard curves in the right half of the figure show the results obtained with antiserum 405 raised to RPT in the reaction mixtures. The vertical axis indicates the percentage of the labelled hormone bound by antibodies in each individual reaction mixture. The horizontal

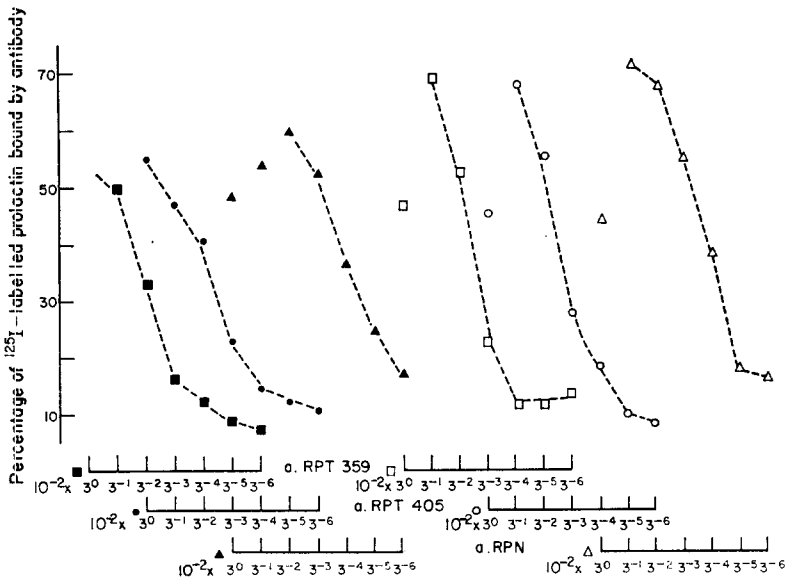

Fig. 2. *Antisera titration curves. For technical details see Material and Methods.*
■ *antiserum* 359 *to RPT* × *labelled RPT;*
● *antiserum* 405 *to RPT*+*labelled RPT;*
▲ *antiserum to RPN*+*labelled RPT;*
□ *antiserum* 359 *to RPT*+*labelled RPN;*
○ *antiserum* 405 *to RPT*+*labelled RPN;*
△ *antiserum to RPN*+*labelled RPN.*

The lower %*-bound of the labelled RPT observed with dilutions* $3^{-0} \times 10^{-2}$ *and* $3^{-1} \times 10^{-2}$ *of antiserum to RPN and the lower* %*-bound of the labelled RPN with all three antisera at dilution* $3^{-0} \times 10^{-2}$ *are presumably due to precipitation of antigen–antibody complex at the application site on the DEAE-c paper strip.*

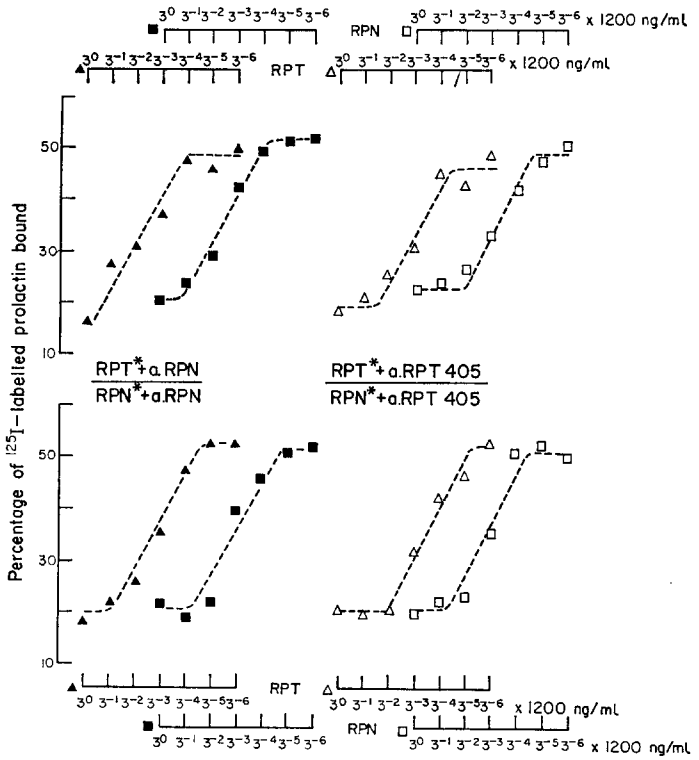

Fig. 3. *Inhibition of the* ^{125}*I-labelled rat prolactin–antiserum to rat prolactin system*
(*a*) *upper left quarter: labelled RPT–antiserum to RPN assay system; upper right quarter: labelled RPT–antiserum* 405 *to RPN assay system;*
(*b*) *lower left quarter: labelled RPN–antiserum to RPN assay system; lower right quarter: labelled RPN–antiserum* 405 *to RPT assay system. Inhibition of binding by addition of serial* (1 :3) *dilutions of respectively:*
1. *unlabelled RPT: open triangles and closed triangles.*
2. *unlabelled RPN: open circles and closed squares.*

bars indicate the dilution of unlabelled RPT (closed and open triangles) or of unlabelled RPN (closed or open circles), which was added to each reaction mixture to compete with the labelled preparations for the specific antibodies.

The regression lines have a striking resemblance to each other and provide a fair indication that the antigenic determinants in the two rat prolactin preparations qualitatively and quantitatively are similar and that there is no indication for striking differences between the two antisera as to their respective composition of antibodies that react specifically with either of the two rat prolactin preparations.

The curves representing the inhibition of binding of either of the two labelled preparations by the specific antibodies in antiserum V-20-B30 (i.e. the four combinations represented in the left half of Fig. 3) were repeated four times during two weeks. The scatter of individaul percentages of the labelled preparations bound by antibodies at each of the 7 dose levels of the unlabelled hormone preparations were pooled for each of the four combinations. The four accumulated regression lines were checked for (1) fitting straight lines; (2) whether all the observations fitted one and the same regression line and when this proved not to be the case; (3) whether the four regression lines were parallel:—

All four accumulated regression lines fitted straight lines; the calculated regression lines are represented in Fig. 4.

The combined scatter of percentages of the labelled hormone bound by antibodies of all four combinations taken together cannot be considered to represent one and the same regression line ($F_{calc.}=10\cdot57$, whereas $F_{95\%}=2\cdot76$); statistically they may be considered to represent parallel regression lines $F_{calc.}=0\cdot99$; $F_{95\%}=2\cdot76$).

Analysis of covariance comparing the scatter diagram for each combination of two regression lines gave the following results ($F_{95\%}$ for identical lines$=3\cdot32$; $F_{95\%}$ for parallel lines$=4\cdot17$):

a. RPN*/RPN and RPN*/RPT coincide ($F=1\cdot95$);

b. RPN*/RPN and RPT*/RPN do not coincide ($F=12\cdot8$), but may be considered parallel ($F=0\cdot32$)

c. RPN*/RPN and RPT*/RPT coincide ($F=2\cdot7$);

d. RPN*/RPT and RPT*/RPN do not coincide ($F=7\cdot56$), but may be considered parallel ($F=0\cdot23$);

e. RPN*/RPT and RPT*/RPT coincide ($F=0\cdot23$)

f. RPT*/RPN and RPT*/RPT do not coincide ($F=13\cdot7$), but may be considered parallel ($F=1\cdot81$).

Apparently the regression line, which does not coincide with any of the 3 other regression lines is the one representing the inhibition by unlabelled RPN of the binding of labelled RPT by the specific antibodies.

Comparison of prolactin estimates of biological material by radioimmunoassays, based respectively on RPT or on RPN

Two plasma samples, known to have relatively high prolactin levels, and one pituitary homogenate were assayed in 'duplicate assays'. Twenty-eight reaction tubes were used for each

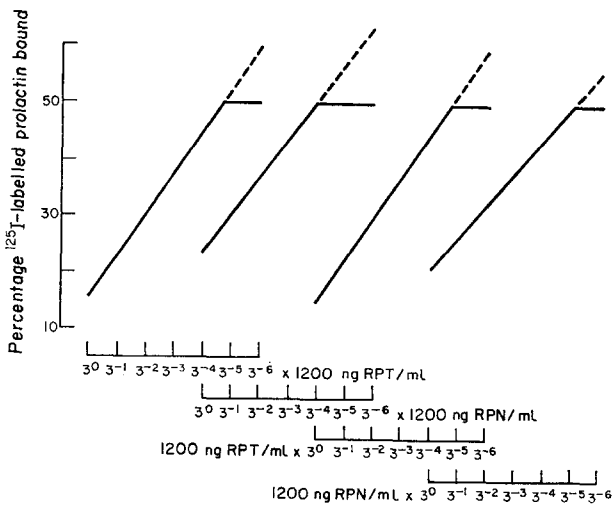

Fig. 4. Calculated regression lines for four repeat inhibition curves. From left to right: labelled RPT/antiserum to RPN/unlabelled RPT; labelled RPT/antiserum to RPN/unlabelled RPN; labelled RPN/antiserum to RPN/unlabelled RPT; labelled RPN/antiserum to RPN/unlabelled RPN. (see text for further details).

duplicate assay for incubation of: (1) one of the biological samples in seven dilutions in duplicate; (2) seven dilutions of unlabelled RPT and (3) seven dilutions of unlabelled RPN. The reaction tubes were preincubated with the standardized amount of antiserum to RPN for 40 hr. Labelled RPT was added to one set of 7 tubes with dilutions of the biological sample and to the 7 tubes containing the unlabelled RPT dilutions. To the remaining 14 tubes labelled RPN was added. This was done on three subsequent days respectively with the plasma samples and with the pituitary homogenate.

The plasma samples were obtained respectively from: (1) a male rat of the highly inbred strain R-Amsterdam that had been orchidectomized at the age of 8 weeks and which had received from that time onward until the sample was taken one pellet (2 mg pellets containing oestrone/cholesterol 1:4—w/w) implanted sub-cutaneously per week for 12 weeks; (2) a male rat of the same strain, orchidectomized at the age of 8 weeks and into which ±10 mg of tissue of a primary prolactin-producing pituitary tumour had been implanted in the calve musculature. The blood sample was taken when the transplant had attained a size of approximately 1½ cm dia. under influence of a monthly implantation of one oestrone/cholesterol pellet. Analysis of covariance indicated that the two sets of three regression lines obtained with the three samples of biological material were parallel to each other and were parallel to their respective regression lines. Thus the extrapolation is allowed and the prolactin contents of the samples may be expressed in nanograms of the respective reference preparations. The results are summarized in Table 1 and gives the values obtained for each sample extrapolated with the reference curves of the same day. As can be seen the assays carried out with labelled RPT were fair duplicates of the assays carried out with labelled RPN when the results were expressed respectively in ng RPT aequivalents per ml (or per mg) and in ng RPN aequivalents per ml (or per mg).

Inhibition of binding by heterologous prolactin preparations

Figure 5 summarizes the results of experiments performed to see whether the binding of either of the two labelled rat prolactin preparations are inhibited in comparable ways and to the same extent by competition of respectively mouse or bovine prolactin. Binding of labelled rat prolactin of around 50% is brought down to less than 20% by inhibition of the binding by excess of unlabelled rat prolactin competing for the antibodies. Mouse prolactin is far less effective in this competition for specific antibodies, since a tenfold amount of mouse prolactin brought the binding of labelled RPT down to just under 30%. It was slightly more effective in reducing the specific binding of labelled RPN, which was brought down to just under 25%. The slopes of the two regression lines representing the inhibition of binding by serial dilutions of mouse prolactin is less steep than that obtained by serial dilutions of rat prolactin. Bovine prolactin was even far less effective in competing for specific antibodies, since up to ±15,000 ng/ml did not noticeably influence the binding of 12 ng/ml of either of the two rat prolactin preparations labelled with ^{125}I. The binding of labelled RPT began to show a decrease when approximately 133,000 ng/ml of the bovine preparation was added to the reaction mixture. The specific binding was further lowered by 1·2 mg/ml of the bovine prolactin preparation; this latter concentration added to the reaction mixture containing labelled RPN lowered the binding of this labelled preparation too.

DISCUSSION

Rat prolactin isolated from the 'granular

Table 1.

Donor of biological sample	Estimates of prolactin by two radioimmunoassay systems based on different rat prolactin preparations	
	RPT	RPN
Castr. ♂ R-Amsterdam after 12 weekly oestrone pellet implantation	1·61 µg/ml plasma	2·06 µg/ml plasma
Castr. ♂ R-Amsterdam bearing a 1st passage pituitary tumour (dia. ±1 p cm)	28·7 µg/ml plasma	23·6 µg/ml plasma
Normal pituitary tissue of 3 month-old ♂♂ R-Amsterdam	3·23 µg/mg wet wt.	2·82 µg/mg wet wt.

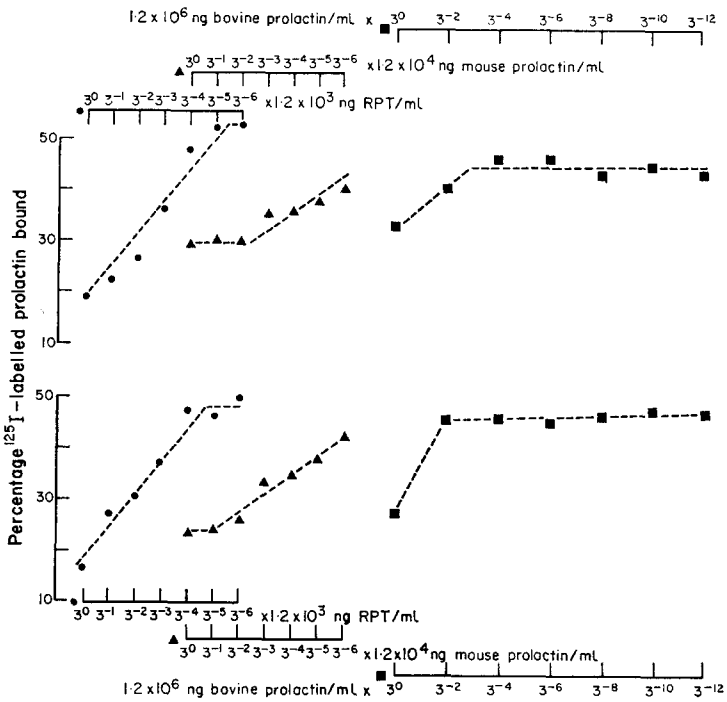

Fig. 5. Inhibition of ^{125}I*-labelled rat prolactin–antiserum to RPN assay systems by heterologous prolactin preparations.*

a: upper part: labelled RPN–antiserum to RPN assay system; b: lower part: labelled RPT–antiserum to RPN assay system.

●—*inhibition by rat prolactin, dilutions employed* $3^0\times$, $3^{-1}\times$, $3^{-2}\times$, $3^{-3}\times$, $3^{-4}\times$, $3^{-5}\times$ *and* $3^{-6}\times 1200$ *ng/ml.*

▲—*inhibition by mouse prolactin, dilutions employed* $3^0\times$, $3^{-1}\times$, $3^{-2}\times$, $3^{-3}\times$, $3^{-4}\times$, $3^{-5}\times$ *and* $3^{-6}\times 12{,}000$ *ng/ml.*

■—*inhibition by bovine prolactin, dilutions employed* $3^0\times$, $3^{-2}\times$, $3^{-4}\times$, $3^{-6}\times$, $3^{-8}\times$, $3^{-10}\times$ *and* $3^{-12}\times$ 1·2 *mg/ml.*

fraction' of pituitary tumour transplants was the first preparation of rodent prolactin available. The rather unorthodox approach of isolation (i.e. extracting an enriched material consisting mainly of secretion granules) on the one hand and the unorthodox starting material (i.e. transplants of oestrone-induced pituitary tumours) are sufficient reasons to suspect that the prolactin preparation so obtained may differ in some aspects from normal rat prolactin.

The first observations, which suggested that RPT may be slightly different from normal prolactin preparations, were observations by Dr. Forsyth (personal communication, see also Kwa *et al.* [5]. When the preparation was applied locally in biological tests (e.g. the local crop sac assay in pigeons, or the intra-ductal mammary gland assay in rabbits) the biological activity was estimated to be about 10% of that of the ovine reference preparation, whereas the biological activity estimated in mammary glands *in vitro* (e.g. using as parameter either the mammotropic response [12, 13] or the galactopoetic response [14] was nearly aequipotent to the same ovine reference preparation. In the only biological test performed, whereby RPT was administered systematically (i.e. using the effect on induction and maintenance of pseudopregnancy in normal female mice as parameter, RPT was superior to the ovine reference preparation [2]. Since the starting material for the isolation of RPT as checked by electron microscopy consisted mainly of prolactin-cells with occasionally a few GH-cells, the other types including the LH-cells being virtually absent, the objection that this test is non-specific and can be induced by HCG (LH) as reported by Weller, Mishkinsky and Sulman [15] is not relevant in this case.

Another difficulty arose when Dr. W. S. Bont (personal communication) failed to find any N-terminal amino acid spot after two-dimensional thin layer chromatography when the usual amount of dansylated-prolactin (RPT) hydrolysate was applied. Only when six to ten times the usual amount was applied instead of a single amino acid spot, six vague spots could be discerned.

Last year two groups reported the isolation of prolactin from normal rat pituitary glands [6].

The first claimed a biological activity of 25 i.u./mg and reported leucine as the sole N-terminal amino acid. The reported pattern after electrophoresis on polyacrylamide gels and the molecular weight estimates by both groups are consistent with those reported earlier for RPT [2]. The N-terminal amino acid was kindly checked by Dr. Ellis' group by a quantitative DNFB-method. This disclosed per mole of 23,000 MW: 0·1 mole of serine, 0·05 mole of leucine and between 0·01 mole of each of Asp, Glu, Ala, Val and Thr. Whereas the N-terminal amino acid analysis indicated that only 5% of the RPT preparation contained material with the same N-terminal amino acid as the rat prolactin preparation isolated from normal pituitary glands of rats, the two preparations resembled each other quite closely qualitatively as well as quantitatively in radioimmunoassay experiments reported in the present paper. More consistent with the latter observations is that the amino acid values of RPT run quite close to that found for RPN (personal communication Dr. S. Ellis; amino acid analysis kindly performed by Dr. P. X. Callahan), even to the characteristically low methionine value (i.e. 2 moles for both rat prolactin preparations v. 7 moles for ovine prolactin).

The comparison in performance of RPT and RPN in radioimmunoassay experiments suggest identical sequences of amino acids at the immunologically active sites of the respective molecules. This is consistent with the earlier reported parallel inhibition curves obtained with respectively unlabelled RPT, dilutions of plasma samples and homogenates of normal pituitaries of the rat [3]. It was gratifying, however, that the reported results seem to warrant the conclusion that radioimmunoassays based on either of the two rat prolactin preparations will give estimates in unit weights that do not differ much, not withstanding the marked discrepancy in biological activity of the two preparations in the local pigeon crop assay.

The antisera tested differed slightly in titre of antibodies. The antiserum to RPN not only showed a better titre of specific antibodies, but apparently had also a better avidity to rat prolactin as may be inferred from its capacity to bind the labelled rat prolactin preparation being less affected by iodination damage of rat prolactin during the labelling procedure. These differences, however, reflect a difference in the immuno-competence of the respective rabbits rather than a difference in antigenicity of the two rat prolactin preparations.

The competitive inhibition curves observed in the different combinations of labelled and unlabelled rat prolactin preparations are all consistent with the assumption of parallel regression lines and extrapolations of these results are therefore valid and allows the relative potency of RPT to be expressed in terms of RPN and vice versa. When RPN is used as labelled preparation analysis of covariance of four repeat assays indicated RPT and RPN aequipotent in inhibiting the binding of labelled RPN by the specific antibodies. When labelled RPT is employed as the labelled preparation, however, unlabelled RPT appeared to be significantly superior to unlabelled RPN in competing for the binding sites of the specific antibodies. This suggested slight differences in the absolute ratio of the various antigenically active sites common to both preparations.

The slightly different extent to which the binding of labelled RPT is inhibited by mouse prolactin compared to the inhibition of binding of labelled RPN also can be ascribed to slight differences between the two rat prolactin preparations. However, the regression lines in both cases have a much flatter slope compared to slopes obtained with rat prolactin, indicating partial identity of antigenically active sites between mouse and rat prolactin molecules. The observations are consistent with earlier reported findings indicating partial immunological identity of prolactin of rat and mouse origin [2, 8].

Apparently bovine prolactin in very high concentrations gave rise to some degree of inhibition. The technique employed for separating bound hormone from free hormone (i.e. under the conditions chosen for separation of 'bound' from 'free' the free hormone remains at the application site on the DEAE-c paper strips) made it unlikely that the observed effect was due to an artefact caused by displacement of rat prolactin by the bovine hormone on the (ion-exchange) binding sites of the strip. The explanation of the observed results as prolactin specific inhibition for the binding sites of the antibodies to prolactin is consistent with the observation of Rumke and Ladiges [16] of cross reactivity between an antiserum raised to ovine prolactin and bovine as well as prolactin in mouse as well as in rat material with the immunofluorescent technique.

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RESUME

Plusieurs techniques de dosage radio-immunologique ont été essayées sur une préparation de prolactine du rat, isolée à partir de la 'fraction granulaire' de transplants de tumeurs hypophysaires, et sur une préparation de prolactine isolée à partir de glandes hypophysaires normales, stockées et congelées.

Les courbes de titration de l'antisérum et les courbes d'inhibition compétitive suggèrent que les deux préparations se ressemblent étroitement. L'analyse de covariance des courbes d'inhibition compététive montre que les petites différences observées au cours des essais répétés entre les deux préparations peuvent être attribuées à l'erreur expérimentale.

Il existe une grande différence entre l'activité biologique de ces deux préparations lorsque celle-ci est exprimée par l'action sur le jabot du pigeon. Les expériences présentées montrent néanmoins que les essais radio-immunologiques sur chacune des deux préparations donnent des valeurs plus ou moins semblables quand les estimations des taux de prolactine sont exprimées en unités de poids équivalents. L'expression en unités de poids équivalents sera donc préférable à une extrapolation de celles-ci en des unités internationales de l'activité biologique de prolactine.

SUMMARY

The performance in various procedures for radioimmunoassay of a rat prolactin preparation isolated from the 'granular fraction' of transplants of prolactin-producing pituitary tumours is compared to that of a prolactin preparation isolated from pooled frozen normal pituitary glands. Antisera titration curves and competitive inhibition curves indicate that the two preparations resemble each other closely. Analysis of covariance of competitive inhibition curves indicates that the small differences observed between the two preparations in recent assays can be ascribed to experimental error. Not withstanding a marked difference in biological activity between the two preparations as measured by the local pigeon crop assay, the reported experiments indicate that radioimmunoassays based on either of the two preparations will give approximately the same values when the prolactin estimates in biological material are expressed in unit weight aequivalents of the preparation employed. Expression in unit weight aequivalents should therefore be preferred to an extrapolation from these to i.u. of biological prolactin activity.

ZUSAMMENFASSUNG

Prolactinpräparate wurden aus der 'Granularfraktion' von prolactinproduzierenden Hypophysentumoren, die auf Ratten transplantiert worden waren, isoliert. Ihr Verhalten in verschiedenen Verfahren der Radioimmunomethode wurde untersucht und mit dem von Prolactinpräparaten verglichen, die aus gesammelten, gefrorenen, normalen Hypophysen isoliert worden waren.

Antiserumtitrationskurven und kompetitive Inhibitionskurven zeigen eine grosse Ähnlichkeit der beiden Prolactinpräparate. Kovarianzanalyse der kompetitiven Inhibitionskurven zeigt, dass die kleinen Unterschiede zwischen den zwei Präparaten in den verschiedenen Proben dem Probenfehler zugeschrieben werden können. Trotz des grossen Unterschiedes in der biologischen Aktivität der beiden Präparate—getestet mit der lokalen Taubenkropfmethode—zeigen die Resultate der Radioimmunomethode auf der Grundlage beider Präparate gleiche Prolactinwerte für verschiedenes biologisches Material, wenn sie in Gewichtseinheiten umgerechnet werden. Wiedergabe in Gewichtseinheitsäquivalenten ist darum einer Extrapolation auf I.E. biologischer Prolactinaktivität vorzuziehen.

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Radioimmunoassay of Rat Prolactin

Prolactin Levels in Plasma of Rats with Spontaneous Pituitary Tumours, Primary Oestrone-Induced Pituitary Tumours or Pituitary Tumour Transplants

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INTRODUCTION

SPONTANEOUS pituitary tumours have been reported in a fairly high incidence in a great number of strains of rats: the Albany strain [1], the Vanderbilt strain and the Wistar strain [2], the Yale strain, the Sherman strain and the Wistar strain [3, 4].

In view of the common occurrence of spontaneous pituitary tumours it is difficult to accept unreservedly the reported incidence in various experimental conditions as a consequence of the treatment, with the exception of long-term oestrogen administration. Since the finding by Zondeck [5] and McEuen, Selye and Collip [6] of pituitary adenomata in rats after oestrone administration, the effectiveness of this procedure has been substantiated by many investigators. Many other experimental conditions resulting in pituitary tumours, e.g. rats bearing ovarian grafts [7], parabiosis experiments joining an ovariectomized rat to a non-ovariectomized partner [8] may be interpreted to prove that long-term hyperoestrogenization results in tumourous enlargement of the hypophysis. Even the spontaneously occurring pituitary tumours may be ascribed to a disruption of the subtle interplay between gonads and pituitary with advancing age, resulting in an undisrupted oestrogenization by the ovaries or by adrenal tissue. The striking morphological similarity of oestrogen-induced and the spontaneously occurring pituitary tumours in rats has been stressed by Wolfe *et al.* [2].

The development of a radioimmunoassay for rat prolactin [9, 10] made it feasible to follow the fluctuations in prolactin levels in plasma during the induction and growth of pituitary tumours under the influence of oestrone pellet implantation and to measure the prolactin levels in the plasma of old female rats and correlate the assay results with the occurrence of spontaneous pituitary tumours and mammary tumours.

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MATERIAL AND METHODS

Animals

Rats of the highly-inbred strain. R-Amsterdam, were used as plasma sample donors for all the experiments reported. The animals were kept in macrolon cages of 38 × 25 × 15 cm; two or three animals to a cage, under controlled illumination (14 hr light and 10 hr darkness). The diet consisted of commercial food pellets (Standard Laboratory Diet, Hope Farms, Woerden, Holland) and tap water *ad libitum*, unless otherwise stated.

Old rats: 25 female rats, which had been

force-bred throughout their reproductive life-span without any additional treatment, were kindly made available to us by Dr. P. A. J. Bentvelzen.

Rats developing primary pituitary tumours: Male rats of 8–10 weeks were orchidectomized and weekly subcutaneous implantation of oestrone-cholesterol pellets was started (one pellet per rat per week). Primary pituitary tumours of approximately 200 mg were detected between days 160–190 after initiation of treatment by signs of disturbed sense of equilibrium. Four animals of the 'regular production line' of primary tumours were followed throughout this period.

Rats bearing transplants of oestrone-induced pituitary tumours: Male rats of 8–10 weeks were orchidectomized. Up to 10 pieces of tumour tissue of approximately 1 mg of the primary pituitary tumours were rinsed free from excess blood in isotonic buffered sucrose (pH 7·4) and introduced intramuscularly half-way between the knee and ankle joint with the aid of a trocar, through a small incision in the skin near the ankle joint. In order to allow the transplanted pituitary tumour tissue to grow, before the rat develops a primary pituitary tumour of its own incompatible with further survival, this group of animals received one oestrone–cholesterol pellet at the time of operation and subsequently one pellet per month. The animals in this group received from the time of operation onward oestrogenized drinking-water.

Oestrone administration

Appropriate amounts of oestrone and cholesterol (1 : 4, w/w) were mixed by dissolving them in benzene, after which the solvent was evaporated at room temperature under a hood. The resulting homogeneous dry mixture was compressed into pellets of approximately 2 mg (dia. 1·1 mm, length approximately 3 mm). Pellets were implanted subcutaneously in the dorsal region of the neck.

Oestrone dissolved in drinking-water was prepared twice weekly by adding 1 ml of a stock solution (200 mg oestrone dissolved in 100 ml ethanol) to 1 l. tap-water.

Collection of blood samples

Heparinized capillary tubes (length 75 mm; i.d. 1·1–1·2 mm) were broken into three approximately equal parts. A rat was brought under light ether anaesthesia. By applying light pressure with thumb and forefinger of the left hand on both sides behind the jaw bones, the venous pressure in the region was increased. A piece of capillary was inserted with its sharp (broken) edge into the eye-socket, laterally and caudally of the right eyeball. By rolling the capillary slightly between thumb and forefinger of the right hand, the edge cut into the retrobulbar venous plexus and blood started to drip from the capillary into a polystyrene tube held under the free end of the capillary. At the tenth drop the capillary was withdrawn from the eye-socket and the engorgement was stopped. The eyelid was closed and gentle pressure was applied on it to stop the bleeding by 'tamponading' the wound with the eyeball. The polystyrene tubes were centrifuged for 10 min at 500 **g**; the plasma was pipetted off into another tube and either used for radioimmunoassay immediately or stored for up to a few months at −20°C.

Rat prolactin preparation

Rat prolactin was isolated as described earlier [11], except that only first passage tumour transplants were employed.

Rabbit antisera

Antiserum 415 was raised in a rabbit to rat prolactin and was used in the dilution 1 : 2700 (i.e. the dilution of antiserum *before* it was added to the reaction mixture); or antiserum 405, which was raised in a rabbit to rat prolactin coupled to bovine γ-globulin (in an effort to raise its antigenicity), was used in the dilution 1 : 900. Immunization procedure as described earlier [11]. Both antisera gave identical results in the mentioned dilutions.

Iodination procedure

The chloroamine-T method of [12] was carried out with minor modifications as described extensively elsewhere [10].

Incubation of reaction mixtures

Enough polystyrene reaction tubes (disposable tubes, size 4 cm, i.d. 8 mm with round bottom) were divided in sets to cover the three or more dilutions of plasma samples and the seven dilutions of unlabelled rat prolactin for the reference curve. Into each reaction tube 0·1 ml of the standardized dilution of the particular antiserum was pipetted. Besides the antiserum the tubes contained one dilution of one of the plasma samples or one of the seven dilutions of the reference rat prolactin preparation. These reaction mixtures were 'pre-incubated' for 40 hr at 4°C. The labelled hormone was then added (0·1 ml of a solution containing 12 ng of labelled hormone per ml of diluent buffer) and the reaction mixtures

were left for a further 24 hr at 4°C. Aliquots of each reaction mixture were taken for chromato-electrophoretic separation of 'bound' and 'free' hormone.

Chromato-electrophoretic separation

The separation of 'bound' hormone from 'free' hormone was carried out on DEAE-c paper strips (Whatman Paper Chromedia DE 81; cat. no. 14813) as described in detail elsewhere [10].

Buffer solutions

Diluent buffer used for diluting antisera, labelled prolactin preparations and for making serial dilutions of reference preparation and of plasma samples was a 0·025M borate/HCl buffer, pH 8·4, to which 0·5 mg bovine serum albumin per ml was added.

A 0·025 M borate/HCl buffer, pH 8·4, with a NaCl concentration of 0·14 N was used for wetting the DEAE-c paper strips and for the electrode compartments for the chromato-electrophoretic runs.

Iodoacetamide horse serum

To prevent excessive trailing of the antigen-antibody complex on the DEAE-c strips, 0·05 ml of a 1 : 3 dilution of IAA-horse serum (1 part of a 5% iodoacetamide solution + 9 parts of horse serum) was applied on the application site on the strip before the aliquot of the reaction mixture was applied.

RESULTS

Old female rats

At the age of 24 months 8 out of 25 rats had palpable mammary tumours. For some unknown reason the plasma samples of these old rats caused 'incubation damage' at the dilution 1 : 3. Therefore only the serial dilutions starting from 1 : 9 of the plasma samples contributed to competitive inhibition curves that run parallel to the reference curve. This resulted in setting the lower limit of detectable prolactin levels in plasma samples of this group of rats at 135 ng/ml. As can be seen in Table 1 only 9 out of 25 plasma samples taken at the age of 24 months exhibited prolactin levels above this value. Only one single rat that had detectable amounts of prolactin in its plasma had no palpable mammary tumour.

The rats with mammary tumours were autopsied one month later; six out of the eight rats having one or more palpable mammary tumours proved to have pituitary tumours. The pituitary weights in six of the rats were in excess of 30 mg. The plasma prolactin values of the blood samples taken at autopsy only in one case showed a considerable increase and this rat had a pituitary tumour of 363 mg. In two of the rats the pituitaries were in the weight range of 12–30 mg and these pituitaries were classified as 'enlarged' [13]. Again in the rats without palpable mammary tumours only one showed prolactin values above the 135 ng/ml level.

At the age of 29 months 3 other rats had developed a palpable mammary tumour. There was no correlation with pituitary weight and the prolactin levels fell within the range of values found in the rats, which had no mammary tumours. The rat, which had measurable amounts in all three assays, had the highest prolactin levels of the 17 animals killed at the age of 29 months. This rat proved to have a pituitary tumour and was found to have a tumour of one of the ovaries.

Rats developing primary tumours

The radioimmunoassay results of following the changes in plasma levels of prolactin induced in the four orchidectomized male rats treated with weekly oestrone–cholesterol pellets until their tumourously enlarged pituitary glands illicited signs of disturbed sense of equilibrium are summarized in Fig. 1.

Within ten days after initiation of treatment plasma levels in the four rats rose from less than 45 ng/ml found in untreated orchidectomized male rats to values around 1000 ng/ml (range: 750–1400 ng/ml). Twenty-four days after starting the weekly implantation of hormone pellets the plasma levels have risen in all four rats to values above 1000 ng/ml (range: 1250–2400 ng/ml). The estimates of the plasma values on day 40 of treatment (range: 850–1900 ng/ml) and day 70 of treatment (range 470–950 ng/ml; one plasma sample had to be discarded because of haemolysis) indicated a temporary decline in prolactin secretion suggestive of an 'exhaustion' of the prolactin-producing cells of the pituitary. Apparently between days 70 and 110 after initiation of treatment the pituitary cells that produce prolactin recovered their ability to maintain a more or less constant and elevated level of this hormone in the circulation: day 70 mean value 1150 ng/ml (range 650–1500 ng/ml); day 85 mean value 1500 ng/ml (range 950–1900 ng/ml) and day 110 mean value 1250 ng/ml (range 850–1700 ng/ml).

Between day 110 and day 150 a progressive increase of prolactin in the circulation started, possibly coinciding in time of onset with the progressive increase in weight of the pituitary

Table 1. *Plasma prolactin levels in old female rats*

Rat	At 24 month of age ma. tum. palpable (+/−)	prolactin level (μg/ml)	At 25 month of age ma. tum. palpable (+/−)	prolactin level (μg/ml)	At 29 month of age ma. tum. palpable (+/−)	prolactin level (μg/ml)	Pituitary wt. mg	Remarks
rats killed at 25 month								
04	+	0·7	+	0·68			110	
10	+	0·55	+	0·65			35	
11	+	0·31	+	1·02			114	
16	+	0·73	+	1·37			38	
21	+	1·3	+	4·5			363	
22	+	1·25	+	0·9			55	
24	+	1·3	+	0·47			16	
27	+	1·0	+	0·55			15	
rats killed at 29 month								
03	—	0·78	—	0·53	—	0·93†	42	ov. tum.
09	—	<0·135*	—	<0·135	+	0·16	15	
12	—	0·135	—	0·135	+	0·13	10	
23	—	0·135	—	0·135	+	0·25	18	
01	—	0·135	—	0·135	—	0·14	15	
02	—	0·135	—	0·135	—	0·24	12	
05	—	0·135	—	0·135	—	0·31	10	
06	—	0·135	—	0·135	—	0·15	10	
07	—	0·135	—	0·135	—	0·32	11	
08	—	0·135	—	0·135	—	0·08	12	salivary gland tum.
13	—	0·135	—	0·135	—	0·14	15	
15	—	0·135	—	0·135	—	0·18	15	
17	—	0·135	—	0·135	—	0·03	11	
18	—	0·135	—	0·135	—	0·29	14	
20	—	0·135	—	0·135	—	0·24	13	
25	—	0·135	—	0·135	—	0·31	14	
26	—	0·135	—	0·135	—	0·09	11	

*All measurements below the limit of sensitivity of the assay.
†Estimated by double-antibody radioimmunoassay, which improved sensitivity.

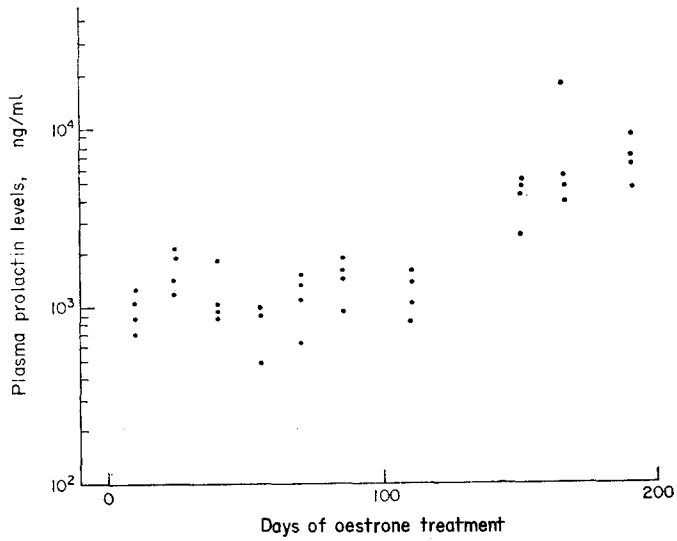

Fig. 1. *Prolactin levels in plasma of orchidectomized rats under continuous oestrone treatment (see text). Each dot represents the plasma prolactin level of an individual rat, assayed at three serial dilutions and extrapolated to three serial dilutions of the reference preparation assayed simultaneously. The values are expressed in ng equivalents of the reference preparation per ml of plasma. Untreated male rats, that are not sexually aroused, and untreated orchidectomized rats have plasma prolactin values below* 100 ng/ml

gland. The levels in the circulation have reached a mean value of 4300 ng/ml (range 2700–5500 ng/ml) on day 150. The plasma levels increased still further to a mean value of 7000 ng/ml on day 165 after the inititation of oestrone administration (range 4000–17,500 ng/ml). On day 190 the animals were autopsied and a last blood sample was taken during the ether narcosis before asphyxiating the animals. The mean value of the four plasma estimates was 7000 ng of prolactin per ml (range 4800–9600 ng/ml). All four pituitary tumours were used for implantation and could therefore not be weighed, but they all were estimated to be in excess of 200 mg.

Rats bearing transplants of oestrone-induced pituitary tumours

To ensure a more or less constant supply of starting material for the isolation of rat prolactin, tumour tissue was grafted at more or less regular intervals into a small number of hosts. The radioimmunoassays of plasma levels in rats bearing pituitary tumour grafts were performed on these rats of the regular 'tumour production line'. Out of the 8 groups of animals with such transplants, whereby the time after the time of of grafting of pituitary tissue and the time of taking the first blood sample varied from 2 to 21 weeks, the prolactin levels in the circulation was followed in two animals per group: at 3, 14 and 21 days after the last oestrone–cholesterol pellet implantation and before the next pellet was implanted. The results of this experiment are summarized in Table 2.

In contrast to the prolactin levels in the animals of which the pituitary gland *in situ* was stimulated by weekly oestrone–cholesterol pellet implantation and in which the assays disclosed a rather uniform response of the pituitary gland to the oestrone stimulation, the animals bearing pituitary tumour transplants show very large fluctuations at the three different times of taking blood samples, e.g. rat B. Although all grafted tumours appeared to respond to the pellet implantation by an increased outflow of prolactin, there seemed to be great individual variations. The majority of the grafts responded with a maximum outflow around 2 weeks after the implantation of the hormone. However, some responded with very large fluctuations; low levels at day 3, very high at day 14 and low again at day 21. Others reach fairly high levels already at day 3, increasing only little further on day 14, to have fallen to low levels again at day 21, e.g. rats G and rat H. Whereas rat C for instance showed high values on all three days of taking blood samples, and rat F responded late to the oestrone challenge having still low values at day 3, the plasma value for prolactin rose by day 14 to 2·65 that value and increased still further by day 21.

Table 2. Orchidectomized ♂♂ rats, R-Amsterdam, bearing oestrone-induced pituitary tumour transplants

Rat	At last oestrone-pellet implantation: estimate of tumour dia. (cm)	age of transplant (weeks)	Estimates of prolactin in μg/ml plasma at three intervals after last oestrone-pellet implantation: 3 days	14 days	21 days	At next oestrone-pellet implantation estimate of tumour dia. (cm)
A	0·5	21	4·7	11·2	4·0	0·7
B	0·4	21	2·6	44·5	2·0	0·5
C	0·6	17	7·1	10·3	9·4	0·8
D	0·8	17	9·6	42·6	10·2	1·1
E	—	14	1·5	2·9	—*	0·3
F	0·3	14	1·7	4·5	5·8	0·6
G	1·0	12	9·9	10·3	1·6	1·1
H	1·2	12	20·0	42·6	0·3	1·4
I	—	11	0·3	2·4	0·4	—
J	—	11	0·5	0·6	0·6	—
K	—†	9	2·4	28·1	6·8	1·0
L	—†	9	2·4	37·3	6·6	1·1
M	—	6	0·5	0·7	2·4	—
N	—	6	0·8	2·5	4·2	—
O	—	2	0·1	0·3	0·2	—
P	—	2	0·2	0·5	0·5	—

*Haemolytic plasma sample, not used for assay.

†Transplanted tumour may have been missed by first palpation, since the graft was situated unusually high up the calve-musculature.

Not only the prolactin output in response to the oestrone challenge varied, the rate of growth of the grafts showed great variations too. For instance the grafts in rats C and D attained a larger size than the grafts in rats A and B although the latter grafts were implanted a month earlier. The transplants, which attained the largest size at the time of blood sampling, had even been grafted 3 weeks later than the grafts in rats C and D.

At the time of collecting the pitutary tumour tissue for extraction of prolactin, i.e. when the grafts attained a size of 10–40 g, almost all rats were found to have developed one or more palpable mammary tumours.

DISCUSSION

When the same classification of pituitary glands is used as was applied in an earlier publication [13] 7 pituitary glands in the group of 25 old female rats of the present experiments were tumours, 10 were enlarged and 8 fell in the normal weight range.

An interesting observation was the close correlation between detecting elevated prolactin levels in the circulation and the presence of spontaneous pituitary tumours on the one hand and the occurrence of spontaneous mammary tumours on the other hand. Especially when the mammary tumours, which had developed already at the age of 24 months, are considered.

Kim, Clifton and Furth [14] noted that in their highly-inbred strain of Wistar rats spontaneous mammary fibroadenomata developed in around one-fourth of the females and they found an equally high incidence of spontaneous mammotropic pituitary tumours. In their group of rats, however, only 3 out of 20 animals with spontaneous tumours also had mammary tumours.

Long sustained oestrogen administration to either mice or rats may have definite effects on mammary tumorigenesis only in animals possessing a pituitary; again administration of oestrogens is an excellent means of inducing pituitary tumours in both these species [15]. The present experiments indicate that oestrone pellet administration has the effect that the rat pituitary secreted prolactin at very high rates. These high rates of secretion apparently were maintained for over 110 days without great fluctuations. At some time thereafter the prolactin levels in the circulation showed a further and more or less progressive increase, which probably was related to the progressive increase in weight of the pituitary noted to occur around this time in rats [13].

These findings suggested that it was the continuously maintained high secretory activity of the prolactin-producing cells, which after a certain period of time was followed by cellular multiplication of these cells. A similar pattern of prolactin secretory response to continuous administration of oestrone in mice was found (Kwa and Verhofstad, unpublished observations).

Already in 1936 Desclin and Grégoire [16] observed that the cells of ectopically transplanted pituitary glands showed signs of functional hyperactivity (e.g. enlarged Golgi rings, basophilic cytoplasm and larger size of the eosinophilic cells) only after rather large doses of oestrone. They concluded that this oestrogen acted directly and not necessarily via the central nervous system. Potvliege [17] described that the renal graft in rats when studied under the electron microscope showed a homogenization of the cellular population; the prolactin-producing cells forming the large majority. In general the observations of Rennels [18] were confirmed. After oestrogen stimulation, however, a considerable hypertrophy of the cytoplasm of the prolactin cells was noted, their ergastoplasm became an extremely complex structure with multiple 'Nebenkerne', suggestive of extreme cellular activity.

The finding in the present experiments that the prolactin-producing pituitary tumour graft only maintain high plasma levels of this hormone in response to a strong oestrogenic challenge is consistent with the morphological evidence cited. Hyperfunction, or at least such a hyperfunction that will maintain sustained high prolactin levels, is not primarily caused by implantation away from the restraining influence of the hypothalamus, as suggested by Everett [19, 20]. At least to induce such an excessive hyperfunction of the prolactin-producing cells that they will respond with cellular multiplication, a direct action of oestrogen at relatively high doses is needed in the rat: implanting pituitary tissue away from the restraining influence of the hypothalamus alone is not sufficient.

It is of interest to emphasize at this point an interesting difference in the regulation of prolactin secretion between rats and mice, which is pertinent to the correlation noticed between long sustained extremely elevated activity of prolactin-producing cells and unrestrained cellular multiplication of these cells. The work of Mühlbock and Boot [21] and more recently of Boot [22] has stressed the importance of prolactin in mammary tumorigenesis:

being effective in mice of low mammary cancer strains. Long sustained high levels of prolactin were obtained in these experiments by isologous implantation of pituitary glands in female mice away from the restraining influence of the hypothalamus. The conclusion of having achieved long sustained high levels of prolactin under these experimental conditions was based on the change of the cyclic pattern of the vaginal smears from the normal 4–5-day oestrous cycle into an uninterrupted sequence of so called pseudopregnancies. It should be pointed out that the isologous pituitary implant in mice show, after a more or less stationary phase, a progressive weight increase and that radioimmunoassay disclosed that prolactin levels were constantly very high during this stationary phase and thereafter increased progressively [23]. Thus the extrapolation made from the observation of a change of the pattern of vaginal smears to the conclusion of continuous hypersecretion of prolactin in mice is further substantiated by the results of radioimmunoassay and the unrestrained cellular multiplication of the secretory cells involved.

In rats, however, although isologous pituitary implantation changes the pattern of vaginal smears like it does in mice, radioimmunoassay failed to reveal continuously elevated levels (implanted under the renal capsule in intact female rats, the levels of prolactin in plasma were only periodically high, more or less like in pseudopregnancy in rats, Kwa and Verhofstad, unpublished results; administration of oestrone in drinking-water in a dose four times higher than was sufficient to restore vaginal cornification in ovariectomized rats was not sufficient to raise the prolactin levels in spayed rats bearing isografts, (Van der Gugten, Sala and Kwa, in preparation) *and* unlike the situation in mice, the implanted rat pituitary fails to go into unrestrained cellular multiplication. This difference between the two species is also reflected in the prolactin levels during pseudopregnancy, which in *mice* were found high throughout its duration [23], whereas in *rats* prolactin levels are high only during the first day or two after the last vaginal cornification, to decline and remain at near basal levels from the fourth to fifth day until the next pro-oestrus [24].

RESUME

Les taux de prolactine dans le plasma des rats ont été estimés par la méthode de radioimmunologie. Dans un groupe de 25 *rats femelles, âgés de* 2 *ans, une corrélation étroite a été observée entre, d'une part, la détection de taux élevés de prolactine dans la circulation et la présence de tumeurs dans l'hypophyse, et, d'autre part, la présence de tumeurs mammaires.*

Dans un groupe de 4 *mâles castrés, recevant une implantation d'oestrone toutes les semaines, les taux de prolactine augmentaient, en moins de* 10 *jours, d'une valeur à peine mesurable à une valeur dix fois plus élevée. Ce niveau élevé restait plus ou moins constant pendant les* 110 *premiers jours du traitement à l'oestrone. Après cette période, le niveau commençait à monter progressivement. Cette seconde augmentation coïncidait probablement avec le commencement d'une croissance progressive de l'hypophyse (qui commençait cette souche de rats pendant cette période d'hyperoestrogénisation).*

Des rats portant un greffon d'une hypophyse rendue tumorale par une hyperoestrogénisation prolongée ont reçu une implantation d'oestrone par mois. Les augmentations du taux de prolactine en réponse au stimulus oestrogénique montraient des variations aussi grandes que celles de la croissance des greffons. La majorité des greffons, néanmoins, répondait par une sécrétion maximale entraînant un taux plasmique élevé, approximativement 2 *semaines après l'implantation de l'oestrone.*

Ces données suggèrent qu'une hyperfonction extrême, qui peut maintenir des taux de prolactine élevés pendant des périodes de longue durée, n'est pas causée principalement par l'implantation dans un site éloigné de l'influence freinatrice de l'hypothalamus, mais est produite par des doses d'oestrogènes relativement hautes.

SUMMARY

In old female rats a close correlation was observed between detecting elevated prolactin levels in the circulation and the presence of spontaneous pituitary tumours on the one hand and mammary tumours on the other hand.

Weekly oestrone–cholesterol pellet implantation in a group of 4 *orchidectomized rats*

resulted in a rapid rise in prolactin plasma levels, indicative of very high secretion rates of this hormone by the pituitary gland as a consequence of oestrone stimulation. These high rates of secretion apparently were maintained for over 110 *days without great fluctuations. Between day* 110 *and day* 150 *a further and more or less progressive increase of plasma prolactin was found to occur, which probably was related to the progressive increase in weight of the pituitary occurring around this time in this strain of rats under this regimen.*

Rats bearing transplants of oestrone-induced pituitary tumours and in which one oestrone-cholesterol pellet was implanted monthly showed great variations in prolactin output in response to the oestrone stimulus as well as great variations in the rate of growth of the grafts. The majority of the grafts, however, responded with a maximum outflow of prolactin reflected in high plasma values around 2 weeks after implantation of the oestrone.

The findings suggested that an extreme hyperfunction that will maintain high prolactin levels over long periods of time was not primarily caused by implantation away from the restraining influence of the hypothalamus, but was consequent to relatively high doses of oestrogen.

ZUSAMMENFASSUNG

Mit Hilfe der Radioimmunomethode wurde der Prolactingehalt von Rattenblutplasma bestimmt. Bei alten Weibchen wurde eine grosse Übereinstimmung zwischen dem erhöhten Prolactinspiegel im Blut und dem Vorhandensein von spontan entwickelten Hypophysentumoren einerseits und Brustgeschwülsten andererseits, festgestellt.

In einer Gruppe von 4 kastrierten Männchen, die jede Woche eine Oestron–cholesteroltablette subkutan implantiert bekamen stieg der Prolactingehalt im Plasma rasch an; das deutet auf eine durch Oestron sehr stark stimulierte Sekretion dieses Hormons durch die Hypophyse hin. Der Plasmaprolactingehalt blieb, abgesehen von kleinen Schwankungen, für ungefähr 110 *Tage konstant erhöht. Danach wurde eine weitere Steigerung festgestellt, welche wahrscheinlich mit einer vorher beobachteten Zunahme des Hypophysengewichtes zu diesem Zeitpunkt der Oestronbehandlung zusammenhängt.*

Bei Ratten, die eine erste Passage der von Oestron induzierten Hypophysentumoren erhalten hatten und jeden Monat eine Oestrontablette bekamen, variierte sowohl der Plasmaprolactingehalt als auch die Wachstumsgeschwindigkeit des Implantats. Die meisten Transplantate jedoch reagierten, den hohen Plasmawerten gemäss, zwei Wochen nach der Oestrongabe mit einer maximalen Prolactinausschüttung.

Die Resultate weisen darauf hin, dass eine extreme Hyperfunktion der Hypophyse und der dadurch bedingte hohe Prolactinspiegel, nicht primär durch die Implantation, ausserhalb der bremsenden Wirkung des Hypothalamus, verursacht wird, sondern eine Konsequenz der relativ hohen Oestrogendosen ist.

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Changes Occurring During the Growth of Ehrlich Ascites Cells *in vivo*

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INTRODUCTION

THE GROWTH characteristics of Ehrlich ascites tumour cells change as the tumour proliferates *in vivo*. In particular, there is a progressive lengthening of cell cycle time as the number of tumour cells in the peritoneal cavity increases and the later stages of tumour growth are associated with a fall in the growth fraction [1–4].

It is probable that other differences between early and late ascites tumour cells will occur as a consequence of the variation in cell growth kinetics. There is general information available about the relationship of DNA content to protein and histone content of individual Ehrlich ascites cells but the observations, so far, have been restricted to samples taken from tumours whose phase of growth was not well defined [5–7].

In this investigation the growth kinetics of tumour cells, 2 and 13 days after transplantation, were determined. The compositions of the two populations were also compared by studying the distribution of cell size, protein content and chromosome number; and by analysing the distribution of cells within the various phases of the cell cycle. The ability of slowly cycling tumour cells, present in the later stages of tumour growth, to resume rapid cyclic activity on transplantation was also examined.

MATERIALS AND METHODS

The Ehrlich ascites tumour was transplanted routinely, approximately every 10 days, into male A mice maintained on Oxo 41B diet and water *ad libitum*. Five million cells were transplanted; the donors' ascitic fluid was diluted when necessary to produce a suitable cell concentration with warm, sterile 0·9% saline.

Size distribution analyses were carried out at room temperature on cells suspended in Hanks' solution within 20 min of sampling using the Coulter Counter model B (Coulter Electronics, Florida, U.S.A.). Absolute cell numbers in the size range covered by 4 instrument units (equivalent to a spherical particle diameter of approximately 0·65 μ) were counted in ½ml samples throughout the size range of the cells. The number of cells in each 4 unit window was expressed as a percentage of the toal cells in the ½ml sample. The instrument was calibrated with a suspension of paper mulberry pollen (dia. 13·5μ) in 0·9% sodium chloride.

Autoradiography and quantitative cytochemical techniques

Radioactive thymidine, (^{3}H-TdR) (Radiochemical Centre, Amersham, England) specific activity 5C/mM, was diluted with isotonic saline to 100μC/ml and used in the amounts detailed in each experiment.

Cells were smeared on clean slides immediately after removal from the peritoneal cavity, were air-dried and then fixed in methanol for 10 min. Smears were stained for DNA by the Feulgen method: a 10 min hydrolysis in 1N HCl at 60°C gave maximal staining of interphase cells. Naphthol yellow S staining for total protein was used in conjunction with Feulgen staining [8]. Light absorption by the Feulgen and naphthol yellow S dyes were measured at 540nm and 435nm respectively, using an integrating microdensitometer (Barr & Stroud, Glasgow, Scotland).

Smears were dipped in Ilford K5 nuclear emulsion, stored at 4°C for 2–4 weeks and then developed in Kodak D19b developer. The use of a photographic map of the cells enabled DNA and protein content and the incorporation of ^{3}H-TdR to be estimated successively for individual cells [9]. Two thousand cells were counted for the determination of the percentage of cells labelled and 100 mitoses were examined to determine the percentage of labelled mitoses.

Chromosome analysis

Mice were injected with colchicine (1 mg/kg body weight) 1 hr before they were killed and the tumour cells

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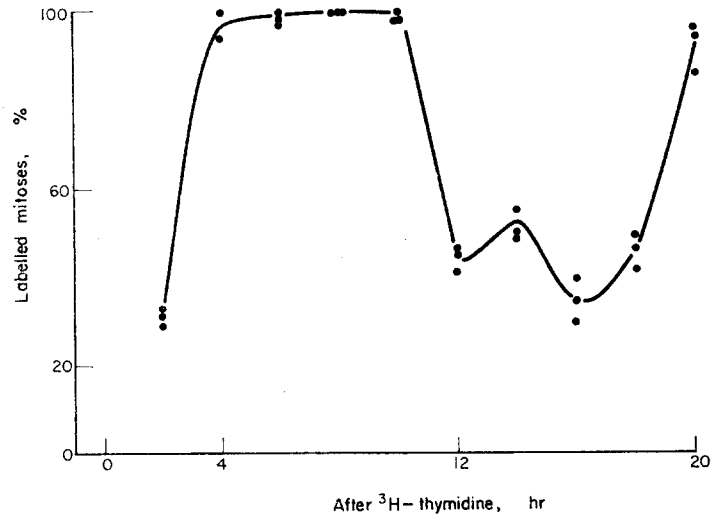

Fig. 1. *Percentage labelled mitoses curve of a* 2*-day Ehrlich ascites tumour.*

removed, or 0·1 ml of ascitic fluid was incubated for 1 hr at 37°C in Hanks' solution containing 1μg of colchicine/ml, before preparing chromosomes by the method of Moorhead *et al.* [10].

RESULTS

Kinetic analysis

A group of 60 mice, with 2-day old tumours was given 0·5μC/g body weight of ^{3}H-TdR and subsequently 3 mice were killed at 2-hr intervals for 20 hr. The proliferation of the cells was studied by the method of the percentage labelled mitoses curve (% LM curve) [11]. The results of this experiment are shown in Fig. 1. Other mice from this group were given colchicine at various times after the ^{3}H-TdR injection and chromosome spreads were then prepared, and the distribution of labelling over individual chromatids was detected by autoradiography.

Three mice with 13-day old tumours were given 1μC/g body weight of ^{3}H-TdR and a small sample of ascitic fluid was removed from each mouse every 4 hr. Part of the sample was used to obtain the % LM curve shown in Fig. 2, and the remainder used to observe chromatid labelling.

The results of analysing these two kinetic experiments are summarised in Table 1. In the 2-day old tumour, the mean cell cycle time was 16 hr and of this, about 9 hr was spent in S. In the 13-day old tumour the mean cell cycle time could not be estimated with accuracy because the %LM curve did not reach a second peak, but a rough estimate of mean cell cycle time suggests a value not less than 80 hr. These results confirm that in the 13-day old tumour G_2 and S periods are much longer than in the 2 day old population.

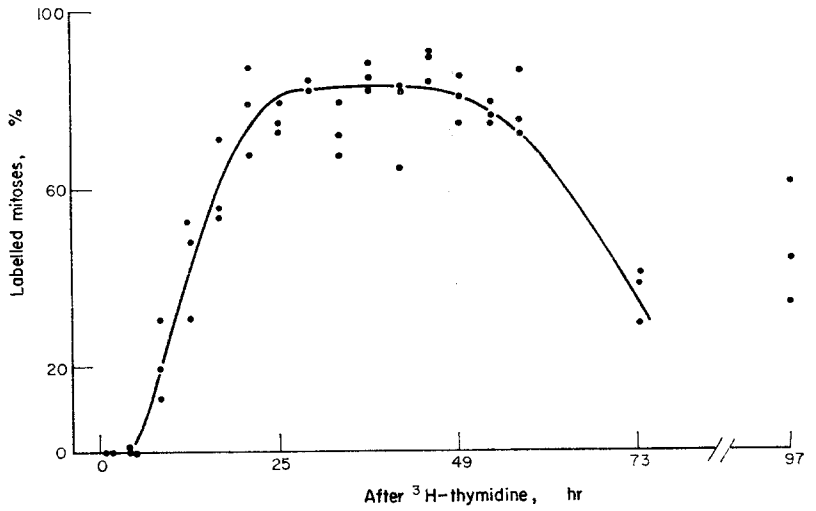

Fig. 2. *Percentage labelled mitoses curve of a* 13*-day Ehrlich ascites tumour.*

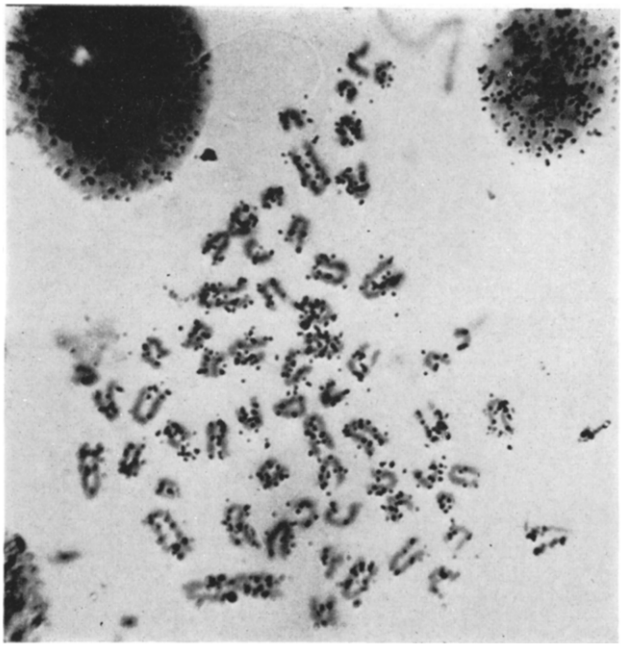

Fig. 3. *Metaphase spread prepared* 10 *hr after* ^{3}*H-TdR injection to a* 2*-day old tumour, showing label over both chromatids of each chromosome.* × 1250.

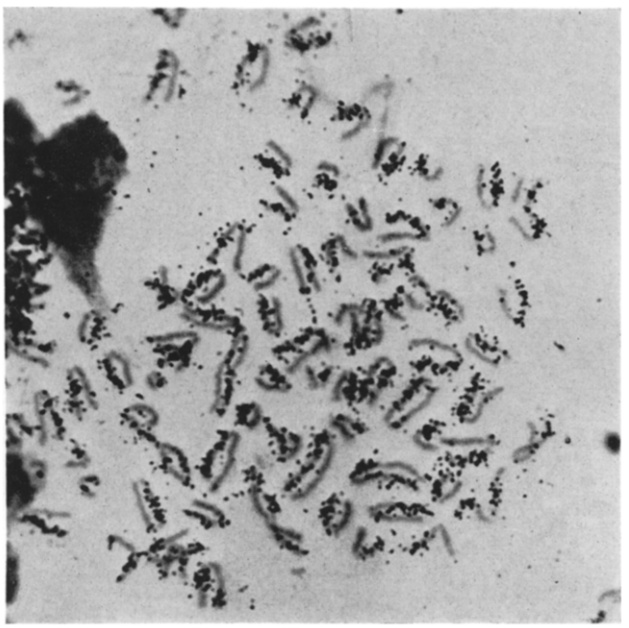

Fig. 4. *Metaphase spread prepared* 20 *hr after* ^{3}*H-TdR injection to a* 2*-day old tumour, showing label over one chromatid of each chromosome* × 1250.

(to face p. 582)

Table 1. *Results from the percentage labelled mitoses curves for tumours* 2 *and* 13 *days after transplantation*

Cell cycle phases (hr)	2-day tumour	13-day tumour
$G_2+\frac{1}{2}m$	2·5	14
S	9	52
$G_1+\frac{1}{2}m$	4·5	14*
Cell cycle time	16	80*
Initial labelling index	60%	30%

*Rough estimates because the % labelled mitoses curve, Fig. 2, did not reach a second peak.

On the second day of tumour growth 100 chromsome spreads, prepared 10–12 hr after ^{3}H-TdR injection, all had label over both chromatids, indicating that the tumour cells were in mitosis for the first time since the ^{3}H-TdR injection (Fig. 3). On the other hand 100 spreads prepared 18–20 hr after ^{3}H-TdR, all had label over only one chromatid of each chromosome showing that these metaphases were all daughter cells of the first division (Fig. 4). Hence, there must be a relatively narrow distribution of cell cycle time in the 2-day old tumour population. However, of 115 chromosome spreads prepared 72 hr after injection of ^{3}H-TdR to a 13-day old tumour, 110 had label over both chromatids whilst 5 had label over only one chromatid of each chromosome. These 5 hemi-labelled metaphases had varied total chromosomes number—115, 81, 54, 60 and 71. The results indicate that in the 13-day old tumour the majority of cells had a truly long cell cycle time, although a heterogeneous minor population of fast dividing cells was present.

Table 2. *Distribution of cells in the main compartments of interphase, determined after a single injection of* ^{3}*H-TdR*

Tumour age	% Cells in G_1	% Cells in S	% Cells in G_2
Day 2	19	60	21
Day 13	58	20	22

The distribution of tumour cell chromosome number was examined 2 and 13 days after transplantation (Fig. 5), but no significant disturbance in the balance of the population was detected.

Distribution of cells within the phases of the cell cycle

Table 2 shows the distribution of the cells in the main compartments of interphase, as determined by microdensitometric measurements of Feulgen stained nuclei followed by autoradiography to identify the cells in S. In the 13-day tumour, the number of cells in DNA synthesis was decreased and there was a build up of cells with post-mitotic, pre-DNA synthetic, DNA values. These cells have been assigned to the G_1 compartment, although it is recognised that some may be out of cycle, i.e. G_0 cells. Despite the wide variation in chromosome number the range of DNA values for cells at the G_1 mode was comparatively small. (G_1 tumour cells 65–85 and normal peritoneal cells 29–34 arbitrary units).

A further comparison of these two populations of tumour cells was made by studying the proportion of cells labelled after multiple injections of ^{3}H-TdR. Four 6-hr injections of

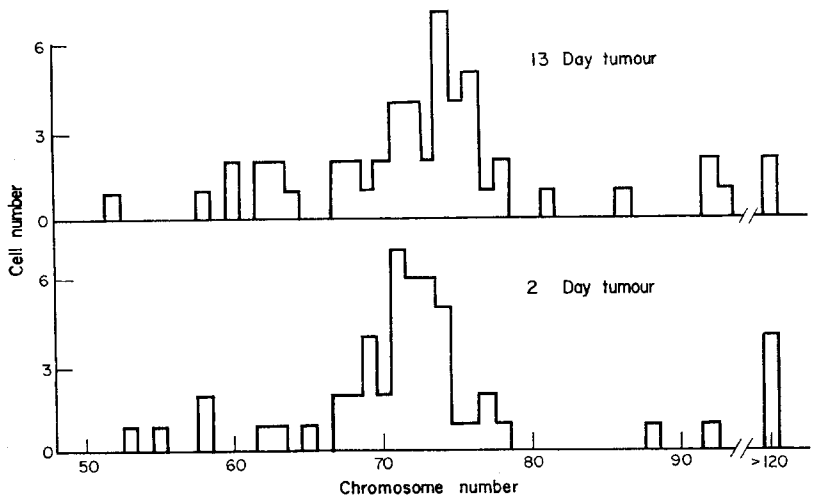

Fig. 5. *Distribution of chromosome number of Ehrlich ascites tumour* 2 *and* 13 *days after transplantation.*

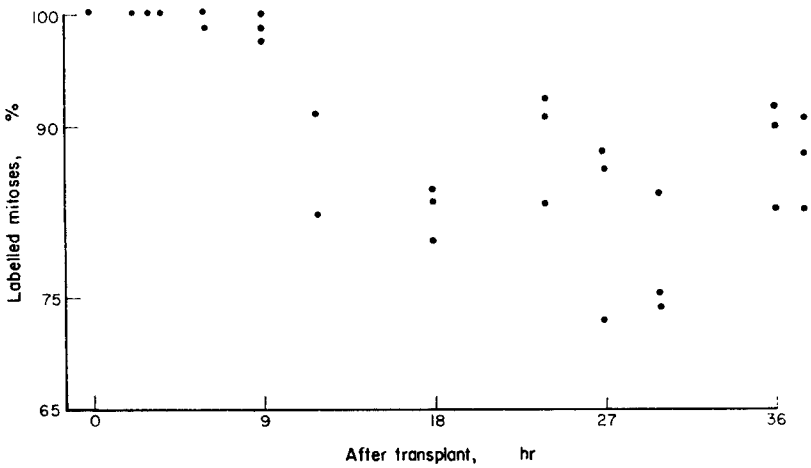

Fig. 6. The results of transplanting tumour cells from a mouse given multiple injections of ^{3}H-TdR between days 13 and 15 of tumour growth and killing the recipients at various times after transplantation.

^{3}H-TdR given to 4 mice between days 2 and 3 of tumour growth labelled 95–100% of the cells, indicating that virtually all the cells were in cycle. However, six 8-hr injections of ^{3}H-TdR given to 4 mice between days 13 and 15 of tumour growth labelled only 49–80% of the cells. Microdensitometric measurements of the DNA content of these unlabelled cells at 15 days showed that the majority of them had G_1 DNA values, and there was no appreciable build up in G_2 or arrest in S. To examine whether this unlabelled portion of the tumour cell population was capable of fast cyclic division, cells from a 15-day old tumour which had received 6 8-hr injections of ^{3}H-TdR were transplanted to new hosts. The recipients were killed at 3-hr intervals and smears of tumour cells were prepared to study the labelling pattern of the mitoses. Figure 6 shows that immediately after transplantation all the mitoses were from the labelled cohort of the population but by 12 hr, the initially unlabelled cells appeared as mitoses. The autoradiographs were exposed for a long time (2 months) to eliminate the possibility that the appearance of unlabelled mitoses at this time was due to apparent loss of label as a result of repeated cell division. A comparable phenomenon was observed in mice with 15-day old tumours whose tumour mass was rapidly reduced by aspirating the ascites. Figure 7 shows the rapidity with which the initially unlabelled cells appeared as mitoses after aspirating the ascites (15–18 ml) from 3 mice on the fifteenth day of tumour growth. Microdensitometric measurement of Feulgen stained, unlabelled mitoses proved that these were tumour cells and not normal peritoneal cells in mitosis.

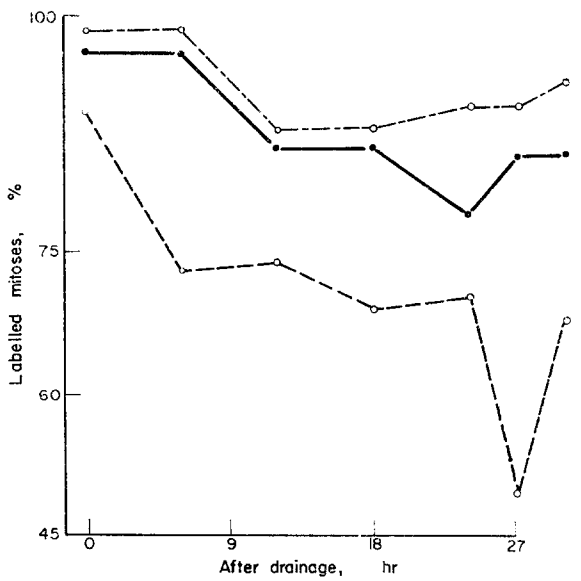

Fig. 7. The appearance of unlabelled mitoses is shown after draining the ascites from three mice given multiple injections of ^{3}H-TdR between days 13 and 15 of tumour growth.

Size and protein content of tumour cells

Coulter counter analysis of the size distribution of 2- and 13-day old tumour cells showed that 13 days after transplantation the modal size of the cells had increased and there was a significant rise in the proportion of large cells in the population (Fig. 8). On transplantation the modal cell size diminished, reaching its minimum value on the second day. This change in size was not due to the higher proportion of peritoneal cells mixed with the 2-day tumour population because adjusting the tumour/peritoneal cell ratio of the 13-day tumour population *in vitro* to that of the *in vivo* 2-day population did not decrease the modal tumour cell size.

Sequential measurements of DNA and pro-

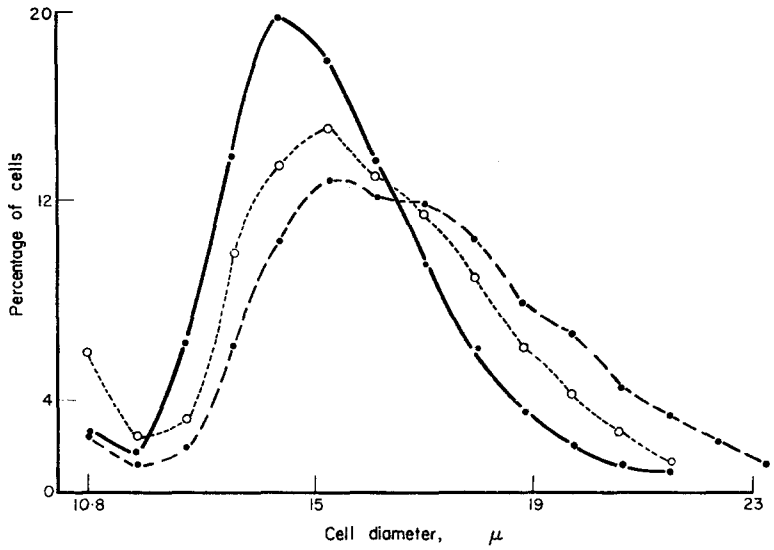

Fig. 8. *Size distribution of Ehrlich ascites tumour cells: . . . . one, —— two and - - - - 13 days after transplantation.*

Table 3. *Cell protein content*

Tumour age	Mean Protein Content (arbitrary units) G_1 cells	G_2 cells
Day 2	23±8	36±11
Day 13	32±11	51±13
Student *t*-test	$p<0{\cdot}001$	$p<0{\cdot}001$

tein contents of individual tumour cells, followed by autoradiography to identify the cells in S, showed that this increase in tumour cell size in the older tumours was associated with a significant increase in the mean protein content of both G_1 and G_2 cells (Table 3). The correlation of individual cell protein and DNA content in a 13-day tumour is shown in Fig. 9. There is considerable variation in protein content of cells at the G_1 mode but cells in S or G_2 have protein values above a certain level. For the 2-day tumour, the shape of the DNA/protein graph is similar, but the protein contents of the G_1, S and G_2 cells are less than those of the corresponding 13-day tumour cells.

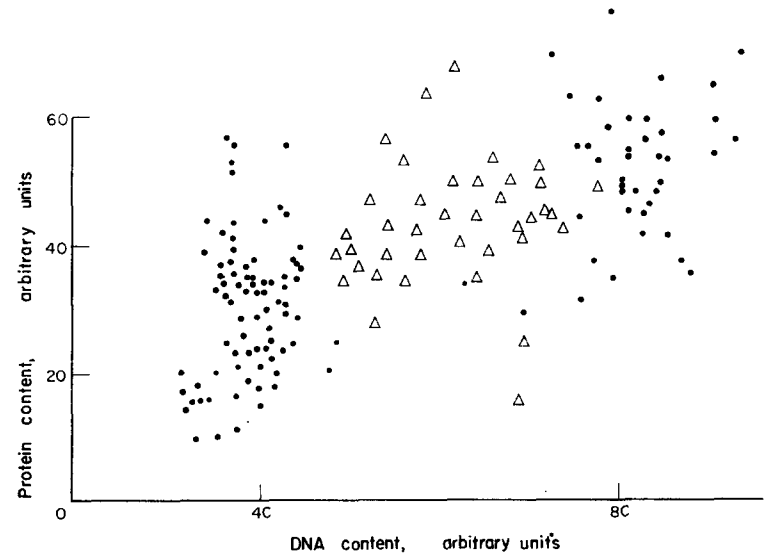

Fig. 9. *Correlation of individual cell DNA and total protein content for tumour cells* 13 *days after transplanation.*
● *cells not labelled with* ^3H*-TdR*
△ *cells labelled with* ^3H*-TdR*

Discussion

This study of Ehrlich ascites tumour cells confirms that as the tumour ages there is an increase in cell cycle time and, in particular, an increase in the S period [1, 3, 4]. However, Ehrlich ascites tumours in various laboratories differ; for example, in this study the G_1 period at 2 days was 4 hr, whereas Lala and Patt [1] report that in their tumour there was no appreciable G_1 period.

Mathematical analyses of %LM curves have been used to assess the variation in cell cycle time occurring within the population [4, 12] but if the %LM curve does not allow an accurate estimate of cell cycle time to be made, the situation is too complex for an accurate analysis.

In these experiments, where the distribution of radioactivity over individual chromatids at various times after injection of ^{3}H-TdR was examined, it was shown that despite the intrinsic heterogeneity of the tumour in terms of chromosome number, there was a narrow range of cell cycle times in the 2-day tumour population. This method also confirmed that the long cell cycle time inferred from the %LM curve for a 13-day tumour was correct for the majority of cells in the population. Furthermore, it was demonstrated unequivocally that in the 13-day tumour a minor fraction (5/110) of the cells was in mitosis for the second time 72 hr after the injection of ^{3}H-TdR. As the chromosome distribution did not differ in the 2- and 13-day old tumours and the rapidly cycling cells in the 13-day tumour had various chromosome numbers, there is no evidence that the changes in cell cycle time as the tumour ages are associated with the selection of cells of a particular genotype more suited to slow proliferation.

The reason for the increase in cell cycle time as ascites tumours age remains obscure. Although in large ascites tumours the cells must be anoxic for a large proportion of time [13] the cells must occasionally be adjacent to a capillary bed. In solid tumours such an intermittency in the availability of oxygen probably does not occur and it is of interest that ageing in solid tumours is associated with a decrease in the growth fraction rather than a large increase in cell cycle time [4, 14]. Whether the intermittent nature of oxygen availability is involved in the increased cell cycle time characterising the later stages of ascites tumours has been considered [4]. It would be of interest to study the effects of intermittent oxygenation on cells growing in tissue culture, to see if the kinetic changes characteristic of ascites cells occurred and, conversely, to try to improve the oxygenation of ascites tumours to see if this would decrease the cell cycle times.

It was confirmed that most cells unlabelled after repeated ^{3}H-TdR injections between days 13 and 15 of growth had G_1 DNA contents. The effect of aspiration or transplantation of such tumours was studied and the appearance of the unlabelled cells as mitotic figures 6–12 hr later, showed that cells which had not synthesised DNA for 48 hr were capable of progressing to mitosis. Most of these cells must have synthesised DNA rapidly following reduction of tumour cell mass by aspiration or transplantation, because most of the cells unlabelled after multiple ^{3}H-TdR injections had G_1 DNA values. However, it is possible that the early appearance of unlabelled mitoses in the case of one mouse (Fig. 7) may be due to cells which had G_2 periods longer than 48 hr, rather than cells with S and G_2 periods less than 6 hr. An increase in the number of cells engaged in DNA synthesis has been reported to occur immediately after transplantation [3] but this was not observed in other studies until 4 hr after transplantation [15]. The difference between these reports may reflect differences in techniques or tumours. Lala and Patt [1, 2] inferred that as their tumour aged, the increased growth fraction was made up of G_0 cells with G_1 DNA contents (they were unable to detect an appreciable G_1 period). They showed that re-cycling after transplantation was not due to cells arrested in S or blocked in G_2 resuming cycling.

Analysis of the size distribution of tumour cells showed that as the tumour aged, modal cell size increased and the distribution was skewed to the right. This change was not associated with an increased proportion of higher ploidy or G_2 cells nor did it reflect the lower proportion of peritoneal cells in the later tumour. It was, however, associated with an increase in mean protein content of the G_1 and G_2 cells in the 13-day tumour.

Although the protein/DNA relationship of individual Ehrlich ascites cells has been described as linear [6, 16] our results suggest that the G_1 cells have a wide range of protein contents and those cells that begin to synthesise DNA only do so above a certain protein level. These observations may be compared with those on mouse fibroblasts grown in tissue culture, from which it was suggested that a critical mass had to be reached before DNA synthesis was initated [17].

Protein synthesis occurs in all Ehrlich

ascites cells but the rates of protein synthesis vary from cell to cell and decrease as the tumour ages [15]. If there is a critical protein content in the 2-day tumour for the initiation of DNA synthesis, then these results suggest that this absolute protein value does not trigger off the 13-day cells into S; instead, protein synthesis continues, size increases and only at a new protein level do the cells enter S. In tumours in which the G_1 period is inappreciable, a straight line DNA/protein relationship would be expected because there is little or no time for varying amounts of protein to be synthesised. No wide scatter in protein content of cells with G_1 DNA values, such as those seen in this study or in dog venereal sarcoma cells [18] would, therefore, be observed.

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RESUME

L'étude cinétique des cellules de la tumeur ascitique d'Ehrlich, faite 2 et 13 jours après la transplantation, montra que la durée moyenne du cycle cellulaire était augmentée après 13 jours, et que la période de synthèse de l'ADN (S) était extrêmement longue. L'étude des chromatines, après injection de thymidine marquée au tritium, montra qu'il y avait peu de variations des temps du cycle cellulaire dans la population de 2 jours, alors que dans celle de 13 jours, la tumeur présentait un nombre réduit de cellules à cycle rapide qui avaient un nombre chromsomial différent. La distribution des nombres chromosomiaux était identique dans les deux populations, ce qui prouve que les modifications cinétiques n'étaient pas dues à la sélection de cellules ayant une ploïdie particulière.

L'observation microdensitométrique des cellules colorées d'après Feulgen, suivie d'une autoradiographie afin d'identifier les cellules en S, a montré que, dans la tumeur de 13 jours, la diminution du nombre des cellules en S était accompagnée d'une augmentation du nombre de cellules dont le contenu en ADN correspond à l'état G_1. Les injections successives de thymidine tritiée, faites aux souris portant une tumeur de 2 jours, marquèrent toutes les cellules de la tumeur, alors que pour le même traitement, appliqué aux souris portant une tumeur de 13 jours, la proportion des cellules non marquées varia de 20 à 51%. La plupart des cellules non marquées possédaient un contenu en ADN correspondant à l'état G_1, et l'on a pu constater qu'elles étaient capables de se diviser rapidement, après réduction de la masse tumorale, soit par transplantation, soit par aspiration.

L'analyse, au compteur Coulter, de la distribution des cellules en fonction de leur dimension a montré que plus la tumeur vieillissait, plus la dimension modale des cellules augmentait, et plus la proportion des cellules plus grandes était élevée. Ceci allait de pair avec une augmentation du contenu moyen en protéines des cellules, liée à l'âge de la tumeur, ce contenu en protéines ayant été déterminé par l'étude microdensitométrique des cellules colorées au naphtol jaune. Cette corrélation entre le contenu en ADN et le contenu en protéines suggérait que toutes les cellules avaient un contenu en protéines important, et la possibilité qu'il faille atteindre un certain contenu minimum en protéines, avant que ne commence la synthèse de l'ADN, est discutée.

SUMMARY

Kinetic studies of Ehrlich ascites tumour cells 2 and 13 days after transplantation showed that at 13 days mean cell cycle time was increased and there was an extremely long DNA synthetic period (S). Examination of the distribution of label over chromatids at various times after injection of tritiated thymidine (^{3}H-TdR) showed that in the 2 day population there was little variation in cell cycle time but in the 13-day tumour there was a minor fraction of fast cycling cells which had different chromosome number. Distribution of chromosome number in the two populations was the same and so the kinetic changes were not the result of selection of cells of particular ploidy.

Microdensitometric measurements of Feulgen stained cells followed by autoradiography to identify cells in S, showed that in the later tumour the decrease in cells in S was accompanied by an increase in cells with G_1 DNA values. Repeated injections of ^{3}H-TdR to mice with 2-day old tumours labelled all the tumour cells but, when given to mice with 13-day old tumours did not label 20–51% of the tumour cells. Most of the cells which

were unlabelled after multiple injections of ^{3}H-TdR had G_1 DNA contents and their ability to divide quickly once the tumour mass was reduced by transplantation or aspiration was demonstrated.

Analysis of the distribution of cell size, using the Coulter counter, showed that as the tumour aged there was an increase in modal cell size and there were more larger cells. This was correlated with an increase in the mean protein content of cells as the tumour aged, as determined by microdensitometric measurements of cells stained with naphthol yellow S. The correlation of individual cell DNA and protein contents suggested a wide range of protein contents for all cells and the possibility of a threshold content which must be reached before DNA synthesis begins is discussed.

ZUSAMMENFASSUNG

Kinetische Studien an Ehrlich Ascites Tumorzellen, 2 und 13 Tage nach der Transplantation, zeigten, dass die durchschnittliche Generationszeit nach 13 Tagen erhöht war und dass dabei eine extrem lange DNS-Syntheseperiode (S) bestand. Die Markierung der Chromatiden, nach, zu verschiedenen Zeiten appliziertem, 3H-Thymidin, lässt in der 2 Tage alten Population auf eine geringe Variationsbreite in der Cyclusdauer schliessen. Im 13 Tage alten Tumor jedoch fand sich eine kleine Fraktion rasch proliferierender Zellen, die sich durch veränderte Chromosomenzahlen unterschieden. Die Verteilung der Chromosomenzahlen war in den beiden Zellpopulationen gleich, und so konnten die kinetischen Veränderungen nicht das Ergebnis einer Selektion von Zellen spezieller Ploidie sein. Mikrodensimetrische Messungen an Feulgen gefärbten Zellen mit nachfolgender Autoradiographie identifizierten die Zellen in S-Phase und zeigten,dass die Verringerung der Zellen in S-Phase beim älteren Tumor begleitet war von einer Zunahme der Zellen mit den G_1 entsprechenden DNS-Werten. Wiederholte Injektionen von 3H Thymidin in Mäuse mit 2 Tage altem Tumor markierten alle Tumorzellen. Bei der gleichen Behandlung blieben jedoch beim 13 Tage alten Tumor 20 bis 51 Prozent der Zellen unmarkiert. Die meisten dieser unmarkierten Zellen hatten den G_1 entsprechenden DNS-Gehalt und ihre Fähigkeit zur schnellen Teilung konnte bei Verminderung der Tumormenge nach Transplantation oder Punktion demonstriert werden. Analysen der Verteilung der Zellgrössen mit dem Coulter Counter zeigten generell eine mit dem Alter des Tumors zunehmende Zellgrösse. Diese war mit einer Zunahme des durchschnittlichen Proteingehalts der Zellem im älteren Tumor verbunden, was durch mikrodensimetrische Messungen nach Färbung der Zellen mit Naphtholgelb S festzustellen war. Dieser Zusammenhang zwischen individueller Zell-DNS und Proteingehalt lässt einen weiten Bereich im Proteingehalt aller Zellen vermuten und die Möglichkeit eines Schwellenwerts, der erreicht sein muss vor Beginn der DNS-Synthese, wird diskutiert.

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Growth of Tumour Cells in Diffusion Chambers on the Chick Chorioallantois

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INTRODUCTION

SINCE the original demonstration by Murphy [1] that the chick chorio-allantoic membrane (CAM) supports the growth of heterologous tumours, a considerable number of studies [2] have shown that this system has a number of potential advantages over growing tumour cells in tissue culture or transplanting tumours to 'conditioned' rodents. One advantage is that the developing chick embryo is an enclosed stable biological system in which there are optimal conditions of sterility, temperature and nutritive supply. A second, and perhaps more important advantage is the inability of the embryo to mount an immune reaction against transplanted heterologous tumour cells. Thirdly, it is also possible to grow tissues of different origins on the same CAM.

Despite these advantages the results of transplanting heterologous tumours to the CAM have been disappointing [3]. One reason is the small spectrum of tumours which can be successfully propagated, and this applies particularly to human tumours. A further complication is the individual variation in growth rate of the tumours. This particular difficulty can be partially overcome if tumour cells are kept within the confines of plastic grids placed on the CAM [4]. While this technique produced favourable results with Walker 256 ascites cells, in further studies only poor or patchy growth could be obtained with a number of murine ascites tumour cells and L1210 leukaemia [5].

However, excellent growth of various normal tissues and isolated cells has recently been obtained in diffusion chambers implanted on the CAM [6, 7]. These chambers gradually fill with nutritive fluid which diffuses through their Millipore filter bases from the underlying extra-embryonic regions. Presumably the diffusion can operate in both directions thereby reducing the amount of toxic products accumulating in the chamber. Such fluid exchange might be considered analogous to continuous perfusion.

In the present study, we have examined the suitability of this technique for growing various murine tumour cells and the ability of the cultured cells to produce tumours when transplanted back into mice.

MATERIAL AND METHODS

Tumours

Lymphoid leukaemia L1210, originally obtained from the Chester Beatty Research Institute, was maintained by weekly transfer of spleen cells in BDF_1 ($C57Bl \times DBA/2$) mice. Cells obtained from the spleen of a leukaemic animal were suspended in 100 ml medium 199 and 0·1 ml of this supension (about 4×10^5 cells) was injected subcutaneously into each new host.

Leukaemic cells for cultivation in diffusion chambers on the CAM were obtained from

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spleens of mice that had been injected with L1210 cells 7 days previously.

Crocker sarcoma (S180), Ehrlich carcinoma and benzpyrene-induced sarcoma BP8/P1, originating from the tumour bank of the Imperial Cancer Research Fund, were carried in ascitic form. The Ehrlich and S180 tumours were maintained in random bred SN (Schneider) mice and the BP8 tumour in C_3H/He mice by weekly intraperitoneal injection of 0·2 ml ascitic fluid into each new host.

Ascites cells for cultivation in diffusion chambers were aspirated from mice which had been injected with tumour cells 5 or 6 days previously.

In experiments where cell doubling times in diffusion chambers were estimated, leukaemia L1210 and the three ascites tumours were transplanted to BALB/C strain mice and were transferred to diffusion chambers 6 days after implantation.

Diffusion chamber technique

Chambers were constructed by cementing Millipore nylon-reinforced (type TW) filter discs to one side of Plexiglass diffusion chamber rings (14 mm dia.) with Millipore MF cement. Known numbers of tumour cells in volumes of 0·05 ml medium 199 were carefully placed on the floor of each chamber which was immediately transferred to the CAM of a host embryo. Chambers were left open and, since they gradually fill with nutritive fluid from host embryos, no further culture medium was added.

The CAMS of eggs of 7 or 8 days incubation were separated from the shell membranes using a technique described previously [8]. Each chamber was carefully placed on to the CAM through a circular hole cut in the shell. Finally, the holes were sealed by waxing around a circular cover-slip and the eggs were incubated in a horizontal position with the diffusion chambers lying on the 'dropped' CAM (Fig. 1).

Implantation into mice of tumour cells from diffusion chambers

In order to determine whether cultivation in diffusion chambers affects the ability of L1210 leukaemia cells to proliferate in and kill mice, known numbers of cells which had been cultured for 3 days were injected subcutaneously into mice of histocompatible (BDF_1 and DBA/2) and allogeneic (C_3H/He, C57B1 and A strong) strains. The survival times of these animals were compared with those of the same strains injected with similar numbers of leukaemic cells taken directly from BDF_1 mouse spleens.

In the case of ascitic tumours, 0·2 ml of a suspension of cells which had been cultured for 3 days in diffusion chambers was injected intraperitoneally into the strains of mice used for routine maintenance of tumours. The ability of these cultured cells to kill mice was compared with that of tumour cells in 0·2 ml ascitic fluid removed directly from tumour-bearing mice.

Cell counts

The total cell number in diffusion chambers at various intervals was obtained by the following method. Each chamber was removed from the CAM and, after removing adhering chick cells, the chamber and its contents were immersed in 0·5% Pronase solution (Calbiochem) in saline so as to give a total volume of 1 ml. The solution was agitated at room temperature for 60 min after which a cell count was made in a haemocytometer chamber under phase contrast. Cell viability was checked by the trypan blue exclusion test [9]. Pronase was found to disperse cells sufficiently from the surface of the Millipore filter and from each other to allow accurate cell counts.

Cytological methods

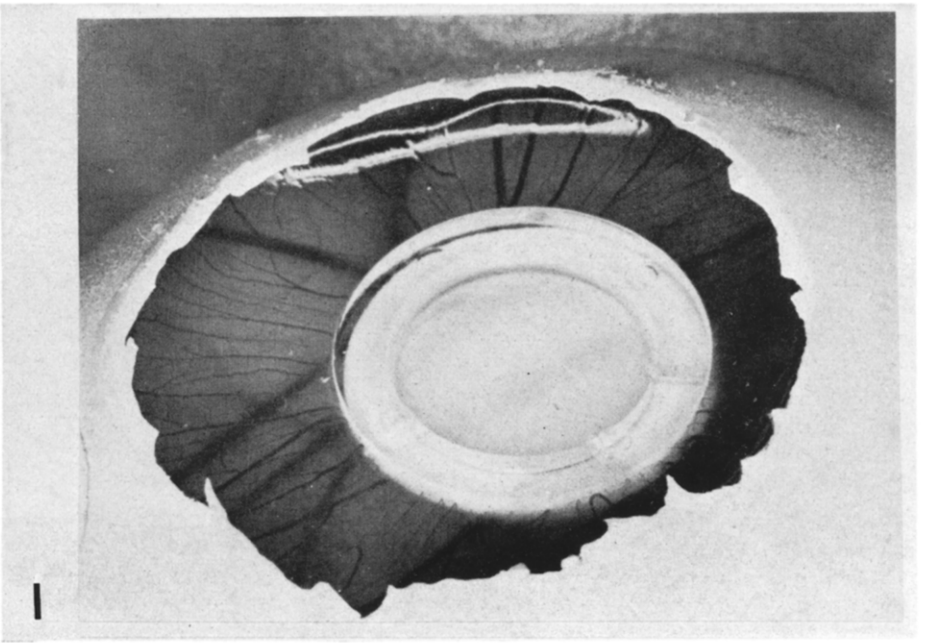
Cell smears were made from the contents of chambers at various stages and stained with Wright's stain. In addition, cells were stained *in situ* on filters in the same way, and the filters were then dehydrated in methyl alcohol and mounted in Euparal. In a few cases, in order to improve cytological detail cells were stained in diffusion chambers constructed with Millipore ultra-thin (type TH) filters.

Chromosome analysis was made on suspensions of L1210 leukaemia cells which had been cultured in diffusion chambers for 3 days. Five micrograms of Colcemid (Ciba) was added to the chambers 3 hr before harvesting the cells. The cell suspension was then processed for chromosome analysis using a hypotonic citrate, acetic–alcohol fixation, air-drying method based on that of Ford [10].

RESULTS

Proliferation of L1210 *leukaemia cells in diffusion chambers*

The results of cell counts made at daily intervals on the contents of diffusion chambers 'seeded' with 100,000 viable L1210 leukaemia cells are shown in Table 1. While the initial rate of cell proliferation is rapid, the rate of increase in cell number over the 3-day culture period is not exponential and counts made on



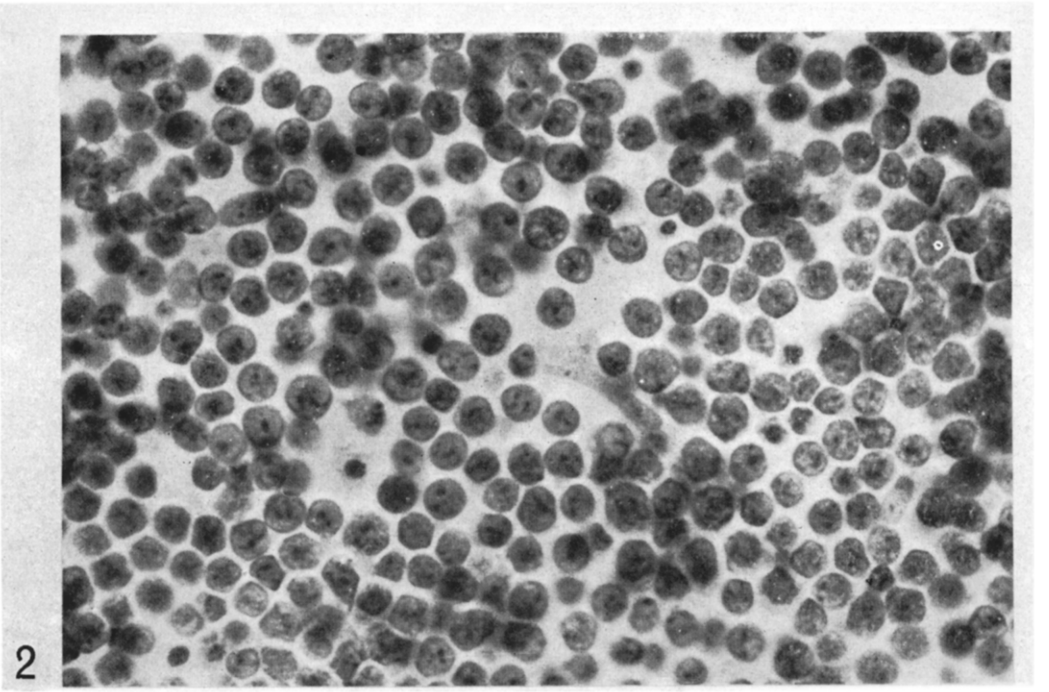


Fig. 1. *Diffusion chamber implanted on chick chorioallantois. The coverslip covering the hole in the shell has been removed and the hole enlarged so as to provide a better view of the chamber.*

Fig. 2. L1210 *leukaemia cells stained* in situ *on the surface of a Millipore filter after* 3 *days culture* (×1200).

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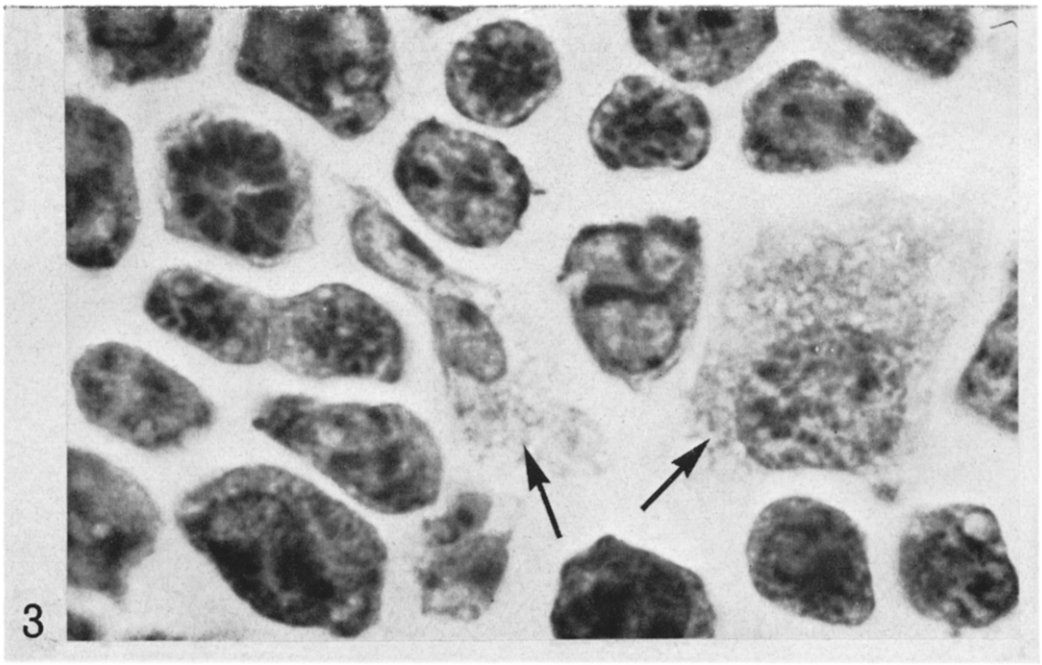

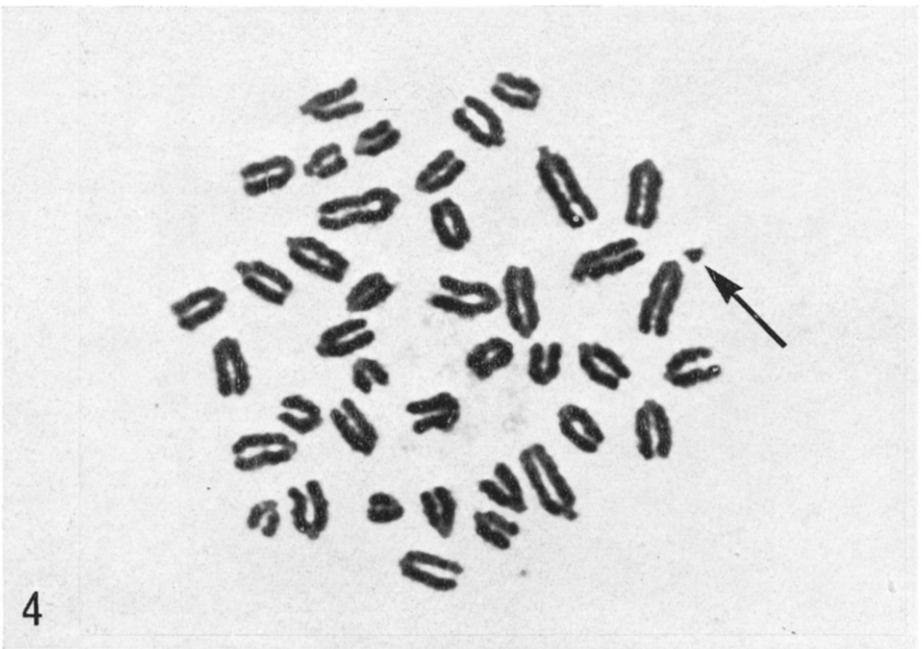

Fig. 3. *High power view of the* L1210 *leukaemia cells shown in Fig.* 2. *A number of mitotic figures can be seen and macrophages* (*arrowed*) *are interspersed among the lymphoid cells* (×4250).

Fig. 4. *Metaphase prepared from* L1210 *leukaemia cells cultured for* 3 *days in a diffusion chamber. Note the minute acrocentric chromosome* (*arrowed*) (×4250).

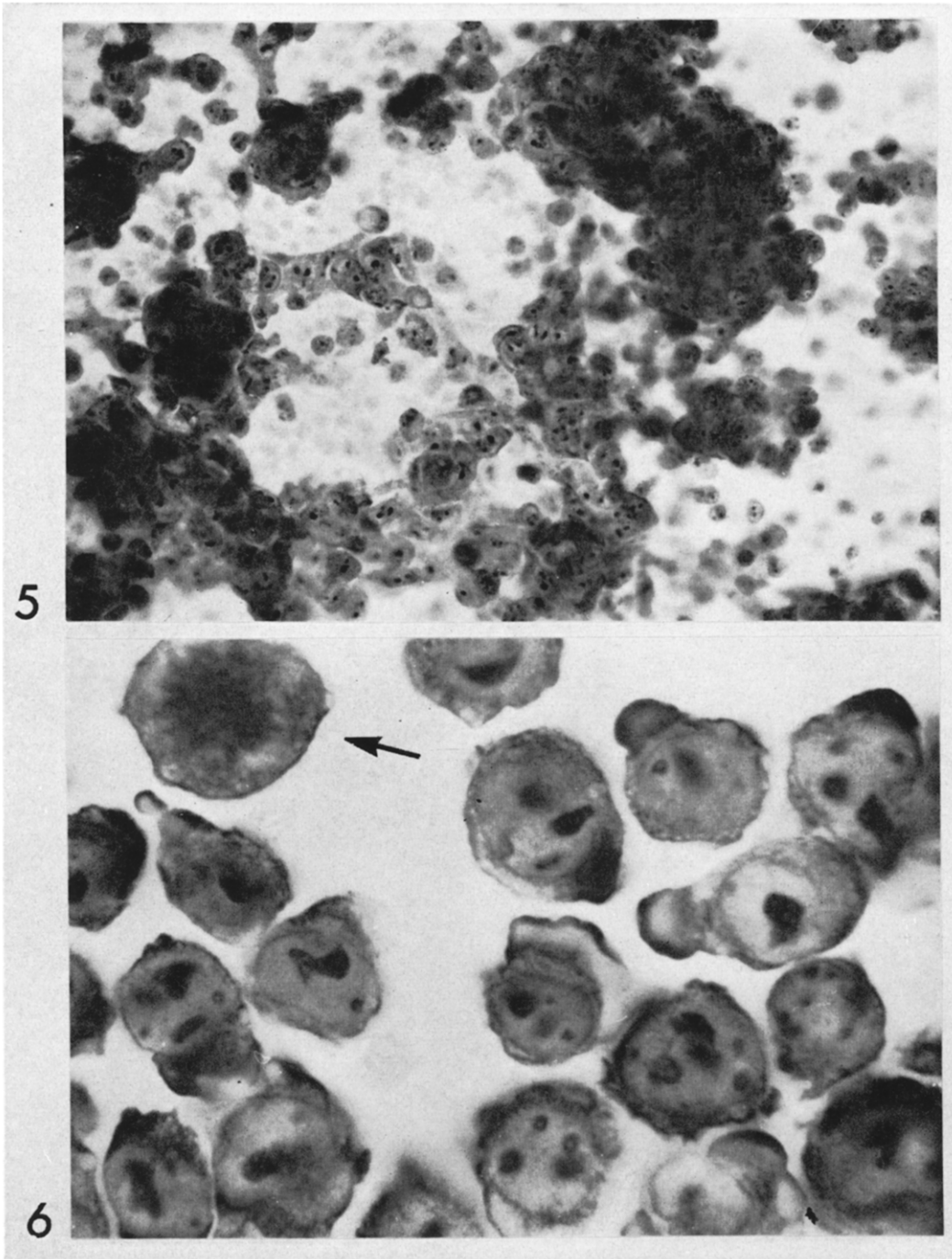

Fig. 5. *Ehrlich ascites cells stained on the surface of a Millipore filter after* 3 *days culture. Note how the tumour cells tend to proliferate in large clumps* ($\times$ 510).

Fig. 6. *High power view of Ehrlich ascites cells stained on a filter* (3 *days culture*). *A dividing cell* (*arrowed*) *can be seen in the upper part of the field* ($\times$ 2550).

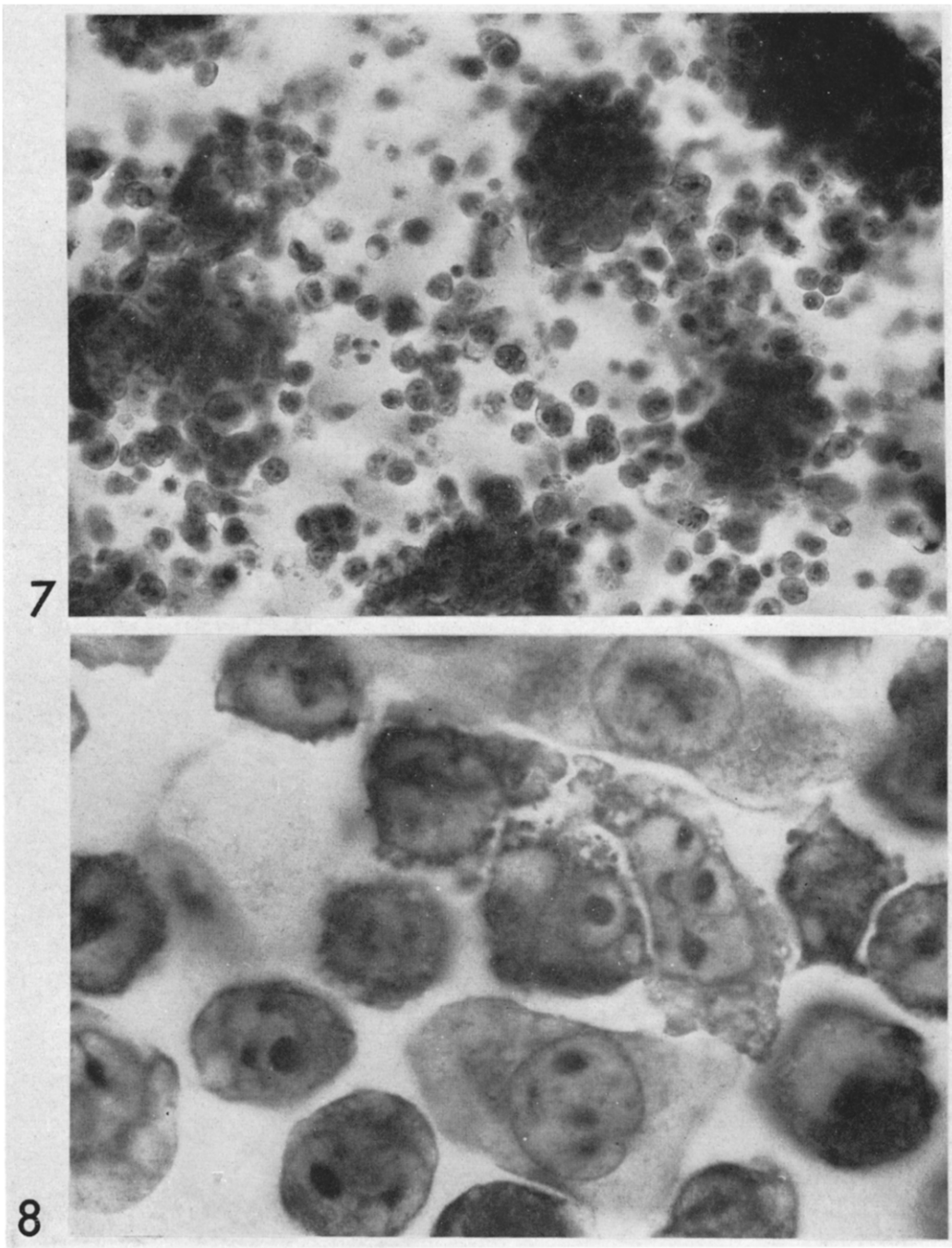

Fig. 7. S180 *cells stained* in situ *on a filter* (3 *days culture*). *The cells tend to grow in large groups similar to those in cultures of Ehrlich ascites cells* (×510).

Fig. 8. *High power view of* S180 *cells seen in Fig.* 7. (×2550).

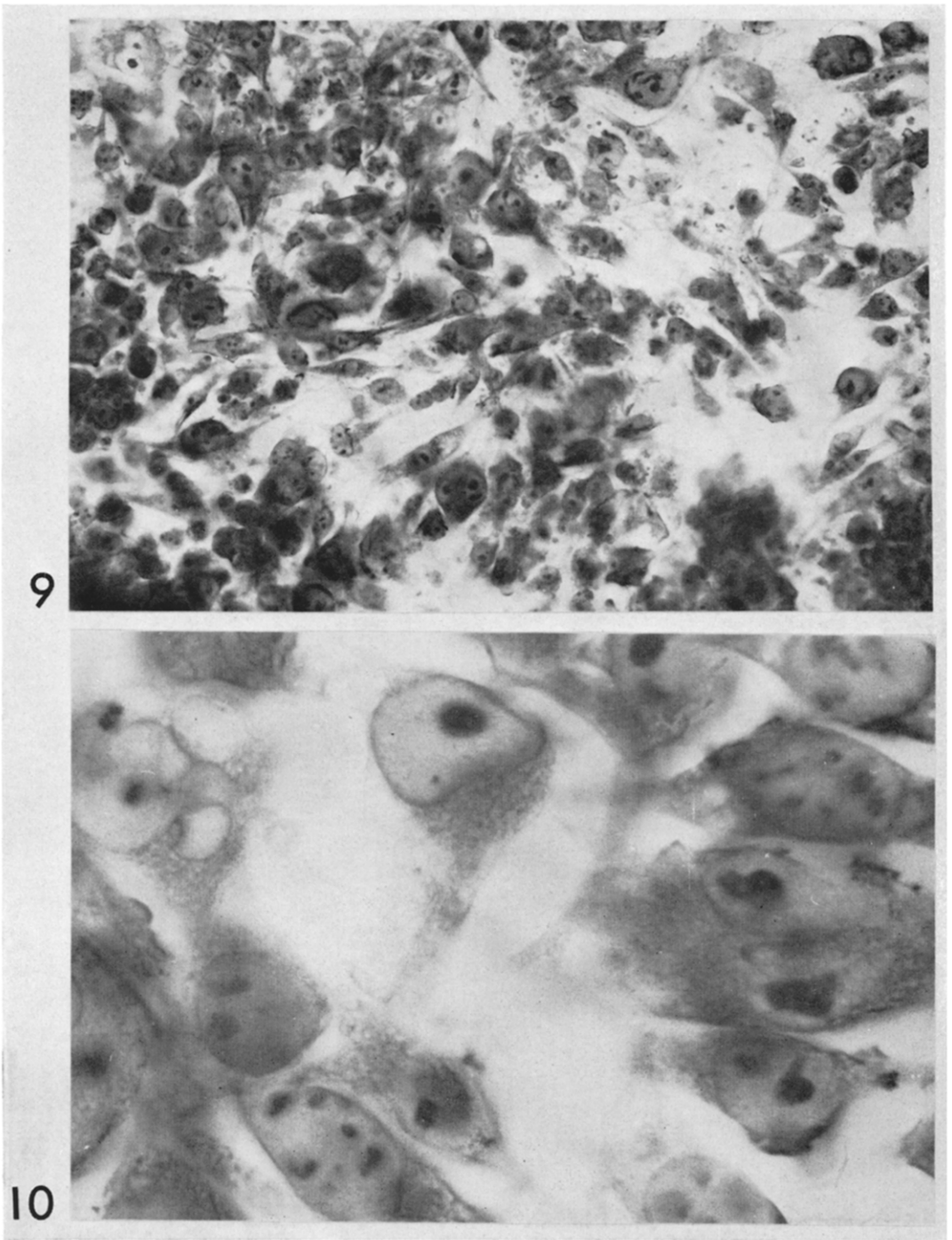

Fig. 9. BP8 *cells cultured for* 3 *days and stained* in situ *on a Millipore filter. The cells show many cytoplasmic processes* (×510).

Fig. 10. *High power view of* BP8 *cells seen in Fig.* 9. (×2550).

*Table 1. Total number of tumour cells in diffusion chambers at various intervals**

Tumour	No. viable cells ($\times 10^5$) start No./chamber	24 hr mean No./chamber (range)	48 hr mean No./chamber (range)	72 hr mean No./chamber (range)
Leukaemia L1210	1·0	2·55 (2·4–2·8)	4·22 (4·0–4·8)	5·90 (5·6–6·2)
Ehrlich carcinoma	1·0	2·17 (1·8–2·4)	4·47 (4·2–4·7)	8·87 (8·2–9·8)
S180	1·0	2·13 (1·8–2·5)	3·73 (3·6–3·8)	7·80 (7·6–8·0)
BP8	1·0	1·57 (1·4–1·7)	3·20 (3·0–3·4)	6·13 (5·4–6·8)

*Counts at each stage are based on 4 chambers in the case of L1210 leukaemia and 3 chambers in the ascites tumours.

chambers after 4 days showed little further increase in cell concentration.

When stained *in situ* in the diffusion chambers, L1210 cells were seen as a continuous layer over the Millipore filter surface (Fig. 2). High power views of this layer (Fig. 3) showed that while the vast majority of cells are lymphoid, macrophages are interspersed throughout.

Analysis of chromosome preparations made from L1210 cells after 3 days culture showed that all cells examined contain a small acrocentric chromosome (Fig. 4). Most of the cells had a chromosome number of 40 or 41.

Survival of mice injected with L1210 *cells*

The survival times of DBA/2 and BDF_1 mice injected withL1210 leukaemia cells derived either directly from BDF_1 spleen or after 3 days culture in diffusion chambers are shown in Table 2. While the survival times of mice injected with leukaemia cells obtained directly from spleen clearly depend upon the number of cells injected, the data suggests that mice survive the injection of equivalent numbers of cells obtained from diffusion chambers for a slightly longer period. However, in both cases all animals die by 10 days.

The survival times of mice of strains (C_3H/He, C57Bl and A Strong), allogeneic to L1210 leukaemic cells, and injected with leukaemic cells obtained directly from BDF_1 spleen or after 3 days culture in diffusion chambers are shown in Table 3. While the survival times in each strain depend upon the number of L1210 cells injected, a proportion of mice do survive a cell number which proves lethal to all histocompatible mice. When some of these survivors were injected with a further number of cultured L1210 cells no additional mortality was observed. Survival times following the injection of cultured L1210 cells into allogeneic hosts suggest that these cells are less lethal than comparable numbers of cells taken directly from mouse spleen.

Proliferation of ascites tumour cells in diffusion chambers

Cell counts made on diffusion chambers containing ascites tumour cells are shown in Table 1. In each of the three tumours studied the rate of increase in cell number is more closely exponential than is found with L1210 leukaemia cells. The rate is greatest in chambers containing Ehrlich ascites cells and slowest in cultures of BP8 tumour cells. The proportion of dead cells in all chambers was of the order of 1%.

Figures 5–10 show the appearance of ascites tumour cells stained *in situ* in diffusion chambers. The cells proliferate in large aggregates so that after 3 days culture masses of tumour cover the surface of the Millipore filter.

Table 2. Survival times of histocompatible (BDF_1 *and* DBA/2) *mice injected with* L1210 *leukaemia cells derived directly from* BDF_1 *mouse spleen or cultured for* 3 *days in diffusion chambers on the chick chorioallantois*

	Cell No. injected	Recipient strain	No. animals injected	No. survivors (days after injection) 6	7	8	9	10	11
	10^3–10^4	BDF_1	36	36	36	31	23	1	0
Mouse spleen	1×10^5	BDF_1	18		14	3	0		
L1210 cells	3×10^5	BDF_1	6	2	0				
	3×10^5	DBA/2	6	2	0				
Cultured	3×10^5	BDF_1	11	11	4	0			
L1210 cells	3×10^5	DBA/2	5	5	5	2	0		

Table 3. Survival times of allogeneic mice injected with L1210 *cells derived directly from* BDF_1 *mouse spleen or cultured for 3 days in diffusion chambers on the chick chorioallantois*

	Recipient strain (H2 locus)	Cell No. injected	No. animals injected	No. survivors (days after injection)										
				6	7	8	9	10	11	12	15	22	30	>30
Mouse spleen		10^3–10^5	54	54	54	53	51	51	49	47	40	38		
L1210 cells	C_3H	3×10^5	6		5	1	1	1	1	1	1	1	1	1
Cultured	(H-2K)	3×10^5	11	11	11	8	8	7	6	6*	6	6	6	6
L1210 cells		$8{\cdot}8\times10^5$	7		6	2	2	2	2	2	2	2	2	
Mouse spleen		10^3–10^5	54	54	54	54	53	51	48	48	45	44		
L1210 cells	A Strong	3×10^5	6	6	4	4	3	3	3	3	3	3	3	3
Cultured	(H-2a)	3×10^5	11	11	10	9	9	7	7	7*	7	7	7	7
L1210 cells														
Mouse spleen		10^3–10^5	54	54	54	54	54	54	54	54	51	50		
L1210 cells		3×10^5	6		5	1	1	1	1	1	1	1	1	1
	C57B1	4×10^4	10	10	10	10	10	10	10	9	9	9		
Cultured	(H-2b)	3×10^5	11	11	11	11	11	10	10	10*	10	10	10	10
L1210 cells		$8{\cdot}8\times10^5$	8	8	8	8	8	8	8	8	8	8	8	8
		4×10^6	10					6	6	6	6	6		

*These groups of animals were injected at this stage with a further 9×10^5 cultured L1210 cells.

Survival of mice injected with ascites tumour cells

S180, BP8 and Ehrlich tumour cells which have been cultured for 3 days in diffusion chambers are as lethal on injection into mice as ascites tumour cells taken directly from the peritoneal cavity. Thus all of 10 mice injected with cultured S180 cells were dead by 20 days, seven out of ten mice injected with cultured BP8 cells were dead by 28 days and all of eight mice injected with cultured Ehrlich cells were dead by 24 days.

DISCUSSION

A considerable number of studies have been made on the growth of tumours from a variety of species in the chick embryo [11]. The chorioallantoic membrane, in particular, has been frequently used as a site for tumour implantation. However, difficulty has been experienced in growing ascites tumours [3] and leukaemias [12] in this situation.

By contrast, the diffusion chamber method used in the present study allows proliferation of murine ascites tumour and leukaemia cells and also enables the degree of proliferation to be estimated. The proliferation of L1210 leukaemia cells during the initial period of growth in diffusion chambers is of the same order as that reported *in vitro* [13]. The fact that growth of this tumour is not exponential over the whole period is probably due to the adverse effect of high cell concentration on cell proliferation. In additional studies it has been found that the exponential growth phase can be extended if chambers are 'seeded' initially with smaller numbers of cells (i.e. 20,000 cells). A similar relationship between initial cell concentration and proliferation rate has also been found to apply to ascites tumour cells. The optimal 'seeding' dose for prolonged exponential growth of the different tumour cells in diffusion chambers is being investigated further.

It is of interest that even when high doses of tumour cells were used for initial inoculation there were no deleterious effects on the development of host embryos. Therefore, possible accumulation of toxic cell products does not seem to present an obstacle to prolonged cultivation of cells in diffusion chambers on the CAM. Nevertheless, cultivation of tumour cells over long periods in diffusion chambers would clearly involve the regular transfer of cells to new chambers.

The morphological and cytogenetic appearance of L1210 leukaemia cells grown in diffusion chambers are similar to those which have been reported following growth *in vitro* [13]. The feature which is characteristic of all cells is the presence of a minute acrocentric chromosome. Rare cells were found which contained a further small acrocentric chromosome of about 3 or 4 times the size of the minute chromosome. These features were also found in metaphases prepared from spleens of leukaemic animals, and it therefore seems likely that despite the considerable number of divisions that occur in the 3 day cultivation period in diffusion chambers, the cells retain a high degree of chromosome stability. Further, the fact that cultured leukaemia cells are only slightly less efficient in killing both histocompatible and allogeneic hosts than leukaemia

cells taken directly from mice suggests that cultured cells retain their transplantation and lethal characteristics in mice.

The failure of L1210 cells taken directly from spleens or after culture to kill some allogeneic mice seems likely to be due to the ability of these mice to mount a homograft reaction against the foreign H2 antigens of the tumour cells. The finding that further injections of tumour cells were without lethal effect adds supporting evidence that these mice had become immune. It is interesting to note that injections of sera from these animals failed to protect DBA/2 mice against the fatal consequences of 4×10^5 L1210 cells. The basis of any immunity is more likely, therefore, to be cellular than humoral.

The ability of cultured ascites cells to match the lethal effects produced in mice by tumour cells taken directly from the peritoneal cavity indicates that there is no loss of malignancy during the culture period. It would be interesting to know whether much longer periods of growth in diffusion chambers would adversely affect the ability of these tumour cells to return to mice.

In conclusion, the diffusion camber method may have wide applicability for the growth of murine tumour cells and possibly for the growth of cells derived from human solid tumours. Preliminary results suggest that cells separated from solid tumours grow well in this system. The technique is easy to perform and requires no special tissue culture media. Finally, although the period of cultivation examined is relatively short, the extent of cell proliferation during this time is considerable and may be sufficient to enable a quicker evaluation than could be achieved previously [4, 14] of the effects of anti-cancer agents on tumour cell growth.

RESUME

On a observé une prolifération considérable de différentes cellules de tumeur de la souris dans des chambres de diffusion implantées dans la membrane chorioallantoïde. Les cellules de tumeur cultivées retiennent leur capacité de tuer des souris de certaines races. Bien qu'il soit possible de cultiver ces cellules cancéreuses in vitro, *notre technique a l'avantage d'être facile, de ne demander aucun milieu de culture spécial, et d'être utilisable pour la croissance des cellules tumorales de différentes espèces.*

SUMMARY

Various murine tumour cells have been shown to proliferate rapidly in diffusion chambers implanted on the chick chorioallantois. The cultured tumour cells retain their capacity to kill mice of certain strains. Although these tumour cells can be grown in vitro, *the diffusion chamber method is easy to carry out, requires no special tissue culture media and may be suitable for the growth of tumour cells from a variety of species.*

ZUSAMMENFASSUNG

Zellen verschiedener Mäusetumoren konnten in Durchströmungskammern auf Hühnerchorionallantois zu raschem Wachstum gebracht werden. Diese in Kultur gezüchteten Tumorzellen behielten ihre Fähigkeit bei, Mäuse bestimmter Stämme zu töten. Die Kulturmethode mit Hilfe der Durchströmungskammer ist leicht durchführbar und braucht keine speziellen Nährmedien, obwohl dabei die Tumorzellen in vitro *wachsen. Sie könnte auch bei der Züchtung von Tumorzellen anderer Spezies Anwendung finden.*

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Changes in the Pyruvate Kinase and Lactate Dehydrogenase Activity in Rat Lung During Cultivation and 'Spontaneous' Development of Malignancy *in vitro**

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IN PERMANENT cultures of animal tissues morphological criteria often fail to distinguish between non-malignant and malignant cells. Such a distinction usually has to be based on the demonstration of tumorigenic properties of the cultured cells after inoculation into susceptible hosts. However, even this criterion is not completely satisfactory partly because susceptible hosts are not always available, as in the case of human tissues and partly because tumour cells often loose their transplantability during propagation *in vitro*. Thus, other criteria of malignancy are needed for cultured cells.

The high rate of glycolysis, which characterizes most tumors, has also been taken as a sign of malignancy in tissue cultures [1]. However, the rapid increase in glycolysis, which has been observed in primary explants of non-malignant tissues [2–7], raises the question whether other explanations might be offered for the high glycolytic metabolism which is found in most tissue cultures both of malignant and non-malignant origin.

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The changes which may occur in the activity of the individual glycolytic enzymes in primary cultures has only received little attention. An increased activity of hexokinase has been observed in primary explants of monkey [4] and rabbit [8] kidney, but a similar enzymatic change could not be demonstrated in primary explants from newborn rat hearts [9], which are characterized by a high hexokinase content.

In the present investigation the activity of two glycolytic enzymes, pyruvate kinase (PK) and lactate dehydrogenase (LDH), in primary and long term cultures of rat lung were studied. In order to clarify whether changes in the activities of these enzymes might be taken as a sign of 'spontaneous' development of malignancy, the tumorigenicity of the cultured cells was tested in newborn rats, and comparisons were made with the metabolism and the enzyme activity of Yoshida's ascites tumor (YAT) cells and normal rat liver.

MATERIAL AND METHODS

The lung tissue was obtained from 2–6 month old inbred rats of the Wistar strain. Primary explants were grown in Fibiger flasks, i.e. a modification of Carrel flasks with a bottom surface of 28 cm^2. Tissue pieces approximately 1 mm dia. were embedded in a drop of chicken plasma which was brought to coagulation with one drop of chicken embryo extract. About

100 explants were placed in each flask. After incubation for 12–24 hr at 37°C, 4 ml of a fluid medium (Fib 14b) were added to each flask. This medium contained 20% bovine embryonic serum and 80% Eagle's MEM [10] modified to contain twice the concentration of amino acids and four times the concentration of glutamine and vitamins recommended. The medium was changed daily in all primary cultures. In long term cultures the medium was changed three times weekly. Each time when the medium was changed the cultures were gassed with a gas mixture containing 5% CO_2, 20% O_2, and 75% N_2. Two permanent cell lines (R 30 and R 121) were established from primary explants prepared as described above. A third permanent cell line (R 131) was derived from a cell suspension prepared from lung tissue cut into small pieces and then treated with a 0·25% solution of collagenase (Nutritional Biochemicals Corp.) and 0·25–0·50% solution of trypsin (Difco 1 : 250) as described by Kawiak *et al.* [11]. All three cell lines were propagated in Fib 14b. The R 121 line received in addition pyruvate at a concentration of 1 mM. During subcultivation the R 30 cultures were divided by trypsinization while the two other cell lines were divided mechanically by gentle scraping. Thus R 121 cells were never exposed to trypsin and the R 131 only during the initial preparation of the primary cell suspension.

All three cell lines eventually underwent apparently spontaneous malignant transformation, which was tested regularly by the subcutaneous inoculation into newborn Wistar rats of a number of cells corresponding to 0·5–5 mg of protein, i.e. approximately $1–10 \times 10^6$ cells. Progressive tumor growth was taken as a sign of malignancy. Invasion and metastasis were occasionally seen.

The cultured material which was used for metabolic or transplantation studies was harvested by gentle scraping with a silicone rubber policeman without the use of trypsin.

The Yoshida ascites tumor (YAT) was propagated in inbred Wistar rats 2–3 months old. The tumor cells were harvested 6–8 days after inoculation when they were still in their exponential growth phase. Only non-hemorrhagic tumors were used.

Rat liver was obtained from 2–6 month old Wistar rats which had been fasted for 24 hr. The animals were killed by decapitation and bled to diminish blood contamination as much as possible.

The material which was prepared for biochemical studies was immediately cooled on crushed ice, and all the following preparative procedures were carried out in a cold room at 2–4°C. Liver and lung tissue was cut into small pieces with a diameter of about 0·5 mm. The YAT and the cultured cells were centrifuged at about 300 **g** for 10 min and the supernatant fluid was discarded. After washing twice in Krebs–Ringer phosphate buffer the materials described were either resuspended in the reaction medium or submitted to further mincing and homogenization. In the latter case the material was first frozen and then homogenized during the subsequent process of thawing in a Potter–Elvehjem glass homogenizer with Teflon pestle. This procedure was repeated twice.

Particle-free supernatants were prepared by centrifugation at 100,000 **g** at 4°C for 45 min.

The PK and LDH activity was determined by spectrophotometric measurements of NADH oxidation at one minute intervals at 340 nm under conditions yielding zero order kinetics. The PK activity was studied in a medium containing 2-phosphoenolpyruvate (PEP), NADH, and LDH, while the LDH activity was measured in a medium containing pyruvate (Pa) as substrate using the method described by Kubowitz and Ott [12]. The results were expressed as mμmoles of NADH oxidized at 25°C/min/mg protein.

Aerobic glycolysis and PEP-metabolism were investigated in Erlenmeyer flasks shaken in a water bath at 37°C. The glycolytic activity was followed for one hour by measurements of the glucose and lactate (La) contents of samples withdrawn at 15 min intervals. Equal volumes of 0·6 M perchloric acid were added to each sample, and the specific enzymatic methods described by Huggett and Nixon [13] and by Horn and Bruns [14] were used for glucose and La determinations respectively.

The conversion of PEP to Pa and La was followed over a period of 6 min using the methods described by Kubowitz and Ott [12] and by Horn and Bruns [14]. Samples for Pa and La determination were withdrawn at 2 min intervals and the linearity of the reaction was ascertained.

Protein was determined with the Folin Ciocalteau reagent as described by Lowry *et al.* [15].

Further details of the conditions of the individual experiments will be presented together with the results.

For the statistical evaluation of the results the Wilcoxon test was used [16–17].

Table 1. *Pyruvate kinase (PK) and lactate dehydrogenase (LDH) activity in rat lung, rat liver, and Yoshida's ascites tumor (YAT)*

	PK activity (mμmoles/mg protein/min)				LDH activity (mμmoles/mg protein/min)			
	n	$\bar{x}$	s	rel.*	n	$\bar{x}$	s	rel.*
Lung	8	154	47	13%	8	711	134	9%
Liver	9	460	176	40%	9	5572	2447	68%
YAT	9	1147	548	100%	9	8144	2276	100%

*Percentage of the activity found in YAT.

Enzyme activities were measured in particle-free supernatants prepared from homogenates suspended in a solution containing tris (20 mM), KCl (115 mM), $MgCl_2$ (10 mM), and EDTA (2mM)—pH 7·5. The PK activity was determined spectrophotometrically at 340 nm in a reaction mixture which in 2·2 ml contained: tris 150 μmoles (pH 7·4), KCl 200 μmoles, $MgSO_4$ 50 μmoles, NADH 0·7 μmoles, LDH 0·033 mg, PEP 10 μmoles, and 50–200 μl of diluted supernatant yielding a $\Delta E = 0{\cdot}015$–$0{\cdot}030$/min. The reaction was started by adding 10 μmoles of ADP. The LDH activity was determined at 340 nm in a reaction mixture which in 3·0 ml contained: tris 1060 μmoles (pH 7·4), $MgCl_2$ 0·1 μmoles, NADH 0·7 μmoles, and 50–200 μl of diluted supernatant as mentioned above. The reaction was started by adding 5 μmoles of Pa.

RESULTS

The PK and LDH activity in the particle-free supernatants of homogenized rat lung, liver, and YAT cells is shown in Table 1. As it is seen, the PK activity in lung tissue was only 13% of the activity in YAT cells. The corresponding value for liver was 40%. The differences between the three materials were statistically significant ($P<0{\cdot}01$). The LDH activity in lung tissue was significantly lower than the activity in liver and YAT cells ($P<0{\cdot}01$) but between the two last mentioned materials no significant difference was found ($P>0{\cdot}1$).

Table 2 shows the results of an experiment in which the conversion of PEP to Pa and La was studied in full homogenates and particle-free supernatants of lung, liver and YAT cells. Without the addition of ADP and NADH only very small amounts of Pa and La were formed. When ADP was added, an accumulation of Pa occurred. Between lung and YAT cells a difference was seen which ran parallel to the difference in PK activity, but between lung

Table 2. *Pyruvate (Pa) and lactate (La) production* from 2-phosphoenolpyruvate (PEP) in full homogenates and particle-free supernatants of rat lung, rat liver and Yoshida's ascites tumor (YAT)*

Substrate		PEP (2mM)		PEP+ADP (2mM) (5mM)				PEP+ADP+NADH (2mM) (5mM) (1·5mM)					
		Pa	La	Pa			La	Pa			La		
Material	n	$\bar{x}$	$\bar{x}$	$\bar{x}$ abs.	s	rel.† (%)	$\bar{x}$	$\bar{x}$ abs	s	rel.† (%)	$\bar{x}$ abs.	s	rel.† (%)
Lung Homogenate	6	<0·1	<0·1	30·7	5·2	14	<0·1	14·9	3·4	16	20·7	9·6	12
Lung Supernatant	6	<0·1	<0·1	62·9	25·9	13	<0·1	34·1	13·0	19	32·6	13·0	9
Liver Homogenate	5	<0·1	<0·1	31·9	8·1	14	<0·1	0·4	0·8	0·4	33·7	8·6	19
Liver Supernatant	5	<0·1	<0·1	64·4	24·2	13	<0·1	2·9	2·8	1·6	59·4	22·0	17
YAT Homogenate	5	<0·1	<0·1	220·1	49·1	100	<0·1	95·2	3·9	100	173·3	60·8	100
YAT Supernatant	5	<0·1	<0·1	495·9	142·3	100	<0·1	177·5	52·9	100	359·1	124·7	100

*μmoles per mg protein per hr.
†Percentage of the Pa and La production found in YAT.

Homogenization was performed in a 0·15 M KCL solution. The incubation mixture contained: PEP (2 mM), $MgCl_2$ (10 mM), KCl (75 mM), tris (50 mM), $KHCO_3$ (25 mM), and in some cases ADP (5 mM) and NADH (1,5 mM). pH was adjusted to 7·4 by gassing with 10% CO_2. Samples of homogenates and supernatants containing 0·25–2·00 mg protein were added to the incubation mixture described to give a final volume of 2 ml.

Table 3. Aerobic glycolysis in rat lung and Yoshida's ascites tumor (YAT)

	Glucose uptake (μmoles/mg protein/hr)			Lactate accumulation (μmoles/mg protein/hr)		
	n	$\bar{x}$	s	n	$\bar{x}$	s
Lung	4	0·06	0·02	11	0·07	0·03
YAT	6	0·93	0·19	6	1·18	0·12

Reaction mixture: Krebs–Ringer phosphate solution with 10 mM glucose, pH=7·4.

and liver no difference in Pa accumulation was observed in spite of the higher PK activity of the latter.

When in addition to ADP, NADH was added, considerable amounts of La accumulated. A comparison between La production in the YAT cells and in lung tissue seemed to reflect the difference in LDH activity shown in Table 1. However, a similar parallelism did not exist between La formation and LDH activity in liver and YAT cells. The sum of Pa and La produced in the presence of ADP and NADH agreed fairly well with the amount of Pa accumulated in the absence of NADH in lung and liver while in the YAT cells it seemed a little higher.

Table 3 shows the difference between aerobic glycolysis in lung and YAT cells. The glycolytic activity of the former was only 6% of that of the latter.

During cultivation *in vitro* a rapid increase in the PK and LDH activity was seen starting as early as the second day after explantation (Table 4). On the seventh day the PK activity

Table 4. Changes in PK and LDH activity in primary explants of rat lung tissue during short term cultivation in vitro

Duration of cultivation (days)	PK activity (mμmoles/mg protein/min)			LDH activity (mμmoles/mg protein/min)		
	Exp. 1.	Exp. 2	Exp. 3	Exp. 1	Exp. 2	Exp. 3
0	137·5	—	—	1100	—	—
1	129·0	105·2	—	1168	710	—
2	—	383·0	—	—	1600	—
3	—	—	—	—	—	—
4	796·2	—	347·0	4820	—	1885
5	—	—	511·0	—	—	3000
6	—	—	—	—	—	—
7	903·4	772·0	—	5870	3220	—

Enzyme activities were measured in particle-free supernatants prepared from homogenates suspended in 0·25 M sucrose. PK and LDH activity was determined as described in Table 1.

Table 5. Changes in aerobic glycolysis in primary explants of rat lung tissue during short term cultivation in vitro

Culture age (days)	No. of experiments	Glucose uptake (μmoles/mg dry wt/hr)		Lactate accumulation (μmoles/mg dry wt/hr)	
		$\bar{x}$	s	$\bar{x}$	s
0	4	0·077	0·010	0·108	0·011
1	4	0·064	0·009	0·084	0·019
2	4	0·077	0·021	0·117	0·021
14	4	0·175	0·053	0·244	0·122
28	4	0·504	0·178	0·951	0·262

Incubation medium: tissue culture medium Fib 14 b containing dialyzed foetal bovine serum and glucose 10mM, and the addition of tris 30 mmoles/l and 7 non-essential amino acids. The medium contained no bicarbonate.

Table 6. Pyruvate kinase (PK) *and lactate dehydrogenase* (*LDH*) *activity in three malignant rat lung cultures*

Cell line	Culture age (months)	Tumorigenecity Percentage of takes (No. of rats inocul.)	PK activity (mμmoles/mg protein/min) n	$\bar{x}$	s	LDH activity (mμmoles/mg protein/min) n	$\bar{x}$	s
R 131	17	100 (21)	8	500	133	8	7287	575
R 121	20	100 (24)	7	1710	151	7	4745	302
R 30	32	78 (18)	6	790	270	6	5047	481

The PK and LDH activity was determined as described in Table 1.

had increased 7-fold and the LDH activity 5-fold. The total glycolytic activity as measured by glucose consumption and La production rose too, but more slowly (Table 5).

The increased rate of PK and LDH activity was maintained during long term cultivation. The results presented in Table 6 were obtained after the development of tumorigenic properties 17–32 month after explantation. The PK values were significantly higher in all three cell lines than in fresh lung tissue ($P<0{\cdot}01$). In R 121 and R 30 they were higher than in liver ($P<0{\cdot}01$ and $<0{\cdot}02$, respectively), and in R 121 even higher than in the YAT cells ($P<0{\cdot}05$), while R 131 showed values which were significantly lower ($P<0{\cdot}02$).

Also the LDH values were significantly higher in all three cell lines than in fresh lung tissue ($P<0{\cdot}01$). In R 131 the LDH activity was higher than in liver ($P<0{\cdot}05$), and it did not differ significantly from that shown by the YAT cells. The two other cell lines showed LDH activities which did not differ significantly from the values seen in liver, but which were below the values found in the YAT cells ($P<0{\cdot}01$).

When the results presented in Tables 4 and 6 are compared it is seen that with the exception of the PK activity in R 121 the malignant cell lines did not show enzyme activities which differed much from what might be found in seven day old non-malignant cultures.

DISCUSSION

In the search for biochemical criteria of malignancy three parameters are usually considered: chemical composition, metabolic pattern and enzyme profile.

Studies of chemical composition have mainly indicated a lack of differentiation [18], but so far have provided no useful criterion of malignancy. Metabolic studies, particularly of glucose metabolism [see 19], have been more encouraging, but the existence of multiple metabolic pathways often complicates the interpretations of such investigations. The enzyme profile offers a chance of a more unequivocal criterion of malignancy, but yields only limited information about the physiological significance of the differences observed.

An increased content of several enzymes including PK and LDH has been found in human [20–22] as well as in animal tumors [see 23–28]. In the present study the PK activity of YAT cells was found to be significantly higher than in both lung and liver, while the LDH activity of the tumor cells only differed significantly from that of lung but not from that of liver. In primary cultures of various non-malignant tissues an increased activity of hexokinase [4, 8], glucose 6-*P*-dehydrogenase [29] and LDH [8] has been reported. In our experiments the PK and LDH activity in rat lung explants rose 5–7-fold in the course of 7 days.

The mechanism of the enzymatic changes in primary explants is not clear, but they are most likely the result of enzyme syntheses. In support of this assumption *p*-fluorphenylalanin [30] and actinomycin-D [7] have been found to block the increase in hexokinase activity and lactate accumulation, respectively. The presence of glucose is required for these changes, but considering the fact that glucose is offered to the cultured tissues at concentrations corresponding to those found in blood, the requirement for glucose can hardly be taken as sign of this substrate being the inducing factor. It is also known that infection of tissue cultures by viruses may be followed by changes in glycolysis and activity of glycolytic enzymes [31–33].

The PK values measured in one week old lung cultures approached the values found in YAT cells, while the LDH activity reached the level of normal liver, which did not differ significantly from that of the YAT cells. At this time no tumors were produced by the inoculation of the primary cultures into newborn rats, indicating that the enzymatic

changes may not be taken as a sign of malignancy. In agreement with this conclusion the 'spontaneous' development of malignancy during prolonged cultivation *in vitro* was not necessarily accompanied by an additional increase of the PK and LDH activity.

In rat hepatomas a correlation between glucose consumption and the activity of hexokinase [28, 34] phosphofructokinase and PK [34] has been demonstrated.

In the present study of non-cultured lung tissue and YAT cells the differences in PK and LDH activity on one hand and PEP metabolism on the other hand seemed to run parallel, while a similar parallelism was not observed in the case of liver. The presence of alternative metabolic pathways may possibly account for the discrepancies observed.

The production of Pa and La from PEP in the particle-free supernatants was about double that in full homogenates. Whether this is a sign of a Pasteur effect mediated by the mitochondria or of enzyme purification cannot be decided on the basis of the present experiments, where the results were calculated per mg protein found in full homogenates and particle-free supernatants, respectively.

The changes in PK and LDH activity in primary explants were followed by an increase in glucose consumption and lactate production as previously observed by other authors [2–7]. However, the increase in the activity of the two enzymes occurred earlier than the changes in total glycolysis, indicating that other reactions are rate limiting in the glycolytic metabolism of primary lung explants.

Previous studies in this laboratory [29] of the glucose-6-P dehydrogenase activity in primary explants have shown that the increase of this enzyme like those of PK and LDH starts as quickly as the second day after explantation. Also the hexokinase activity of primary explants seems subjected to more rapid changes than glycolysis. Thus Lieberman *et al.* [30] found a doubling of the hexokinase activity in primary cultures of rabbit kidney on the third day after explantation. Other enzymes like phosphofructokinase might be rate limiting, but it is also conceivable that the increased rate of glycolysis is triggered by other factors than enzyme induction.

Even if the increased activity of glycolytic enzymes cannot be used as a sign of malignancy *in vitro*, it is likely that these enzymatic changes are necessary for the uncontrolled growth of cultured cells. In the present experiments the highest PK value was found in the R 121 cell line which showed a doubling time of 29–40 hr. The doubling time of the R 30 and R 131 cell line was 63 and 55 hr respectively, and the PK activity $\frac{1}{3}$–$\frac{1}{2}$ of that of the R 121 cells. Further studies are required before the question can be answered whether one or several glycolytic enzymes control the growth rate. A correlation between growth rate and rate of glycolysis has been reported in rat hepatomas [see 23] but similar observations have not been made in cultured tissues and cells, in which the rate of glycolysis could be increased considerably by raising the glucose concentration from 2–10 mM without any influence on the growth rate. Similarly the glycolysis of cultured ascites tumor cells could be depressed 50 per cent by oxamic acid, before any growth inhibition was seen [35]. Thus, a correlation between growth rate and the activity of glycolytic enzymes is hardly to be expected in cells and tissues grown *in vitro*.

RESUME

L'activité de la pyruvate kinase (PK) et de la déshydrogénase lactique (LDH) a augmenté rapidement au cours de la culture in vitro *du tissu pulmonaire du rat, et s'est rapprochée des valeurs trouvées dans les cellules YAT. Ces altérations ont précédé l'augmentation de la glycolyse observée dans les explants primaires et se sont produites beaucoup plus tôt que le développement spontané de la malignité. Ainsi, des activités élevées de pyruvate kinase et de déshydrogénase lactique n'ont pas pu être utilisées comme critères permettant de déterminer une transformation maligne* in vitro.

SUMMARY

During cultivation in vitro *the pyruvate kinase (PK) and lactate-dehydrogenase (LDH) activity of rat lung tissue increased rapidly, approaching the values found in YAT cells. These changes preceded the increase in glycolysis seen in primary explants and occurred much earlier than the 'spontaneous' development of malignancy* in vitro. *Thus, high PK and LDH activities could not be used as a criterion of malignant transformation* in vitro.

ZUSAMMENFASSUNG

Während der Züchtung von Rattenlungengewebe in Vitro hat man eine schnelle Steigerung der Aktivität von Pyruvat Kinase (PK) und Laktatdehydrogenase (LDH) bis zu einem Niveau nicht weit von den Werten, die in YAT Zellen gefunden werden können, beobachtet. Diese Änderungen gehen der Steigerung der glykolytischen Aktivität, wie sie in Primärexplantaten beobachtet wird, voraus, und sie treten lange bevor des "spontanen" Entwicklung von Malignität in Vitro in Erscheinung. Eben deshalb können die Aktivitäten von PK und LDH nicht als ein Kriterium der bösartigen Transformation in vitro *gebraucht werden.*

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The Frequency of Constitutional Chromosome Abnormalities in Patients with Malignant Disease

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INTRODUCTION

In 1958 Stewart, Webb and Hewitt [1] reported that the incidence of leukaemia was significantly increased in children with Down's syndrome. The subsequent demonstration that this syndrome was associated with a constitutional chromosome abnormality (trisomy 21) led to a consideration of whether or not there was any direct relationship between the abnormal chromosome constitution and susceptibility to the development of malignant disease. Many other chromosome abnormalities, often without phenotypic expression, have now been described but little information is yet available on their significance in terms of morbidity and mortality. The possibility therefore exists that other chromosome abnormalities may be associated with an increased incidence of malignant disease.

In 1959 a Registry of Abnormal Karyotypes was established by the late Professor W. M. Court Brown in Edinburgh in which records are kept of all the individuals discovered to have an abnormal chromosome constitution and also of controls in the case of familial abnormalities [2]. All these cases are subject to an annual follow-up, and one of the objects of the Registry is to determine morbidity and mortality patterns in chromosomally abnormal individuals. Although by September 1968 over 1000 cases had been recorded in the Registry it has always been recognised that, using this approach, it will take a long time for meaningful results to be obtained. Recent data on the frequency of chromosome abnormalities in the general population [3–6] make it possible to evaluate information obtained from a cytogenetic survey of patients with cancer, and in this way is it possible to find out relatively rapidly whether or not there is an unusually high proportion of individuals with a constitutional chromosome abnormality in a population studied because they had a malignant disease.

Study population

Blood samples were obtained from over 95% of new cases of malignant disease admitted to the Department of Radiotherapy, Western General Hospital, Edinburgh, during the period 30 January 1968–29 January 1969. Blood samples from a total of 1185 patients were examined. In 36 instances it was not possible to get a chromosome count from the cultured blood, so that successful preparations were made from 571 males, 578 females, a total of 1149 patients. The distribution of the types of malignancy included in this group are indicated in Table 1. Of the 36 failures, eight had disorders of the reticuloendothelial system (three cases of Hodgkin's disease, two of

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Table 1. *Distribution of malignancies by site in the study population*

Type of malignancy*	Males	Females	Total
Bronchus including trachea	243†	34	277
Cervix	0	100	100
Breast	2	177	179
Thyroid	3	11	14
Gastro intestinal tract excluding mouth and oesophagus	6	4	10
Oesophagus	30	28	58
Sarcomata, including bone sarcomata	12	4	16
Leukaemia, reticuloses, myeloma	38	34	72
Mouth, oro-naso-pharynx, salivary glands para-nasal sinuses, larynx, etc.	85	53	138
Central nervous system	9	10	19
Ovary, vagina, uterus, vulva	0	63	63
Testis	7	0	7
Kidney, ureter and bladder	96	30	126
Skin, including lip	28†	26	54
Miscellaneous	13	4	17
Total patients studied	571	578	1149
Cultures failed			36
Total patients			1185

*Categories were arbitrarily chosen to give an indication of the distribution of the tumours observed.

†One patient with a double primary tumour included in two categories.

reticulum cell sarcoma, and one each of myeloma, chronic myeloid leukaemia and chronic lymphatic leukaemia). This distribution is disproportionate to the total number of cases in this category and could be a source of bias in the survey.

MATERIAL AND METHODS

Peripheral blood lymphocytes were cultured by a modification of the method of Moorhead *et al.* [7]. All cells examined were counted and analysed. For each patient two cells were examined in the first instance. If these were the same and normal, this was taken to indicate a normal chromosome constitution in the patient. If either of the first two cells was abnormal a further eight cells were counted and analysed and a decision taken on the basis of 10 cells whether or not to accept the patient as normal. If the patient was thought to have an abnormal karyotype a total of 30 cells was examined. Where mosaicism was suspected a total of 100 cells was counted, if possible from two independent cultures. If the first two cells were both abnormal, a total of 30 cells was examined and a further blood sample was obtained to confirm the findings. When the patient had received the first fraction of a course of X-ray treatment before the blood sample was obtained, 25 cells were scanned to ensure that any aberrations seen were consistent with the dose of radiation known to have been received.

RESULTS

Eight patients (3 males and 5 females) were found to have a clear constitutional chromosome abnormality (0·69%). These are listed in Table 2. The abnormal marker chromosomes in cases 3 and 6 are shown in Figs. 1a and b. The abnormality was confirmed by observations on skin fibroblasts in cases 1–7. Fibroblasts of case 8 were not examined.

In each instance family studies were made and in three cases (Nos. 1, 5 and 7) the abnormality was found to be familial. These observtions are not yet complete, but in none of these eight cases have we any reason to believe that the chromosome abnormality is associated with an unusual familial incidence of a specific cancer or malignant disease in general (Table 3).

Three other patients (Table 4) had an abnormal chromosome count distribution which make it necessary to consider whether or not these are mosaics.

Case 9 has a total of 13 cells out of 100 that are lacking a C group chromosome, which does not differ significantly ($P<0·1$) from the

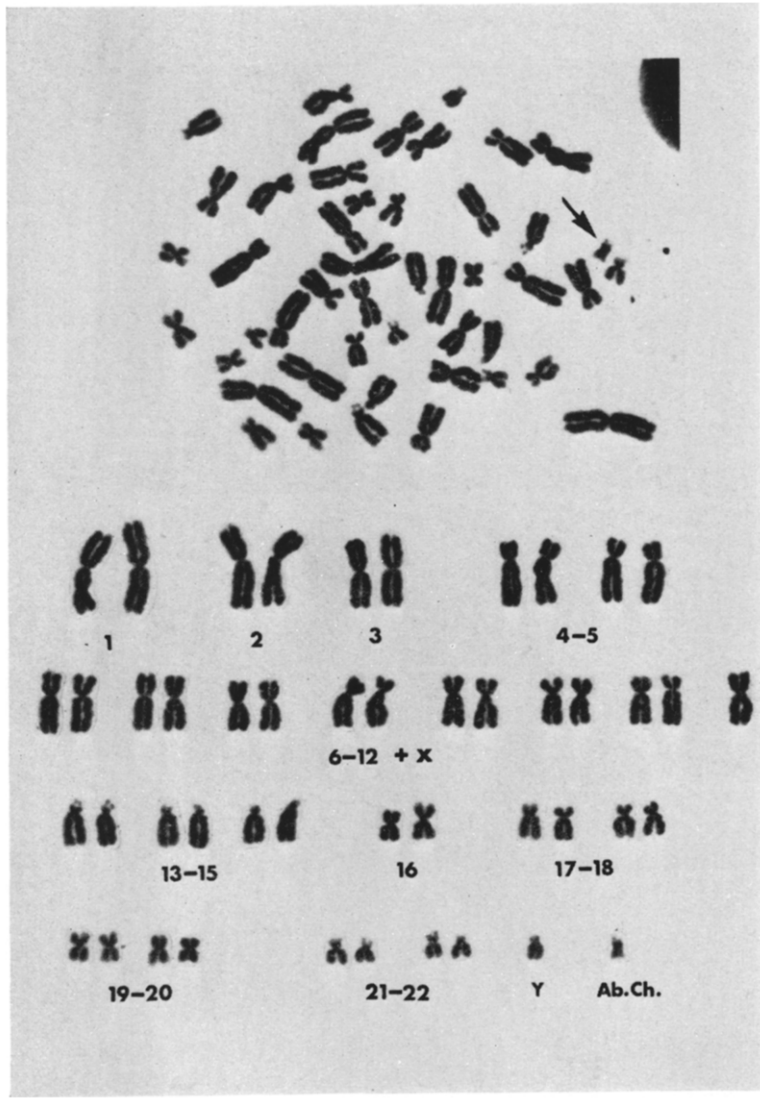

Fig. 1*a. Cell and karyotype from patient No.* 3 *showing the additional small marker chromosome.* ×2000 *approx.*

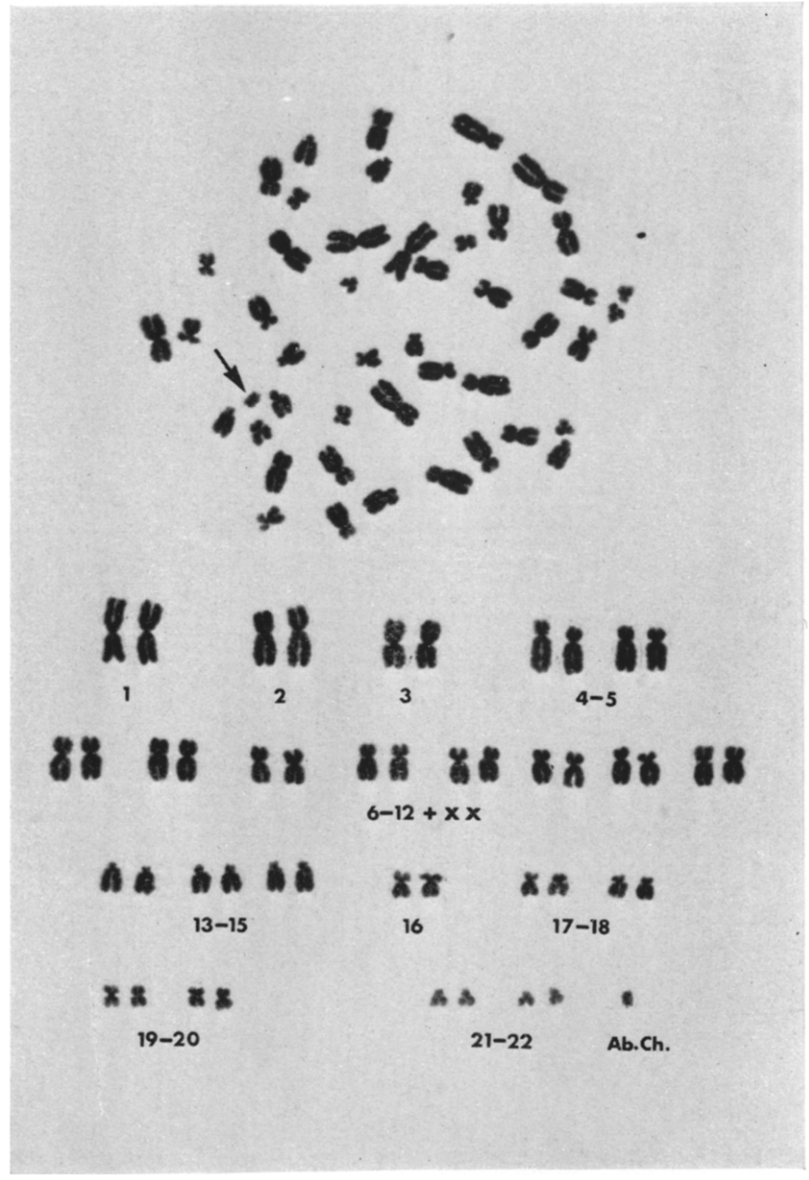

Fig. 1*b. Cell and karyotype from patient No.* 6 *showing the additional small marker chromosome.* ×2000 *approx.*

(to face p. 609)

Table 2. Patients with a constitutional chromosome abnormality

Case No.	Registry MRC No.	Age	Sex	Chromosomes	Diagnosis
1	K52/226/68	57	F	45, XX, D-, D-, t(DqDq)+	Well differentiated lymphocytic lymphosarcoma, right axilla, right supraclavicular region
2	A/10/69	59	M	46, XY, inv(Cp-q+)	Carcinoma right lung*
3	A/11/69	60	M	47, XY, mar+	Well differentiated squamous carcinoma mouth (right lower alveolus), pernicious anaemia
4	A/13/69	52	F	47, XXX	Poorly differentiated squamous carcinoma uterine cervix (Int. stage II)
5	K46/105/68	38	F	45, XX, D-, D-, t(DqDq)+	Spheroidal cell carcinoma right breast (Int. stage I)
6	A/73/68	69	F	46, XX/47, XX, mar+	Poorly differentiated adeno-carcinoma right breast (Int. stage III)
7	K48/135/68	67	M	46, X, inv(Yp+q-)	Generalised superficial lymphadenopathy. Histologically "most likely to be a lymphoblastic lymphosarcoma though Hodgkin's disease cannot be excluded"
8	A/227/68	71	F	46, XX, t(2q-Cq+)	Papillary adenocarcinoma right ovary

*Clinical diagnosis only.

Table 3. Summary of family studies on the abnormal patients

Case No.	Relatives found abnormal	Relatives found normal	Relatives possibly informative who remain to be examined	Total	No. of deaths in family where cause of death known					
					Not malignant	Malignant disease	Sex	Age	Diagnosis	Relationship to propositus
1	4	2	11*	18	16	2	M	81	Ca. stomach	Paternal uncle
							F	77	Epithelioma of tongue	Maternal aunt
2	0	0	0	5	5	0				
3	0	0	3	3	3	0				
4	0	2	2	2	2	0				
5	5	9	3†	7	7	0				
6	0	2	2‡	4	4	0				
7	10	0	20	8	7	1	F	38	Ca. Breast; secondary carcinomata	1st cousin
8	0	1	1§	12	10	2	M	75	Ca. thyroid; liver metastasis	Father
							F	45	Ca. stomach liver metastasis	Maternal aunt

*2 others overseas.
†Overseas.
‡Decision taken not to examine these.
§Whereabouts unkown.

Table 4. Patients with a possible mosaic sex chromosome constitution

Case No.	Type of malignancy	Age	Sex	Date	Count Distribution of A and B Cells*						C* cells	Total cells examined	Probable chromosome constitution
					<44	44	45	46	47	>47			
9	Ca. Oesophagus	75	F	30 April 68	—	3	9(8)*	37	—	—	1	50	46, XX
				13 April 68	—	2	8(5)	30	1	—	9	50	
10	Ca. Lung	53	F	18 April 68	1	—	4	42	3(3)	—	0	50	46, XX/47, XXX
				26 April 68	—	1	3(2)	44	1(1)	—	1	50	
11	Ca. Breast	61	F	2 April 68	—	—	14(13)	81	3(3)	—	2	100	45, X/46, XX

*A cells are those with no chromatid or chromosome aberrations.
B cells are those with chromatid aberrations only.
C cells are those with chromosomes aberrations [3].
†In each case the figure in parenthesis indicates the number of cells lacking or having an additional C group chromosome.

figure given for females in this age group in a population selected at random from the general population [8]. The count distribution could therefore be attributed to the age of the individual. In case 10, however, it can be shown that the four cells found to have an extra C group chromosome out of 100 examined is a significant excess ($P<0\cdot01$). In case 11, the finding of 13 cells lacking a C group chromosome in 100 examined is a significant departure from the expectation for the age group ($P<0\cdot01$) but three cells with an extra C group chromosome could be attributed to chance. No double sex chromatin bodies were detected in buccal smears of either case 10 or case 11. No clinical abnormalities suggestive of a sex chromosome aberration were seen in any of these patients. Thus case 9 must be considered as a normal female while case 10 is probably an XX/XXX mosaic and case 11 is probably an XO/XX mosaic. In a study where only two cells are being examined it is obvious that mosaics will be missed and this should not be taken as an indication of the frequency of mosaicism in this population.

Ten cases were observed where there was a chromosome which was considered a 'normal variant'. These are detailed in Table 5. The definition of a normal variant is difficult since they are at the extreme ends of a range of normal variation [3]. In the present study they are defined as chromosomes which show a variation which in the opinion of two independent observers would serve as an unequivocal marker in a family study.

In addition an unusual result was obtained in one 84 year old woman with breast cancer. When she was first examined it was found that so many chromosome and chromatid aberrations were present that it was difficult to carry out a full analysis. The basic chromosome complement was 46,XX but in scanning a total of 100 cells, 58 were found to be abnormal. Seven cells were polyploid and of these one was endoreduplicated and two partially endoreduplicated. Of the remainder the majority had chromatid aberrations, no fewer than 15 having chromatid interchanges. In a second blood sample examined 43 days later only one chromatid aberration was found in 25 cells examined. A culture from the patient's brother was also found to be normal. These findings seem to rule out the possibility that the abnormalities observed were of the hereditary type such as are seen in Fanconi's anaemia and Bloom's syndrome, and there is no obvious explanation of this curious observation.

DISCUSSION

In the study of the frequency of aberrations in populations it is never possible to entirely eliminate bias in the selection of these populations and it is therefore necessary to be conscious that it exists. In the present study population there is a bias against malignancies which are not normally treated by radiotherapy and therefore, for example, very few cases of carcinoma of the gastro-intestinal tract are included. The frequency of the various types of malignancy is not representative of the frequency of these malignancies in the local population. It is also true in general that the incidence of cancer increases with age. Therefore the possibility exists that any chromosome abnormality which leads to a shortening of life expectation may be under-represented while any excess of chromosomally abnormal individuals could reflect an enhanced life expectation rather than an unusual liability to malignant disease. Further, any chromosome abnormality that leads to serious congenital abnormality is unlikely to be represented in this population.

Similarly no one control population will give

Table 5. *Distribution of malignancies in patients with a chromosome variant*

Type of malignancy	Age	Sex	Type of variant
Ca. cervix	41	F	Long short arms on a G
Ca. bladder	71	M	Long short arms on a G
Ca. bladder	74	M	Long short arms on a D
Ca. oesophagus	68	M	Long short arms on a D
Ca. bladder	57	M	Long short arms on a D
Rodent ulcer	80	F	Large 16
Ca. lung	63	M	Large Y
Ca. bladder	70	M	Large Y
Ca. bladder	72	M	Large Y
Ca. vallecula	77	M	Large Y

Table 6. Female patients with breast cancer and a constitutional chromosome abnormality

Case No.	Edinburgh Registry No.	Age at diagnosis of breast carcinoma	Chromosome constitution	Method of ascertainment
12	K3/135/61	65	46, XX, t(1?-; Cq+)	Unpublished observation by K. E. Buckton. Fourteen females with cancer were studied, 10 of these with breast cancer. This was the only abnormal [29]
13	K24/60/66	46	46, XX, t(Cq-; Dq+)-	Unpublished observation by E. Day. Fourteen females, all of whom had international stage I breast cancer, were studied. This was the only abnormal
6	A/73/68	69	46, XX/47, XX, mar1+	Present study
5	K46/105/68	38	45, XX, D-, D-,t(DqDq)+	Present study
14	K20/146/65	33	46, XX, t(Dp-; Gp+)	Relative of propositus with the same abnormality found during a population survey*
15	K26/252/66	37	46, XX, t(1?+Cq-)	Relative of propositus with the same abnormality found during a population survey

*In September 1968 there were in the Edinburgh Registry of Abnormal Karyotypes 168 females with autosome abnormalities who had been first ascertained because of their chromosome abnormality. Six of these are known to have a malignant disease and of these two have breast cancer. Follow-up studies are also carried out on 164 cytogenetically normal first degree relatives of individuals with such aberrations. Of these two are known to have cancer one of them having breast cancer. These figures are at present too small to have any significance.

a satisfactory incidence of chromosome abnormalities against which our figures can be compared since these also represent one sector of the population rather than a true overall incidence. Bearing these difficulties in mind, the best current estimate for the frequency of autosomal aberrations in a general population is that of Court Brown and Smith [9] who found 12 abnormal individuals in 3045 adult and young males (approximately 0·4%). This figure of course does not include trisomies and rearrangements which will lead to severe congenital malformations but does include individuals with an additional marker chromosome. Another possible figure for comparison is that from the combined data of surveys carried out on newborn populations excluding those abnormalities which lead to severe malformations. These combined data [4–6, 10] give a frequency of approximately 0·15% for roughly 10,500 babies examined. The finding of six autosomal abnormalities in the present population of 1149 individuals (approximately 0·5%) is therefore slightly higher than would be expected, but the figures are so small and the variation between individual surveys so great that it would not be wise to attach too much significance to this observation at present but it does suggest that a continuation of the survey is necessary. The only single autosomal abnormality which is frequent enough to consider separately is the D/D translocation. Our finding of two such translocations in just over 1000 subjects examined (0·2%) is in accord with Court Brown and Smith's estimate of a frequency of 1 in 800 (0·13%).

The numbers of cases is too small for any useful comment to be made on the frequency of sex chromosome abnormalities. The frequency of triple X females is roughly 1 in 1000 females at birth (0·1%) [11]. Our finding of one case in 578 females examined (0·2%) is in accord with this frequency. The frequency of pericentric inversions of the Y chromosome is not known.

Variants of the normal chromosomes can occur in some individuals without obvious phenotypic expression. Gunz, Fitzgerald and Adams [12] described an apparent association between chronic lymphatic leukaemia and an abnormal G group chromosome which was lacking the short arms. The chromosome described by Gunz *et al.* was probably such a variant [13] and the associations with chronic lymphatic leukaemia may have been fortuitous but until further data have been accumulated the possibility of a true association cannot be entirely discounted. No variant of this type was observed in the present study although others were observed (Table 5). Since the classification of variants is very arbitary any comments about frequencies must be considered with caution but we have no evidence that the number of variants seen in this population differs radically from the numbers seen in other populations studied in this laboratory. We have noted with interest that five out of the ten variants were observed in men with carcinoma of the bladder, but, on such small numbers, this is not a significant departure from a random distribution.

The present study was primarily designed to look for constitutional chromosome aberrations in cancer patients but because of a report [14] that unusually long No. 1 and No. 2 chromosomes were seen in the lymphocytes of cancer patients with a family history of cancer a special check was made on these chromosomes. We found no evidence that these chromosomes were abnormal in any patient in this series. Neither have we found any such evidence in four patients with double primary tumours, three female patients with breast cancer (not in the series) who had first degree relatives with breast cancer, two affected members of a family with polyposis coli, four members (two affected) of a family with epithelioma adenoides cysticum, and two members (one affected) of a family with Gorlin's syndrome.

One of the stimuli to the present study was the finding of two cases of breast cancer with autosomal translocations in small surveys (a total of 24 cases of breast cancer) carried out in this Unit some time ago (cases 12 and 13, Table 6). A sister (age 59) of case 13 who also has a chromosome abnormality has now been found to have a tumour of the breast and a maternal aunt whose chromosomes were not examined died of carcinoma of the breast (aged 66 years). This family will be published in detail elsewhere. In the present study 177 females with breast cancer have been examined and two have been found to have chromosome abnormalities. An additional 27 women with breast cancer who are not included in the present series, but who were attending for treatment as outpatients were also examined and none was found to have a chromosome abnormality. Thus out of a total of 228 patients examined because they had breast cancer four have been found to have a constitutional chromosome abnormality. In addition in the M.R.C. Registry of Abnormal Karyotypes two female patients with breast cancer are recorded who were first ascertained because of their chromosome abnormality. A total of six

patients with breast cancer and an autosome abnormality are thus now recorded in the Edinburgh Registry. These are detailed in Table 6. Because of the varied mode of ascertainment it is not possible to draw any firm conclusions about the frequency of these associations, but the early age of onset in four out of the six cases is unusual. Pergament [15] mentions a case of breast cancer in a woman with a translocation where the age of onset was 31 years. It would be clearly profitable to extend the present study by examining the frequency of chromosome aberrations in specific types of cancer and in particular a study of women with breast cancer would be desirable.

There is now a well established association between leukaemia and Down's syndrome [1, 16]. An association between cancer of the breast in the male and Klinefelter's syndrome has been suggested [17], but an independent study [18] failed to confirm these findings. Our own unpublished observations and other isolated reports in the literature indicate however that this may be a real correlation. Abnormalities of sex chromosome constituion in the female are associated with an increased incidence of malignancies of gonadal origin but these appear to occur only in females where there is a Y chromosome present in all the cells or at least in one cell line of a mosaic individual [19]. In the latter two instances there is a specific hormonal disturbance which could play a part in the development of malignant disease. Further studies in this field can be extended in two quite different directions. Firstly further elucidation of the association between constitutional chromosome aberrations and malignant disease must be based on epidemiologic studies such as that from the the data in the Edinburgh Registry of Abnormal Karyotypes, or from a continuation of population cytogenetic studies such as the present one. While the description of a familial association of chromosomal abnormalities with malignant disease [20–22], and also of isolated cases of the association of chromosome abnormality with malignant disease [15, 23–25] are of interest, they do not replace formal population studies. The second method of approach is to try to determine mechanisms by which constitutional chromosome abnormalities would predispose to malignant disease. One possible approach has been outlined by Miller and Todaro [26], who suggest that the SV40 transformation assay previously described [27] and which has been used to show an unusual sensitivity of mongol cells to transformation by SV40 virus [28] could be used to screen individuals with chromosome abnormalities to determine their sensitivity to virus transformation. It must be remembered however that a constitutional chromosome aberration may predispose to malignancy not because there is an unusual susceptibility to malignant transformation at the cellular level but because there may be a deficiency in the immune response or in some other systemic mechanism.

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NOTE ADDED IN PROOF

The chromosome abnormality has now been found to be familial in one additional case. A marker chromosome identical to that found in the propositus has been found in the peripheral blood cells of a sister of case 3. One brother has been found to have normal chromosomes while a second brother died aged 24 yr in 1937 from appendicitis and peritonitis.

The presence of the marker chromosome in case 3 was confirmed by examination of 50 cells from a bone marrow sample taken prior to any radiation therapy or treatment with vitamin B12. In addition 14 cells out of 50 lacked a small acrocentric chromosome and in 10 of these the missing chromosome was considered to be the Y.

RESUME

Des études de chromosomes furent entreprises sur le sang de 1.149 *malades atteints de cancer. Huit d'entre eux montrèrent des anomalies chromosomiques constitutionnelles.*

Cette proportion est à peine plus élevée que celle que l'on trouve dans la population normale, et aucune signification particulière ne doit être attachée à cet accroissement. On suggère que la poursuite des observations serait utile, et qu'à la place d'une étude globale du cancer, il y aurait avantage à entreprendre des études particulières de syndromes malins bien spécifiques.

SUMMARY

Chromosome studies were carried out on peripheral blood lymphocytes of 1149 *patients with cancer. Eight patients were found to have a constitutional chromosome abnormality. This frequency is only slightly higher than that found in normal populations and no special significance is attached to this increase. The study does suggest that further observations would be of value and in particular studies should be made on specific malignancies rather than cancer in general.*

ZUSAMMENFASSUNG

An Lymphozyten aus dem peripheren Blut von 1149 *Tumorpatienten wurden Chromosomenuntersuchungen durchgeführt. Bei acht Patienten wurde eine konstitutionelle Chromosomenanomalie gefunden. Diese Häufigkeitsquote liegt nur geringfügig über den bei einer normalen Bevölkerung gefundenen Zahlen und hat keinerlei signifikante Bedeutung. Es wird darauf hingewiesen, daß weitere Untersuchungen von Nutzen sein könnten, und daß insbesondere Studien an spezifischen Krebserkrankungen den Studien am Krebs im allgemeinen vorgezogen werden sollten.*

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Transplantation of Canine Osteosarcoma

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INTRODUCTION

A DESCRIPTION of the transplantation of canine lymphosarcoma has been previously given [1]. Following laparotomy in bitches 46–48 days pregnant foetuses were injected with suspensions of lymphosarcoma cells. A high percentage of puppies died *post partum* with widespread lymphosarcoma. In the present experiments transplantation of a canine osteosarcoma has been made by intra-foetal injections or following immunosuppression by azathioprine.

Description of donor dog

The donor animal was a cross-bred Alsatian male aged 8 yr with an osteosarcoma of the left proximal radius. Histologically there was a frankly sarcomatous stroma giving rise to tumour osteoid and new bone (Fig. 1). Some parts of the tumour showed cartilage formation.

METHODS

First transplantation

The donor animal was premedicated with 0·6 mg atropine and 2 mg acetyl promazine; anaesthesia was induced with thiopentone sodium and maintained with nitrous oxide and halothane. A hemi-splenectomy was then performed, the excised splenic tissue being passed through a Craigie grinder and a suspension of spleen cells made in Hank's solution. Histological examination of adjacent spleen showed it to be normal and free of tumour.

Following laparotomy in a cross-bred bitch of large type (54 days pregnant) foetuses were palpated through the uterine wall and each foetus was injected intra-peritoneally with 10^8 of spleen cells from the donor dog. The volume injected was 0·1 ml.

On the 63rd day of pregnancy the bitch whelped 6 puppies over a 2 hr period and half an hour later 5 of these 6 puppies were injected subcutaneously with diced small pieces of osteosarcoma obtained from the primary tumour of the donor animal. Some tumour was also placed in the intramedullary cavity of the long bones and in one animal a cell suspension was injected intravenously. Whole thickness skin grafts from the donor were made in the one puppy not injected with tumour and in a second puppy in which tumour was injected subcutaneously. Skin grafts were sutured with 5/0 silk, covered with Penicillin Vaseline gauze and bandaged.

Subsequent intra-foetal transplantations

Tumour tissue was cut into small pieces about 0·1 ml volume and trypsinised using 0·25% Trypsin in Hank's solution at 39°C. Trypsinised cells were washed in tissue culture fluid T.C.199 and injected into foetuses either intraperitoneally or subcutaneously. The number of cells injected per foetus varied from 2×10^6 to 1×10^9. Viability of cells was assessed by the Trypan Blue test [2]. To mark foetuses in a litter where not all foetuses had been treated in the same manner 0·1 ml Thorotrast was injected subcutaneously. A radiographic examination of the puppies was then made soon after birth to detect the thorotrast injected animals.

In some instances puppies which had received

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tumour cells when *in utero* were also inoculated with tumour cells or implanted with tumour fragments at birth.

Abortion occasionally occurred following intra-foetal injection and appeared to be of three types: early abortion occurring within 48 hr of operation and often associated with uterine contractions during handling of the uterus; a later abortion following death of a foetus and abortion due to infection.

In an attempt to reduce the incidence of early abortion bitches were injected intravenously before and during operation with 8% ethyl alcohol (20 ml/kg.BWt.) in isotonic dextrose solution. Mechanical injury to the foetus with subsequent death can be reduced by using a fine needle (No. 25) and by injecting subcutaneously. Infection has been controlled by strict aseptic technique and by injecting the donor tumour bearing animal with antibiotics for 2–3 days before surgery. The cell suspensions in T.C.199 which have been injected into foetuses have also contained penicillin and streptomycin.

Transplantation after immunosuppression with azathioprine or whole body X-irradiation

New-born puppies were implanted subcutaneously with tumour tissue and dosed with oral azathioprine (5 mg/kg.BWt.) daily.

New-born or young puppies were given whole body X-irradiation the dose usually being between 75 rad and 150 rad. Tumour tissue was transplanted the following day.

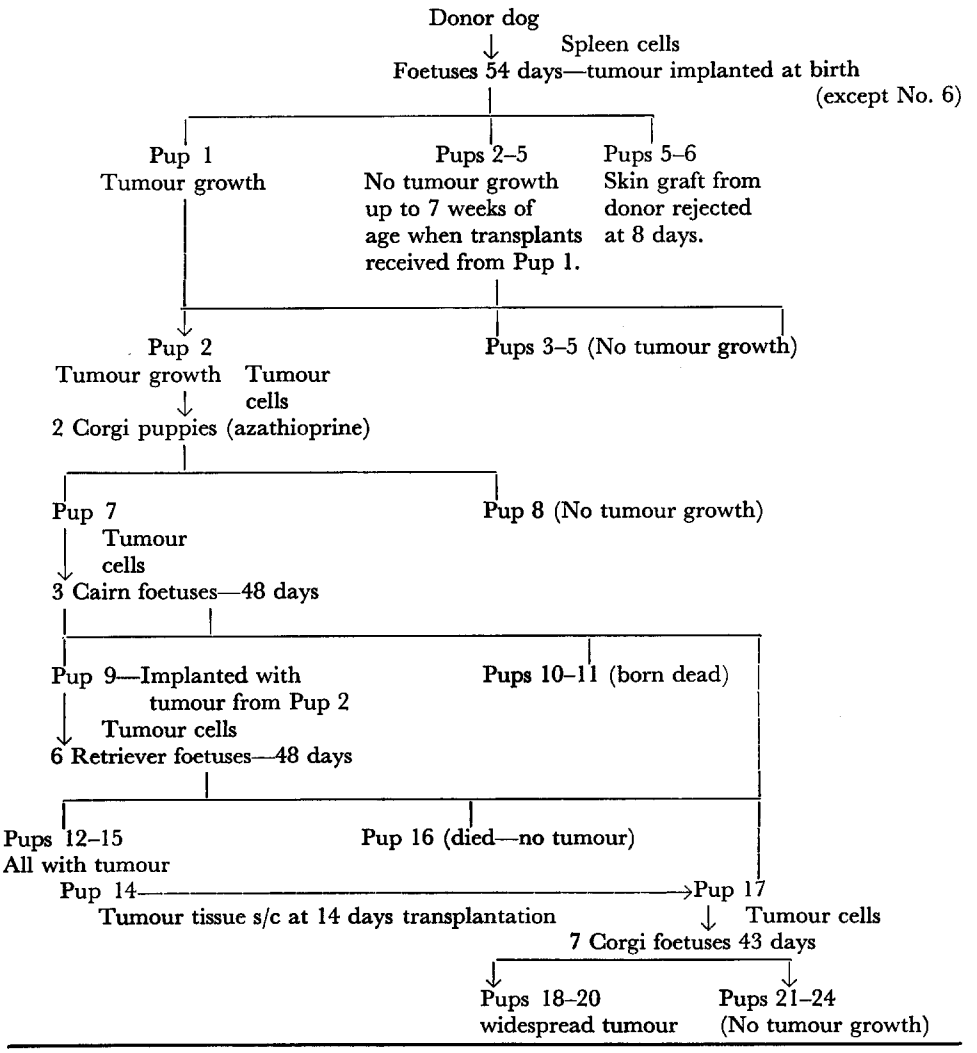
The scheme of transplantation using intra-foetal injections or transplantation into animals immunosuppressed with azathioprine is shown in Table 1.

Histology and histochemistry

The detection of osteoid was aided by staining 1 : 9 dimethymethylene blue, Weigert's haematoxylin, phloxine and tartrazine and Herovici's picro-polychrome stains.

Enzyme studies (made by Dr. G. M. Jeffree) were made as described by Jeffree and Price [3].

Table 1. *Transplantation of osteosarcoma*



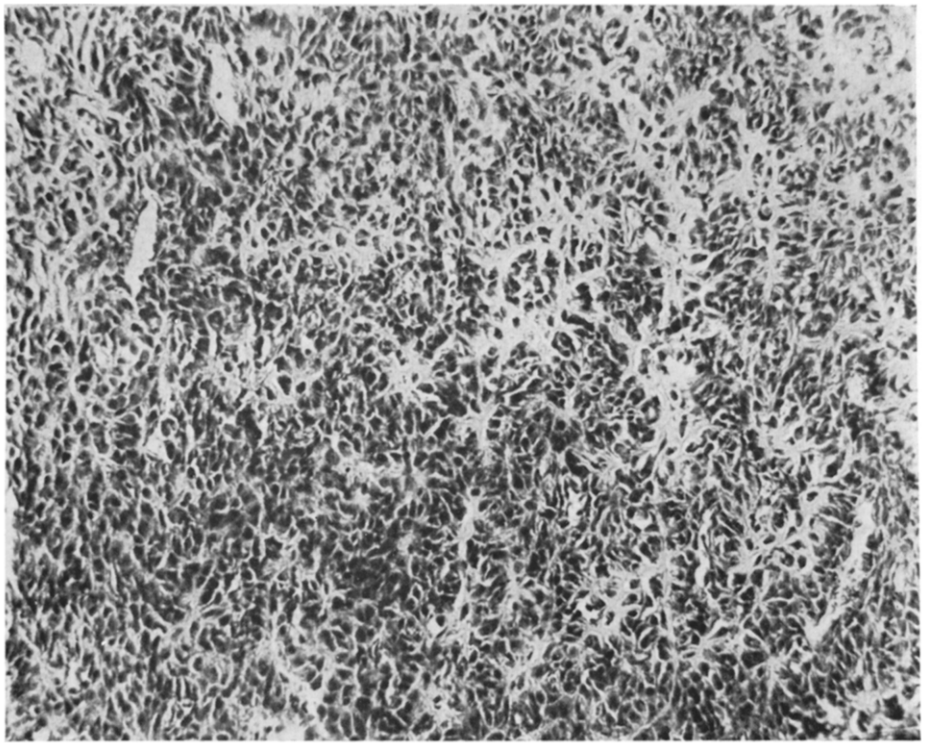


Fig. 1. *Photomicrograph of the primary tumour in the donor animal showing darkly staining sarcoma cells and areas of osteoid and new bone.*

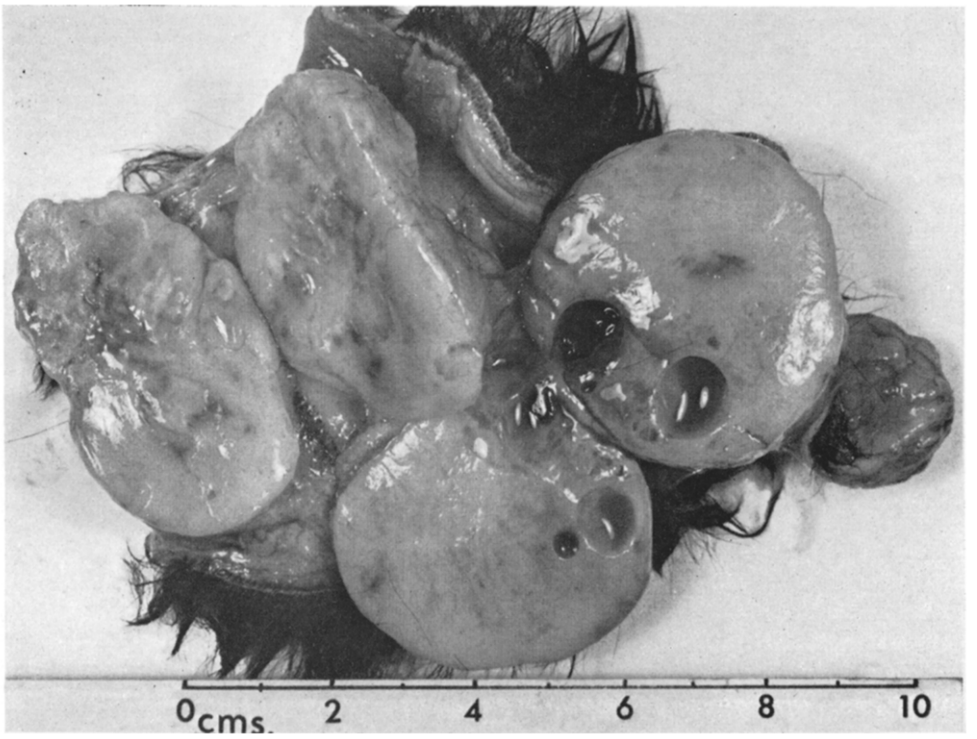


Fig. 2. *Subcutaneous osteosarcoma showing cystic and haemorrhagic areas. First transplantation. Pup* 1.

(to face p. 616)

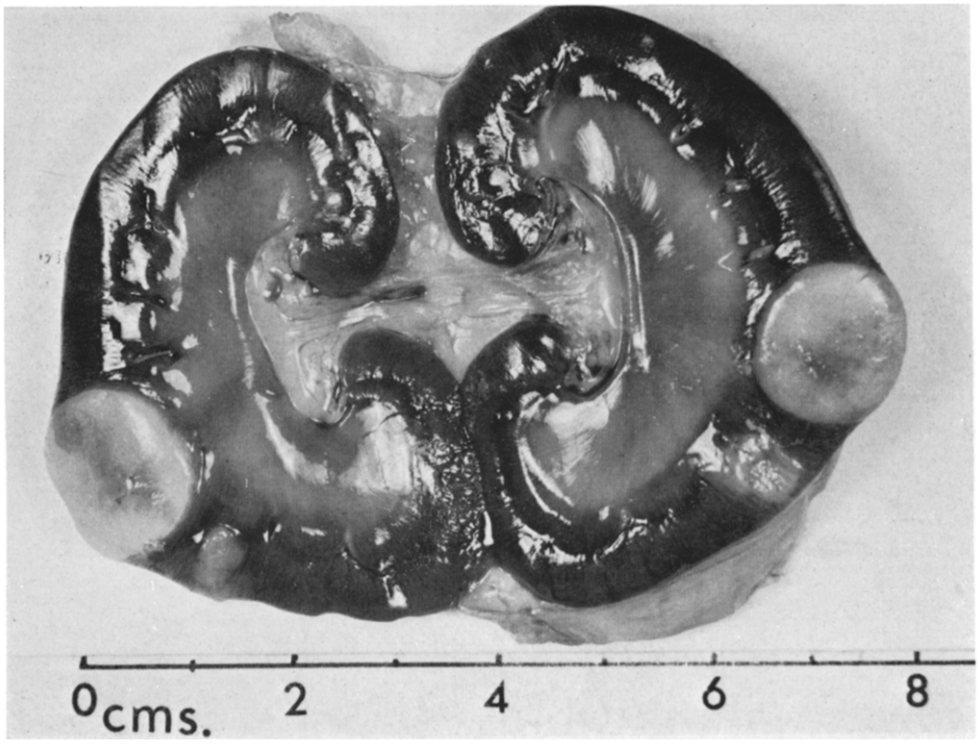


Fig. 3. *Metastatic tumours in the kidney. First transplantation. Pup* 1.

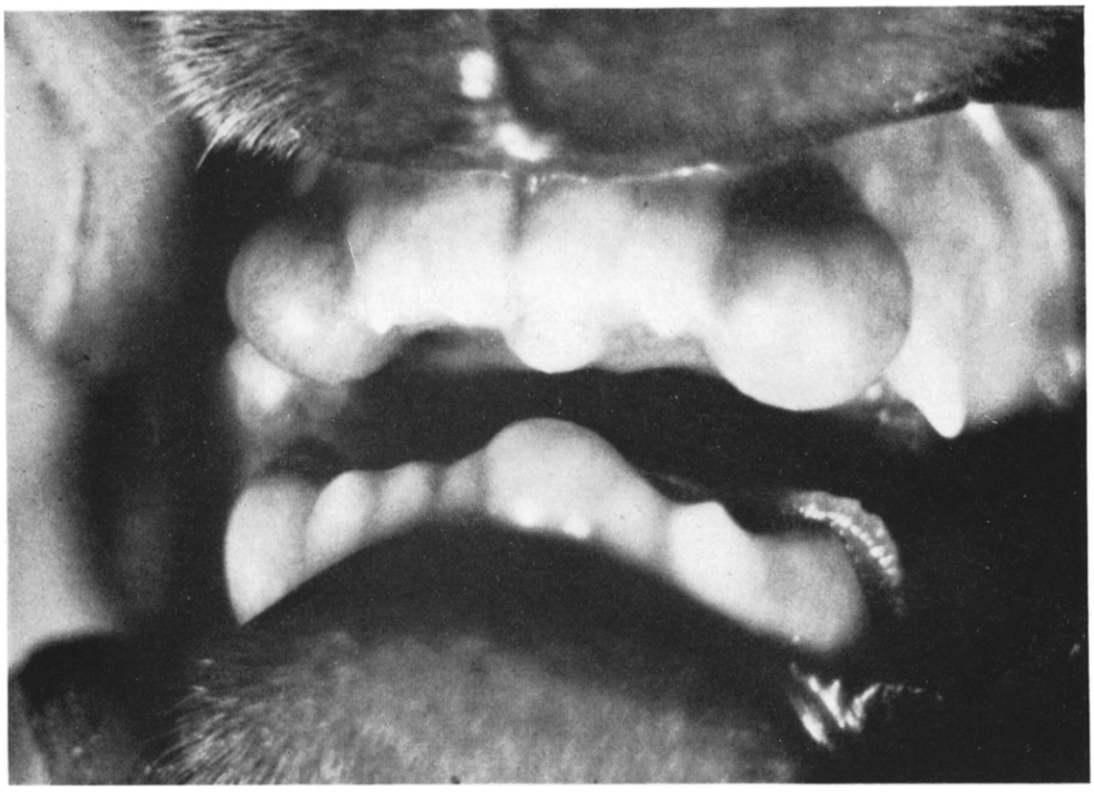


Fig. 4. *Retriever puppy* (*Pup* 14) *at* 3 *weeks of age showing osteosarcomas of the gums.*

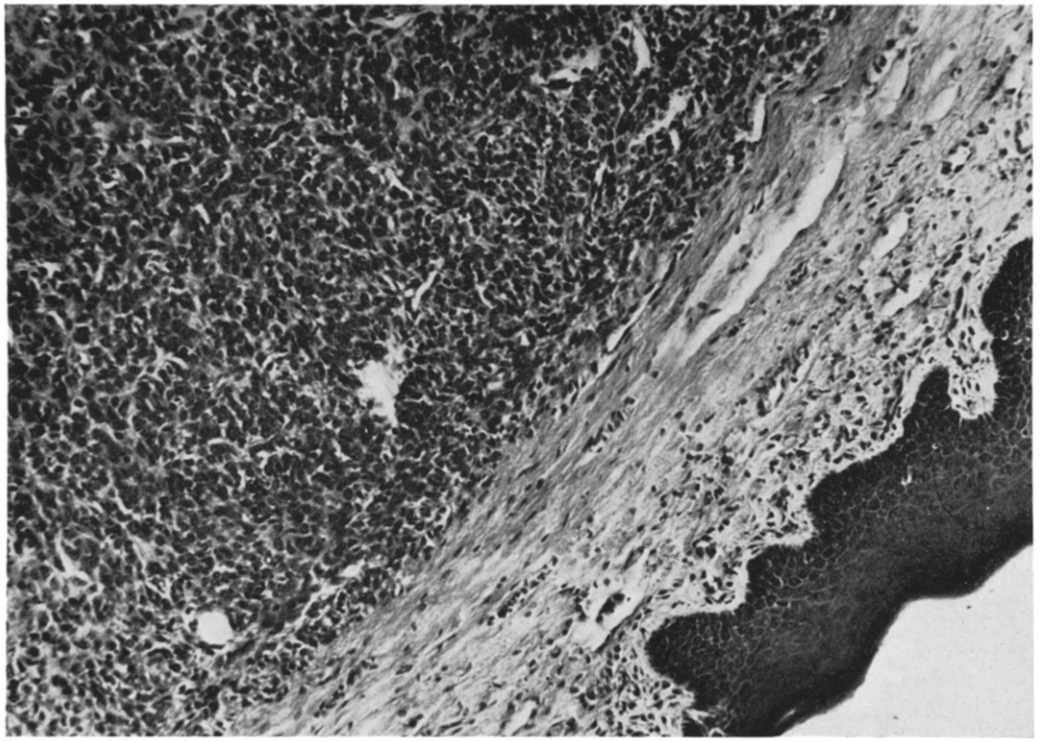


Fig. 5. *Histological appearance of the gum tumour over the right upper canine tooth from Pup* 14 *showing a mass of sarcoma cells with some areas of osteoid also present.*

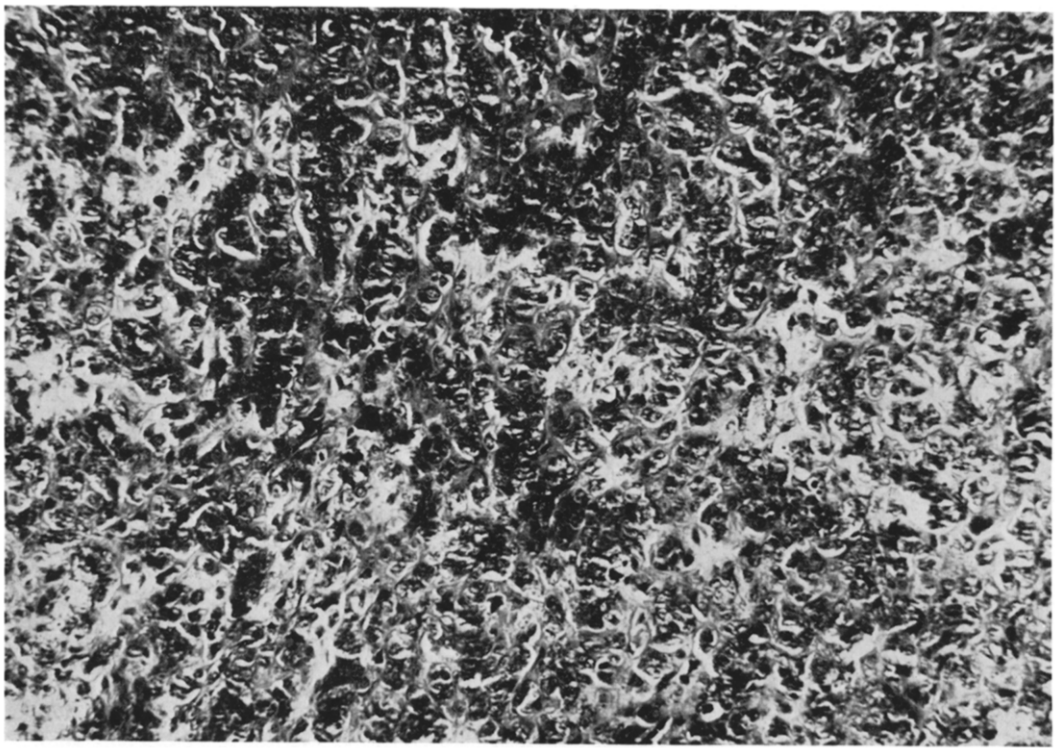


Fig. 6. *Photomicrograph of the sixth transplant osteosarcoma showing formation of osteoid and early new bone.*

RESULTS

1. *Transplantation and distribution of tumours*

The results of transplantation are shown in Table 1. At 54 days from conception the intraperitoneal injection of 10^8 spleen cells into canine foetuses failed to induce tolerance to skin grafts in two puppies. Whole thickness skin grafts from the donor were made when the puppies were 36 hr old but rejection occurred approximately 8 days later. In only one of five puppies in the litter, all of which had been injected intraperitoneally with 10^8 spleen cells when *in utero* and implanted with tumour from the donor immediately after birth, was there progressive growth of the tumour. This animal (Pup 1, Table 1) died 18 weeks after transplantation with extensive subcutaneous tumours on both flanks just behind the rib cage (Fig. 2). The lungs contained multiple hard shot-like tumour nodules the left diaphragmatic lobe being particularly affected. Tumour masses were also present in the anterior mediastinum, the bronchial lymph nodes, the ileo-colic and sublumbar lymph nodes and the kidneys (Fig. 3). The right popliteal lymph node was also greatly enlarged and tumorous with calcareous deposits. Tumour tissue was present adjacent to the right femur which was one of the original injection sites. Histology of the tumour showed two distinct areas, one extremely cellular with very little intracellular matrix and a second area where there was much osteoid and new bone formation. This new bone was visible radiographically in the larger tumours.

At 7 weeks of age fresh diced tumour tissue from Pup 1 was implanted subcutaneously into 4 littermates with progressive growth of tumour in one (Pup 2). This latter pup was killed 17 weeks after tumour transplantation. Apart from large subcutaneous tumours the only other osteosarcoma tissue found in this animal was in the pancreas.

Diced tumour tissue from Pup 2 was implanted subcutaneously into 2 normal Corgi puppies a few hours after birth. There was no apparent tumour growth. The tumour was also implanted into two littermates of the Corgis treated with azathioprine and in one of these the tumour grew slowly reaching a volume of approximately 1·9 ml one month after transplantation (Pup 7).

This latter tumour was excised and trypsinised, the cells having 65% viability as judged by the Trypan Blue test. Three Cairn foetuses *in utero* on the 48th day of pregnancy were each injected intraperitoneally with $2{\cdot}3 \times 10^6$ of these cells suspended in 0·1 ml T.C.199 solution. At parturition 2 puppies were born dead and nothing abnormal was found on post-mortem examination. The remaining puppy (Pup 9) which appeared normal was implanted subcutaneously in both flanks on the 6th day *post partum* with diced tumour tissue from Pup 2. Tumour growth was rapid and death of the animal occurred when 6 weeks old. On post-mortem examination large tumours were found in both flanks and in the superficial inguinal lymph nodes. Smaller tumours 0·4–1·8 cm dia. were found on the pleura, in the left apical lobe of the lung, the peritoneum and the sublumbar lymph nodes. Tumours on the gums were present.

The fourth transplantation was made into Retriever foetuses *in utero* on the 48th day of pregnancy and the results are shown in Table 2. The most interesting finding in Pup 14 and Pup 15 was the appearance of osteosarcomas in the gums, an observation which had also been previously noted in Pup 9 (third trans-

Table 2. Fourth transplantation

Retriever puppies injected *in utero* on 48th day of pregnancy		
Pup	Death (days)	Main tumour sites
12	2	Pleura, mediastinum, peritoneum.
13	14	Pleura, lungs, gastric lymph node.
14	35	Gums. Nasal chamber, pericardium, pleura over ribs.
15	35	Gums. Subcutaneous. Not elsewhere.
16	3	No tumour.
17	Alive	Transplanted at 14 days Pup 14.
	4 months old	Progressive growth 6 weeks.
		Biopsy. Regression.

plantation). Between 2–3 weeks after birth and a few days before normal tooth eruption would be expected to occur tumorous nodules were noticed on the gums of the upper and lower incisors, upper canines and the carnassials (Fig. 4). The large swelling over the right carnassial in Pup 14 was cystic. Histological examination showed the tumorous masses to be osteosarcomas of similar histological appearance to tumours growing elsewhere in these puppies (Fig. 5). By 5 weeks of age the tumours seriously interfered with eating in Pups 14 and 15 and euthanasia was performed.

Tumour from Pup 14 was successfully transplanted subcutaneously into a littermate (Pup 17) and progressive growth occurred for 6 weeks. At this time some of the tumour was excised and used in the sixth transplantation. Shortly after this operation on Pup 17 the remaining tumour, which had an approximate volume of 50 ml immediately post operation, regressed over a 2–3 weeks period.

A trypsinised cell suspension was made from the tumour of Pup 17 and injected either intraperitoneally or subcutaneously into 7 Corgi foetuses *in utero* on the 43rd day of pregnancy (10^7 cells per foetus). During the first 10 days *post partum* 2 puppies died and one was killed. All contained widespread osteosarcomas still showing the formation of osteoid and new bone (Fig. 6).

2. *Histology and histochemistry*

The histological appearance of transplanted tumours was very similar to the histological appearance of tumour from the original donor. Osteoid or new bone was present in all transplants. In some cellular areas of transplanted tumour the mitotic index was very high. Chromosome studies from the tumour in Pup 14 (female) showed the tumour to be male, the same sex as the donor, indicating that cellular transplantation had occurred rather than viral transmission.

Enzyme studies on fourth transplant tumour material (Pup 14) showed the characteristically high alkaline phosphatase activity of an osteosarcoma. Lactic acid dehydrogenase and β-glucuronidase activities were rather high. There was a moderate amount of non-specific esterase, but little acid phosphatase except in a few intensely active cells.

From trypsinised cells of tumours in Pups 13 and 14 the tumour has been established in tissue culture [4].

3. *Partially successful and unsuccessful transplants*

(a) Normal new born puppies. Transplants, both intravenous and subcutaneous, of diced or trypsinised tumour tissue made into normal new born puppies were unsuccessful except in two instances when progressive tumour growth occurred for a period of 20 days (third transplant tumour). At this time biopsy showed no evidence of histological rejection but shortly after this biopsy tumour regression occurred.

(b) No tumour grew in new-born or older puppies which were implanted with tumour 24 hours after whole body X-irradiation. No growth of tumour occurred in puppies injected with trypsinised tumour cells at birth when the dam had been given 60 rads whole body X-irradiation on the 47th day of pregnancy.

(c) When a pregnant bitch was given azathioprine orally (5 mg/kg) during the last 8 days of pregnancy and 3 new born puppies were injected with tumour this grew in 2 puppies. Both bitch and puppies however died of distemper when the puppies were 4 weeks old. At this time there was no histological evidence of tumour regression. It is not known if immunosuppression was a factor in the occurrence of distemper.

DISCUSSION

The natural history and behaviour of osteosarcoma in dog and man is essentially similar [5]. The dog, because of its large size, is better suited for tumour therapy using surgical, irradiation and perfusion techniques than the smaller laboratory animals and a transplantable osteosarcoma in the dog would be of considerable value for furthering such studies.

It is hoped that by further selective *in vivo* passages and by animal inoculation of the tumour growing in tissue culture that progressive growth of the tumour will eventually be established in normal neonatal dogs.

The appearance of tumours in the gums in 3 puppies is worthy of note especially as in one animal (Pup 15) the only other tumour found was a subcutaneous tumour on the back. It may be that just before eruption of the milk teeth the vascularity and other unknown factors in the gum make it a particularly favourable site for tumour development. Further experiments are planned, including chromosome studies, on tumours appearing in this site.

Following successful results in the transplantation of canine lymphosarcoma [1] and the transplantation of osteosarcoma described above it seems likely that any spontaneous canine tumour could be transplanted either by (a) the injection of spleen cells into foetuses *in utero* to produce tolerance followed by implanta-

tion of the tumour from the same donor after birth, or (b) the direct injection of tumour cells into the foetuses. The age at which cellular "immunological maturity" develops in the foetal dog is not known although studies on this using specific pathogen-free dogs are in progress [6].

In two non specific pathogen-free puppies injected intraperitoneally with 10^9 spleen cells at 41 days of pregnancy and transplanted with skin from the same normal donor 36 hr after birth a successful graft occurred in one puppy. The graft remained healthy for 8 months at which time rejection occurred [7]. Skin grafts made from the donor osteosarcoma dog described in this article to two puppies previously injected with spleen cells on the 54th day of pregnancy were rejected within the normal expected period for homologous skin grafts. Preliminary experiments on tolerance to skin grafts in cats following the injection of spleen cells to foetuses *in utero* were successful in one instance when kittens were injected at 39 days of pregnancy [7]. It seems likely that in this species as well as the dog that tumour transplantation using the intra-foetal method could be made. The most valuable feline tumour to study in this way would be the mammary carcinoma as the pathology and behaviour are very similar to carcinoma of the breast in women.

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RESUME

Des foetus de chien furent inoculés in utero *avec des cellules encore vivantes de la rate d'un chien non apparenté et porteur d'un ostéosarcome d'origine spontanée. L'injection de l'ostéosarcome aux jeunes chiens, lors de leur naissance, produisit une tumeur chez un des chiens. La transplantation de la tumeur put être continuée, soit par injection de cellules trypsinisées à des foetus, soit, dans de jeunes chiens, avec immuno-suppression à l'azathioprine. Une tumeur rarement observée des gencives fut découverte chez trois jeunes chiens.*

SUMMARY

Canine foetuses in utero *were inoculated with living spleen cells from an unrelated dog bearing a spontaneous osteosarcoma. At birth osteosarcoma from the donor was inoculated into the puppies and successful transplantation occurred in one dog. Using the inoculation of trypsinised cells into foetuses* in utero *or by transplantation into puppies whose immunity was suppressed with azathioprine successive transplants have been made. An unusual occurrence of tumours in the gums is described in* 3 *puppies.*

ZUSAMMENFASSUNG

Hundeföten wurden in utero *mit lebenden Milzzellen von einem, nicht verwandten, an einem spontanen Osteosarkom erkrankten Hund, geimpft. Bei der Geburt wurde das Osteosarkom von dem Spendertier auf die Welpen transplantiert. In einem Hund war die Transplantation erfolgreich. Weitere Tumorpassagen durch Inokulation trypsinierter Zellen in Föten* in utero *oder in Welpen, deren Immunitat durch Azathioprin herabgesetzt war, wurden durchgeführt. Ein ungewöhnliches Auftreten von Gingivatumoren bei* 3 *Welpen wird beschrieben.*

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A Replication Technique for the Identification of Asbestos Fibres in Mesotheliomas

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INTRODUCTION

THERE has been a great deal of interest over the past decade in the fact that the incidence of pleural and peritoneal mesotheliomas increases when there is an exposure to crocidolite asbestos. The relationship between this exposure and tumour incidence in South Africa was described by Wagner and his colleagues [1] and for the English population by Elwood and Cochrane [2]. It was also shown by Elwood and his colleagues that as well as an increased tumour incidence in workers concerned with asbestos in industry, there was a corresponding higher incidence in the local non-industrial population.

A number of other reports have been concerned with the relationship between asbestos and carcinogenesis [3–5] but it has always been difficult to obtain an accurate identification of the type of asbestos particle considered responsible for the tumour development. Histological studies are difficult, since fine particles embedded in the tumour tissue are often beyond the resolution limits of the optical microscope. Incineration of tumour tissue with subsequent examination in the electron microscope of the isolated mineral particles can produce misleading information due to chemical changes induced by the ashing procedure. Furthermore, the 'thin-sectioning' technique used for the investigation of biological tissues by electron microscopy, is of little value in studying the localisation of such particles *in situ*, in relation to the surrounding tumour tissue, since the sections tend to disintegrate as hard particles embedded in the tissue are caught on the ultramicrotome knife. Also the possible deposition of the particles in a false position or the disintegration of the finer crystals resulting in larger surface areas being in contact with the tissue, also make the sectioning technique unreliable.

The present report describes the application of a simple replication procedure to the identification of asbestos particles in tissue from various mesotheliomas, and illustrates the use of the technique for studying the location of the particle in relation to surrounding tissue.

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MATERIAL AND METHODS

Tissues

The tissue studied was obtained from three patients with mesotheliomas. It was prepared as a paraffin section for normal routine histological examination, but was unstained.

Case histories

The first patient, aged 40, presented with productive cough and chest pain. X-ray studies showed a right pleural effusion and a biopsy was taken since tuberculosis was suspected. A typical mesothelioma, confirmed histologically, was found and the patient died one month later. The biopsy samples were used for the electron microscopy studies. Exposure to as-

bestos had been for the eighteen years prior to the patient's death, being concerned with the handling of asbestos brake drum linings.

The second patient died at 65 yr of age, one year after presenting with severe chest pain, dysphagia and cough. Pleural and oesophagoscopic biopsy revealed no malignancy. The patient had been employed for 50 years of his life in the chemical industry, insulating pipes with asbestos. At necropsy a pleural mass was found, which after histology and extensive consideration was identified as a mesothelioma.

The third patient had been employed as a boiler maker for a number of years and had been concerned with asbestos lagging. He presented at 60 years of age with cachexia and a persistent dry cough. There was a right pleural effusion and enlarged hilar glands. At necropsy, a mesothelioma was found.

Replication Technique

The procedure used was that described by Henderson [6]. Paraffin was removed from the sections by immersion in xylene, and the tissue dehydrated in ethanol. The dehydrated tissue was embedded by impressing the section onto the surface of a sheet of acetone softened cellulose acetate (0·5 mm thick 25 × 25 mm sq. mounted on a glass slide for ease of handling) (Fig. 1a) which was then left to harden (Fig. 1b). The slide was removed, leaving behind most of the tissue embedded in the cellulose acetate. The tissue was then outlined with strips of Scotch tape (Fig. 1c) to form a shallow well, and a 10% (v/v) solution of polyvinyl alcohol (PVA) was applied to the tissue (Fig. 1d). Once the PVA hardened, it was stripped from the section, providing a replica of the tissue surface (Fig. 1e). At the same time, embedded particles are often removed with the PVA during this stripping process.

This procedure allows successive strippings to be taken and a complete sequential examination through the embedded tissue. Surface replicas were preshadowed with platinum, a carbon film deposited for strength, and the PVA removed by floating the replica in a hot water bath. The replicas were then mounted on electron microscope grids for examination using the AEI-6B microscope.

RESULTS

Figure 2 illustrates a number of asbestos fibres *extracted* from the mesothelioma from case one, by the replication technique. The irregular broken-end fracture of the fibres is characteristic of the crocidolite particle, and is distinct from the amosite fibre which fractures with a clean regular 90°C break, perpendicular to the axis of the fibre. A *replica* of a crocidolite particle in association with collagen fibres is shown in Fig. 3. Other mineral particles were sometimes observed in these tumour tissues and Fig. 4 shows a particle of a kaolin-type mineral, with its typical 120°C fracture angle, in association with collagen. The kaolin-particle was seen in studies with tissue from case one.

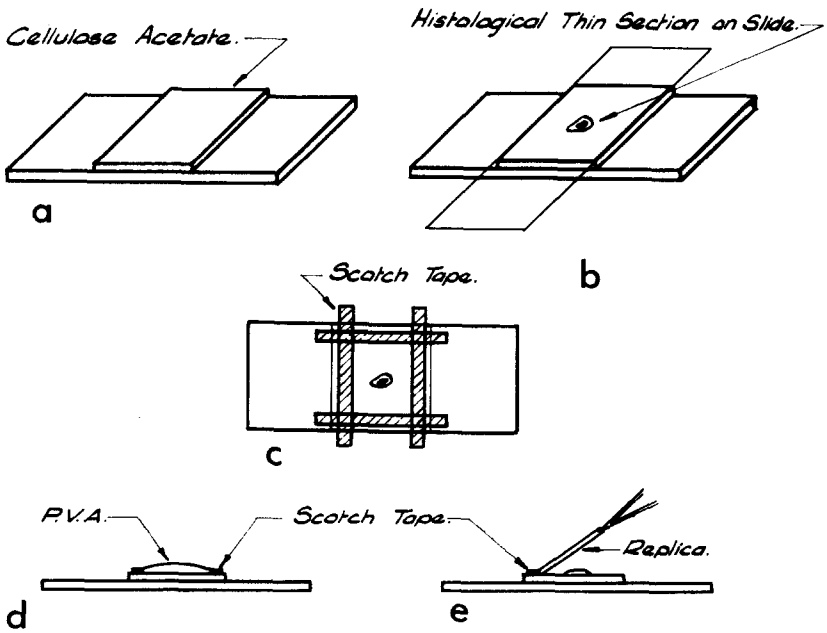

Fig. 1. *Stages of the replication technique.*

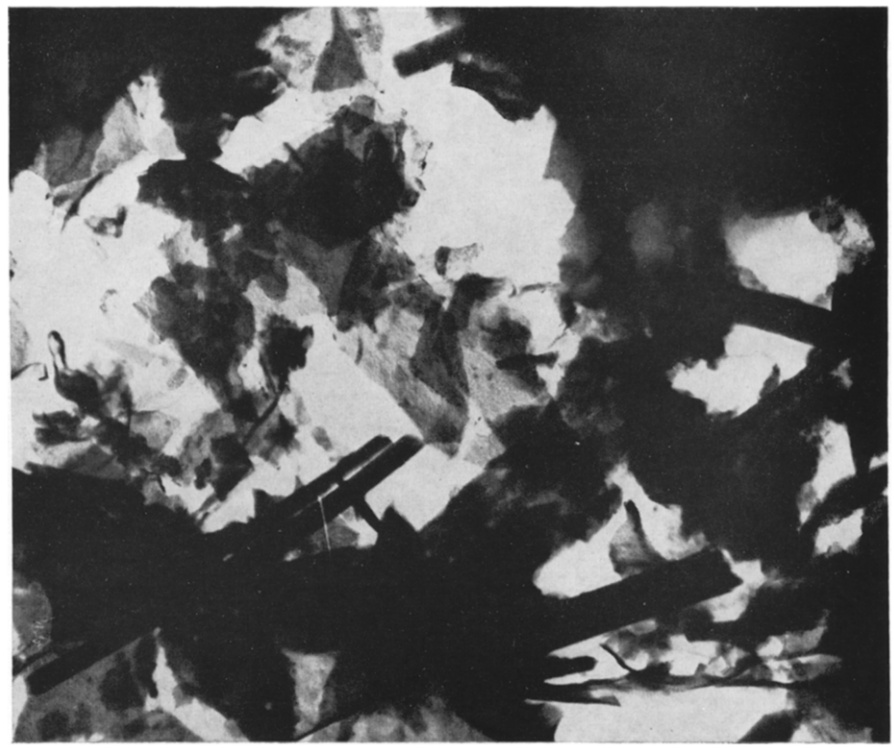

Fig. 2. *Illustrates a number of asbestos fibres extracted from the mesothelioma.* ×10,000

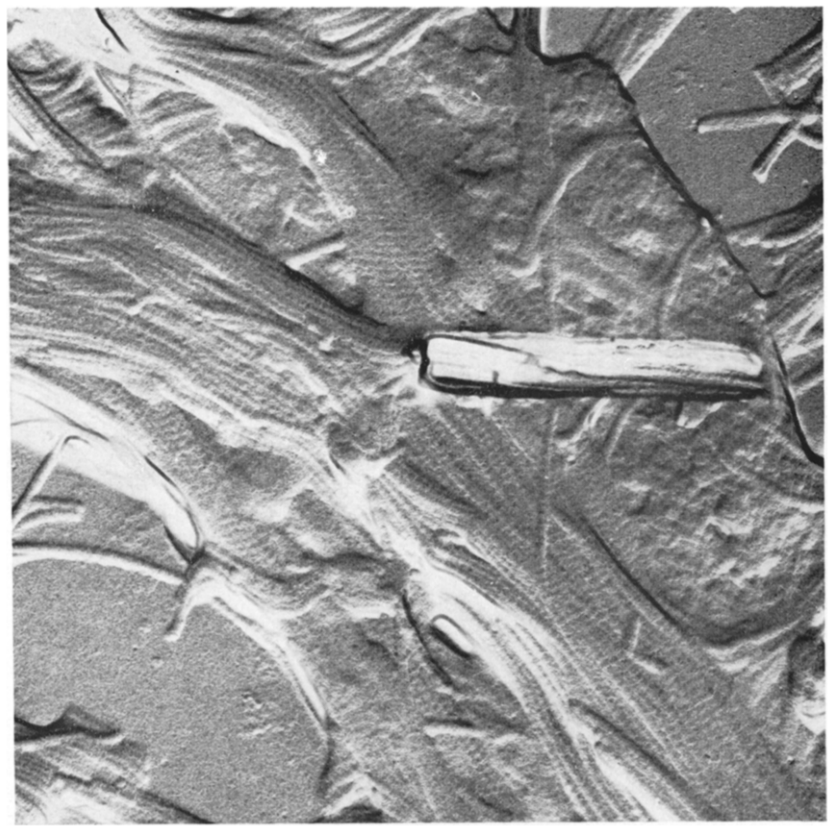

Fig. 3. *Replica of a fibre in relation to collagen tissue.* ×20,000.

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Fig. 4. *Particles of Kaolin type crystals in association with collagen observed in the mesothelioma.* ×40,000.

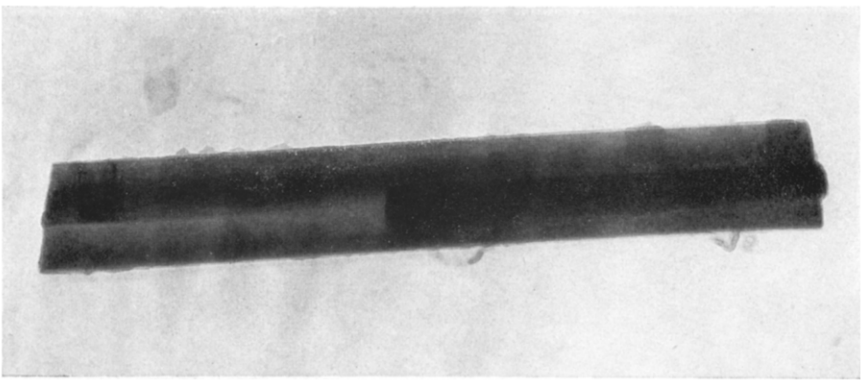

Fig. 5. *Typical crocidolite fibre showing the irregular end fracture of this type of asbestos.* ×40,000.

Fig. 6. *Crocidolite fibre with unknown mineral crystal adjacent to it.* ×20,000.

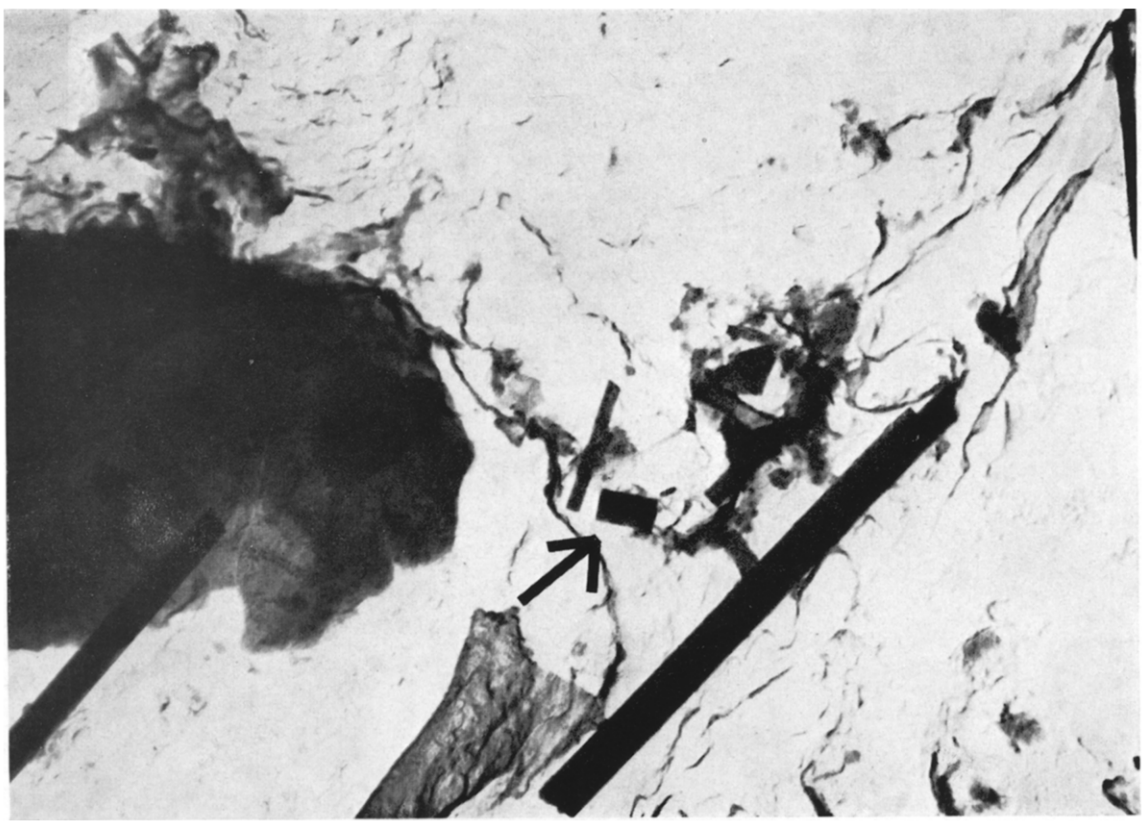

Fig. 7. *Group of crocidolite particles extracted from the tissue: present is a typical amosite fibre (marked by arrow).* ×10,000.

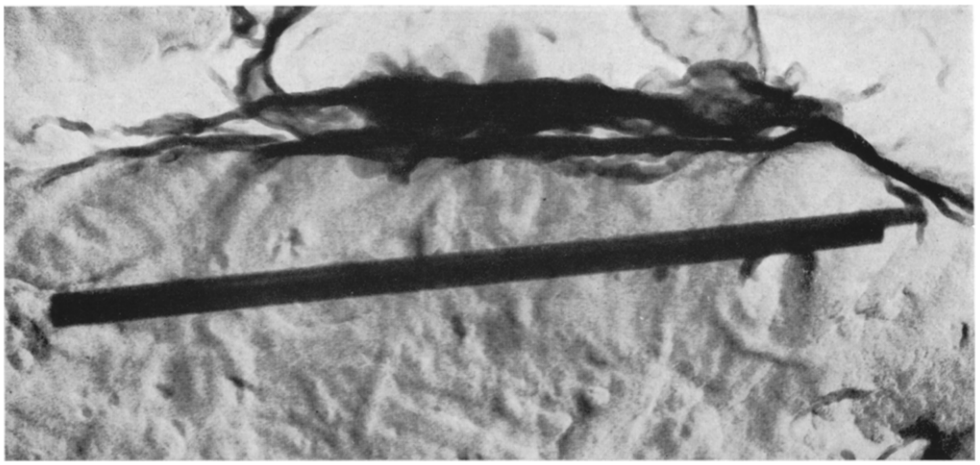

Fig. 8. *Crocidolite particle.* ×40,000.

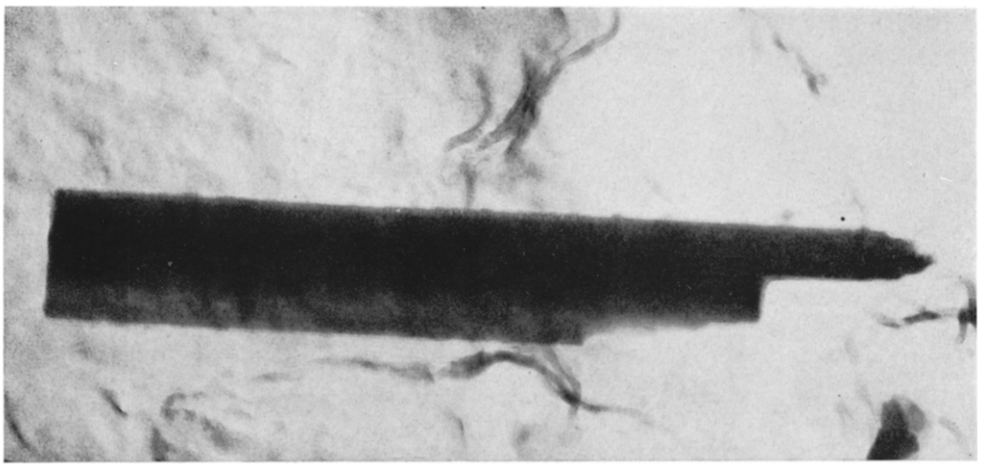

Fig. 9. *Crocidolite particle.* ×40,000.

Fig. 10. *Characteristic ferritin bodies on asbestos fibres from ashed lung material.* ×4500.

Similar fibres with irregular end fractures, and typical of crocidolite, were observed in tissue from the mesothelioma of case two (Figs. 5 and 6). An unknown mineral particle is also seen associated with the crocidolite in Fig. 6. Figures 7, 8 and 9 provide further examples of the use of the technique in identifying crocidolite particles in the tissue from the mesothelioma of case three. In the centre of the group of fibres in Fig. 7 can be seen one which is characteristic of amosite. It is rectangular and exhibits the typical 90°C angle of fracture across the plane of the fibre.

DISCUSSION

The potential hazards associated with exposure to asbestos are now well accepted, and there is little doubt that such exposure is related to neoplasia, particularly to the development of pleural and peritoneal mesotheliomas [7].

It has been considered nearly impossible to show the presence of asbestos fibres in these mesotheliomas. The size of the particle is beyond the resolution limits of the optical microscope, although a birefringent crystal such as asbestos appears brightly illuminated in polarized light. Thin-sectioning techniques are of little value in the localization of such particles, and ashing studies could provide misleading information. Normal histological studies on the identification of asbestos fibres in tissues is based on the presence of characteristic ferritin bodies on some of the fibres (Fig. 10). The bodies are usually the principal means of identification of asbestos by optical microscopy, but are extremely difficult to differentiate from similar bodies observed around elastin fibres [8]. The technique used in these studies, described first by Henderson [4] and used for the identification of mineral particles in pneumoconiotic lungs [9] provides a simple replication-extraction procedure for the study of asbestos in tissue from mesotheliomas. The studies described in this communication indicate that crocidolite would appear to be the principal asbestos-type associated with the mesotheliomas reported herein. As well as the identification of the particle, the replication technique also shows in some instances, the relationship of the particle to the surrounding tissue (Fig. 2), and can provide valuable information in investigations of patients who have been exposed to asbestos, or the particular mineral which might have produced the disorder. Since asbestos is a substance of the modern western civilization, used in many aspects of life [10], the problem of its uptake by tissues may become more serious. The application of procedures such as the replication technique to these tissues, possibly relating it to laser diffractometry, may well be of great value.

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RESUME

Une technique d'extraction et de réplication a été utilisée pour l'étude, au microscope électronique, du tissu de trois mésothéliomes. La technique offre le moyen d'étudier la surface des tissus répliqués, et les particules étrangères présentes sont soit répliquées, soit extraites du tissu, et peuvent être identifiées.

La présence de fibres d'asbeste, ayant des particularités caractéristiques de crocidolithe, a été établie dans le tissu tumoral étudié.

SUMMARY

An extraction replication technique has been used for the study, by electron microscopy, of tissue from three mesotheliomas. The technique provides a means of studying the surface area of replicated tissues, and foreign particles present are either replicated or extracted from the tissue, and can be identified.

The presence of asbestos fibres with features characteristic of crocidolite was established in the tumour tissue studied.

ZUSAMMENFASSUNG

Das Gewebe von drei Mesotheliomas wurde mit Hilfe einer Extraktions- und Replikationstechnik elektronenoptisch untersucht. Die Replikationstechnik erlaubt ein genaues Studium der Gewebeoberfläche und vorhandene fremde Partikel bleiben entweder im PVA haften oder sie werden aus dem Gewebe extrahiert und können so identifiziert werden. Das Vorhandensein von Asbestfasern mit den charakteristischen Merkmalen von Crocidolit wurde in den untersuchten Tumoren nachgewiesen.

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Survival Gain and Volume Gain

Mathematical Tools in Evaluating Treatments

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As we intend to show in the next paper, the way in which screening tests and controlled trials are performed does not meet the requirements for accurate quantitative evaluations. The present study is devoted to the task of setting up a simple mathematical apparatus allowing such evaluations to be made.

The exponential rate of growth of human solid tumors, first established by Collins [1, 2] on the basis of the experimental work of Mottram [3] has been verified for pulmonary metastases and peripheric bronchogenic carcinomas by Schwartz [4], Nathan [5], Garland [6], Spratt [7], Weiss [8], Gerstenberg [9], Breur [10], Bru [11], Combes [12] and for cases of other carcinomas by Welin [13], McDonald [14], Spratt [15], Berger [16], Ingleby [17], Philippe [18] and others. We were able to confirm this model and we discussed the reasons for the apparent discrepancy between the gompertzian model observed in transplanted tumors and the exponential one observed in man during the clinical period [19, 20]. This exponential way of growth leads to some simple algebraic and trigonometric considerations which will be discussed around three theoretical models of therapeutic response to chemotherapy.

METHODS

Construction of growth curves

The method we used is the graphic one proposed by Collins [1, 2]. The mean diameter of a measurable tumor is plotted against time on a semi-logarithmic scale. The curve obtained is a straight line, which means that the doubling time is constant. The volume doubling time is one-third of the diameter doubling time, and can be read directly from the curve.

Mathematical relations between the volume doubling time and the slope of the growth curve

The function describing the growth being of the shape:

$$V = V_0\, e^{bt}$$

where V_0 is the volume at the time t_0 and b a constant of the system choosing for t the value of the doubling time (T_2) gives for b the value:

$$b = \frac{\text{Log } 2}{T_2}$$

Diameters and volumes being bound by the formula:

$$\frac{V}{V_0} = \left(\frac{d}{d_0}\right)^3$$

it follows that:

$$\text{Log } d = \text{Log } d_0 + \frac{\text{Log } 2}{3\ T_2}$$

or, in terms of decimal logarithms:

$$\log d = \log d_0 + \frac{0{\cdot}3013}{3\ T_2}\, t$$

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or, to a good approximation:

$$\log d = \log d_0 + \frac{1}{10\ T_2}\ t$$

Then the function of the growth curve on the semi-logarithmic scale is of the shape:

$$y = \log d_0 + \frac{1}{10\ T_2}\ t$$

and the slope of the curve $t_g\theta$ (see Fig. 1) is:

$$t_g\theta = \frac{1}{10\ T_2}$$

Graphical expression of the concept survival gain compared to total survival time

Figure 1 shows a theoretical therapeutic growth curve, together with the extrapolated

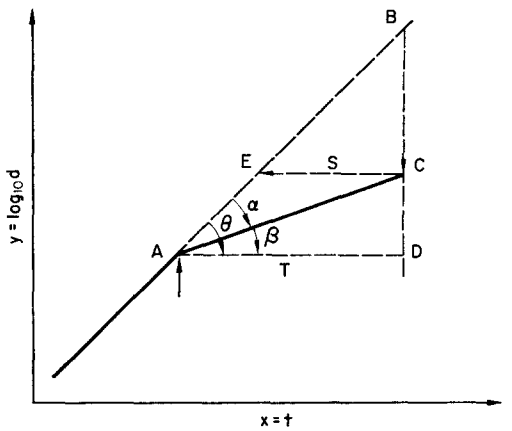

Fig. 1. Graphic expression of spontaneous doubling time, therapeutic doubling time, duration of treatment (T), *survival gain* (S) *volume gain* (BC). *The arrow shows the start of the treatment.*

spontaneous one, in a case of lengthening of T_2. It appears that there is a difference between the total duration of treatment (or the total survival time) T and the benefit induced by this treatment as far as survival and volume are concerned. If the death were to occur when the diameter of the tumor was the one reached in *C*, this diameter would have been reached without treatment in *E*. The survival gain S_g is, in this case of lengthening, smaller than T.

Mathematical relations between S_g *and* T

Considering Fig. 1 again, it appears that the slope of the therapeutic growth curve, $t_g\beta$, is:

$$t_g\beta = \frac{1}{10\ T'_2}$$

where T'_2 is the therapeutic doubling time. Trigonometric consideration of the triangles *ADB*, *BCE* and *ADC* leads to the formula:

$$S_g = T\left(1 - \frac{T_2}{T'_2}\right)$$

So, there is a relationship between the survival gain and the total survival which depends upon the spontaneous and the therapeutically modified doubling times, which is not surprising. The ratio S_g/T provides a quantitative approach towards the evaluation of the effectiveness of a given treatment in cases of different T_2.

Validity of the S_g *formula in different response patterns*

Three different possibilities are outlined in Fig. 2: shrinkage, equilibrium and lengthening. These events are described by the

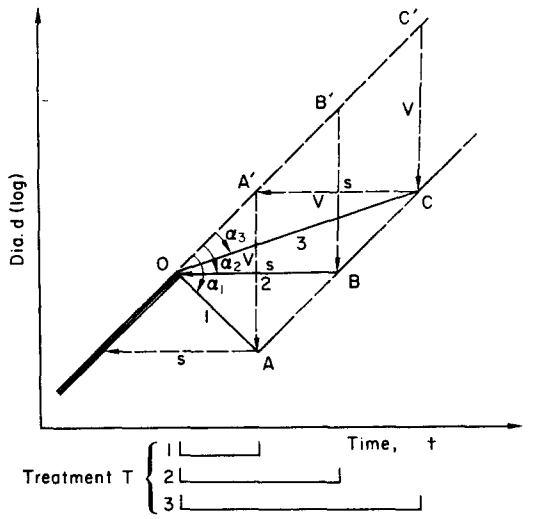

Fig. 2. Theoretical effects of three different kinds of treatment on the therapeutic growth curve. 1: *shrinkage;* 2: *equilibrium;* 3: *lengthening.*

different values of the angle α, with $\beta = \theta - \alpha$. In the case of the figure the same value for S_g results from three different therapeutic responses showing that treatments with different effectiveness and different duration can induce the same S_g. It can be seen that the formula holds true not only when the response is a lengthening or an equilibrium, but also when it has the shape of a shrinkage. In this particular case, T_2 must be changed for $T_{1/2}$ (semi-reduction time) and counted negatively in the formula. This gives for S_g/T a value greater than 1, and $T_{1/2}$ can be read directly on the growth curve in the same manner as T_2.

Validity of the S_g *formula when effectiveness is varying along time*

Figure 3 shows a model of such a response

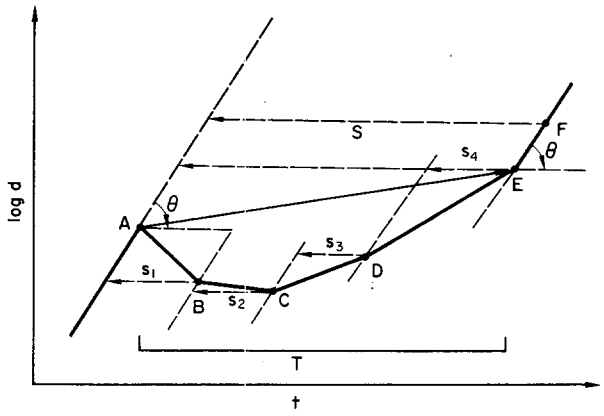

Fig. 3. *Therapeutic growth curve obtained with varying successive effects of the same treatment. Resultant doubling time.*

which is in agreement with most clinical curves registered by us. It can be seen that the vectorial sum of *AB*, *BC*, *CD* and *DE* is equal to *AE*. Then the final survival gain can be calculated directly in *E*, drawing from *E* a horizontal line towards the extrapolated spontaneous growth curve.

Mathematical evaluation of the volume gain

The segment BC in Fig. 1 represents the volume gain from the onset of the treatment to the end point. If d_1 and V_1 are the diameter and the volume at *C*, and d_2 and V_2 the diameter and the volume at *B*, the residual volume in percentage is:

$$\text{R.V.}\% = 100.\frac{V_1}{V_2} = 100\left(\frac{d_1}{d_2}\right)^3$$

The value for BC being $S_g.t_g.\theta$, it follows that

$$\text{R.V.}\% = 10e^{2{\cdot}3 S_g t_g \theta}$$

Thus, for a given survival gain, the shorter the doubling time the more important is the volume gain; and, for a given volume gain, the longer the doubling time the more important is the survival gain. In such a case, even a weak therapeutic effect can induce an important survival gain. This formula may be used to estimate which duration of treatment should be reached in order to produce a given effect, assuming that its effectiveness will be constant. For example, if R.V.% is 1%—i.e. if the reduction in volume induced by the treatment is 99%, the resulting survival gain is:

$$S_g = \frac{20T_2}{3}$$

that is equal to approximately 7 times the doubling time. In tumors with very short doubling times, such a dramatic achievement as far as volume is concerned remains of a somewhat poor value.

DISCUSSION

It is not possible to describe here all the various formulae which may be found in [19]. We shall only underline the following two points:

(a) The calculations discussed here were performed on theoretical models, and it may be difficult in some cases to apply them in human tumors. Most tumors are not measurable. Metastases in different organs can have different growth rates and can be reached differently by cytostatic drugs. Furthermore, small tumors may kill due to their site. Nevertheless, the formulae developed here may prove of some value to the therapist, as we intend to show in the next paper.

(b) The total survival time from the start of chemotherapy (or, for that matter, after the completion of X-ray therapy or surgery) is of little informational value, since the spontaneous survival time that the patient would have reached without therapy remains unknown. The concept of the survival gain induced by the treatment, the value of which is bound to the total survival time (or the total duration of the treatment) and to the spontaneous doubling time, allows evaluation of treatments to be made accurately even in patients whose tumors have different T_2. This may lead to more information being gained from controlled trials.

RESUME

Les essais contrôlés de chimiothérapie des tumeurs humaines comparent des résultats obtenus sur des tumeurs de volume et de vitesse de croissance différents.

Ils évaluent la survie totale qui confond bénéfice du traitement et le temps pendant lequel le malade aurait survécu spontanément.

Les auteurs proposent de remédier à ces insuffisances au moyen de la mesure du gain en survie et en volume. Ces paramètres sont liés, par une formule simple, au temps de doublement spontané et à l'effet du traitement sur la croissance. Des exemples sont donnés à l'aide de modèles théoriques de réponse thérapeutique.

SUMMARY

The usual controls on the effects of chemotherapy on solid human tumors compare the evolution of tumors of different initial volumes and growth rate. They rely on the evaluation of the total survival time, in which the benefit from the treatment and the time of survival without treatment are not distinguishable.

The authors propose a better quantitative appreciation by measuring the gain in survival time and in volume, which are defined in the article. These parameters are connected by simple formulae to the spontaneous doubling time and the effect of the treatment on the growth. Examples are discussed in the light of theoretical models of therapeutic response.

ZUSAMMENFASSUNG

Serienuntersuchungen an Tumoren mit chemotherapeutischen Mitteln vergleichen die Entwicklung anfänglich verschieden grosser Tumore und ihr Wachstumsverhältnis miteinander. Diese Untersuchungen erlauben es die totale Überlebenszeit, in welcher der Erfolg der Behandlungsmethode und die Überlebenszeit ohne jegliche Behandlung als Parameter eingehen, zu bestimmen.

Die Autoren schlagen in diesem Bericht eine bessere, quantitative Methode vor, die es erlaubt den Gewinn an Überlebenszeit und die Volumenänderung des Tumors direkt zu bestimmen. Diese beiden Parameter kann man durch einfache Formeln mit der spontanen Verdoppelungszeit, d.h. der Zeit, in der sich der Tumor um das doppelte vergrössert hätte, und der Wirkung der Behandlung auf die Grösse des Tumors in Verbindung setzen. Beispiele werden an Hand eines Modells für die therapeutische Ansprechwahrscheinlichkeit diskutiert.

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Evaluation of the Survival Gain in 22 Measurable Lung Tumors Treated with Chemotherapy

An Approach Towards Quantitative Evaluation of Cancer Treatments in Man

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THE EVALUATION of chemotherapeutic treatments relies generally on the total survival time from the onset of treatment, and on approximative measurement of the shrinkage of the tumor (25% or 50% of the initial volume). When considering the growth curves of measurable tumors prior to and during the treatment one can, however, easily reach the conclusion that these parameters are not significative. The total survival time does not take into account the spontaneous survival time that the patient would have reached without treatment—which remains unknown, nor the volume reached by the tumor at the onset of treatment, though the volume reduction has different meanings as far as survival time is concerned, whether the spontaneous doubling time has a small or a great value, as is shown in our first paper. Furthermore, it appears that some valuable results, from the survival point of view, may be reached without any shrinkage. It occurred to us, considering the therapeutic growth curves of our patients, that it is possible to gain more information using such quantitative parameters as survival gain and the ratio of survival gain to the total duration of treatment, the values of which are bound together and to the spontaneous and therapeutic doubling times. Calculations for these parameters are given in the first paper. In the present study, we intend to show how they may apply to clinical cases, and what may be their impact in such fields as evaluation of clinical resistance, screening tests for new drugs and evaluation of controlled trials.

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CLINICAL MATERIAL AND METHODS

(a) Twenty-two patients were retained for this study according to the following criteria: unresectability, primary or secondary lung tumors, measurability on standard X-ray films, grossly spherical shape, no important changes in therapy during the observed period, and at least three measurements prior to and during treatment.

(b) Cases were distributed as follows: 9 bronchogenic peripheric carcinomas and 13 secondary lung tumors—the primary of which being: kidney, 3 cases; oro-pharynx, 3 cases; breast, 2 cases; uterus, 1 case; hepatoma, 1 case; stomach, 1 case; myosarcoma, 1 case; bone sarcoma, 1 case.

(c) Four patients were given cyclophosphamid, 200 mg daily by intraveinous route, the treatment being stopped when the white blood count dropped below 3000 mm^3, and resumed when it rose above 4000. Eighteen

Table 1. *Cyclophosphamid treated patients*

Case No.	Site and histology	Doubling time (days)	Duration of treatment (months)	Survival gain	Sg/T
1	Larynx	41	5·5	5·5	1
2	Bronch.	62	6·1	2·8	0·46
3	Bronch.	13	2·8	0·7	0·25
4	Bronch.	37	3·1	2·6	0·84

Table 2. *Combination group. Patients treated till death*

Case No.	Site of primary	Doubling time t (days)	Duration of treatment (months)	Survival gain (months)	Sg/T
5	Stomach	67	10·3	12·8	1·24
6	Bronch.	36	8·6	6·1	0·71
7	Bronch.	56	7·3	13·5	1·85
8	Hepatoma	45	2·6	1·5	0·57
9	Kidney	7	1·7	1·3	0·76
10	Uterus	14	therapy discontinued 3 months before death		
11	Pharynx	25	7·2	5·4	0·75

Table 3. *Surviving cases from the combination group. Bronchogenic carcinomas*

Case No.	Site of	Doubling time t (days)	Duration of treatment (months)	Survival gain (months)	Sg/T
12	Bronch.	70	2·2	24·5	11
13	Bronch.	150	1·6	7·6	4·8
14	Bronch.	21	15·5	16	1·03
15	Bronch.	38	13·8	15	1·08
16	Bronch.	56	4·5	5	1·1

Table 4. *Surviving cases from the combination group. Secondary tumors*

Case No.	Site of primary	Doubling time t (days)	Duration of treatment (months)	Survival gain (months)	Sg/T
17	Myosarcoma	140	11	13	1·18
18	Pharynx	270	6·9	15	2·2
19	Kidney	63	10·2	7·8	0·76
20	Breast	100	2·7	8	3
21	Bone	37	4·1	3·5	0·85
22	Bronch. met. from bronch.	9	5·2	5·6	1·08

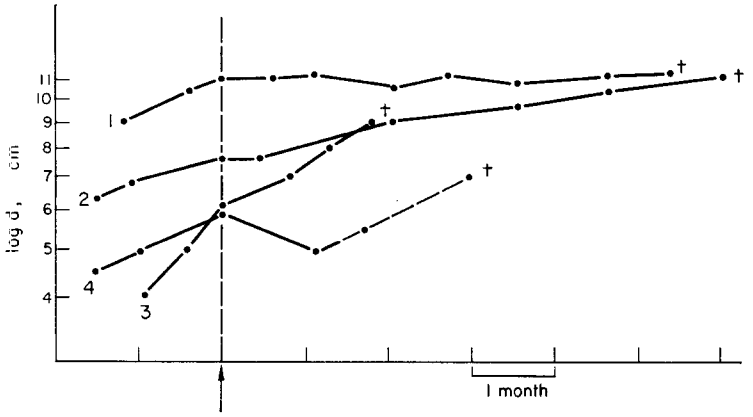

Fig. 1. *Growth curves from the one drug group. The arrow shows the start of the treatment and the cross the time of death.*

patients were given a five drug combination including: cyclophosphamid, 12 mg/kg; 5-fluorouracil, 12 mg/kg; methotrexate, 0·5 mg/kg; vinblastin, 0·1 mg/kg; and rufocromomycin, 0·003 mg/kg. These drugs were given simultaneously by intraveinous route every 7, 10 or 14 days according to the blood count, and for as long as it was possible.

(d) In Tables 1–4 for each case are indicated the nature of the tumor, the spontaneous doubling time, the total duration of the treatment (T) till the death occurred or, for the surviving cases till May 1969, the value for the survival gain (Sg) induced by the treatment and the ratio Sg/T.

(e) The growth curves are given in Figs. 1–4. They were constructed as shown in our previous paper. Each figure shows the cases for which calculations were made in the corresponding tables. Patients described in Figs. 1 and 2 died, and the calculations are made for the real total survival time. Patients described in Figs. 3 and 4 are alive and the calculations describe the results registered at the point of the last measurement in May 1969.

RESULTS

(a) As was shown by Collins (see the first paper) no case survived the 41st doubling and most deaths occurred around or before the 40th doubling when the tumor has a diameter of 10 cm.

(b) As can be seen in case 1 (Fig. 1), in cases 6, 9 and 10 (Fig. 2), and in cases 14, 15 and 16 (Fig. 3), the tumor resumes its previous growth rate as soon as the treatment has been discontinued, as was shown by Breur [1] and Bru [2].

(c) The different patterns induced by the treatment can be categorized in 5 groups:

no effect: cases 3 and 8

prolonged lengthening: cases 2, 11 and 19

shrinkage: cases 4, 5, 7, 12–16, 20 and 22

effect varying along time: mainly case 21, but many cases classified in the other groups displayed some varying response.

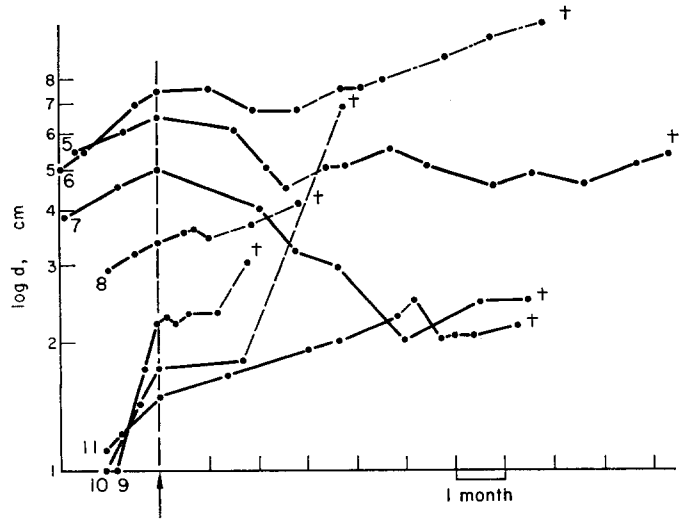

Fig. 2. *Growth curves from the combination group. Patients deceased.*

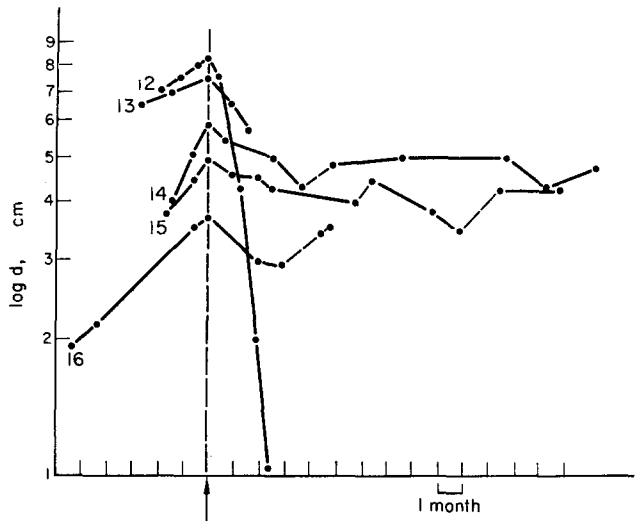

Fig. 3. Growth curves from the combination group. Bronchogenic carcinomas surviving.

(d) In the first group, treated with cyclophosphamid, one patient only reached for Sg/T a value of one—i.e. a plateau on the growth curve. In the three other groups treated with combination therapy, 10 patients out of 18 reached an Sg/T greater than 1, which means a shrinkage; and 7 out of the remaining 8 reached a value greater than 0·75, which means that at the end point where the calculation was made, the final doubling time during treatment had been multiplied by at least a factor of 4. The case of patient number 12 must be set apart, the dramatic and fast improvement seen on the growth-curve resulting from 1500 rads in addition to chemotherapy.

(e) In the two groups of patients who died (Figs. 1 and 2), we registered four cases in which the tumor resumed its previous spontaneous growth rate in spite of the continuation of the treatment (cases 2, 3, 5 and 7). The possible meaning of this fact will be discussed later.

DISCUSSION

(a) The first point we would like to comment on deals with the aid these growth curves can give to the therapist. For one thing, it appears that stopping the treatment when a given dose has been reached is dangerous, and makes no sense since the growth resumes immediately at its previous rate. The only case in which therapy could be discontinued without risk would be total eradication. It follows that the search for toxicity as the limit for the application of the treatment is also dangerous since toxicity means discontinuation of treatment. A lengthening, obtained with comparatively low doses, and which can be maintained over months—as in case 19, for example—is of more benefit

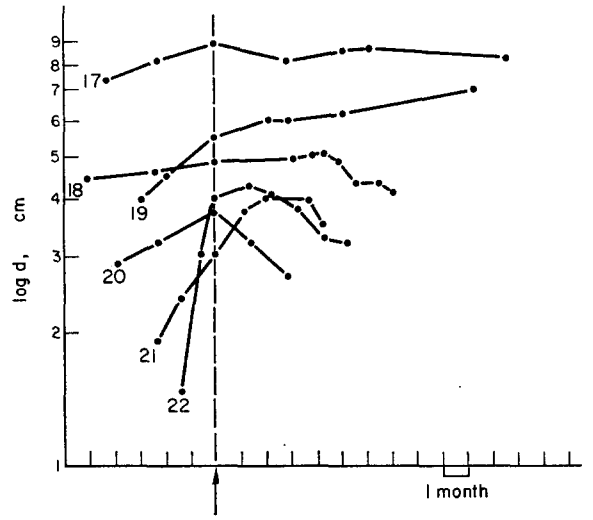

Fig. 4. Growth curves from the combination group. Secondary carcinomas. Patients surviving.

than a shrinkage obtained at the cost of toxicity leading to the discontinuation of therapy as seen in case 16.

As far as the comparison between one drug and combination schedules is concerned, this study was not performed in order to give an answer to this important question. Other works [3, 4] have led us to the conclusion that one drug therapy is not the best way to use cytostatic drugs. In the cases presented here, it appears that the tumor resumed its previous growth rate in spite of continued therapy in 2 of 4 patients treated with one drug, and in 2 out of 18 patients treated with combination therapy. The disappearance of the effectiveness of the treatment, as measured on the growth curve, thus parallels the distribution of the survival gains in the two groups of patients who died.

(b) Apart from the discussion of one drug versus combinations, it seems important to consider the possible meaning of such an event as the disappearance of effectiveness during therapy. It may result from many different causes, namely:

(1) diminishing cells losses
(2) changes in the percentage of proliferating cells
(3) changes in the accessibility of drugs to the tumor
(4) disappearance of all sensitive cells and selection of an entirely resistant population.

Causes (1) and (2) seem to us to have but a faint possibility since no such changes were detected in tumors growing without being treated, the doubling time remaining constant for years or lengthening slightly at the end of the evolution but in no case becoming shorter. The third possibility may certainly be considered. In case 5, however, the event considered was encountered after 4 months' therapy, and as no other drug was available to us, doses of cytostatics were multiplied by a factor of 1·5. It proved possible to reach an equilibrium during a further 4 months. In our opinion, possibility (4) is the more likely to be true in most cases, and the moment at which the therapeutic doubling time becomes identical to the spontaneous one is presumably the moment at which the treatment has killed all the sensitive strain cells in the tumor. If that proved true experimentally, which remains to be shown, the calculation of the time elapsed between the onset of the treatment and the appearance of clinical resistance would provide another valuable parameter to compare various treatments. As it is, it remains a good indication for the therapist in individual cases as to the time the therapy must be changed for another one when possible.

(c) Considered from the point of view of growth curves, the conventional screening tests for new drugs deserve some comments. In three of our cases, as said before, the only result was a lengthening of the spontaneous doubling time, which was multiplied respectively by a factor of 2, 4 and 5. In six other cases, the result was a more or less prolonged plateau, indicating that as many new cells were destroyed per doubling time as were incorporated into the tumor during the same period of time.

Such results are commonly overlooked when the growth curve is not constructed and when only a shrinkage is taken into account. So drugs are possibly missed which would prove able to induce significant lengthenings when used alone and which would possibly, when used in combinations, prove very useful. In our opinion, clinical screening tests should be performed with measurable tumors and growth curves constructed so that such possibilities are not overlooked.

(d) The point we would like to underline, however, deals with the way controlled trials are generally performed. Their inaccuracy has recently been discussed by Feinstein [5, 6]. This author states that controlled trials compare treatments from a static and not from a dynamic point of view. We are in complete agreement with these criticisms for the following reasons:

As we have said before, the total survival time from the onset of treatment (in case of chemotherapy) or from the end of treatment (in case of X-ray therapy or surgery) is a parameter of little informational value since the time the patient would have survived without therapy and the survival gain induced by the treatment are not distinguishable. It is then necessary to work with measurable tumors in order to ascertain the spontaneous doubling time of the cases treated.

However, spontaneous doubling times vary, in human solid tumors, approximately from 10–400 days. Registering a one year total survival time when the initial doubling time is 10 days or when it is 400 days is not at all the same thing. In the first case, the treatment proved extremely active. In the second, its effectiveness remains to be shown. The only way to compare them safely is to calculate the survival gain induced in each case, since this value is bound to the spontaneous doubling time as well as to the duration of treatment.

Furthermore, the volume reached by the

tumor at the onset of treatment has an important meaning, whatever its growth rate is. Assuming with all the authors quoted in our first paper, and whose observations proved true in our cases, that death is to occur around the 40th doubling, a one year total survival time has very different meanings when the treatment is started at the 33rd doubling (the diameter being 2 cm) or when it is started at the 39th (the diameter being then 8 cm). The chance for a spontaneous one year survival time was far greater in the first case than in the second one, and the treatment proved clearly more effective in the second than in the first. Here again, the survival gain is more valuable a parameter than the total survival time, but other values need to be considered in addition, namely the volume gain, as calculated in our first paper, and the time elapsed between the death and the moment at which the tumor would have spontaneously reached the 40th doubling (assuming, of course, that there are no other metastases). Another way to achieve more satisfying comparisons would be to match together tumors having reached identical diameter when the treatment is started.

We are, of course, conscious of the fact that a correct randomisation leads to fairly identical distributions in the two (or more) groups submitted for comparison, but considering all the different doubling times involved and all the different diameters reached at the onset of treatment, it may be shown that the number of cases necessary to detect relatively small differences (and in the field of chemotherapy small differences are what we are working with at the present time) has been but rarely reached in previous studies.

Assuming then, that effectiveness of treatments does not depend on the measurability of tumors, it would, in our opinion, be safer to perform controlled trials with measurable tumors in order to assess differences in a more scientific and quantitative way than before.

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RESUME

Etudiant les courbes de croissance avant et pendant la chimiothérapie de 22 tumeurs mesurables, les auteurs distinguent les divers types de réponse thérapeutique et calculent, dans chaque cas, le gain en survie et le rapport du gain à la durée du traitement. Ils en concluent:

(a) *que le moment où la résistance clinique est attenuée peut être décelé grâce aux courbes de croissance.*

(b) *que les tests de screening méconnaissent des drogues qui, sans déterminer une réduction de volume, sont capables de multiplier par un facteur 5 le temps de doublement spontané, résultat cependant très important. Ils critiquent la valeur des essais comparatifs, actuellement menés, qui ne tiennent compte ni de la vitesse initiale de croissance, ni du volume initial des tumeurs soumises aux traitements à comparer. Ils montrent que seules les tumeurs mesurables permettant le calcul du gain en survie, qu'ils ont montré être lié au temps de doublement et à la durée du traitement, doivent être utilisés pour de tels essais.*

SUMMARY

Investigating the growth curves before and during chemotherapy of 22 measurable tumors, the authors separate the different types of therapeutic response and calculate for each case the survival gain and its ratio to the time of treatment. They conclude from this:

(a) *that the growth curves reveal the time when the clinical resistance is reached;*

(b) *that the screening tests overlook drugs which, although not producing a volume reduction, are capable of multiplying by a factor of 5 the time of spontaneous doubling, a result which can hardly be ignored.*

They criticize the value of comparative tests currently undertaken which do not take into account either the initial growth rate or the initial volume of the tumors submitted for the treatments under comparison. They show that only measurable tumors, permitting the calculation of the survival gain, which they have shown to be connected to the doubling time and the treatment time, should be used for such comparisons.

ZUSAMMENFASSUNG

Durch die Untersuchung von Wachstumskurven vor und während chemotherapeutischer Behandlung 22 messbarer Tumore, unterscheiden die Autoren verschiedene Arten thera-

peutischer Ansprechswahrscheinlichkeit und berechnen für jeden Fall den Gewinn an Überlebenszeit und sein Verhältnis zu der gesamten Behandlungszeit. Sie schliessen hieraus:

(a) *dass die Wachstumskurven den Zeitpunkt anzeigen, wo klinische Immunität gegen die gewählte Behandlungsweise erreicht ist,*

(b) *dass Screening Tests Medikamente übersehen können, die, obwohl sie keine Volumenverkleinerung des Tumors bewirken, die spontane Verdopplungszeit des Tumors um einen Faktor* 5 *vergrössern können, was eine äusserst wichtige Tatsache ist.*

Sie kritisieren die Werte heutiger Vergleichstests, die weder die Anfangswachstumsrate, noch die Anfangsausdehnung des Tumors, der verschiedenen Behandlungsweisen unterzogen wird, die untereinander verglichen werden, berücksichtigen.

Sie zeigen ferner, dass nur messbare Tumore die Berechnung eines Gewinns an Lebenszeit erlauben und dass dieser Gewinn, der, was bewiesen wird, mit der Verdopplungszeit verknüpft ist, für solche Vergleichstest benützt werden sollte.

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Letter to the Editor

Considerations on Factors Influencing the Oxygenation of Ascites Tumours

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DURING the past years the environmental conditions under which the ascites tumour cells are living in the host have been investigated in several laboratories. As far as oxygen tension (pO_2) in ascites tumours is concerned, some data published in a recent issue of this journal [1], and re-opening a question that seemed settled, make this subject worthy of a brief comment.

Studies with ascites tumours of the mouse and of the rat led to the conclusion that in the tumour-bearing animal the intraperitoneal pO_2 is very low. Indirect evidence for this came from experiments with Ehrlich ascites carcinoma demonstrating: (i) the presence in the ascitic plasma of high levels of aldolase, an enzyme leaking out from the ascites cells only under anaerobiosis without glucose [2], (ii) the germination in the ascitic fluid, but not in the organs, of the spores of anaerobic bacteria injected intravenously into tumour-bearing mice [3], and (iii) the hydration *in vivo* of the cytostatic quinone E39 injected in the ascitic fluid [4]. Another fact suggesting that in ascites tumours oxygen is scarce was that following an injection of glucose into the ascitic fluid of rats bearing the Yoshida ascites hepatoma the ascites tumour cells display *in vivo* a rate of glycolysis higher than they do *in vitro* under aerobic conditions [5]. More recently, using an oxygen microelectrode inserted in the abdomen of mice bearing the Ehrlich ascites carcinoma a very low pO_2 was found [6]. These and some other data show that the ascites tumour cells take up all, or almost all, the oxygen reaching the ascitic fluid, but they do not answer the question whether the ascites cells are subjected to a marked oxygen shortage during a considerable part of the life cycle of the tumour. This point was investigated by myself by measuring polarographically the intraperitoneal pO_2 in normal rats and in rats bearing the Yoshida ascites hepatoma at three different stages of growth [7]. The intraperitoneal pO_2 was already lowered 2 days after transplantation of 20×10^6 cells and it decreased down to very low levels in the subsequent days. In 5 day-old hepatomas, however, the oxygen current although much lowered, still raised promptly when the rats were breathing oxygen instead of air, suggesting that at least up to this stage of tumour growth the ascites cells were not subjected to a substantial oxygen shortage. In fact, hypoxic conditions were supposed to prevail in the peritoneal environment only one week or more after tumour transplantation, at which time the oxygen level in the ascitic fluid was not increased by oxygen inhalation. Likely, at this time all the extra oxygen reaching the tumour cells was readily taken up.

The conclusion that oxygenation of large ascites hepatomas of the rat is rather poor [7] does probably apply also to the Ehrlich ascites carcinoma of the mouse. Conceivably, in the mouse tumour the more favourable nutrition conditions (higher ratio of peritoneal surface to tumour volume [5]) are counterbalanced by its higher metabolic capacity and by its relatively larger cells mass (Ehrlich: Q_{O_2} −5 to −15;

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cell dry weight 220 mg/5 ml ascitic fluid [2]. Yoshida: QO_2—7; cell dry weight 1·2 g/50 ml ascitic fluid [5].

In contrast with polarographic measurements *in vivo* showing a very low pO_2 both in the Ehrlich ascites carcinoma [6] and in the Yoshida ascites hepatoma [7], Frindel, Blayo and Pocidalo [1] by using an *ex vivo* technique recently found a fairly high pO_2 in the ascitic fluid taken from 12 day-old ascites sarcoma NCTC 2472 of the mouse, but they concluded that oxygen availability may be limiting for ascites cell respiration even in their case. However, since they state that the dissimilarity between their *ex vivo* results with the mouse sarcoma and my data *in vivo* with the rat hepatoma may be related to difficulties of measuring pO_2 *in vivo*, it should be emphasized that pO_2 of the ascitic fluid of the Yoshida hepatoma (and of the Ehrlich carcinoma too) can be hardly underestimated by the polarographic techniques used as, in case, the oxygen current can be raised but not lowered by movements of the ascitic fluid which may occur *in vivo* (and which are not present during electrode calibration *in vitro*). Disregarding the possibility that the pO_2 data concerning the NCTC 2472 ascites sarcoma may be related to the measurement procedure *ex vivo* which, although useful for blood gas determinations, seems to be somewhat less appropriate for measuring pO_2 in an haemoglobin-free fluid (in which case 1 µl O_2 brought into solution during handling of a 1 ml oxygen-free sample would raise its pO_2 to about 32 mm Hg at 37° C), it is apparent that pO_2 differences among different tumours may be related to several factors. For instance, the cellularity of the tumours is certainly important. However, because of differences in cell size from one tumour to another, even the total cell number could be a misleading basis of reference unless the cell dry mass is known. Biochemical properties of tumour cells may be important too. Probably, the need of oxygen by Yoshida hepatoma and Ehrlich carcinoma cells, which are known to display *in vitro* a marked Crabtree effect, may increase *in vivo* during tumour enlargement owing to the increased capacity of these cells to take up oxygen at the low glucose levels prevailing in old ascitic fluids. On the other hand, since in 12 day-old NCTC 2472 ascites sarcomas the rather high pO_2 is accompanied by unusually high glucose levels (up to 40 mg%) [1] it is quite possible that at least *in vivo* this tumour uses up oxygen and glucose at lower rates than the two other ascites tumours do. Finally, nutritional differences depending either on the volume of the ascitic fluid [7] or on the state of the animal may also affect tumour oxygenation. In conclusion, the ascites tumour oxygenation may be influenced by a number of factors concerning both the tumour and the host so that some differences among different tumours are not surprising at all.

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Communication

"Prix Annie-Dalsace"

L'INSTITUT GUSTAVE-ROUSSY dispose des moyens lui permettant d'attribuer un Prix annuel qui porte le nom de "Prix Annie-Dalsace".

Ce Prix, de 10.000-francs, est attribué à un jeune chercheur français ou étranger, dont les travaux de recherche clinique ou expérimentale seraient particulièrement intéressants, dans le but de lui faciliter la poursuite de ses recherches.

Ce Prix est décerné par un jury de quatre membres: 2 cliniciens, chefs de services, et 2 directeurs de recherches expérimentales.

Il a été attribué, pour 1969, à Mademoiselle Irma Deleón Rodriguez, chercheur de nationalité mexicaine.

Le "Prix Annie-Dalsace" sera décerné, en principe, tous les ans, au cours de 1er trimestre; les candidatures pour l'année 1970 doivent parvenir au Directeur de l'Institut Gustave-Roussy, *avant le premier décembre* de l'année en cours.

Professeur Pierre Denoix
Directeur de l'Institut Gustave-Roussy

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