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Metabolic Controls in Precancerous Liver—III. Further Studies on the Control of Lipid Synthesis During *N*-2-Fluorenylacetamide Feeding

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Abstract—*We have examined, both* in vivo *and* in vitro, *various aspects of the control of cholesterol and fatty-acid synthesis in the livers of rats fed the hepatocarcinogen,* N-2-*fluorenylacetamide.*

Within 1–4 weeks of beginning carcinogen treatment "feedback" control of cholesterol synthesis was partially or completely lost. Other controls over lipogenesis, however—fasting inhibition of cholesterol synthesis and dietary control of fatty-acid synthesis (fasting, refeeding after fasting, high-fat diet)—were retained. The normal diurnal rhythm of hepatic cholesterol synthesis was missing from carcinogen-treated animals, but this was due to an alteration in their daily pattern of voluntary food intake. The loss of "feedback" control of cholesterol synthesis was found to be not due to the reduction in food intake that accompanied carcinogen feeding.

This work is further evidence in support of the hypothesis that a breakdown in "feedback" control of cholesterol synthesis is an important feature in the development of liver cancer.

INTRODUCTION

Loss of control of fatty-acid and cholesterol synthesis is a consistent feature of every hepatoma studied, both primary and transplanted [e.g. 1, 2]. Furthermore, "feedback"* control of cholesterol synthesis is absent also from "precancerous" livers, i.e. livers of animals either genetically prone to develop hepatomas [3], or that have been treated with hepatocarcinogens [4–9].

In the previous papers in this series [7, 8] we reported that, feeding rats, the carcinogen *N*-2-fluorenylacetamide (FAA) disrupted feedback control of hepatic cholesterol synthesis, measured both *in vitro* and *in vivo*. The actual degree of loss of control was very variable between individual animals—only a few showed complete loss of control and there was a wide range of partial control.

The experiments presented in this paper were designed to examine the following questions concerning the metabolic behaviour of rat liver during carcinogen treatment:

(i) is the consistent loss of feedback control of cholesterol synthesis simply a result of the reduction in feed intake which invariably accompanies such treatment?

(ii) is feedback inhibition of cholesterol synthesis the only control lost, or are other controls of lipid synthesis also abnormal? Our earlier work [7] suggested that FAA-treated livers retained their normal response to fasting at a time when feedback control was lost.

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*Inhibition of cholesterol synthesis by cholesterol feeding is often referred to as "feedback" inhibition [3]. We use this term in a general sense only, and do not intend to imply any particular mechanism of action.

MATERIAL AND METHODS

Animals and diets

Male, hooded-Wistar rats were used, aged 2–3 months at the beginning of each experiment. All animals were subjected to a 12 hr day : 12 hr night (lights on at 6.00 a.m., off at 6.00 p.m.). They were fed a commercial laboratory ration *ad libitum* (Charlicks M164 mouse cubes, either powdered or as cubes). The cholesterol content of this feed, determined as digitonin-precipitable sterols, was $0{\cdot}07 \pm 0{\cdot}01\%$. This diet was supplemented, where indicated, with 0·05% FAA (K & K Laboratories, Inc., Plainview, N.Y.), 5% cholesterol (Pierce Chemical Co., Rockford, Ill.), 2% cholestyramine (a gift from Mead Johnson & Co., Evansville, Ind.) or 15% maize oil (Clifford Love & Co. Ltd., Australia).

Vitral assay for cholesterol and fatty-acid synthesis*

Animals were killed by decapitation and their livers quickly removed, placed in ice-cold saline (0·9%) and then sliced with a McIlwain tissue chopper. The incubation of tissue slices (200 mg) with 4μ moles of 1-^{14}C-acetate (0·2 μC) and the subsequent extraction and measurement of ^{14}C-labelled cholesterol and fatty acids were carried out as described previously [2].

Vival assay for liver cholesterol synthesis*

We have described this assay previously [11]. Briefly, rats were given either 10 or 20 μmole/kg body weight of AY–9944† (the gift of Dr. Morris Givner, of Ayerst Research Laboratories, Montreal, Quebec, Canada). This was given by intraperitoneal injection, rather than by stomach tube as described previously, since we found this route more convenient and comparable results are obtained with both methods [12]. Twenty four hours after AY–9944 treatment blood was collected from the tail vein, saponified, and then extracted with petroleum spirit (b.p. 40–60°C). After evaporation of the petroleum spirit, 7-dehydrocholesterol was measured by colour development with Liebermann–Burchard reagent [13]. The blood level of 7-dehydrocholesterol under these conditions is indicative of liver sterol synthesis.

RESULTS

The first two experiments we are reporting here were designed to assess the relationship, if any, between reduced food intake and loss of feedback control of cholesterol synthesis, both of which effects follow carcinogen treatment.

Cholesterol synthesis during controlled feeding

Five rats were placed in individual cages and fed powdered chow, *ad libitum*, for 5 days. For the following 4 weeks each animal was offered each day (at the commencement of the dark period) half the average amount consumed daily over the last 3 days of the pre-trial period. This dietary restriction is approximately equivalent to that induced by FAA-feeding [7]. A further 5 rats, as controls, were fed *ad libitum* throughout the entire experiment. All rats were tested *in vivo* before the trial period commenced and also after 1, 2, 3 and 4 weeks, with 5% cholesterol given for 2 days at the 2nd and 4th weeks.

The results (Table 1) indicate an apparent loss of control of cholesterol synthesis. But this was due entirely to a reduction in synthesis while on chow and not to increased synthesis while on the high-cholesterol diet, the effect which invariably occurred in carcinogen-treated animals [7, 8]. Therefore, although reduced food intake may seem to cause some loss of feedback control of cholesterol synthesis, it does not explain the results obtained during carcinogen treatment.

Relationship between weight loss during FAA feeding and feedback control of cholesterol synthesis

As another attempt to find a possible relationship between loss of feedback control of cholesterogenesis and altered food intake, we have re-analysed the data from a previous experiment. All results from the FAA-fed rats, as reported in the preceding paper in this series [8], were grouped into four sets, depending upon the initial weight lost by each animal and with each set containing results from approximately the same number of animals.

(i) and (ii)—High weight loss: results from rats, in experimental groups C and D respectively, which lost the most weight during the first 3 weeks of FAA feeding.

(iii) and (iv)—Low weight loss: results from the remaining rats, i.e. those which lost the least weight.

These animals had been fed the 5% cholesterol diet and the low-cholesterol (chow) diet at each alternate assay, with groups C and D receiving the particular diet on alternate weeks. The percentage of feedback inhibition of cholesterol synthesis was calculated for each individual animal by comparing its synthesis while on the 5% cholesterol diet with synthesis

*Green [10] has suggested that the terms "vival" and "vitral" should be used in place of "*in vivo*" and "*in vitro*" when these are required as adjectives.

†AY–9944: Trans-1,4-*bis*(2-chlorobenzylaminomethyl)-cyclohexane dihydrochloride.

Table 1. *Effect of reduced food intake on feedback control of cholesterol synthesis*

Week	−1	0	1	2	3	4
Diet	Chow	Chol.	Chow	Chol.	Chow	Chol.
	Blood 7-dehydrocholesterol					
Ad libitum	29·6 ± 2·8	7·2 ± 1·4	28·3 ± 2·9	8·4 ± 1·3	31·9 ± 2·4	10·9 ± 2·6
½ rations	33·4 ± 3·1	7·7 ± 2·1	19·3 ± 3·6	9·6 ± 2·1	26·6 ± 4·7	12·8 ± 4·7
		% Inhibition by cholesterol feeding				
Ad libitum		23·9 ± 3·8		30·7 ± 5·5		33·7 ± 9·1
½ rations		23·5 ± 5·9		49·6 ± 3·0*		42·1 ± 12·2

Five rats were fed half their normal daily feed intake each day for 4 weeks, while 5 control rats were fed *ad libitum*. All rats were tested weekly by the vival assay, with 5% cholesterol fed for 2 days before and including each alternate assay period.
*Significantly different from control group ($P < 0·02$).

Table 2. *Weight changes for animals showing high and low weight loss during the first* 3 *weeks of FAA feeding*

Set	Experimental group	No. rats	Weight (g) Week 0	Weight (g) Week 3	Weight loss (g)	% Loss
(i) High weight loss	C	5	262 ± 18	223 ± 17	39 ± 3*	15·0 ± 0·8*
(iii) Low weight loss	C	6	228 ± 10	211 ± 8	17 ± 3	7·2 ± 1·2
(ii) High weight loss	D	5	249 ± 9	221 ± 8	28 ± 5*	11·4 ± 1·9*
(iv) Low weight loss	D	6	237 ± 5	229 ± 3	8 ± 4	3·2 ± 1·6

*Significantly different from corresponding low-weight-loss groups ($P < 0·01$).

measured in the previous assay and the following assay (i.e. when on the low-cholesterol diet).

Table 2 gives the actual weight changes by these rats, and Fig. 1 shows the percentage of feedback inhibition of cholesterol synthesis during the first 14 weeks of FAA feeding. The difference in control between the high- and low-weight-loss groups was significant only at 11 weeks ($P < 0·02$) and in this case the rats showing greatest loss of control were actually those that had lost the least weight. Since weight change presumably is related primarily to feed intake, this analysis confirms that loss of control is not due simply to an alteration in feed intake.

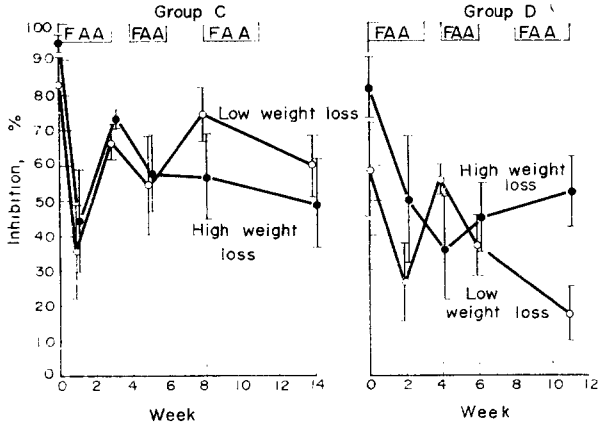

Fig. 1. *Relation of pecentage of feedback inhibition of cholesterol synthesis to weight loss during the first* 3 *weeks of FAA feeding. These results are from experiments described previously* [8]. *Sterol synthesis was measured by the vival assay described in materials and methods.*

Effect of FAA feeding on various controls of lipid synthesis, as measured in vitro.

A number of rats were fed FAA (0·05%) for 2 weeks, then fed also cholestyramine (2%), high-fat (15% maize oil) or fasted for 3 days, or fasted 2 days and refed chow for 2 days (Table 3) before measurement of cholesterol and fatty-acid synthesis *in vitro*. Another group were fed FAA for 4 weeks, then fasted for 3 days (Table 4). Liver cholesterol synthesis, but not fatty-acid synthesis, in the latter rats has been reported previously [7] and normal fasting inhibition of cholesterol synthesis was apparent in all animals.

The results already shown in Fig. 1 clearly indicate a defective feedback control of hepatic cholesterol synthesis at both 2 and 4 weeks of FAA feeding. Control of fatty-acid synthesis at these times, however, as measured by the effects

Table 3. Control of lipid synthesis (measured in vitro) after 2 weeks of FAA feeding (0·05%).

FAA	Cholesterol synthesis		
	Chow-fed	Fasted	Cholestyramine-fed
–	0·33	0·02	0·70
	0·19	0·06	1·12
	0·38	0·14	
	0·14	0·02	
	(0·26 ± 0·06)	(0·06 ± 0·03)*	(0·91)*
+	0·29	0·18	1·71
	0·59	0·19	9·67
	1·92	0·19	4·23
	1·40	0·39	3·21
		0·05	0·09
		0·07	3·05
			1·52
	(1·05 ± 0·37)	(0·18 ± 0·05)*	(3·35 ± 1·17)

	Fatty-acid synthesis			
	Chow-fed	Fasted	Refed after fasting	High-fat
–	1·33	0·09	8·38	0·62
	1·12	0·12	2·94	0·49
	1·34	0·32		
	1·32	0·23		
	(1·26 ± 0·08)	(0·19 ± 0·05)†	(5·66)	(0·56)†
+	0·67	0·84	5·19	0·49
	1·14	0·58	4·22	0·86
	1·48	0·24		
	1·59	0·53		
		0·64		
		0·23		
	(1·22 ± 0·20)	(0·51 ± 0·10)†	(4·71)†	(0·65)

The results (means of duplicate incubations) are shown for each animal, and are expressed as percentage of incorporation of ^{14}C from acetate-1-^{14}C into ^{14}C-labelled cholesterol and fatty acids.
*Significantly different from chow values ($P < 0·05$).
†Significantly different from chow values ($P < 0·02$).

Table 4. Fasting inhibition of fatty-acid synthesis after 4 weeks of FAA feeding (0·05%).

FAA	Fatty-acid synthesis	
	Fed	Fasted
–	2·08	0·25
	2·86	0·34
	2·04	0·15
		0·14
	(2·33 ± 0·27)	(0·22 ± 0·05)*
+	1·56	0·34
	0·75	0·29
	0·81	0·28
	0·51	0·32
	1·14	0·23
	1·00	0·29
		0·31
		0·24
	(0·96 ± 0·15)	(0·29 ± 0·01)*

Results were obtained and are expressed as in Table 3.
*Significantly different from fed values ($P < 0·001$).

of fasting, refeeding after fasting and a high-fat diet, appeared to be normal (Tables 3 and 4). Fasting also resulted in a normal reduction of cholesterol synthesis (Table 3 and ref. 7) in all animals, but cholestyramine on the other hand did not increase cholesterol synthesis in all rats (Table 3), indicating a partial loss of this particular control. This latter result is in agreement with the suggestion that cholestyramine acts by relieving the continuous feedback inhibition of cholesterol synthesis caused by endogenous cholesterol [14]. In view of the large number of rats we have now tested by fasting, it is clear that all rats fed 0·05% FAA retain fasting inhibition of both fatty-acid and cholesterol synthesis, at least at 2 and 4 weeks of the carcinogen treatment. In fact, of all the controls tested in FAA-fed animals only feedback inhibition and cholestyramine activation of cholesterol synthesis were ab-

normal. These results also confirm the suggestion that inhibition of cholesterol synthesis by fasting acts independently of feedback control [15].

Effect of FAA feeding on fasting inhibition of cholesterol synthesis, as measured in vivo

Thirty rats were tested *in vivo* for feedback control of cholesterol synthesis and were then divided into 3 groups, one of which was fed chow and the other two chow plus 0·05% FAA.

Group A—Fed chow, but fasted for 2 days at 1 week and fed 5% cholesterol for 2 days at 3 weeks.

Group B—Fed FAA, but supplemented with 5% cholesterol for 2 days at 1 and 3 weeks.

Group C—Fed FAA, but fasted for 2 days at 1 and 3 weeks.

This schedule allowed a comparison of fasting inhibition with feedback inhibition at the same stage of FAA feeding. The rats were all tested for cholesterol synthesis *in vivo* each week, and we found (Table 5) that the FAA-fed rats exhibited an essentially-normal fasting inhibition (Group C) at both 1 and 3 weeks when there was substantial loss of feedback control (Group B) on both occasions.

These results confirm the results of our vitral experiments, presented previously [7] and in Table 3, i.e. that fasting inhibiton of cholesterol synthesis is retained during carcinogenesis at a time when feedback control is lost.

Effect of FAA feeding on the diurnal rhythm of cholesterol synthesis, measured in vivo

Our previous studies [16], together with those of Goldfarb and Pitot [17], have shown that the normal diurnal rhythm of hepatic cholesterol synthesis is missing from some fully-developed hepatomas. Hence we decided to test for this control in the livers of carcinogen-treated animals.

Seven rats, while on the standard chow diet, were tested for normal diurnal rhythm *in vivo*. Four were then fed FAA (0·05%) for 1 week, after which all rats were retested. These studies on diurnal rhythm were carried out by treating the rats with AY-9944 (20 μmole/kg) at 6.00 a.m. and then collecting blood from each animal at both 6.00 p.m. on the same day and at 6.00 a.m. the following day.

The results (Table 6) indicate that the diurnal rhythm in the FAA-fed rats was significantly *more* pronounced than the rhythm in the control group, and that none of the individual FAA-fed rats showed any loss of rhythm when compared with their initial values.

In a second experiment 14 rats were examined for normal diurnal rhythm, both on the chow and the 5% cholesterol diets. Ten rats were then fed 0·05% FAA, while 4 were retained on chow as controls. All rats were then tested at 7, 16 and 25 days, for both feedback control (days 7 and 25) and diurnal rhythm (days, 7, 16 and 25).

The results (Table 7) again indicate a

Table 5. *Effect of FAA feeding on fasting inhibition of cholesterol synthesis, as measured* in vivo.

Weeks of FAA feeding	Blood 7-dehydrocholesterol (mg/100 ml)					
	−1	0	1	2	3	4
A −FAA	Chol. 4·7 ± 0·9	Chow 26·3 ± 1·8	Fast 7·4 ± 1·1	Chow 32·4 ± 2·4	Chol. 4·7 ± 0·7	Chow 24·7 ± 1·9
B +FAA	Chol. 4·6 ± 0·8	Chow 27·5 ± 1·7	FAA-Chol. 14·9 ± 2·1	FAA 41·3 ± 2·7	FAA-Chol. 13·9 ± 1·4	FAA 33·6 ± 2·3
C +FAA	Chol. 4·5 ± 0·7	Chow 25·2 ± 1·2	Fast 11·0 ± 0·8	FAA 42·8 ± 1·6	Fast 10·3 ± 0·8	FAA 39·0 ± 1·2
	% inhibition of cholesterol synthesis by diet					
A − FAA	83·1 ± 2·5 Chol.		77·0 ± 3·2 Fast		81·1 ± 1·7 Chol.	
B + FAA	83·9 ± 2·3 Chol.		63·1 ± 5·1* Chol.		57·6 ± 4·5* Chol.	
C + FAA	81·6 ± 2·7 Chol.		74·3 ± 1·4† Fast		73·4 ± 2·1† Fast	

*Significantly less than control values ($P < 0·01$).
†Not significantly different from control values.

Table 6. Diurnal rhythm of cholesterol synthesis during FAA feeding

	Blood 7-dehydrocholesterol (mg/100 ml)					
Days of FAA feeding	0			7		
FAA	12 hr.	24 hr.	% day	12 hr.	24 hr.	% day
–	7·4	27·5	26·9	9·3	19·8	47·0
	21·2	47·6	44·5	12·2	26·2	46·6
	11·9	31·2	38·1	7·9	28·8	42·0
	(13·5 ± 4·1)	(35·4 ± 6·2)	(36·5 ± 5·1)	(9·8 ± 1·3)	(21·6 ± 2·3)	(45·2 ± 1·6)
+	15·6	50·8	30·7	13·2	59·3	22·3
	10·1	27·5	36·7	8·5	19·8	42·9
	10·1	24·9	40·6	8·7	24·9	34·9
	19·8	49·0	40·4	13·8	43·4	31·8
	(13·7 ± 2·2)	(38·1 + 6·9)	(37·1 + 2·3)	(11·1 ± 1·4)	(36·9 ± 9·0)	(33·0 ± 4·3)*

All rats were dosed with 20μmoles/kg AY-9944 at 6.00 a.m., and blood was collected from each rat both at 6.00 p.m. on the same day (12 hr.) and at 6.00 a.m. on the following morning (24 hr). "% day" is the 7-dehydrocholesterol production during the first 12 hr (the light period), expressed as a percentage of the total production in 24 hr.
*Significantly different from controls ($P < 0{\cdot}05$).

Table 7. Effect of FAA feeding on the diurnal rhythm of cholesterol synthesis

Days of FAA feeding		−11	−2	7	16	25
FAA		Chol.	Chow	Chol.	Chow	Chol.
		Blood 7-dehydrocholesterol (mg/100 ml)				
–	Day	5·2 ± 1·8	17·5 ± 5·2	8·3 ± 2·0	12·2 ± 2·8	4·5 ± 2·0
	24 hr	17·9 ± 5·9	46·8 ± 7·7	19·8 ± 5·2	37·5 ± 6·5	13·6 ± 4·4
+	Day	4·0 ± 0·6	12·1 ± 1·6	6·6 ± 1·6	38·7 ± 3·3	18·6 ± 2·1
	24 hr	13·7 ± 2·2	37·9 ± 2·9	24·1 ± 2·5	58·9 ± 2·0	32·6 ± 2·8
		% synthesis during the day				
–		30·7 ± 2·7	34·9 ± 5·3	42·0 ± 1·6	31·5 ± 5·5	28·6 ± 5·9
+		29·6 ± 2·7	31·4 ± 2·2	25·2 ± 3·5*	64·9 ± 3·9*	55·7 ± 2·9*
		% feedback inhibition				
–		64·7 ± 6·8		54·9 ± 5·2		65·5 ± 6·4
+		64·1 ± 4·5		50·8 ± 3·9†		44·6 ± 4·7‡

Cholesterol synthesis was measured *in vivo* (as in Table 6) in 4 control and 10 FAA-fed rats. 5% cholesterol was included in the diet for 2 days at each alternate assay.
*Significantly different from control group ($P < 0{\cdot}01$).
†Significantly different from first assay (Day −11) ($P < 0{\cdot}05$).
‡Significantly different from control group ($P < 0{\cdot}02$).

significant loss of feedback control, at both 7 and 25 days. The FAA-treated rats were not significantly different from the controls at day 7, but they were significantly different from their initial value whereas the control rats were not.

At day 7 the diurnal rhythm in the FAA-fed rats was again significantly *more* pronounced than that in the control animals, but at 16 and 25 days it was significantly *less*. These results suggest that perhaps the diurnal rhythm was retained in the early stages of carcinogen feeding but lost later. It also was possible, however, that FAA feeding had altered the pattern of food intake, and this in turn could readily have altered the rhythm. Rats have a markedly reduced food intake when offered an FAA diet, but as soon as normal chow is offered they eat readily [7]. This suggests that they dislike the FAA feed, even though they

are hungry, and consequently they probably nibble their food continuously, without regard for the lighting schedule [18]. Such a change in feeding habits would undoubtedly alter the normal rhythm of cholesterol synthesis.

The pattern of food intake for these last 14 rats was in fact studied, by measuring the food eaten from 6.00 a.m. to 6.00 p.m. and from 6.00 p.m. to 6.00 a.m. during days 27 to 29 of FAA feeding. As can be seen in Table 8 the FAA-fed rats did indeed eat continually throughout the 24 hr.

It thus seems clear that the lack of diurnal rhythm of cholesterol synthesis in these carcinogen-treated rats is related to a lack of rhythm of food intake. Presumably this latter effect removes or overrides the normal rhythm of production of the adrenal hormone considered primarily responsible for the daily fluctuation in cholesterol synthesis [19], and the lack of rhythm in these animals is probably not a sign of a loss of metabolic control.

Table 8. Feed intake after 4 weeks of FAA feeding

Day of FAA feeding	Food consumed (g/rat)			
	Controls		FAA-fed	
	Night	Day	Night	Day
27	18·2	7·5	9·0	11·3
28	18·0	8·2	13·2	9·9
29	17·3	7·3	11·4	9·1
Mean ± S.E.	17·8 ± 0·3	7·7 ± 0·3	11·2 ± 1·2	10·1 ± 0·6

Results obtained from the animals described in Table 7.

DISCUSSION

These studies have shown that the loss of feedback control of cholesterol synthesis that invariably occurs during carcinogen treatment cannot be due simply to the reduction in food intake that also accompanies such treatment. This has been shown both by the use of controlled feeding and by a comparison of feedback control in animals with different voluntary food consumptions (as judged by different changes in body weight).

These present results are also confirmed by those obtained previously with aflatoxin [8]. After a single LD50 dose of aflatoxin the greatest loss of feedback control of cholesterol synthesis occurred between 3 and 5 weeks later. This was well after the rats had recovered from the immediate toxic effects of the aflatoxin, and they were gaining weight steadily and eating normally by this time.

It is interesting to note that in the carcinogen-treated animals both diurnal rhythm and fasting control of cholesterol synthesis, plus certain dietary controls over fatty-acid synthesis, were retained at times when other identically-treated rats had lost, at least partially, feedback control of cholesterol synthesis. (These results confirm our earlier suggestion [15] that the mechanisms controlling diurnal rhythm and fasting inhibition of cholesterol synthesis are independent of the mechanism of feedback control.)

On the other hand, fully-developed hepatomas are reported to have lost both fasting control of cholesterol synthesis and all dietary control of fatty-acid synthesis [2], suggesting that the loss of control we have observed in precancerous liver may not be directly comparable with that observed in fully-developed hepatomas.

It has been shown by Goldfarb and Pitot [17] and confirmed in our laboratory [16] that a diurnal rhythm of cholesterol synthesis is present in some hepatomas (nos. 5123C and 7800), though it is absent from others (nos. 7794A and 9618A). Therefore it is possible that when the other controls mentioned above, i.e. relating to fasting and to fatty-acid synthesis, are tested in a larger number of hepatomas they may be found to be present in some and absent from others.

Thus, feedback control of cholesterol synthesis, as distinct from all other controls of lipid synthesis, remains as the only control lost from as many as 14 transplantable hepatomas [1], several primary hepatomas of man and rainbow trout [1], and livers of hepatocarcinogen-treated rats.

Despite all the studies that have now been carried out on feedback control of cholesterol synthesis in normal liver, precancerous liver and hepatomas, no satisfactory explanation has yet been given as to why this particular control should invariably be lost, when other

control mechanisms may or may not be retained. If it is accepted, on the basis of these studies, that feedback control of cholesterol synthesis is of particular importance in the carcinogenic process, at least in the liver, then there appear to be only two possible general explanations for this phenomenon.

(i) For any given liver cell the carcinogenic process is dependent at some stage upon the loss of feedback control of cholesterol synthesis, and this process cannot proceed until control has been lost.

(ii) A fundamental change which is essential to the carcinogenic process invariably gives rise to loss of feedback control of cholesterol synthesis, but as a side effect and not directly involved in tumour development.

Whichever of these alternatives is correct, it is clearly desirable to learn the reason, at the biochemical level, for loss of control. In either case this would reveal a fundamental cellular change which is essential to the carcinogenic process, and knowledge of this change in biochemical terms may make it possible to reverse and/or prevent it.

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Metabolic Controls in Precancerous Liver—IV. Loss of Feedback Control of Cholesterol Synthesis and Impaired Cholesterol Uptake in Ethionine-fed Rats

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Abstract—*We have examined several aspects of the control of cholesterol synthesis* in vivo (*measured as plasma levels of 7-dehydrocholesterol following a single dose of AY-9944, an inhibitor of cholesterogenesis*) *in livers of rats fed the hepatocarcinogen, ethionine.*

Within two weeks of beginning ethionine treatment hepatic cholesterol synthesis was increased and feedback control over this synthesis was lost. This confirms our earlier studies with two other carcinogens, N-2-*fluorenylacetamide and aflatoxin. The degree of loss of control of cholesterol synthesis did not correlate with the variable toxicity of the ethionine to the test rats.*

The hepatic uptake of ^{14}*C-cholesterol, particularly as cholesterol ester, following a single, intragastric dose of* 4-^{14}*C-cholesterol was considerably reduced by ethionine feeding. This suggests that the ethionine-treated liver is defective in its mechanism for removing chylomicron-cholesterol from the plasma, an essential requirement for effective feedback control of cholesterol synthesis. Defective uptake of dietary cholesterol has also been observed in fully-developed hepatomas, all of which similarly lack feedback control of cholesterol synthesis.*

Loss of "feedback"* control of cholesterol synthesis is a consistent feature both of primary and transplantable hepatomas [2], and also of livers from hepatocarcinogen-treated animals [3–8].

In the preceding papers in this series [6–8] we have shown that loss of feedback control of cholesterol synthesis regularly occurs as a result of both aflatoxin injection and *N*-2-fluorenylacetamide (FAA) feeding. We have shown also that this loss of control is not due simply to the alteration in feed intake which invariably accompanies carcinogen treatment. Furthermore, most other controls of lipid synthesis appear to be retained at a time, early in carcinogen treatment, when feedback control of cholesterol synthesis is lost, suggesting that the latter control may be unique in its importance in the carcinogenic process.

We present here the results of experiments conducted during the course of ethionine feeding. These studies indicate that ethionine also causes a loss of control of cholesterol synthesis, similar to that observed with aflatoxin and FAA, and that this loss of control is not related to the gross toxicity of the carcinogen.

We also examined, in ethionine-treated animals, the uptake of dietary cholesterol into the liver, and found that this was impaired.

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*Inhibition of cholesterol synthesis by cholesterol feeding is often referred to as "feedback" inhibition [1]. We use this term in a general sense only, and do not intend to imply any particular mechanism of action.

This second part of the study was modelled on the experiments of Harry *et al.* [9], who observed that the entry of dietary ^{14}C-cholesterol into transplantable hepatomas was very much slower than the rate of entry into the host liver, results that confirmed our earlier observations [10].

MATERIAL AND METHODS

Animals and diets

Male, hooded-Wistar rats were used, aged 2–3 months at the beginning of each experiment. All animals were subjected to a 12 hr day: 12 hr night regime—lights on at 6.00 a.m., off at 6.00 p.m. They were fed a commercial laboratory ration *ad libitum* (powdered Charlicks M164 mouse cubes), the cholesterol content of which, measured as digitonin-precipitable sterols, was 0·07 ± 0·01%. This diet was supplemented, where indicated, with DL-ethionine (Koch-Light Laboratories, Colnbrook, Bucks., England).

Vival assay for liver cholesterogenesis*

We have described this assay in detail previously [12]. Briefly, rats were injected intraperitoneally with 10 μmoles/kg AY–9944† (the gift of Dr. Morris Givner, of Ayerst Research Laboratories, Montreal, Canada). Twenty four hours later blood was collected from the tail vein, saponified, and extracted with petroleum spirit (b.p. 40–60°C). After evaporation of the petroleum spirit, 7-dehydrocholesterol was measured by colour development with Liebermann-Burchard reagent [13].

For 2 days, before and including each alternate test period, the diet of all the animals was supplemented with 5% cholesterol (Pierce Chemical Co., Rockford, Ill.). Thus for each animal, each assay measured cholesterol synthesis while it was receiving alternately a low-cholesterol and a high-cholesterol diet. The percentage of feedback inhibition of cholesterol synthesis was calculated for each animal by comparing synthesis while on the 5% cholesterol diet with synthesis measured in the previous assay and the following assay (i.e. on the low-cholesterol diet).

Extraction of free and esterified cholesterol

Free and esterified cholesterol were extracted from plasma, liver and kidney by the method of Sperry and Webb [14]. After dissolving the final digitonin precipitates in 2 ml methanol, 1 ml was removed from each for counting and the remainder evaporated for cholesterol estimation, as described by Kim and Goldberg [13]. Esterified cholesterol was estimated as the difference between total and free cholesterol.

RESULTS

Ethionine feeding: 20 weeks

Thirty eight rats were fed ethionine according to the schedule shown in Table 1, as used by Epstein *et al.* [15] to obtain large, hyperplastic liver nodules. The rats studied here, however, did not develop nodules and we have consistently observed [6, 7, 16] that tumour development occurs more slowly and to a lesser extent in our animals than in those reported elsewhere. This is possibly due to a difference in composition of the basal diet and we are currently investigating this aspect.

Table 1. *Dietary regime for obtaining hyperplastic liver nodules with ethionine* [15]

Diet	Weeks
Chow + 0·25% ethionine	5
Chow + 0·5% ethionine	3
Chow + 0·8% ethionine	5
Chow	9

Although Epstein *et al.* [15] found a mortality of only 6% in their studies, in this present experiment 30/38 animals died; i.e. 79% mortality. Only those 8 rats which survived throughout the 13 weeks of ethionine feeding are shown in this section of results.

Figure 1 shows that ethionine caused a marked increase in cholesterol synthesis while the animals were on the low-cholesterol diet.

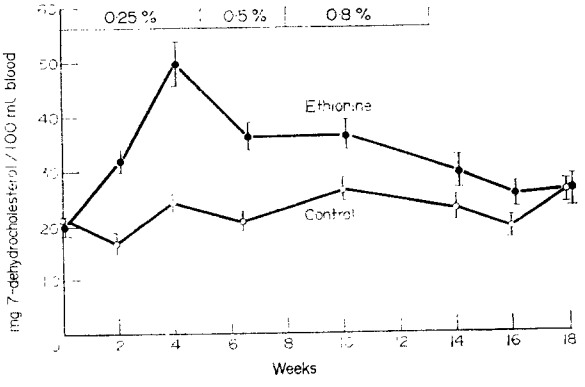

Fig. 1. *Sterol synthesis in ethionine-fed rats while on a low-cholesterol diet. Synthesis was measured* in vivo, *as described in Material and Methods.*

*Green [11] has suggested that the terms "vival" and "vitral" should be used in place of "*in vivo*" and "*in vitro*" when these are required as adjectives.

†AY–9944: Trans-1,4-*bis*(2-chlorobenzylaminomethyl)-cyclohexane dihydrochloride.

This increase was most marked at 4 weeks, but continued throughout ethionine feeding.

The percentage of feedback inhibition of cholesterol synthesis is shown in Fig. 2. The degree of loss of control seen in these rats, and the time for which control was lost, are both greater than that observed following aflatoxin-injection or during FAA feeding [7].

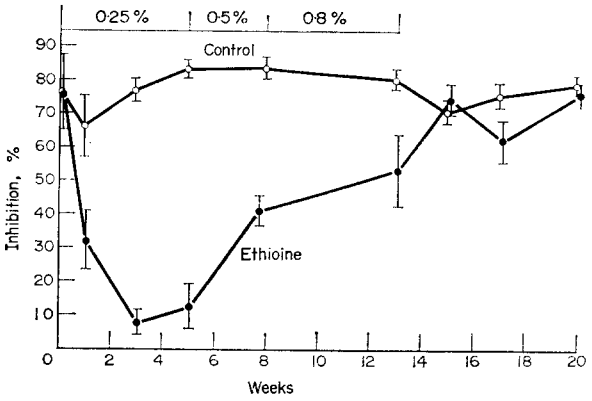

Fig. 2. Percentage of feedback inhibition of cholesterol synthesis in ethionine-fed rats.

Individual values for percentage of feedback inhibition (Fig. 3) show the marked variation characteristic of previous carcinogen studies [6, 7]. In these present experiments, however, most of the treated rats showed a near-complete loss of control, which loss was generally maintained throughout carcinogen feeding.

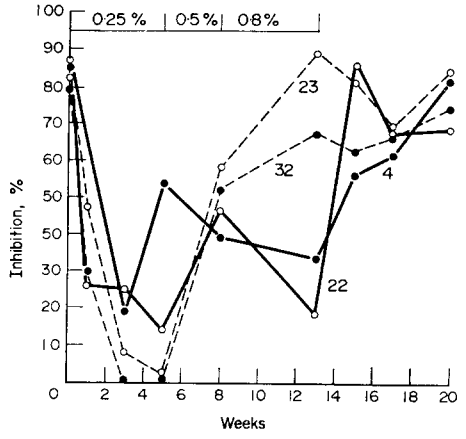

Fig. 3. Individual values for percentage of feedback inhibition of cholesterol synthesis in ethionine-fed rats.

Ethionine toxicity

It could be argued that loss of control of cholesterol synthesis during carcinogen treatment may be merely a toxic side effect and unrelated to the actual mechanism of carcinogenesis. Since about 80% of the ethionine-fed rats had died during the course of treatment, we decided to determine whether those that died had exhibited any difference in control from those that survived.

The results from all the ethionine-fed rats were therefore divided into 3 groups:

High toxicity. Rats which died between the 5th and 7th week (inclusive) of ethionine feeding (13 animals).

Moderate toxicity. Rats which died between the 8th week and the end of ethionine feeding (17 animals).

Low toxicity. Rats which survived throughout ethionine feeding (8 animals).

The percentage of feedback inhibition for these rats during the first 8 weeks of ethionine feeding is shown in Fig. 4. There appeared to be little relationship between mortality and the degree of loss of feedback control. Indeed if there were any relationship it was an inverse one, in that the high-toxicity group showed the least loss of control.

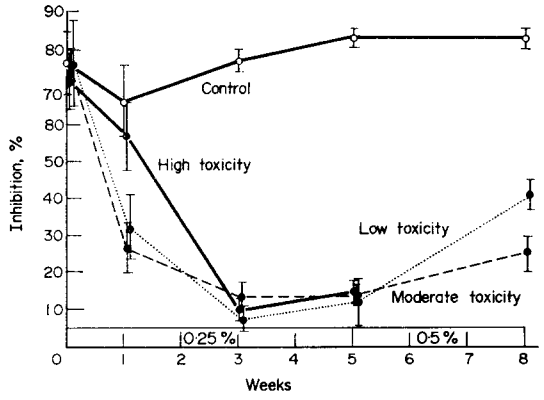

Fig. 4. Effect of ethionine toxicity on percentage of feedback inhibition of cholesterol synthesis.

Entry of ^{14}C-cholesterol into livers of ethionine-fed rats

Following our earlier, preliminary results [10], Harry *et al.* [9] have shown, and we have confirmed [17], that orally-administered ^{14}C-cholesterol is taken up by the fully-developed hepatoma at a much slower rate than by the host liver. We therefore decided to examine the uptake of dietary cholesterol by livers of ethionine-fed rats. These livers, like hepatomas, lack feedback control of cholesterol synthesis.

In order to ensure a complete loss of feedback control in the test rats they were fed ethionine (0·25%) for 2 weeks. A sample of 10 other rats, similarly fed, were tested for cholesterol synthesis *in vivo* at 1, 2 and 3 weeks (5% cholesterol diet at 2 weeks) in order to determine what was the actual degree of loss of control. As can be seen in Table 2, the loss was virtually complete.

The 12 ethionine-fed and 9 control rats were dosed orally with ^{14}C-cholesterol, dissolved in maize oil, between 4.15 p.m. and 4.45 p.m. At 2, 4 and 6 hr later 3 control and 4 ethionine

Table 2. Feedback control of cholesterol synthesis in ethionine fed rats

Weeks of ethionine feeding	Cholesterol supplement	Sterol synthesis	% feedback inhibition
−1	−	29·8 ± 3·5	
0	+	9·8 ± 1·7	67·6 ± 3·7
1	−	33·3 ± 2·7	
2	+	57·7 ± 5·5	7·3 ± 3·7
3	−	72·8 ± 5·6	

Sterol synthesis in 10 rats was measured using the vival assay described in materials and methods, and is expressed as mg 7-dehydrocholesterol/100 ml blood. Cholesterol was fed for 2 days before and including each alternate assay period.

rats were killed and samples of plasma, liver and kidney cortex collected. In all samples free and total (i.e. free + esterified) cholesterol were measured. The esterified cholesterol was estimated by difference.

Figures 5, 6, 7 show the uptake of ^{14}C-cholesterol, as cpm/g tissue, into total, free and esterified cholesterol in plasma, liver and kidney, except that esterified ^{14}C-cholesterol could not be accurately estimated for the kidney, due to the small amount of radioactivity present. Ethionine feeding had little effect on the level of ^{14}C-cholesterol in the plasma, although uptake into the liver was delayed. Entry of ^{14}C-cholesterol into the livers of ethionine-treated rats was, however, much faster than entry into the kidney, which we have shown previously [17] to take up dietary ^{14}C-cholesterol at the same rate as transplantable hepatomas.

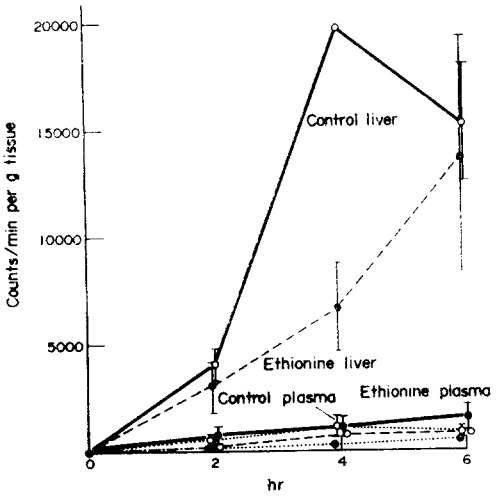

Fig. 6. Appearance of free ^{14}C-cholesterol, following a single oral dose 4-^{14}C-cholesterol, in livers of rats fed ethionine (0·25%) for 2 weeks. Animals as in Fig. 5.

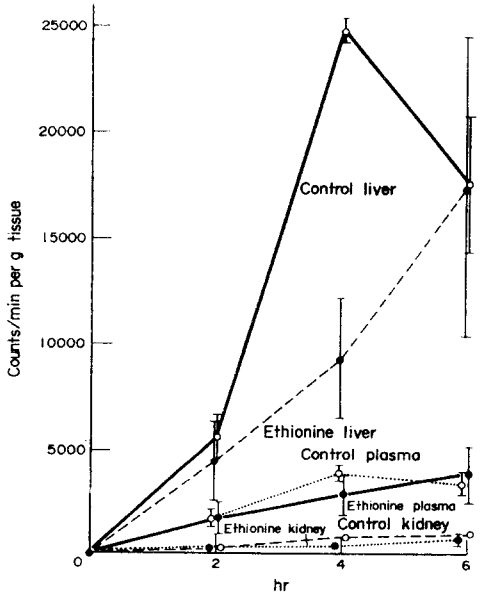

Fig. 5. Entry of ^{14}C-cholesterol, following a single oral dose of 4-^{14}C-cholesterol, into livers of rats fed ethionine (0.25%) for 2 weeks. Each point represents 3 control or 4 ethionine-fed animals.

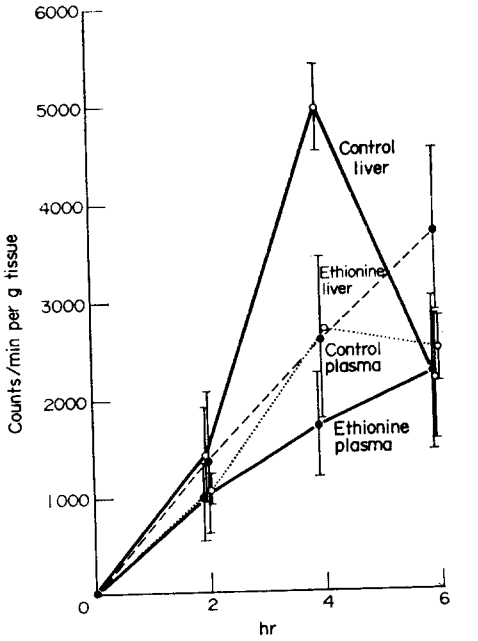

Fig. 7. Appearance of esterified ^{14}C-cholesterol, following a single oral dose of 4-^{14}C-cholesterol, in livers of rats fed ethionine (0·25%) for 2 weeks. Animals as in Fig. 5.

In Fig. 8 the same results are expressed as: specific activity of liver cholesterol/specific activity of plasma cholesterol. This ratio provides a measure of the rate of equilibrium of liver cholesterol with plasma cholesterol, independent of the rate of ^{14}C-cholesterol uptake into the plasma from the gut.

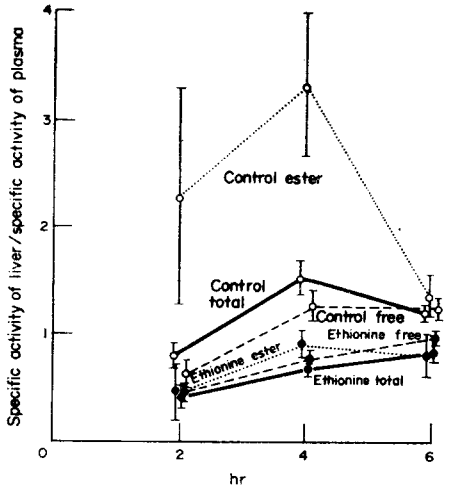

Fig. 8. Uptake of ^{14}C-cholesterol into the livers of ethionine-fed rats, relative to the amounts of ^{14}C-cholesterol in the plasma, following a single oral dose of 4-^{14}C-cholesterol. Animals as in Fig. 5.

In the control animals free cholesterol in the liver equilibrated with plasma free cholesterol within 4 hr, reaching a specific activity slightly higher than in the plasma at that time. Esterified cholesterol, however, equilibrated more rapidly, reaching a specific activity in the liver twice that of the plasma in only 2 hr. This seems to indicate that there is an efficient transport system for rapid removal of esterified cholesterol from the plasma, i.e. assuming that free cholesterol is not absorbed as such and re-esterified inside the liver cells.

In the ethionine-treated livers free and esterified cholesterol were both taken up at a slower rate than in the normal liver, reaching an equilibrium with the plasma in 4–6 hr. In addition, there was no apparent difference between the uptake ratios of free and esterified cholesterol in these animals as there had been in the controls. Furthermore, the specific activity of liver cholesterol never reached a level higher than that of the plasma cholesterol. These results all suggest that the mechanism for rapid transport of esterified cholesterol into normal liver is defective in ethionine-treated livers.

Since esterified cholesterol in the control livers obviously reached an equilibrium with plasma esterified cholesterol in less than 2 hr, the earliest time measured, we were not able in this experiment to determine accurately the exact time required for this equilibrium. Some indication, however, of the time required can be gained from the experiments of Redgrave [18] who found that when ^{14}C-cholesterol-chylomicrons are injected (i.v.) into normal rats, 80–90% of the ^{14}C-cholesterol is taken up by the liver in 10 min, by which time the specific activity of liver cholesterol would approximately equal that of the plasma. Therefore, the time periods under comparison here for equilibration of liver and plasma esterified cholesterol between normal and ethionine-treated animals are approximately 10 min and 4 hr.

It is interesting to note that the liver total cholesterol levels of the ethionine-treated rats were slightly elevated in comparison with the control group (Table 3). This was caused by a 13% increase in free cholesterol and a 120% increase in esterified cholesterol. These changes in liver cholesterol could be a result of either an alteration in uptake of dietary cholesterol or, more likely, the increased hepatic cholesterol synthesis which invariably occurred in these rats (Table 2).

Table 3. Free and esterified cholesterol in liver and plasma of ethionine-fed rats

		mg/g liver or ml plasma	
Tissue	Cholesterol	Control [9]	Ethionine-fed [12]
Liver	Total	1·98 ± 0·03	2·47 ± 0·05*
	Free	1·78 ± 0·02	2·00 ± 0·03*
	Esterified	0·21 ± 0·03	0·47 ± 0·04*
	% Esterified	11·0 ± 1·1	18·9 ± 1·2*
Plasma	Total	0·48 ± 0·02	0·47 ± 0·03
	Free	0·13 ± 0·01	0·24 ± 0·02*
	Esterified	0·35 ± 0·01	0·24 ± 0·02*
	% Esterified	72·7 ± 1·3	49·7 ± 2·5*

The number of animals in each group is shown in parenthesis.
*Significantly different from the control group ($P < 0·01$).

A further unexpected result arising from these studies concerns the relative proportions of free and esterified cholesterol in the plasma (Table 3). In the control rats plasma cholesterol was strictly regulated at 71–76% esterified, with only 1/9 animals outside this range. Total plasma cholesterol in the ethionine-fed rats was not significantly different from that in the controls, but the percentage of esterified cholesterol varied markedly from 40% to 70%. If the liver is responsible for maintaining the constant ratio of free/esterified cholesterol in the plasma, then this finding may indicate another breakdown of control in these livers. Unfortunately, little is known of the role of the liver in controlling the relative proportions of plasma free and esterified cholesterol, though it does play a major role in regulating the total cholesterol level [19, 20].

DISCUSSION

The studies presented here indicate that feeding the hepato-carcinogen, ethionine, causes extensive loss of control of hepatic cholesterol synthesis. These results are similar to those obtained with aflatoxin and *N*-2-fluorenylacetamide [6, 7], in that cholesterol synthesis was significantly increased and feedback control was both variable and either partially or completely lost.

We have shown here that the loss of feedback control is not related to the toxicity of ethionine. This is in agreement with previous studies [7], in which the greatest loss of feedback control after aflatoxin treatment occurred several weeks after the cessation of obvious toxic effects. In addition, loss of feedback control during FAA feeding was not related to the loss of weight caused by the carcinogen treatment [8].

In these present animals we have studied also a possible mechanism for loss of feedback control. Dietary ^{14}C-cholesterol entered the livers of ethionine-treated animals at a significantly slower rate than it entered the livers of normal animals. In particular, esterified cholesterol in the plasma equilibrated with liver esterified cholesterol in ethionine-treated rats at a much slower rate than in normal rats. And furthermore, a significant finding was the fact that although esterified cholesterol in normal liver, following an oral dose of ^{14}C-cholesterol, reached a specific activity 3-fold greater than that, of the plasma, esterified cholesterol in the ethionine livers never reached a specific activity higher than that of the plasma.

These results indicate to us that, whereas the normal liver appears to have an efficient transport system for the uptake of esterified chylomicron cholesterol, this system is missing, or at least seriously impaired, in the ethionine-treated liver.

There appear to be two possible explanations for these effects.

(i) Alteration in the external cell membrane. There may be sites, in or on the external cell membrane, which act as an active transport system for uptake of chylomicron cholesterol. These sites may be modified or inactivated in some way by carcinogens, resulting in impaired permeability of dietary cholesterol and hence no feedback control, as suggested previously by Sabine *et al.* [21, 22].

(ii) Alteration in the microsomal membrane. The sites primarily concerned in uptake of dietary cholesterol may be entirely within the cell. These sites may bind esterified chylomicron-cholesterol as it enters the cell, thus resulting in its concentration there. Feedback control of the enzyme most responsible for regulation of cholesterol synthesis, i.e. β-hydroxy-β-methylglutaryl coenzyme A reductase [2] is mediated specifically by dietary cholesterol (1). Therefore the same sites may be involved in both uptake of dietary cholesterol and inhibition of βHMG–CoA reductase (activity or synthesis), since this enzyme is bound to the microsomal fraction [2]. Carcinogen treatment may cause a loss or modification of these sites, preventing them from recognizing and taking up dietary cholesterol.

In conclusion, it is clear that the most likely cause of the loss of feedback control of cholesterol synthesis in carcinogen-treated livers, as in fully-developed hepatomas, is an alteration in either external or internal cell membranes, resulting in reduced uptake of esterified cholesterol from the chylomicrons.

Acknowledgements—We thank Jeanette Horton and Marian Wilson for expert technical assistance, and the Australian Research Grants Committee for financial support.

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7S Anti-Tumour Antibodies and Activated Fc in Macrophage Tumour Cell Interaction

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Abstract—*Coating tumour cells with 7S anti-tumour antibodies prevented development of ascitic plasmacytoma* 5563 *following inocula of up to* 500 *times the control* LD_{50}. *It seems that with adequate prior coating the peritoneal macrophages of the average mouse can eliminate* 200,000 *tumour cells but cannot cope with a load of* 500,000. *The role of activated Fc in attracting macrophages was shown, and added complement was not necessary for tumour cell killing which occurred within one hour.*

INTRODUCTION

TUMOUR growth *in vivo* has been shown to be inhibited either temporarily or permanently by administration of specifically sensitised immunologically competent cells [1, 2]. Best results are obtained when tumour cells and immunocytes are administered at the same time.

Complement-fixing antibodies have been shown to exert a cytotoxic effect on tumour cells *in vitro* [3, 4], but *in vivo* specific antibodies have been shown to facilitate tumour growth in some instances [5], although retardation of growth was obtained when specific immune antiserum was administered not later than 2 days after the transplantation of mouse leukemia [6].

In these experiments we have shown that if prior to transplantation tumour cells are coated with 7S antibodies, which even with complement cannot effectively kill tumour cells [7], mice are protected against inocula 500 times the control LD_{50}. *In vitro* and *in vivo* reactions between antibody-coated tumour cells and non-sensitized macrophages have been demonstrated. The sequence of reactions involved in this interaction has been demonstrated.

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MATERIAL AND METHODS

The mouse tumour preparation, immunization procedure for rats and isolation of recycled 7S fraction, are described elsewhere [7]. Macrophages were harvested from the peritoneal cavity of normal mice and rats. The first two groups of experiments assessed co-operation of the 7S antibodies with macrophages *in vitro* and *in vivo*. The third showed improved survival after prior coating of inoculated tumour cells. The fourth tested for any residual immunity after such passive transfer. The fifth demonstrated the importance of the Fc fragment in this reaction. The sixth confirmed immunological specificity of the coating of the target cell for the macrophages to then interact. All of the above experiments were conducted in the absence of added fresh complement and used thoroughly washed cells.

1. *The* in vitro *effect of 7S fraction on the interaction between tumour cells and macrophages*

Macrophages and tumour cells were mixed in a proportion of 5:1 and incubated for up to 1 hr with and without specific 7S antibody fraction.

2. *The* in vivo *effect of 7S fraction on the interactiou between tumour cells and macrophages*

Tumour cells previously incubated with 7S antibodies were inoculated into the peritoneal cavities of mice and after 1 hr were aspirated. Controls without antibodies were similarly inoculated.

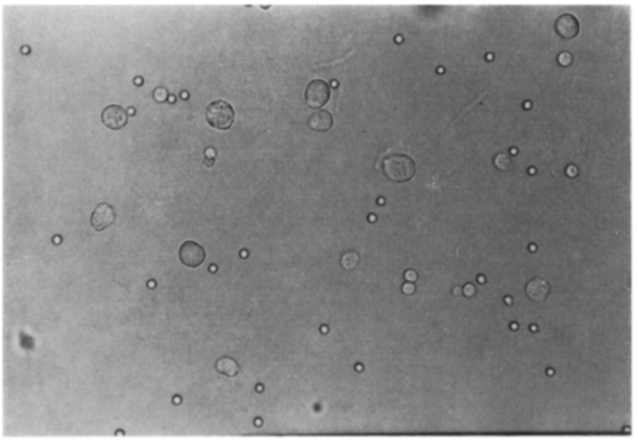

Fig. 1. *Uncoated control. The large cells are the target tumour cells, the smaller cells are mainly macrophages. No aggregation.*

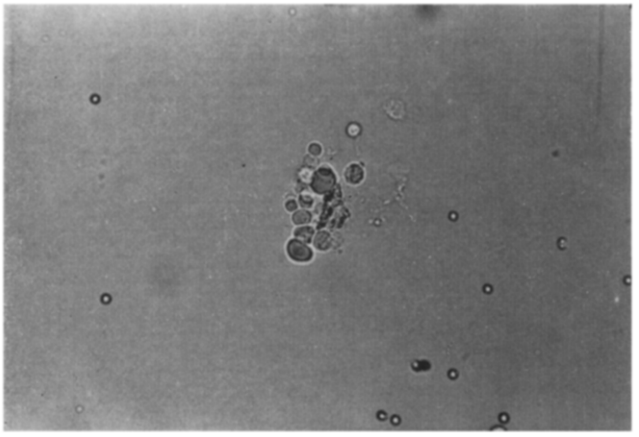

Fig. 2. *Coated test at* 1 *hr. All the target tumour cells are aggregated with macrophages. Many target cells are already dead.*

(to face p. 20)

3. *Transplantation into C_3H mice of tumour cells with and without 7S fraction*

In control animals, 1,000, 3,000 and 10,000 tumour cells were transplanted intraperitoneally into groups of 4 mice. In the test mice tumour cells were mixed with 7S fraction immediately prior to transplantation of doses up to 500,000 tumour cells.

4. *Effect of subsequent tumour cell transplantation into mice which had successfully rejected tumour preincubated with 7S fraction*

Mice which had received 50,000 tumour cells + 7S were challenged one month later with a dose of 10,000 tumour cells without specific antibody.

5. *The role of activated Fc in reactions* 1, 2

Rabbit anti-Rat Ig antiserum (Behringwerke) was used. Fab fragments of this antiserum and of the 7S rat antibodies were obtained by Papain digestion. Incubation for 15 min was followed by washing in excess normal saline. Macrophages were of mouse origin. Table 1 describes the experiments.

6. *The need for activated Fc to interact with the macrophages*

Mouse and rat macrophages were incubated separately for 15 min (at room temperature) with recycled 7S antibodies, which contained no known aggregates or activated Fc, were then washed with normal saline and then added to tumour cells (see Table 2).

RESULTS

1. *The* in vitro *effect of 7S fraction on the interaction between tumour cells and macrophages*

Figures 1, 2 show the sequence of events that occur after addition of macrophages to the tumour cells which had been previously incubated with 7S antibodies. Aggregation was seen to occur within 30 min and was maximal after 1 hr. After 1 hr 100% of target cells were shown to be dead by Trypan blue exclusion as compared to less than 20% in the controls. After 2 hr disintegration of tumour cells occurred and macrophages began to detach themselves from the dead tumour cells.

2. *The* in vivo *effect of 7S fraction on the interaction between tumour cells and macrophages*

Aspiration 1 hr after peritoneal inoculation of coated cells demonstrated a similar aggregation to that seen *in vitro* in those mice which had received tumour cells with prior coating of 7S antibodies. In the controls, using uncoated tumour cells, no aggregation at all was seen.

3. *Transplantation into C_3H mice of tumour cells with and without 7S fraction*

All animals transplanted with more than 1,000 cells in the absence of 7S fraction, developed tumours. 50% of those transplanted with 1,000 tumour cells did so, i.e. the LD_{50} was 1,000 cells.

In animals receiving tumour cells + 7S fraction, no tumour developed in mice receiving up to 200,000 tumour cells. Those transplanted with 500,000 tumour cells all developed tumours, i.e. 500 times the control LD_{50}.

4. *Effect of subsequent tumour cell transplantation into mice which had successfully rejected tumour preincubated with 7S fraction*

Mice which had successfully rejected 50,000 tumour cells with the aid of specific 7S antibodies were challenged one month later by a dose of 10,000 cells. Tumours developed in all the mice.

5. *The role of activated Fc in reactions* 1, 2

Table 1 shows the results:

1a confirms that whole rat-fraction is effective, and 1d shows that coating of tumour-cells by Fab alone cannot attract macrophages. 1b might suggest anti-Fc is without effect, but of course the combination of the rabbit antiserum while covering the rat Fc, results in activation of the rabbit Fc. This can then replace activated rat Fc. By using only the Fab of the rabbit antiserum, 1c, this second activated Fc (rabbit) is avoided and indeed the

Table 1. *Experiments to determine the role of activated Fc in macrophage–tumour cell interaction*

	Experiment		Result
(*a*)	Tumour cells + 7S fraction	incubated and washed + macrophages	AGGREGATION
(*b*)	Tumour cells + 7S fraction	incubated and washed + whole anti-Fc, washed + macrophages	AGGREGATION
(*c*)	Tumour cells + 7S fraction	incubated and washed + Fab anti-Fc, washed + macrophages	NO AGGREGATION
(*d*)	Tumour cells + Fab of 7S fraction	incubated and washed + macrophages	NO AGGREGATION
(*e*)	Tumour cells + whole anti-Fc	incubated and washed + macrophages	NO AGGREGATION
(*f*)	Tumour cells + Fab anti-Fc	incubated and washed + macrophages	NO AGGREGATION

interaction is abolished. 1e and 1f show that the rabbit antiserum alone cannot coat the target cells, and that reactions 1b, 1c do depend on the immunological specificity of the rat 7S fraction.

6. *The need for activated Fc to interact with the macrophages*

Table 2 shows that no reaction occurs when rat or mouse macrophages are incubated with recycled 7S antibodies prior to the addition of tumour cells. The recycled fraction being free of aggregated 7S antibodies or immune complexes is unable to condition the macrophages. This must mean that this ordinary Fc is unable to result in interaction until the antibodies are combined to their target, i.e. until Fc is activated.

DISCUSSION

The above results show how rat 7S antibodies, only when bound to target cells, can attract macrophages with subsequent death of the target cells within 1 hr. This reaction is 100% effective under the ideal conditions of *in vitro* admixture, does not seem to require complement (removed as far as possible), and under the near ideal conditions of prior coating of target cells before inoculation into the macrophage-rich peritoneal cavity can be very effective *in vivo*.

The use of Fab fragments and a second rabbit antiserum against rat immunoglobulins, together with all the controls, affords good evidence that the Fc of the rat antibodies must be activated by prior combination to its target. Presumably the macrophages have a receptor specific for activated Fc (but the latter can cross-species) which cannot be blocked by native Fc. Such immunoglobulin receptors were postulated by Huber and Fudenberg [8] and follow from the classical descriptions of opsonization preceding phagocytosis.

It is generally believed that while opsonins are most important to neutrophil phagocytosis, macrophages can do without them (but not in the present model), although the concept of cytophilic antibodies [9] (with adhesion to the macrophage surface prior to subsequent adhesion to the target) offered a means of improving the appetite of macrophages. In the present series of carefully conducted experiments (using pure recycled 7S antibodies) we could find no evidence of cytophilic antibodies, nor of any "arming" [10] of the macrophages. It is possible that by using whole spleen extracts [10], or less well prepared antibodies [9], activation of the Fc of such antibodies, either by the formation of immune complexes or aggregated antibodies, etc. could result in prior conditioning of macrophages in a manner analogous to "cytophilic antibodies" [9] or "arming" [10].

That Fc receptors could well fail to distinguish between the Fc of other species and the host's own is supported by the way in which rheumatoid factors cross the species, i.e. cannot distinguish the Fc of man from that of rabbit, ox, rat and hen [11]. Rat, mouse and man have, in our experience [12], often shown this for activated Fc. The reactions to activated Fc seem the non-specific part of this process, and it is the actual activation of the Fc (under physiological conditions, see 6 above) which can confer the specificity of such interactions.

Table 2

(*a*)	Mouse macrophages + recycled rat 7S fraction (no antigen)	incubated and washed + tumour cells	NO AGGREGATION
(*b*)	Rat macrophages + recycled rat 7S fraction (no antigen)	incubated and washed + tumour cells	NO AGGREGATION

Furthermore, we could find no evidence of any persisting effect of such 7S antibodies in leaving a persisting population of "armed" or activated macrophages. This could easily be due to the turnover of tissue macrophages [13] together with presumed absence in the present clean model of any transfer factor, etc.

Our analyses [7, 12] of our existing model have shown that the rat's immune reactions to kill the mouse tumour cells can be mediated through:

(i) Complement-fixing IgM antibodies which can be cytotoxic within 20–30 min [7].

(ii) 7S antibodies, which with added complement are largely ineffective by themselves [7] but which without added complement can:

(*a*) call in macrophages, with target cell death within 1 hr;

(*b*) call in non-immune lymphocytes [12], with target cell death by 20 hr.

(iii) Immune lymphocytes (presumably T-) which can result in death of tumour cells after 48 hr (classical delayed hypersensitivity) but which "*in vivo*" have not by themselves been curative.

Measurements in our model have shown (i), (ii)a, and (ii)b can be curative when the tumour cells are confined to the peritoneal cavity—that (ii)a fails first (possibly due to a shortage of macrophages), then (ii)b and finally (i) with target cells becoming less accessible to the reactions.

To conclude the fundamental immunological role of activation of the Fc of antibodies has been further clarified by our own and current experiments:

(1) Activated Fc (especially of IgM) can initiate complement fixation through the normal sequence, $C1_q$ onwards.

(2) Activated Fc (opsonically) can attract phagocytes (neutrophils and Macrophages) and spur them to destroy targets.

(3) As a by-product of such activation, it can be seen how immune complexes (whether IgM, or IgG or even IgA) can encourage phagocytes to release proteases which can then directly act at the level of C3 to activate the further complement sequence, i.e. the C3 pro-activation by-pass [14].

(4) Activated Fc can coopt non-immune lymphocytes and spur them to local cytotoxic activity.

(5) Activation of the Fc of IgE molecules adjacent on mast cell surfaces [15] can trigger immediate hypersensitivity.

(6) Activated Fc serves as antigen for rheumatoid factors, which thereafter can fix to such sites and probably amplify such reactions.

(7) If surface immunogen receptors on lymphocytes are 7S units, then their aggregation [16] by antigen or second antibody (rabbit anti-mouse, etc.) can result in adjacent activated Fc of such receptors and this could trigger the immune response.

(8) It is possible that where an excess of free circulating antigen occurs from the surface of tumour cells, that small immune complexes could result, with activated Fc, which could divert macrophages, non-immune lymphocytes, etc., away from actual target cells, i.e. such complexes would in fact act as "blocking" antibodies.

There remains much work to be done, especially in finding out which classes of immunoglobulins, as it were, prefer, if they do, their activated Fc to take which of the above 8 pathways! Certainly the scope for the non-complement fixing antibodies has increased immensely!

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Effects of Arabinofuranosylcytosine on the DNA Synthesis of Mammalian Cells Treated with bleomycin

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Abstract—*The antitumor peptide-mixture bleomycin inhibits the DNA synthesis of L-929 fibroblasts* in vitro. *The inhibition results in a parasynchronization of the cells in G_1- early S-phase. Following release from bleomycin more successive waves of DNA synthesis are observed, as the cells proceed into S-phase. By treating the released cells for 1 hr with ARA-C (0·05 myg/ml) a considerable reduction of the DNA synthesis of the released cells can be obtained. The combination of bleomycin treatment with short pulses of ARA-C at low concentrations is proposed for the clinical management of bleomycin sensitive human tumors.*

INTRODUCTION

L-CELLS treated with bleomycin, will, on release, exhibit synchronous waves of DNA-synthesis [1]. Also microfluorometric measurements of the nuclear Feulgen-DNA content shows a gradual normalization of the distribution of cells within the S-phase [1]. The mitotic index increases within 4 hours after release from bleomycin from 2·1 to 3·6 (normal: 4·5–5·0) and the number of clonogenic cells, in soft agar, increases from 30% under bleomycin treatment to 70% upon release [2]. These observations may constitute major draw-backs to an effective chemotherapy with bleomycin. We have tried to take advantage from the said parasynchronization of the nuclear DNA-synthesis [1] by successive application of bleomycin and the S-phase "specific" drug arabinosylcytosine [3]. As will be shown, cells treated with bleomycin are highly susceptible to ARA-C.

MATERIAL

Bleomycin mixture (more than 80% A_2, less than 4% B_1) was obtained as a lyophilized preparation in sterile ampules from Fa. H. Lundbeck, Copenhagen. The mixture which is readily soluble in water and aqueous solutions, was dissolved in sterile serum-free medium to yield the proper concentration immediately before use. In most experiments bleomycin mixture was added to the cultures (20 ml) in a final volume of 0·5 ml serum-free medium.

ARA-C (1-beta-D-arabinofuranosylcytosine, Cytosar®) was obtained as sterile standard lyophilized preparation from either Merck, Sharpe and Dohme, or from Upjohn. No difference could be found between the two preparations in parallel experiments, using DNA synthesis inhibition as parameter.

All radiochemicals used in these investigations were obtained through The Radiochemical Centre, Amersham, U.K. The rest of the chemicals used were analytical grade or of the highest purity available.

METHODS

Throughout these experiments a line of L-cells (L-929, The American Type Tissue Collection), growing in monolayer cultures in 250 cc plastic bottles (Falcon Plastics Inc., U.S.A.), was used. The cultures were grown in a modified Eagle's MEM (4), supplemented with 4·5% heat inactivated calf serum (Flow

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Laboratories, Scotland, U.K.). The cultures were propagated by trypsinization (Trypure NOVO, 0·02% in phosphate buffer pH 7·2). Following propagation, the cultures were left undisturbed for 18–24 hr in a climate room, before being used for experiments. No medium change was performed prior to addition of cytostatics and/or labelled precursors.

TREATMENT

For the initial treatment of tissue-cultures with bleomycin we chose a concentration of 4 myg/ml which will inhibit 35–40% of the DNA synthesis of our cell line [1], when treated for 24 hr. After this period the cultures to receive ARA-C were washed 3 times with 20 ml 37°C, conditioned medium, refed and given ARA-C dissolved in 0·5 ml serum-free medium to the appropriate concentrations. At the end of ARA-C treatment the cultures were washed as above, and ^{3}H-TdR, 1 myC/ml (5 C/mM), added and allowed to incorporate for 30 min at 37°C. The reaction was terminated by washing 3 times with 0·27 N PCA (20 ml, icecold) for 5 min. Then 5 ml 1 N PCA was added and the cells were rubbed off with a rubber policeman. The precipitated cells were hydrolysed for 20 min at 70°C followed by centrifugation for 10 min at approximately 2000 **g**. From the supernatant aliquods were taken for DNA estimation a.m. Burton [5] and for counting of the activity incorporated into acid insoluble material.

This was done by mixing 500 myl of the hydrolysate with 15 ml of Bray's scintillation fluid [6] after neutralization with 5 N NaOH (100–150 myl). Counting was done in a Beckman 250S liquid scintillation system. Calculation and conversion of cpm to Pmoles (10^{-12} moles) ^{3}H-TdR incorporated per OD_{595} = 1 per 30 min was performed on a Wang 700 B programable calculator (Wang Laboratories Ltd., U.S.A.).

Cytophotometry

The preparation of coverslips for quantitative microfluorometry has been published earlier [7]. For the measurements reported here a MPV (Microscopephotometer with variable measuring diaphragm, E. Leitz, Germany) was used. A detailed description of this instrument is given by Böhm and Sprenger [8].

RESULTS

Microfluorometry

Microfluorometric measurements of the nuclear Feulgen-DNA content of L-cells subjected to treatment with bleomycin for various periods are given in Fig. 1. From the histogram of the control culture (A) it is seen that the diploid (2n) value corresponds to 60–70 working units (WU), while the tetraploid value (4n) is 120 to 140 WU. Since 2n is the value of cells in G_1-phase and 4n is that of G_2-phases the intermediary values represent the S-phase cells. After 24 hr treatment with bleomycin the profile has changed considerably, showing a high proportion of the cells accumulated in G_1 and early S-phase while late S-phases and G_2-phases are reduced. Histogram C of Fig. 1 shows the situation 45 min after release from bleomycin. At this time a high number of S-phases are present corresponding to the

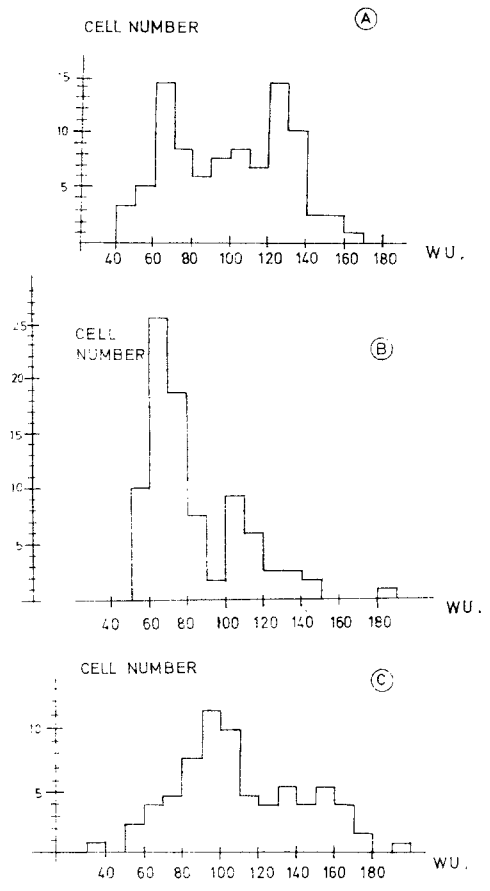


Fig. 1.

(*a*) *Relative nuclear-Feulgen-DNA content of* 200 *acriflavine stained cells from an untreated control culture.*

(*b*) *Relative nuclear Feulgen-DNA content of* 200 *acriflavine stained cells from a culture treated with* 4 *myg/ml of bleomycin for* 24 *hr.*

(*c*) *Relative nuclear Feulgen-DNA content of* 200 *cells from a culture* 45 *min after release from bleomycin.*

Ordinates: cell number.

Abscissas: DNA content in arbitrary working units (WU).

2n: diploid DNA content.

4n: tetraploid DNA content.

parasynchronized population of G_1- and early S-phase cells seen in Fig. 1(*b*). The results will be published in greater detail elsewhere.

DNA synthesis after release from bleomycin

Upon release from bleomycin, 4 myg/ml for 24 hr, equal to an inhibition in the actual experiment of 60% of the DNA synthesis (Fig. 2) the capacity of the released cultures for DNA synthesis was followed by pulse labeling with ^{3}H-TdR of the acid insoluble material. Cultures labeled from time: 0 after release, to time 60 min after release, were labeled for 5 min, while later cultures were labeled for 30 min. After counting all activities were corrected to incorporation per 30 min. This was found justified since a preliminary experiment had shown a linear correlation between ^{3}H-TdR incorporation and time in bleomycin-treated cells for up to 45 min.

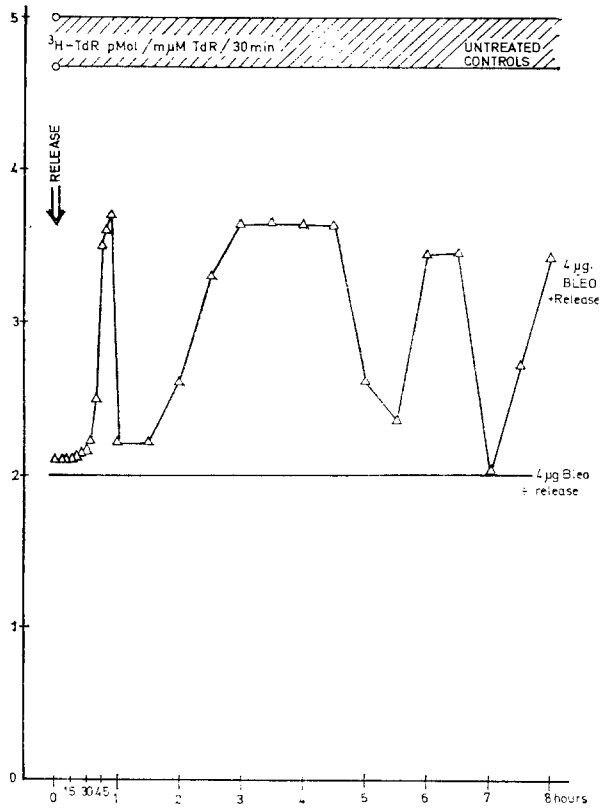


Fig. 2. DNA synthesis of L-cell cultures after release from bleomycin. Cultures were incubated for 24 hr with 4 myg bleomycin/ml medium. Then the cultures were washed 3 times with conditioned medium and refed with 20 ml of conditioned modified MEM containing 4·5% heat inactivated calf serum. At the indicated times 4 released, 2 untreated and 2 unreleased cultures were labeled with ^{3}H-TdR (1 myC/ml, 5C/mM) as described under methods. The values given for released cultures are the mean of the 4 cultures corresponding to each point. Ordinate: Pmoles (10^{-12} moles) of ^{3}H-TdR incorporated per mmymoles TdR per 30 min.

Abscissa: Time.

From Fig. 2 it appears that the release from bleomycin is followed by more successive waves of DNA synthesis, one from 30 to 60 min, the next from 150 to 300 min and a third between 360 and 390 min.

The nature of the DNA synthesized in the different periods will be the subject of a subsequent paper.

Effect of ARA-C on the DNA synthesis of bleomycin-treated and released cells.

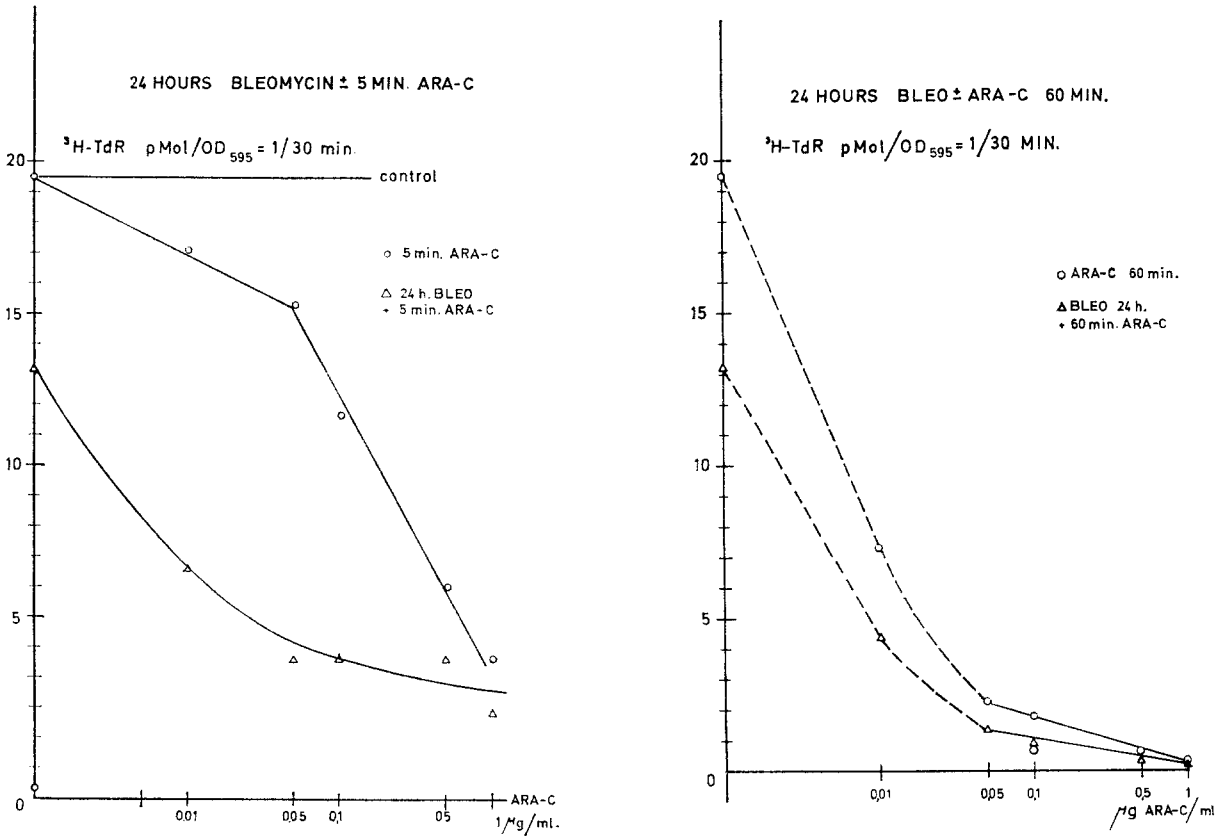
In this experiment one set of cultures received bleomycin 4 myg/ml for 24 hr, while another set had 0·5 ml serum-free medium. After 24 hr the medium was changed on all cultures (4 cultures/experimental point) and ARA-C was applied as indicated in Figs. 3 and 4. These figures thus illustrate the combined effects of a pre-treatment with bleomycin and a post-release treatment with ARA-C, versus the effects of ARA-C alone. It appears that ARA-C is able to abolish the increase in DNA synthesis of released cells which would be expected from the results given in Fig. 2. Furthermore, the parallel decline of the DNA synthesis of pre-treated and released cells and that of cells treated with ARA-C alone, indicate that the effects of ARA-C are additive to those of bleomycin. These effects are relatively more marked for smaller concentrations of ARA-C and for shorter treatments.

Effect of ARA-C on the DNA synthesis of released cells

Figure 5 illustrates the time-course of DNA synthesis in L-cells released from bleomycin but subjected to continued treatment with ARA-C, 0·05 myg/ml from time: 0–60 min after release. The pattern shows some similarity to that of released cells without subsequent ARA-C treatment, although the extent to which DNA is synthesized is considerably smaller. However, even the combination of ARA-C to bleomycin will not abolish the synthesis of some DNA after release from ARA-C.

DISCUSSION

During the last few years the present laboratory has been investigating the mechanism of action of bleomycin. As a means of getting insight into the intracellular processes influenced by bleomycin a number of release experiments have been performed with special reference to DNA synthesis regulation. As shown in Fig. 2 the release from bleomycin is followed by more successive waves of DNA synthesis. This observation may explain some of the difficulties experienced in the clinical



Figs. 3–4. *Combined effect of* 24 *hr treatment with bleomycin* (4 *myg/ml*) *and ARA–C for various periods compared with treatment with either bleomycin or ARA–C alone.* ○———○: *Incorporation of* ^{3}*H-TdR into acid insoluble material of cells treated with ARA–C without pretreatment with bleomycin.*

△———△: *Incorporation of* ^{3}*H-TdR into acid insoluble material of cells pretreated with bleomycin* (4 *myg/ml,* 24 *hr*) *followed by ARA–C for various periods.*

Abscissas: Concentration of ARA–C (logarithmic scale).

Ordinates: 10^{-12} *moles* ^{3}*H-TdR* $\times$ $OD_{595}^{-1} \times time^{-1}$.

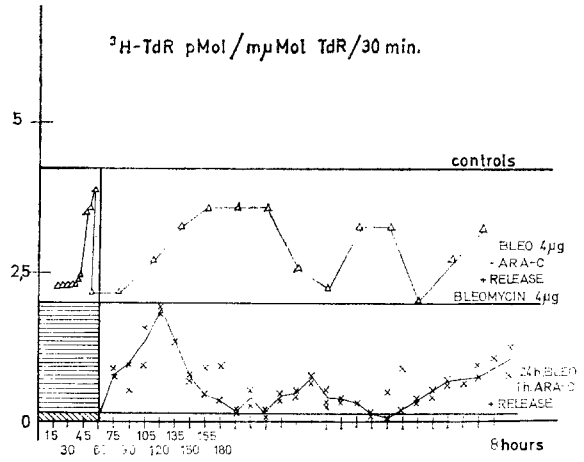


Fig. 5. *Effect of ARA–C* (0·05 *myg/ml,* 1 *hr*) *on the DNA synthesis of cells pretreated with bleomycin* (4 *myg/ml,* 24 *hr*) *compared to the DNA synthesis of cells released from bleomycin treatment.*

△———△: *For details see legend to Fig.* 2.

×———×: ^{3}*H-TdR incorporation into acid insoluble material of cultures treated with bleomycin, released (see methods), treated with ARA–C for* 1 *hr and released once more.*

Controls: Control values for DNA synthesis indicated by horizontal lines:

at 0·08: *for cells treated with ARA–C only;*

at 2·0: *for cells treated with bleomycin only;*

at 4·25: *for untreated controls.*

Abscissa: time after release from bleomycin.

Ordinate: ^{3}*H-TdR incorporation* (10^{-12} *moles* $\times$ *mmymoles* $TdR^{-1} \times time^{-1}$).

treatment of malignant neoplasms with bleomycin. We have tried to take advantage of the observation that bleomycin causes some degree of parasynchronisation of the cell population (Fig. 1) by adding ARA-C, a potent S-phase inhibitor [3], to the cells after release from the block caused by bleomycin. As shown in Fig. 5 this treatment reduces quite considerably the capacity of the cells for DNA synthesis. With the reservations necessitated in any conclusion from *in vitro* experiments the present observations seem to suggest that a combination of bleomycin with short pulses of ARA-C in small concentrations may be of use in the clinical therapy.

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ABBREVIATIONS

G_1: presynthetic phase
S: synthetic phase
G_2: postsynthetic phase
MEM: minimum essential medium
TdR: thymidine
PCA: perchloric acid

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Decreased Formation of Syncytial Plaques in the XC-test with a Double-cloned Hypotetraploid XC-cell Line and Evidence of Budding C-type Particles in This and Ordinary Hypodiploid XC-cell Lines

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Abstract—*Two uncloned hypodiploid XC-cell lines, a double-cloned hypodiploid subline and a double-cloned subline consisting of a mixture of hypodiploid and hypotetraploid cells, were found equally efficient in syncytial formation upon contact with murine leukemia virus in the XC-test of Rowe* et al. (1970). *A double-cloned subline apparently solely composed of hypotetraploid cells showed a one log reduction in plaque count in the XC-test. The ultrastructure was identical in hypodiploid and hypotetraploid cells and both cells types contained budding C-type particles.*

INTRODUCTION

THE XC-TEST [1, 2] for detection of murine leukemia virus has the advantage that it does not require specific immunologic reagents. Other neoplastic cell lines reacting in a morphologically similar way towards leukemia virus of other species may well be found in the future. Since the mechanism behind syncytial formation in XC-cells exposed to murine leukemia virus is unresolved an apparent connection between karyotype and efficiency in the XC-test is reported here.

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MATERIAL AND METHODS

Minimum Essential Medium (Eagle) with Hanks' Balanced Salt Solution pH 7·2 and 10% fetal calf serum was used throughout. The cultures were kept in closed bottles and passed once a week after trypsinization.

The XC-cell line [3, 4] was received from Dr. Wallace P. Rowe, NIH, and kept in two lines, A and B.

Cloning

Cloning was performed with trypsinized cells in a concentration of 0·8 cell per 0·2 ml, the volume being added to each of the 96 wells contained in the plastic micro-test plates (Falcon). The plates were kept in a CO_2 incubator. The actual number of cells per well was determined by microscopy within one hour of plating. When numbering about 100 cells the cloned culture was transferred to a closed bottle. After another passage the cloning was repeated.

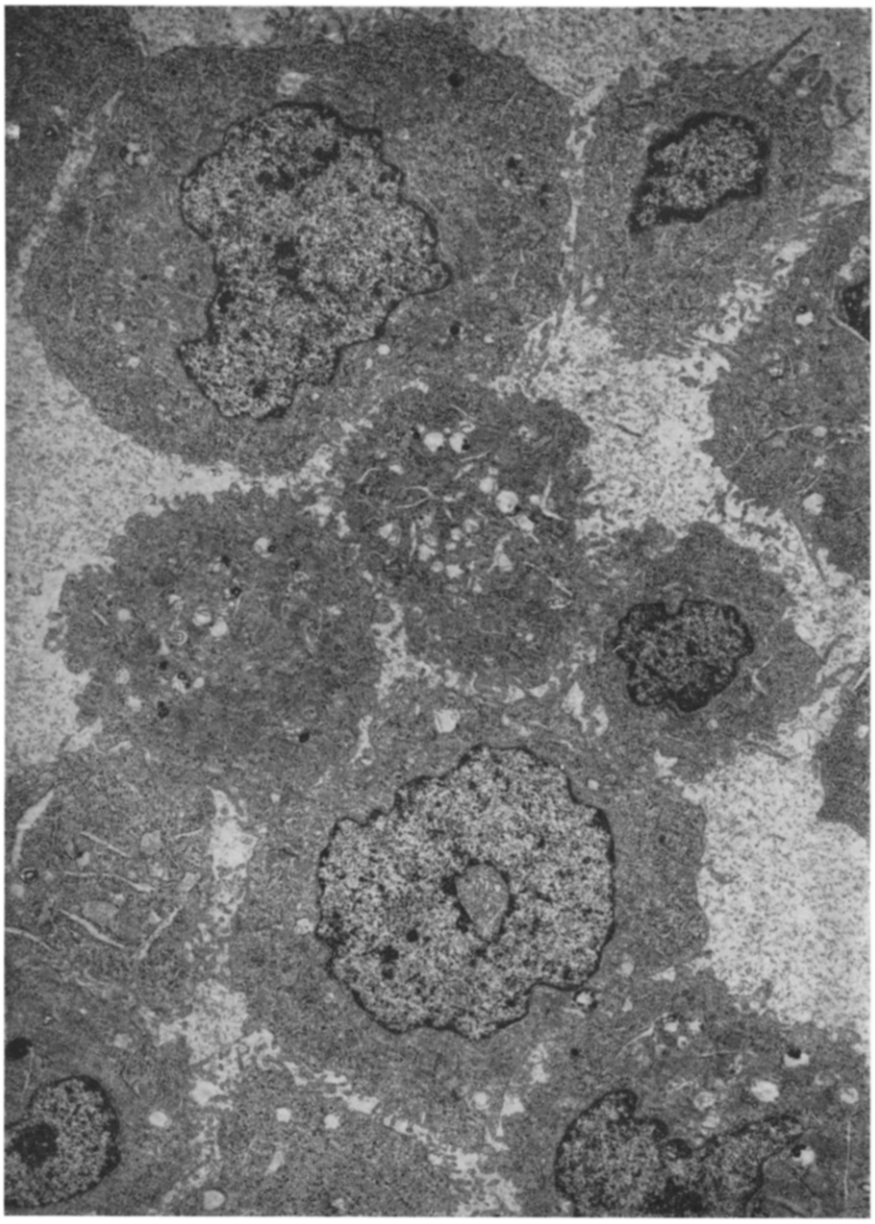

Fig. 3. *XC-cells with morphologic features common to all lines investigated.* × 6300.

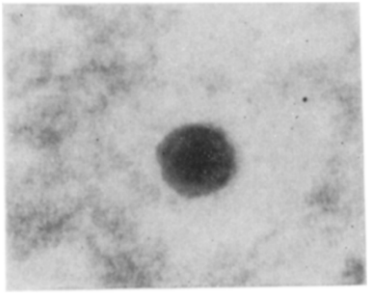

Fig. 4. *Mature C-type particle in cytoplasm.* × 120 000.

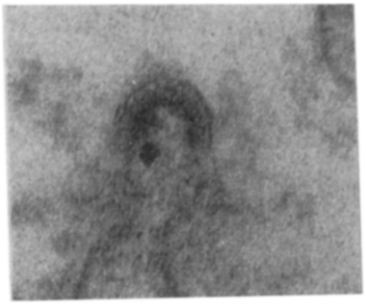

Fig. 5. *C-type particle budding from cell membrane.*

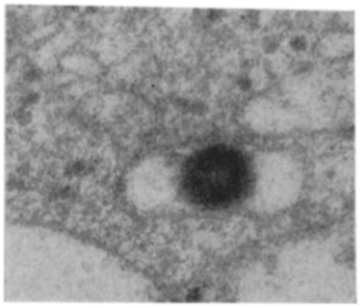

Fig. 6. *Intracisternal A-type particle with surface coating.*

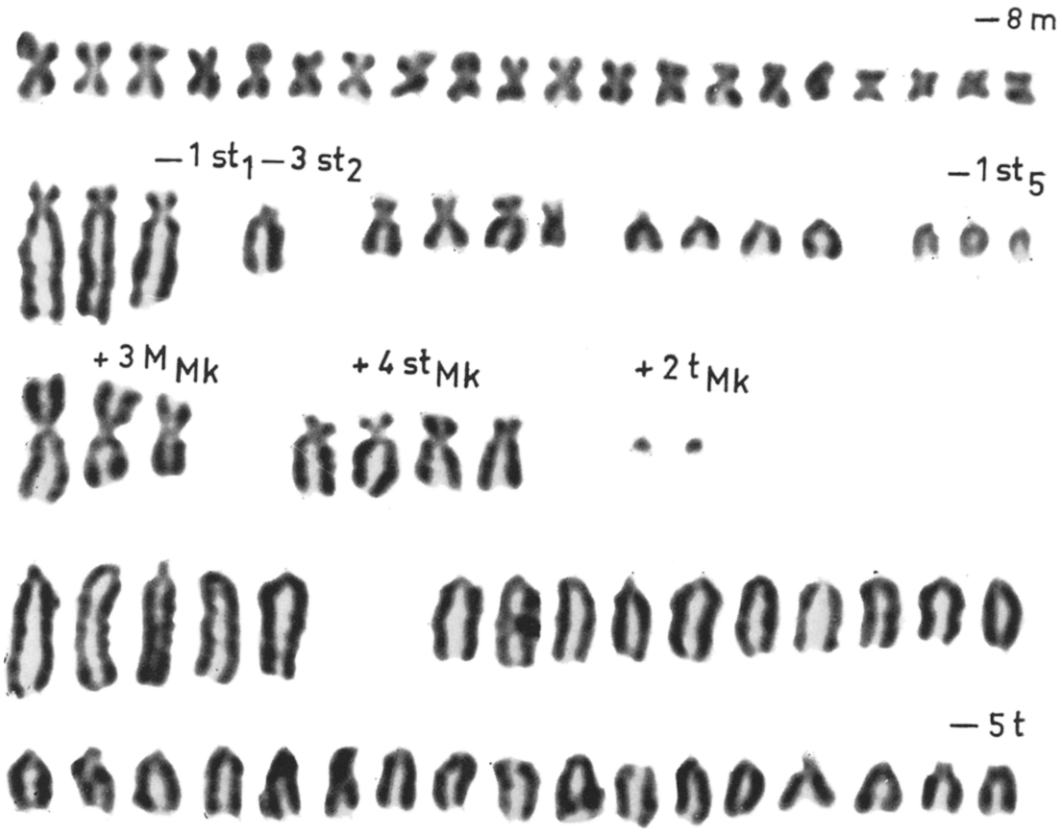

Fig. 2(*a*), (*b*). *Karyotype of cell from double-cloned subline* a_1. 76 *chromosomes*, 9 *marker chromosomes.*

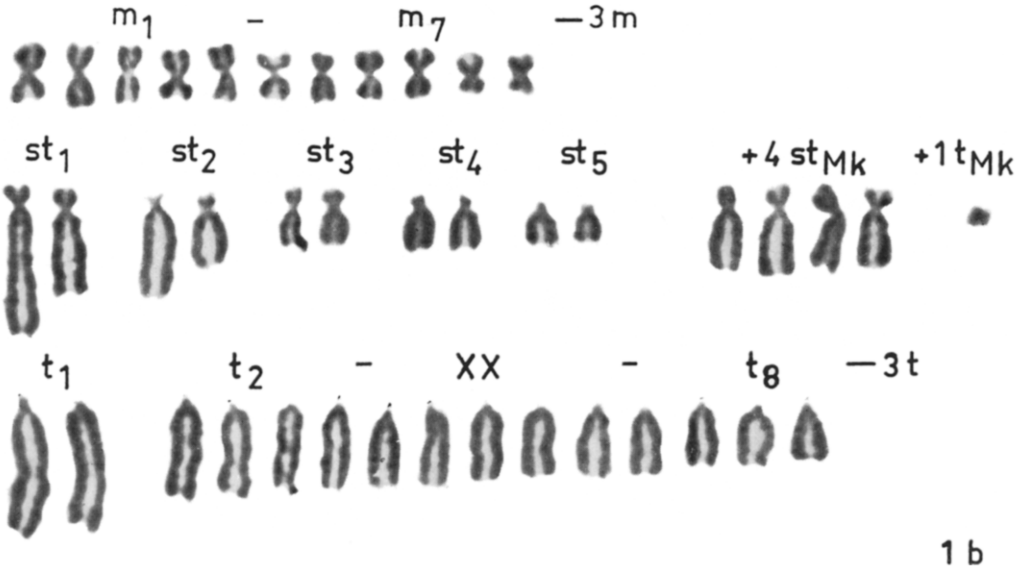

Fig. 1(*a*), (*b*). *Karyotype of cell from line A.* 41 *chromosomes,* 5 *marker chromosomes.*

(to face p. 32)

Virus

The fourth tissue culture passage in BALB/c cells of a BALB-tropic [5] field murine leukemia virus B_1 [6] was used.

XC-test [2]

Five times 10^5 secondary BALB/c mouse embryo fibroblasts were seeded into Falcon plastic dishes with a diameter of 60 mm and kept in 5% CO_2 at 37°C. The following day the subconfluent cultures were pretreated with 2 ml 25 μg/ml DEAE-dextran (M.W. 2×10^6) for 60 min at 37°C. After washing twice virus was added in 5 ml medium. Change of medium was done every day. Four days after infection the fibroblasts were killed with u.v.-light overlayered with 10^6 XC-cells. Three days later the syncytial plaques were counted.

Cytogenetic methods

Our five XC-cell lines were investigated after 10 passages following double cloning. After incubation with Colcemid the cells were trypsinized, hypotonized, and finally fixed in a solution containing 3 parts of ethanol and 1 part of glacial acetic acid. The chromosome slides were then prepared from the cell suspension by air-drying [7]. The chromosome staining was performed with a modified Giemsa procedure [8].

The slides were examined under code. Complete analysis was performed on 20 metaphases from each cell line. Furthermore, a large number of cells were counted and screened for marker chromosomes.

Rat chromosomes and marker chromosomes were classified according to the system of Mitelman [9].

Electron microscopy

Cultures approaching confluence were trypsinized and the cells washed twice in the standard medium, and finally resuspended in 2·5% glutaraldehyde for 30 min at 4°C followed by post-fixation in 1% osmium tetraoxyde. After embedding in Epon ultrathin sections were cut and stained with 5% aqueous uranyl acetate and lead citrate [10].

RESULTS

Double cloning was performed on two cell samples from line A, thereby establishing sublines a_1 and a_2. Double cloning was performed on one cell sample from line B giving rise to subline b_1.

All the XC-lines gave nearly the same plaque count except for subline a_1 with which a one log reduction in efficiency was found (Table 1). The plaque size was the same in all lines, and the doubling time about 21 hr for all lines.

Cytogenetic investigations

Chromosome analysis of line A and line B showed karyotypes with the same chromosome constitution. The chromosome number was 41 in the majority of cells. The karyotypes of most of these cells were identical. The typical karyotype (Fig. 1) contained: 11 small metacentric chromosomes (group $m_1 - m_7$, 3 missing), 2 large subtelocentric (st_1), 2 subtelocentric (st_2), 2 (st_3), 2 (st_4), 2 (st_5), 2 large telocentric (t_1), 13 telocentric ($t_2 - XX - t_8$, 3 missing) chromosomes. A Y chromosome could not be recognized. Four additional medium-sized subtelocentric marker chromosomes and one minute, probably telocentric, marker chromosome were present. A slight difference in size of the two st_1, st_2 and st_3 chromosomes seems a constant feature. The remaining analyzed cells from lines A and B showed minor deviations only from the above described karyotype. Except for one or two cells all had a chromosome number of 41 ± 2. The few structural deviations found were different from cell to cell.

Table 1. *XC-cell lines investigated for plaque count in XC-test, karyotype and ultrastructure*

	XC-test Virus dilution	XC-plaques (two tests)		Karyotype	Budding C-type particle
Line A	10^{-0}	198,	148	Hypodiploid	
	10^{-1}	32,	24		
Subline a_1	10^{-0}	13,	2	Hypo-tetraploid	+
	10^{-1}	0,	0		
Subline a_2	10^{-0}	178,	252	Hypodiploid and Hypo-tetraploid	
	10^{-1}	48,	36		
Line B	10^{-0}	194,	202	Hypodiploid	
	10^{-1}	34,	48		
Subline b_1	10^{-0}	133,	94	Hypodiploid	+
	10^{-1}	8,	10		

The double-cloned subline a_1 showed karyotypes in the hypotetraploid range with chromosome number 72–79 in nearly all cells with approximately double chromosomes in the different groups. In most cells one or two of the marker chromosomes were classified as metacentric, the morphology of the rest of the chromosomes corresponding to the cells from the A and B lines (Fig. 2).

The double-cloned subline a_2 revealed a mixture of hypodiploid and hypotetraploid cells, the morphology of the chromosomes corresponding in all details to the cells from the A and B lines.

The double-cloned subline b_1 showed karyotypes identical to those found in lines A and B apart from a constant lack of the minute marker chromosome. Among the large number of screened cells one endoreduplicated and a few tetraploid cells derived from this cell type (without the minute marker) were detected.

Sera known to react with Epstein–Barr virus antigens [11] did not react in the fluorescence test with the XC-cells.

Attempts to isolate mycoplasms from sublines a_1 and b_1 were kindly performed by Dr. K. Lind, The State Serum Institute, Copenhagen, Denmark. Neither "classical' mycoplasms [12] nor T-mycoplasms [13] were found.

Electron microscopy

The chromosome make-up of the cells did not influence cellular morphology.

All cells had a large lobulated chromatine-rich nucleus, often with a distinct nucleolus. Practically all cells had ample amounts of cytoplasm with numerous free ribosomes. The rough endoplasmic reticulum was sparsely developed and had rather few attached ribosomes. A moderately developed Golgi apparatus and some empty vesicles were found in many cells. Fibrillar material was occasionally found and in a few cases extensive foldings of lysosomes were found scattered in the cytoplasm. In many cells microvilli were seen projecting from the cell surfaces (Fig. 3).

Particles resembling herpes type virus were not found. Both immature and mature C-type particles were observed in the cytoplasm (Fig. 4). C-type particles were furthermore seen budding from the cell membrane in both sublines (Fig. 5). A-type particles coated with electron-dense material were also found in the cytoplasm (Fig. 6).

DISCUSSION

The hypodiploid cells found in the two parental lines and one cloned line must represent the bulk of XC-cells. The modal chromosome number is 41.

In a publication from 1962 Landa *et al.* [14] found tumour XC *in vitro* to be characterized by a basic diploid stem-line with a modal chromosome number of 42. Nelson-Rees [15] found the tumour cells to be hypodiploid with 41 chromosomes, cells with 41 ± 1 chromosomes accounted for 85%. The longest chromosome, commonly unpaired was subtelocentric—a characteristic finding also noticed by us, cf. Fig. 1a, b. Nelson-Rees registered approximately 7 non-rat-like meta-, submeta-, or subtelocentric chromosomes; we found about 4, but the evaluation of these marker chromosomes is very often difficult. The author also described one or two minute chromosomes considerably smaller than the next largest. We found one minute, probably telocentric marker chromosome in the typical karyotype (Fig. 1a, b).

Thus, the typical karyotype of our XC tumour cells closely resembles that of the modal XC tumour cells described by Nelson-Rees.

Occurrence of hypotetraploid cells in a double-cloned line might result from selection of a member of a minority population in the original cell line. Transformation after cloning is also possible. The presence of hypodiploid and hypotetraploid cells in another double-cloned subline derived from the same parental line may reflect an unsuccessful cloning, but transformation after the cloning seems more likely. Prior cloning experiments with subsequent tests for *in vivo* growth indicated an essential homogeneous cell population in non-cloned XC-lines [16].

Co-occurrence of decreased plaque formation upon contact with leukemia virus and tetraploidy makes a chromosome-related alteration in function possible. The average doubling time of the various XC-cell lines, however, did not differ enough to explain the difference in function. Coexistence of hypotetraploid and hypodiploid cells in one subline also speaks against much difference in mitotic rate. If syncytia are only formed by hypodiploid cells a residual population of hypodiploid cells in the hypotetraploid culture might explain the low plaque count. The size of the plaques, however, was not decreased as might be expected if only scattered cells were reactive. Lacking mitosis makes it difficult to determine whether one or both cell types are involved in formation of syncytia which takes place so rapidly that they must arise by cell fusion [2]. The fusion-inducing factor to be registered by the XC-cell membrane may be a lipoprotein [17]. It is

therefore hoped that experimental alteration of membranes in hypodiploid and hypotetraploid XC-cells may give information on the mechanism of syncytial formation.

XC-cells derive from a rat tumour induced by inoculation of Rous sarcoma virus (Prague strain). The Rous virus genome can be rescued from the cells [18] but attempts to demonstrate release of infectious virus have been unsuccessful [19]. Our electron microscopic examinations, however, indicate a production of C-type particles. Since C-type particles were present in both hypodiploid and hypotetraploid lines no lead as to their importance for syncytial formation was given.

A-type particles are often reported present in various cell lines. The particles found revealed an electron-dense coating reminiscent of the coating present on A-type particles in mice [20].

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Modification of Dimethylnitrosamine-induced Changes in Renal Metabolism and Subsequent Effect on Carcinogenic Activity of Actinomycin D and Cycloheximide

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Abstract—*This paper concerns further investigation of nucleic acid and protein metabolism in the kidney of protein-depleted rats during the week following a single injection of dimethylnitrosamine that would cause a high incidence of renal tumours. The maximum doses of actinomycin D and cycloheximide that could be tolerated when administered to rats simultaneously with or shortly after 40 mg/kg of body weight dimethylnitrosamine were determined. When administered to dimethylnitrosamine-treated rats the inhibitors did not affect the rate of metabolism of the nitrosamine or the level of alkylation of liver and kidney nucleic acids. These doses of actinomycin D and cycloheximide were shown to be sufficient to inhibit renal RNA, protein and DNA synthesis in otherwise untreated rats, and were found to have a similar effect on RNA and protein synthesis in dimethylnitrosamine-treated rats. Stimulation of renal DNA synthesis by dimethylnitrosamine 2–3 days after administration was prevented by injection of actinomycin D and cycloheximide. Renal DNA synthesis at other times was also affected by these compounds but to a lesser extent. Relationships between periods of DNA, RNA and protein synthesis in the kidney of the nitrosamine-treated rat are considered.*

The inhibitors did not modify the incidence of dimethylnitrosamine-induced renal tumours but actinomycin D did affect the survival time of rats bearing the tumours. The relationship that biochemical responses to the administration of dimethylnitrosamine bear to its carcinogenic activity is discussed.

INTRODUCTION

A SINGLE median lethal dose of dimethylnitrosamine given to rats will induce kidney tumours in about 20% of surviving animals [1]. The incidence of kidney tumours may be increased to 100% by temporary modification of dietary conditions [2]. Swann and McLean demonstrated that rats fed a diet deficient in protein become resistant to the acute toxic effects of dimethylnitrosamine permitting administration of a larger amount at a single dose [3]. Production of tumours in this way when the carcinogen is metabolized within 24 hr after administration, provides a useful experimental model for the investigation of the earliest stages of tumour induction. Morphological changes occurring within the kidney one week after administra-

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tion of dimethylnitrosamine have been related to the ultimate development of kidney tumours. Hard and Butler [4, 5], who have made a detailed light and electron microscopic study of these changes, concluded that the nitrosamine-induced mesenchymal tumours originate from interstitial lesions which persist from hypercellular aggregations within the kidney, first seen 2–4 days after administration of the carcinogen.

This paper includes the results of further study of the changes in nucleic acid and protein synthesis seen in the kidney of the protein-depleted rat within days of administration of a carcinogenic dose of dimethylnitrosamine [6].

The development of tumours in some animals exposed to carcinogenic stimuli may be modified by secondary treatments [7]. In particular the production of mouse skin tumours by 7,12-dimethylbenz(a)anthracene may be prevented by local application of a variety of agents including actinomycin D either simultaneously with or up to several days after the carcinogen [8–11]. Available biochemical evidence suggests that these agents may exert their effects by interference with intracellular macromolecular metabolism involved in the carcinogenic process. The present paper includes results of analogous experiments using rats, and is based on a study of the production of renal tumours by a single dose of dimethylnitrosamine. Actinomycin D and cycloheximide have been used in an attempt to establish relationships between different periods of RNA and protein synthesis on the one hand and DNA and cell growth on the other [6]. These compounds were administered in single doses either simultaneously with or up to 6 days after the nitrosamine. The doses of the antibiotics were restricted to permit survival of the treated animals so that any modification of the carcinogenic activity of dimethylnitrosamine by the secondary treatment might be determined. This restriction meant that the doses of actinomycin D and cycloheximide employed were lower than those generally used for biochemical investigation. Therefore, to permit interpretation of experiments involving these antibiotics, it was necessary to determine the extent of inhibition produced by them under the present experimental conditions. Furthermore, the effect of the antibiotics on the metabolism of dimethylnitrosamine was examined since available evidence indicates that dimethylnitrosamine requires metabolic activation to exert its carcinogenic as well as its hepatotoxic activity [12].

MATERIAL AND METHODS

Chemicals

Dimethylnitrosamine was purchased from Eastman Organic Chemicals (Rochester 3. N.Y., U.S.A.), cycloheximide from the Upjohn Company (Kalamazoo, Michigan, U.S.A.); and laboratory chemicals (A.R. grade) from B.D.H. Chemicals Ltd. (Poole, Dorset) or Fisons Scientific Apparatus Ltd. (Loughborough, Leicestershire). Actinomycin D was kindly donated by Merck, Sharp and Dohme (Rahway, New Jersey, U.S.A.). [^{14}C]Dimethylnitrosamine was prepared by Dr. P. F. Swann by the method of Dutton and Heath [13]. The radioactive material (11·8 mCi/mmol; 1·18 mg/ml of water) was diluted with unlabelled dimethylnitrosamine before use. The labelled starting material was [^{14}C]dimethylamine hydrochloride purchased from the Radiochemical Centre (Amersham, Bucks).

Animals

Female Wistar rats of the Courtauld Institute colony were maintained on Rowett Research Institute diet 86 till 5–7 weeks old (weight 125–150 g) when they were placed in grid bottom cages and fed a protein-deficient diet [14]. Dimethylnitrosamine (40 mg/kg of body wt.) dissolved in 0·9% NaCl solution was administered by intraperitoneal injection. Control animals received an equal volume of saline by the same route. Cycloheximide (0·25 mg/kg body wt.) and actinomycin D (varying doses) in saline solution were injected intraperitoneally. The rats were fed the protein-deficient diet for a further week after the administration of the nitrosamine before being returned to the complete diet. Organs from rats used to determine renal tumour induction and also from rats killed after acute exposure to different compounds were examined by light microscopy. Tissue was fixed in 10% formol–calcium, and paraffin sections cut at 7 nm were stained with haematoxylin and eosin.

Procedures

The time course of dimethylnitrosamine metabolism in the intact rat was determined by polarographic measurement of the concentration in the blood stream [15] and also by the rate of exhalation of $^{14}CO_2$ following administration of [^{14}C]dimethylnitrosamine [16]. Total nucleic acids and proteins were extracted from livers and kidneys of rats treated with [^{14}C] dimethylnitrosamine by the method of Schneider [17]. The nucleic acids were hydro-

Table 1. *Effect of actinomycin D and cycloheximide on the survival of rats treated with dimethylnitrosamine*

Antibiotic treatment*			No. of animals		
Antibiotic	Dose (μg/kg)	Interval between DMN† & antibiotic (hr)	Treated	Surviving more than 14 days	Surviving fraction (%)
—(None)	—	—	40	29	72
Actinomycin D	120	0	5	0	0
	30	0	5	1	20
	12	0	38	6	16
	6	0	15	10	67
	6	9	25	9	36
	12	24	21	10	48
	12	48	15	12	80
Cycloheximide	500	0	5	0	0
	250	0	20	13	65
	250	9	15	9	60
	250	24	15	9	60
	250	48	14	12	86

*All rats received an initial injection of 40 mg/kg of body wt. dimethylnitrosamine followed by the antibiotic treatment shown.

†Dimethylnitrosamine.

lysed in 1·0M HCl for 1 hr at 100° and then chromatographed to determine the extent of conversion of guanine to 7-methylguanine [18]. The rates of synthesis of nucleic acids and protein *in vivo* were measured using radioactive precursors. The specific radioactivity of DNA, RNA or protein following administration of [^{3}H]thymidine, [^{14}C]-orotate or [^{14}C]leucine respectively was determined as previously described [6].

RESULTS AND DISCUSSION

Toxicity of actinomycin D and cycloheximide administered to dimethylnitrosamine-treated rats

The effect of varying doses of actinomycin D and cycloheximide, administered simultaneously with or shortly after dimethylnitosamine. on the survival of rats is indicated in Table 1. Virtually all animals that died following treatment with antibiotics and dimethylnitrosamine succumbed 3–6 days after administration of the nitrosamine. Such animal showed signs of dimethylnitrosamine poisoning [19]. Histological examination of the liver did not indicate any modification of the dimethylnitrosamine-induced lesion by either cycloheximide or actinomycin D when these inhibitors were administered in amounts that were near-lethal in the present experimental conditions. At this time (3–6 days after dimethylnitrosamine) cellular damage could not be detected in the kidneys of these rats with conventional histological techniques, whether treated with dimethylnitrosamine alone or with the addition of the antibiotics. The rat was most sensitive to actinomycin D when it was administered 9 hr after injection of the nitrosamine (Table 1). Under the same conditions cycloheximide was not as toxic as actinomycin D, nor was the time of administration relative to dimethylnitrosamine as critical.

The histopathological effects of actinomycin D and cycloheximide have been studied by several workers [20–22]. The doses of actinomycin D and cycloheximide used in the present experiments were much less than those required to produce acute toxic symptoms and are unlikely to cause necrosis in liver or kidneys, the tissues most sensitive to dimethylnitrosamine.

Effect of actinomycin D and cycloheximide on the metabolism of dimethylnitrosamine in vivo

The rate of exhalation of $^{14}CO_2$ following administration of [^{14}C]dimethylnitrosamine was not changed by simultaneous injection of actinomycin D (Fig. 1). However, cycloheximide caused a slight reduction in the rate. A more direct assay of nitrosamine decomposition—polarographic determination of the concentration of dimethylnitrosamine in the blood—was therefore employed. Blood levels

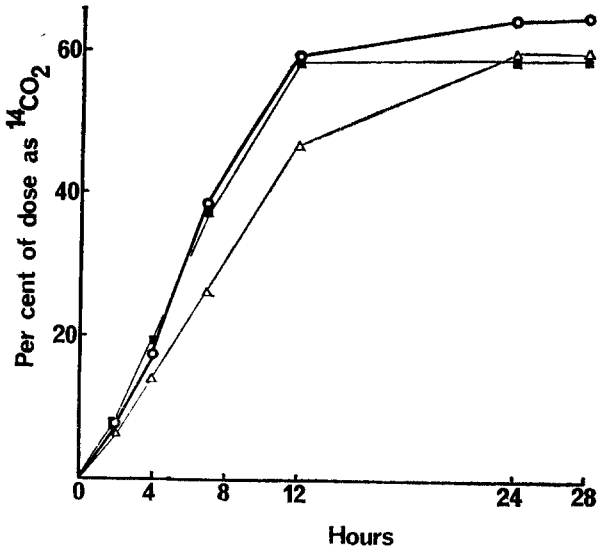

Fig. 1. Effect of actinomycin D and cycloheximide on the exhalation of $^{14}CO_2$ after injection of [^{14}C]dimethylnitrosamine (40 mg, 16 μCi/kg body wt.). Each point represents the mean values from two rats ○, [^{14}C]*dimethylnitrosamine alone;* ■, [^{14}C]*dimethylnitrosamine and actinomycin D (12 μg/kg body wt.);* △, [^{14}C]*dimethylnitrosamine and cycloheximide (0·25 mg/kg body wt.).*

were determined 3½, 5 and 8 hr after injection; at each time there was no significant difference between the mean concentration determined from 5 animals treated with dimethylnitrosamine and cycloheximide and the result from an equal number treated with dimethylnitrosamine alone. The quantity of dimethylnitrosamine metabolized by either liver or kidneys was not affected by either of the inhibitors as indicated by the production of similar levels of 7-methylguanine in the nucleic acids of the respective tissues after various treatments (Table 2). The data suggest that neither actinomycin D nor cycloheximide affected the rate or site of degradation of dimethylnitrosamine in the intact rat.

Table 2. Effect of cycloheximide and actinomycin D, administered simultaneously with dimethylnitrosamine, on the level of 7-methylguanine in liver and kidney nucleic acids

Treatment	Amount of 7-methylguanine* Liver	Kidney
Dimethylnitrosamine (40 mg/kg)	1·12†	0·22
Dimethylnitrosamine + cycloheximide (0·25 mg/kg)	1·08	0·20
Dimethylnitrosamine + actinomycin D (12 μg/kg)	0·96	0·22

*Expressed as a percentage of the total guanine and determined after injection of [^{14}C]dimethylnitrosamine as described under Methods.

†Each result is from the pooled organs of three animals, killed 12 hr after treatment.

Effect of actinomycin D and cycloheximide on nucleic acid and protein synthesis in the protein-depleted rat

Incorporation of [^{14}C]orotate into liver and kidney RNA of protein-depleted rats was inhibited by administration of 12–60 μg/kg of body wt. actinomycin D (Table 3). A dose-dependent reduction in RNA content of liver and kidney was a further indication of the biological activity of the low doses of actinomycin D in

Table 3. Inhibition of [^{14}C]orotate incorporation into liver and kidney RNA by low doses of actinomycin D

Experiment No.	Dose of actinomycin D (μg/kg body wt.)	Duration*	Specific radioactivity Liver	Kidney
1	0 (Control)	—	4·49 ± 0·55	16·47 ± 2·20
	60	5 hr	3·40 ± 0·57‡	8·84 ± 1·29‡
	30	2 hr	5·32 ± 0·36	17·37 ± 3·34
	12	15 min	4·09 ± 1·11	15·30 ± 2·28
2	0 (Control)	—	3·64 ± 0·35	13·11 ± 3·55
	12	15 min	2·73 ± 1·09	9·89 ± 2·42
	12	5 hr	2·70 ± 0·68†	9·43 ± 1·93‡

*Interval between administration of actinomycin D and administration of the labelled orotate.

†, ‡Specific activities significantly less than the respective controls by Student's *t*-test: † $p < 0·05$; ‡ $p < 0·01$.

Rats were killed 30 min (Experiment 1) or 15 min (Experiment 2) after injection of [^{14}C]orotate. Each result is the mean ± standard deviation specific radioactivity, expressed in dpm/μg, of RNA from five individual experiments.

Table 4. Effect of low doses of actinomycin D on the RNA content of liver and kidney from protein depleted rats

Organ	Time after dose (hr)	Dose of actinomycin D (μg/kg body wt.)			
		0 (control)	12	30	60
Liver	2	6·24 ± 0·28*	5·89 ± 0·68	5·50 ± 0·69†	—
	5	6·69 ± 0·59	6·74 ± 0·83	6·41 ± 0·45	4·97 ± 0·27‡
Kidney	2	4·08 ± 0·35	3·66 ± 0·22†	3·26 ± 0·24‡	—
	5	4·22 ± 0·22	3·78 ± 0·22‡	3·94 ± 0·27	3·63 ± 0·37‡

*Each result is the mean ± standard deviation RNA content expressed in mg/g wet wt. of five experiments.
†, ‡Results significantly less than respective controls by Student's *t*-test: † $p < 0.05$; ‡ $p < 0.01$.

these tissues (Table 4). The breakdown of some nuclear RNA species is known to be selectively accelerated in the liver by treatment with actinomycin D [23]; rats given 2·5 mg/kg of body wt. showed a very rapid 30% decrease in nRNA concentration which persisted for at least 18 hr. Since protein depletion is characterized by an extensive loss of microsomal RNA [24], a decrease in nuclear RNA content is more easily detected in the tissues of such animals. Thus the data of Tables 3 and 4 imply synthesis of RNA in liver and kidney of protein-depleted rats is reduced for at least 5 hr following injection of 12 μg/kg of body wt. actinomycin D.

The rate of [^{14}C]leucine incorporation into liver and kidney protein at various times after injection of 250 μg/kg of body wt. cycloheximide is shown in Fig. 2. Lowest activity was recorded 2 hr after administration after which protein synthesis gradually returned to control level. This degree of partial inhibition of protein synthesis is similar to that observed after administration of a similar dose of cycloheximide to rats fed a complete diet [21].

As well as their respective effects on protein and RNA synthesis, both cycloheximide and actinomycin D inhibited DNA synthesis in protein-depleted rats at the dose levels used. Incorporation of [^{3}H]thymidine into renal DNA was reduced by 45% 3 hr after injection of 250 μg/kg of body wt. cycloheximide. Twenty-four hours after administration there was no significant difference between the specific radioactivity of DNA from the kidneys of saline-treated control rats and those treated with the antibiotic. In mammalian cells in culture Bennet *et al.* [25] observed that cycloheximide markedly inhibited incorporation of precursors into DNA under conditions that produced little or no inhibition of incorporation into RNA; under all experimental conditions employed the synthesis of both protein and DNA were rapidly and apparently simultaneously depressed. Such parallel inhibition occurred in the rat kidney in the present experiments. In this case the inhibition of DNA and of protein synthesis persisted for about 9 hr which is consistent with the results obtained by Threlfall and Taylor [26] who monitored the

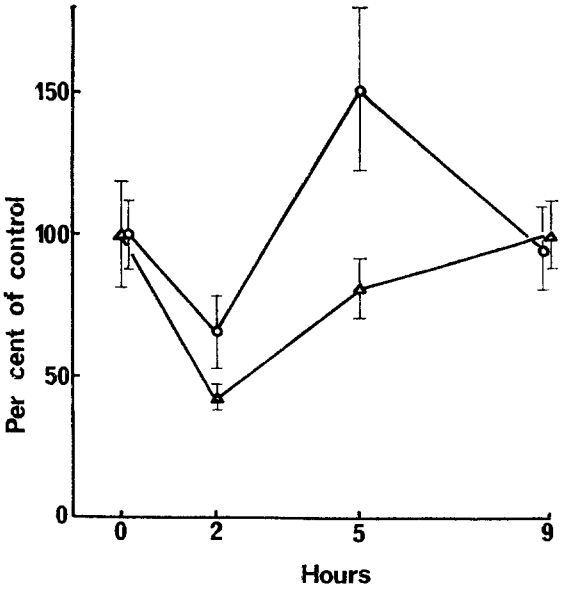

Fig. 2. Inhibition of incorporation of [^{14}C]leucine into liver and kidney protein by cycloheximide. Rats, in groups of 5, were each injected with 0·25 mg/kg of body wt. cycloheximide. After varying intervals each group was injected with [^{14}C]leucine and the level of incorporation into liver and kidney protein determined. Results, the mean ± 1 S.D. of these estimates are expressed as a percentage of the mean specific radioactivity of protein from control animals ○, liver; △, kidney.

effect of a single dose of 1·0 mg/kg of body wt. cycloheximide on renal DNA synthesis. A dose of 60 μg/kg of body wt. actinomycin D reduced slightly the level of incorporation of [^{3}H] thymidine into liver and kidney DNA (Table 5). Such inhibition by a low dose of actinomycin D is presumably dependent on the inhibition of antecedent RNA synthesis by the antibiotic. Thus doses of actinomycin D and cycloheximide which are tolerated by the dimethylnitrosamine-treated rats are sufficient

Table 5. Effect of actinomycin D (60 μg/kg of body wt.) on incorporation of [^3H]thymidine into liver and kidney DNA

Time after actinomycin D (hr)	Specific radioactivity (dpm/mg DNA)	
	Liver	Kidney
0	1646 ± 406*	1046 ± 246
5	1053 ± 553	733 ± 367
24	1240 ± 380	687 ± 127

*Each result is the mean ± standard deviation of five determinations.

to inhibit partially certain renal macromolecular syntheses. The effect of these inhibitors on the biochemical changes produced by the carcinogen [6] is described in the next section.

Modification of dimethylnitrosamine-induced changes in renal nucleic acid and protein synthesis by actinomycin D and cycloheximide

The effect of a single dose of dimethylnitrosamine on protein and nucleic acid synthesis in liver and kidneys of the protein-depleted rat has been described [6]. Administration of 12 μg/kg of body wt. actinomycin D to rats previously treated with dimethylnitrosamine had no detectable effect on the sequence of nitrosamine-induced changes in orotate or leucine incorporation. For example, injection of actinomycin D 9 hr after dimethylnitrosamine, a time at which RNA and protein syntheses are strongly inhibited, did not prevent the recovery in RNA and protein synthesis seen at 14 hr in both liver and kidney. Similarly, administration of actinomycin D, 24 or 48 hr after dimethylnitrosamine did not change the extent of inhibition of kidney RNA synthesis assayed a further 24 hr later.

The extent of inhibition of protein synthesis in rats treated with dimethylnitrosamine was slightly enhanced by administration of cycloheximide. When these compounds were administered simultaneously and protein synthesis assayed after 9 hr the level of incorporation of [^{14}C]leucine was similar to that detected in rats treated with dimethylnitrosamine alone (Table 6). However, the high "acid soluble" radioactivity of the cycloheximide-treated group shown in Table 6 suggests that protein synthesis was more severely inhibited in these rats. Reduction in incorporation of radioactive leucine as the result of injection of cycloheximide was obtained when the antibiotic was administered at a time of minimal protein synthesis, 9 hr after dimethylnitrosamine [6]. The subsequent recovery in activity was less rapid: incorporation of [^{14}C]leucine into both liver and kidney, assayed 24 hr after dimethylnitrosamine, was reduced by about 20%. Treatment with cycloheximide 24 hr after dimethylnitrosamine had no detectable effect on protein synthesis assayed a further 24 hr later.

The above data suggest that, when given to dimethylnitrosamine-treated rats, these doses of actinomycin D and cycloheximide probably inhibit RNA and protein synthesis respectively to an extent not greatly different from that observed when the same doses of antibiotics were administered to otherwise untreated rats.

Administration of 12 μg/kg of body wt. actinomycin D to dimethylnitrosamine-treated rats did not prevent the nitrosamine-induced hepatic regeneration [6]. Though a larger dose of actinomycin D might have been expected to inhibit stimulated DNA synthesis under the same conditions, such an insult would probably prove lethal to the rat already suffering from

Table 6. Effect of cycloheximide and dimethylnitrosamine on protein synthesis in liver and kidney of protein-depleted rats

Treatment*	Acid-soluble radioactivity (dpm/g of wet wt.)		Radioactivity incorporated (dpm/mg of protein)	
	Liver	Kidney	Liver	Kidney
None (control)	69 ± 12	158 ± 15	230 ± 27	141 ± 24
Dimethylnitrosamine (40 mg/kg)	81 ± 19	150 ± 29	150 ± 21	106 ± 26
Dimethylnitrosamine + cycloheximide (0·25 mg/kg)	124 ± 29	242 ± 46	143 ± 9	109 ± 12

*[^{14}C]leucine was administered to rats in groups of 5, 9 hr after the treatment indicated.

severe liver injury. Since the low dose of actinomycin D employed was defined in terms of the survival of the animal, the lack of sustained effect on the liver is not surprising.

Three periods of stimulated renal DNA synthesis follow administration of dimethylnitrosamine under the present experimental conditions [6]. The second phase of stimulated DNA synthesis (1–3 days after dimethylnitrosamine) was particularly sensitive to cycloheximide, and under certain circumstances, to actinomycin D. Figure 3 indicates the effect of a single dose of cycloheximide or actinomycin D administered before or during the period of increased thymidine incorporation occurring 1–3 days after injection of the carcinogen. Stimulated incorporation of thymidine is completely inhibited by cycloheximide whether administered before (24 hr) or during (48 hr) this period of increased DNA synthesis. In contrast actinomycin D is only effective when administered 24 hr after dimethylnitrosamine. The inhibition by this antibiotic at this time is dose-dependent. Figure 4 indicates that 12 μg/kg of body wt. actinomycin D is insufficient to prevent dimethylnitrosamine-stimulated DNA synthesis. On the other hand 120 μg/kg of body wt. reduced thymidine incorporation to a level below that detected in untreated animals. Injection of

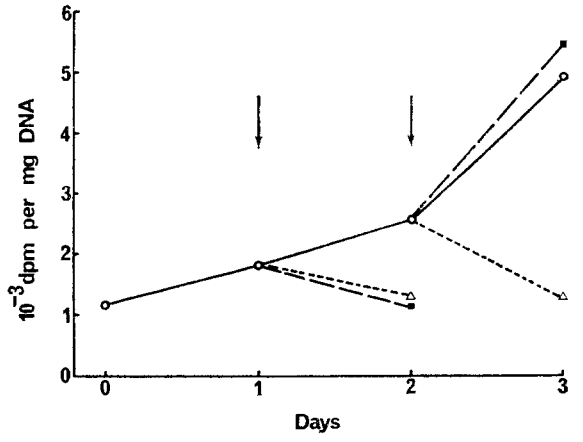

Fig. 3. Effect of cycloheximide and actinomycin D on DMN-stimulated DNA synthesis in the rat kidney. The time course of [^{3}H]thymidine incorporation, following injection of 40 mg/kg of body wt. DMN is indicated (○———○), though changes known to occur in the first 24 hr after administration are not shown. At the points indicated by arrows, the inhibitors were administered to the DMN-treated rats. The level of incorporation of [^{3}H]thymidine into renal DNA was assayed a further 24 hr after △ cycloheximide, ■ actinomycin D.

actinomycin D 48 hr after dimethylnitrosamine did not alter the course of stimulated thymidine incorporation (Fig. 3). The sensitivity to cycloheximide illustrated in Fig. 3 distinguishes this "second" phase of dimethylnitrosamine-stimu-

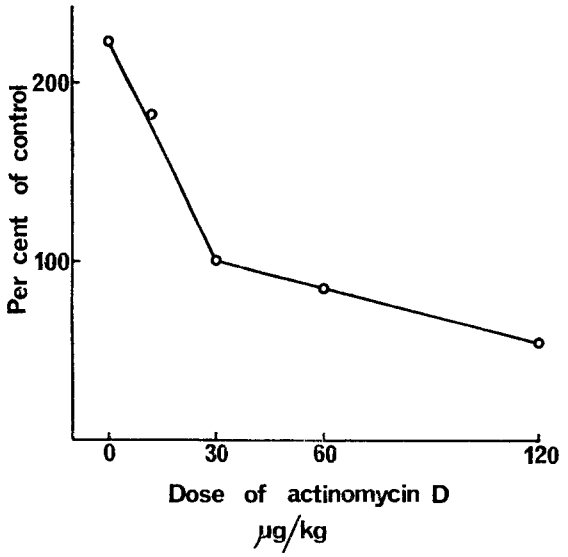

Fig. 4. Inhibition of DMN-stimulated DNA synthesis by actinomycin D. Twenty-four hours after treatment with DMN varying doses of actinomycin D were administered; [^{3}H]thymidine incorporation with kidney DNA was assayed 48 hr after DMN. The mean specific radioactivity of DNA from groups of 4 rats is expressed as a percentage of that in saline-treated control animals.

lated thymidine incorporation from the other two which are observed 3 hr and 6 days after dimethylnitrosamine. In neither case was [^{3}H]thymidine incorporation significantly inhibited by the injection of 0·25 mg/kg of body wt. cycloheximide 24 hr before the precursor (Table 7).

Hence the most conspicuous modification of the sequence of dimethylnitrosamine-induced biochemical changes by actinomycin D and cycloheximide was observed in kidney 1–3 days after administration of the carcinogen. Prior administration of actinomycin D prevented the stimulated DNA synthesis. Furthermore, this stimulated [^{3}H]thymidine incorporation appeared more susceptible to inhibition by actinomycin D than the background level of incorporation detected in untreated rats. These effects of actinomycin D on DNA synthesis probably depend on inhibition of RNA synthesis. The increased DNA synthesis observed 2–3 days after dimethylnitrosamine may be dependent on the synthesis of RNA 24–30 hr after administration of the nitrosamine. If so, this RNA synthesis must be particularly sensitive to actinomycin D. Data are published supporting both these hypotheses. Baserga [27] has summarized the biochemical changes seen in certain populations of cells that become actively proliferating when exposed to specific stimuli. He noted a common increase in RNA synthesis, particularly rRNA, prior to the onset of DNA synthesis in such populations. A three-

Table 7. Effect of cycloheximide on control and DMN-stimulated renal DNA synthesis

Cycloheximide treatment	Control (no DMN)	DMN stimulated	
		3 hr after DMN	6 days after DMN
— (no cycloheximide)	1144	2154	13176
3 hr before [^{3}H]thymidine	629	867	1239
24 hr before [^{3}H]thymidine	1281	2147	10510

Cycloheximide was administered either 3 or 24 hr before the rate of [^{3}H]thymidine incorporation into renal DNA was determined in control rats or rats treated with DMN. Each result, expressed as dpm/mg of DNA, is the mean specific radioactivity of DNA from 5 rats treated as described in the table.

fold increase in renal rRNA synthesis occurs 24 hr after administration of dimethylnitrosamine under the present experimental conditions [6]. There is no evidence of an increase in the rate of degradation of rRNA immediately after administration of dimethylnitrosamine and, therefore, the sucrose gradient analysis of cytoplasmic RNA apparently indicates a net increase in the synthesis of rRNA in the kidney 24 hr after administration of the carcinogen [6]. Perry [28] has shown that the synthesis of rRNA is specifically inhibited by low doses of actinomycin D. There is also evidence that RNA synthesis associated with stimulated cell division is more sensitive to inhibition by actinomycin D than is the synthesis that occurs in the non-stimulated condition [29].

In the present experiments, stimulated DNA synthesis was not affected by 30 μg/kg of body wt. actinomycin D administered 48 hr after the nitrosamine; apparently RNA necessary for increased DNA synthesis has been synthesized by this time. Application of an analogous argument to the cycloheximide data suggests there is a continuous requirement for newly synthesized protein both before and during the period of increased DNA synthesis. The stimulated DNA synthesis is presumably dependent on prior synthesis of protein which is itself sensitive to cycloheximide and this dependence continues until at least 48 hr after dimethylnitrosamine. Verbin *et al.* [30] reached a similar conclusion following their studies on cycloheximide. They reported administration of the antibiotic to partially hepatectomized rats prior to, during or after the DNA synthetic phase resulted in a complete abolition of the first wave of mitosis normally seen in the regenerating liver.

Administration of folic acid to rats produces a proliferative response in renal cells [31]. Using the increased rate of DNA synthesis seen 26 hr after folate as reference, Threlfall and Taylor [26] examined the effect of actinomycin D and cycloheximide. The authors found actinomycin D produced a marked depression when administered 0–4 hr after folate, though when given after 20 hr, actinomycin D had no effect. In contrast, cycloheximide produced a decrease in the rate of DNA synthesis when the drug was given at any time between 0 and 25 hr after folate. Thus the folate-stimulated DNA synthesis, which is correlated with mitotic activity, responded to these antibiotics in a manner similar to that of stimulated [^{3}H]thymidine incorporation observed 1–3 days after injection of dimethylnitrosamine. This is consistent with the relationship of the nitrosamine-stimulated DNA synthesis to mitotic activity observed in kidney 2–4 days after administration of the carcinogen [4] that has been previously suggested [6].

Proliferation of interstitial cells of kidneys of protein-depleted rats treated with dimethylnitrosamine, as reported by Hard and Butler, was observed in the course of the present experiments. The effect of administration of cycloheximide to dimethylnitrosamine-treated rats on this lesion was examined. Rats, in groups of 4, previously treated with 40 mg/kg body wt. dimethylnitrosamine were each injected once with 0·25 mg/kg of body wt. cycloheximide either 4, 5, 6 or 7 days after the carcinogen; a further group of 4 received two injections, each of 0·25 mg/kg of body wt. cycloheximide, 2 and 3 days after the carcinogen. Rats surviving until 8 days after dimethylnitrosamine (at least 2 per group) were killed and the kidneys compared histologically with those from rats treated with dimethylnitrosamine alone as well as from control (saline-treated) rats. In no case (either individually or on a group basis) had the administration of cycloheximide completely prevented the growth of interstitial cells.

Effect of actinomycin D and cycloheximide on the carcinogenic activity of dimethylnitrosamine

As has been previously described [1, 32], renal tumours in rats treated with dimethylnitrosamine are of two distinct types, of mesenchymal and epithelial origin respectively. Classification was often possible at necropsy whilst the two types were readily distinguishable using conventional histological preparations. Administration of actinomycin D or cycloheximide did not modify either the macroscopic or microscopic appearance of the tumours. The results of experiments to test the effect of actinomycin D and cycloheximide on the carcinogenic activity of dimethylnitrosamine in protein-depleted rats are given in Table 8. The data refer to the incidence of tumours of the kidney in the treated animals.

No attempt was made to determine the carcinogenic potency of single injections of the antibiotics. Actinomycin D is carcinogenic [33]. However, Svoboda, Reddy and Harris observed that in rats given single doses of actinomycin D (0·5, 1·0 or 2·0 mg/kg of body wt.) there was only one tumour (mesothelioma) in one of a group of 9 rats receiving the highest dose. In the same paper it is reported that a single dose of cycloheximide (1·0 or 2·0 mg/kg) did not produce any tumours in rats. The administration of low doses of inhibitors in the present experiments almost certainly did not cause the development of renal tumours.

The results obtained indicate that none of the treatments with actinomycin D or cycloheximide significantly reduced the numbers of kidney tumours induced by dimethylnitrosamine. The survival time of rats treated with actinomycin D 9 or 24 hr after dimethylnitrosamine having mesenchymal tumours of the kidney was significantly reduced. Actinomycin D is known to have inhibitory effects on the induction of the immune response [34] and it seems possible that this antibiotic may have modified an immune response in the present experiments and so shortened the survival time of the tumour-bearing host.

Local application of actinomycin D to the skin of mice previously treated with certain polycyclic hydrocarbons may prevent the subsequent development of skin tumours [8–11]. This effect has been interpreted in terms of inhibition of DNA synthesis in the skin [9, 10]. It has been suggested, on the basis of these experiments [10] and also from work on transformation *in vitro* by hydrocarbons [35] and X-irradiation [36], that if cell division at a critical time after exposure to a carcinogen is inhibited some form of "repair" mechanism [37] may function and transformation (whether *in vivo* or *in vitro*) to the neoplastic state may not occur. Administration of cycloheximide to dimethylnitrosamine-treated rats in the course of the present experiments completely inhibited DNA synthesis at a time when renal cell growth

Table 8. *Effect of actinomycin D and cycloheximide on the carcinogenic activity of dimethylnitrosamine in protein-depleted rats*

Treatment*	No. of rats†	No. of rats with tumours		Tumour incidence‡ (%)	Survival time (weeks) §	
		Mesenchymal	Epithelial		Mesenchymal	Epithelial
Saline (2 × 0·5 ml)	10	0	0	0	—	—
Dimethylnitrosamine	29	14	4	62	62 ± 19	86 ± 6
DMN + actinomycin D (0)	15	9	0	60	48 ± 15 ‖	—
DMN + actinomycin D (9)	8	6	0	75	47 ± 10 ‖	—
DMN + actinomycin D (24)	13	7	1	62	45 ± 14 ‖	82
DMN + actinomycin D (48)	10	3	2	50	50 ± 9	96
DMN + cycloheximide (0)	11	5	2	64	53 ± 23	78
DMN + cycloheximide (9)	8	4	1	62	46 ± 16	69
DMN + cycloheximide (24)	8	4	1	62	56 ± 16	91
DMN + cycloheximide (48)	10	6	1	70	62 ± 17	96

*The interval in hours between administration of dimethylnitrosamine (abbrev. DMN, 40 mg/kg) and the respective inhibitor is shown in parenthesis. The doses of inhibitors were actinomycin D (simultaneously or 9 hr after DMN) 6 μg/kg or otherwise 12 μg/kg; cycloheximide 250 μg/kg.

†No. of rats that survived at least 20 weeks after treatment.

‡The fraction of each experimental group having renal tumours, either mesenchymal or epithelial expressed as a percentage.

§Interval between administration of DMN and death of the animal expressed as a mean (± standard deviation where appropriate) for each experimental group.

‖Survival time significantly less than animals receiving DMN alone.

has been directly associated with the development of neoplasia [4, 6]. The subsequent growth of renal tumours suggests that the principle stated above in terms of a repair mechanism may not be extended to the induction of kidney tumours in the rat by dimethylnitrosamine. A positive correlation between interstitial cell growth one week after administration of dimethylnitrosamine and tumour development was observed by Hard and Butler [4]. This growth was also observed in kidneys of dimethylnitrosamine-treated rats in spite of administration of cycloheximide at various times after the carcinogen. It is possible that inhibition of discrete biochemical reactions in the kidney, perhaps before the first cell division after administration of dimethylnitrosamine, will prevent the induction of cancer. The available evidence, however, suggests that once this period has passed the elimination of a specific cell population, already morphologically different from normal interstitial cells [4, 5], is necessary to prevent neoplasia.

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The Selective Concentration of Sulphadiazine and Related Compounds in Malignant Tissue

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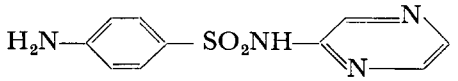
Abstract—*Compared to liver and bone marrow the concentration of sulphadiazine in a number of transplanted animal tumours is very high. Measurements using low levels of labelled compound have shown that this effect cannot be due to selective deposition in tumours of low pH. This affinity for malignant tissue is highly dependent on structure, since, while it is seen with sulphadiazine and three related sulphapyrimidines it does not occur with sulphathiazole or sulphadiazine mustard.*

SULPHAPYRAZINE (I) has been shown to concentrate selectively in the Walker carcinoma growing in rats. Following repeated administration, the presence of deposits of this material was observed in tumour tissue and analysis of various organs of the rat at this time showed that the concentration of drug in the tumour compared to liver was about 4:1 [1]. In no tissue was the concentration of sulphapyrazine more than a third of the concentration found in the tumour. In an earlier paper we have shown that the Yoshida sarcoma similarly concentrates sulphadiazine (II) the tumour/liver ratio being 3:1 [2]. These findings are of considerable interest since tumours generally only take up injected materials to a lesser extent than liver, even though the compounds in question may be active anti-tumour agents.



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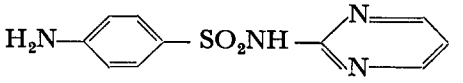
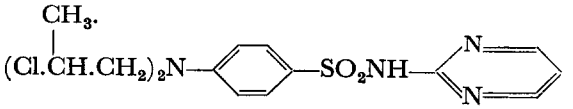
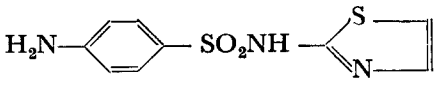


(II)

The selective concentration of these sulphonamides in tumours was thought to be related to (*a*) the difference in pH between tumours and normal tissue and (*b*) to the physico-chemical properties of the sulphonamide molecule. Sulphadiazine in solution exists as an equilibrium mixture of the relatively soluble ionic form and the extremely insoluble unionized form. The proportion of the two compounds in cells is dependent on the intracellular pH and the pKa of the material. In more acid cancer cells there is a larger proportion of the insoluble material at equilibrium. More of the insoluble form is therefore precipitated in tumours with low pH values and this remains, only slowly redissolving after excretion of the soluble ionised material. Some time after injection this results in a higher concentration in tumours compared to normal tissues.



(III)



(IV)

While sulphadiazine is not itself an anti-cancer agent an attempt was made to exploit its ability to concentrate in tumour cells by designing a cytotoxic derivative based on the sulphadiazine molecule [2]. Sulphadiazine mustard (III) was prepared which had the same pK_a as sulphadiazine and was similarly insoluble when unionized. This compound was shown to be selective as an anti-tumour agent [2] but it was not possible to measure its tissue distribution since the Bratton-Marshall method [3] used to estimate sulphadiazine is not applicable to sulphadiazine mustard which does not contain a primary aromatic amino group. More recently C^{14} labelled sulphadiazine mustard has been synthesised [4] and its distribution in tumour bearing animals measured. The surprising finding that this compound was only taken up poorly by those tumours which concentrate sulphadiazine has led to a re-examination of the mechanism by which sulphadiazine concentrates in tumour cells.

MATERIAL AND METHODS

The Sarcoma 180 and ADJ/PC6 plasma cell tumour were transplanted in female BALB/c̄ mice by subcutaneous implantation of tumour fragments under sterile conditions. The NK lymphoma in female BALB/c̄ mice and the Walker carcinoma in female Wistar rats of the Chester Beatty strain were transplanted by subcutaneous injection of ascites cells in the inguinal region. All animals were between 6 and 8 weeks old at the time of transplantation.

Sulphadiazine-2-C^{14} (14·7 mCi/mM) and the N-acetyl, 2-p-tolyl and 2-phenyl analogues of specific activity 14·7, 11·7 and 11·7 mCi/mM respectively and of radiochemical purity greater than 99% were prepared by the action of the appropriate sulphonyl chloride on 2-amino-pyrimidine-2-C^{14} that was obtained (4) from guanidine-C^{14} hydrochloride with a radiochemical yield of 86%.

The sulphonyl chloride (1·0 mmole) in dry pyridine (1 ml) was added to a stirred solution of 2-amino-pyrimidine-2-C^{14} (0·7 mmole) in dry pyridine (1 ml). The mixture was stirred for 24 hr at room temperature. Then the pyridine was removed under reduced pressure. The crude sulphonamide was purified either by column chromatography or by thin-layer chromatography. The radiochemical purity was checked by thin-layer radiochromatography and the chemical purity was determined by u.v. spectrophotometry.

Glycine-1-C^{14} (55 mCi/mM) was obtained from the Radiochemical Centre, Amersham. Chlorambucil (82·4 mCi/mM) tritiated in the aromatic ring and CB 1954 (25 mCi/mM) also tritiated in the aromatic ring were prepared by Jarman and Khan of the Chester Beatty Research Institute. The radiochemical purity of chlorambucil was 96% and CB 1954 99%. Unlabelled sulphadiazine and sulphathiazole were obtained from British Drug Houses. All compounds were administered intraperitoneally, dissolved in dimethylsulphoxide (DMSO).

For determination of sulphonamides in tissues following intraperitoneal injection of relatively high doses the method of Bratton and Marshall was used [3]. Tissues were removed at intervals after injection and homogenised in distilled water (200 mg wet wt/ml). A 4·0 ml aliquot was digested in 0·5 ml N NaOH for 1 hr in a water bath at 90°C to hydrolyse any acetylated material. After precipitation of proteins by addition of 2·0 ml of 15% trichloroacetic acid, the supernatant was used for determination of the total sulphonamide content of the tissue by the method of Bratton and Marshall.

Following the administration of labelled sulphadiazine or its derivatives, tissues were homogenized in distilled water (300 mg wet wt/ml). A 1ml aliquot of the homogenate was digested in 1·0 ml tetraethyl ammonium hydroxide overnight at 50°C; 0·1 ml of the clear digest was added to 10 ml of scintillation fluid (AR toluene 385 g, dioxan 285 g, ethanol 99·5% 230 ml, naphthalene 80 g/l and butyl-PPO 7 g/l) and counted in a scintillation counter (Packard Tricarb, Model 3375).

For both estimations results were expressed in terms of the dry weight obtained from the evaporation to constant weight of 1 ml aliquots taken from the initial tissue homogenates in distilled water.

RESULTS

Figure 1 compares the uptake of sulphadiazine by liver and Walker tumour measured by the Bratton–Marshall method, with the uptake of the same compound but labelled with C^{14} and estimated by scintillation counting. It also compares the distribution of drug after high and low dose levels since, in

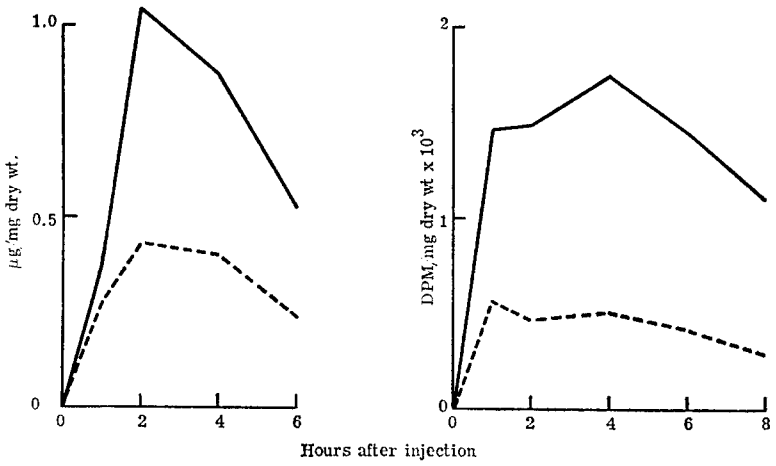


Fig. 1. Uptake of sulphadiazine by liver (– – – – – –) and by Walker tumour (————). In the first graph sulphadiazine was injected at time 0 at 250 mg/kg, i.p. in DMSO and measured by the Bratton–Marshall method. In the second graph sulphadiazine-2-C^{14} was injected at time 0 at 1·7 mg/kg, i.p. in DMSO and measured by scintillation counting. Each point is the mean value for two animals.

the first case, a high dose level was used (250 mg/kg, i.p. in DMSO) and in the second case an extremely low dose level (1·7 mg/kg, i.p. in DMSO).

Figure 2 shows that, whereas sulphadiazine is taken up selectively by the Walker carcinoma, there is only a poor uptake of the related sulphadiazine mustard. In fact, anti-cancer agents even if highly selective in their effects against the Walker carcinoma, are not well concentrated in this tumour. From Fig. 3 it can be seen that chlorambucil is not taken up well by the Walker carcinoma compared with liver, yet it is a highly selective anti-tumour agent in this system with a therapeutic index of 10 [5]. CB 1954 (2, 4-dinitro-5-aziridinylbenzamide) which has a therapeutic index of 70 against the Walker tumour, the highest ever recorded [6], is still no more concentrated in the malignant tissue than in the liver. Even glycine which is readily diffusible, is still incorporated to a lesser extent by the tumour.

A most important finding has been the demonstration that the distribution of sulphadiazine in the bone marrow with time follows a similar pattern to that seen in liver, and at any one time is much less than the amount found in the tumour (Fig. 4). This is of parti-

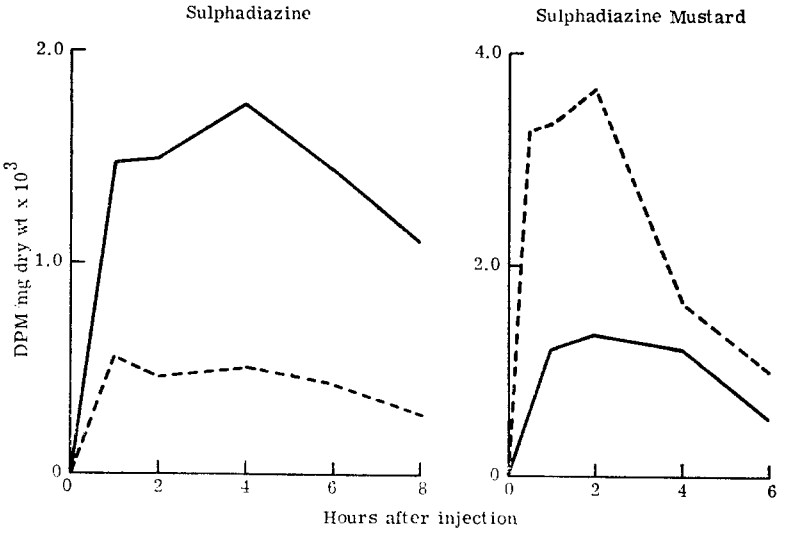


Fig. 2. Uptake of sulphadiazine-2-C^{14} and sulphadiazine mustard-2-C^{14} by liver (– – – – – –) and by Walker tumour (————). Both compounds were administered at time 0 at 1·7 mg/kg, i.p. in DMSO. Each point is the mean value for at least two animals.

cular interest since it is bone marrow toxicity that often limits the amounts of anti-tumour agents that can be given clinically.

The affinity of sulphadiazine already demonstrated for the Walker and Yoshida tumours appears to be a property common to many experimental tumours, since, as shown in Fig. 5, all tumours concentrate sulphadiazine to a greater extent than the liver. This affinity for tumours, which has also previously been seen with sulphapyrazine, is not, however, seen with the related sulphonamide, sulphathiazole (IV). Figure 6 demonstrates that sulphathiazole, in contrast to sulphadiazine, does not concentrate

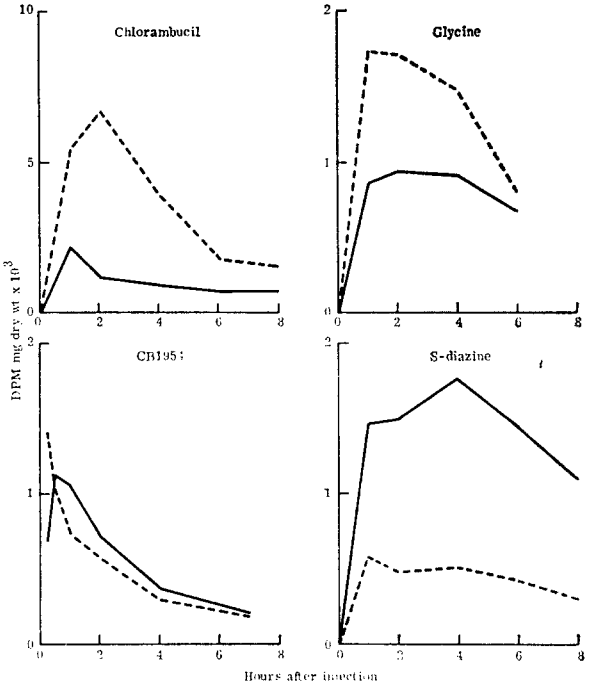


Fig. 3. Uptake of various chemicals by liver (– – – – – –) and by Walker tumour (————). Each point is the mean value for at least two animals. Chlorambucil 3·7 mg/kg, glycine 0·14 mg/kg, CB 1954 10 mg/kg and sulphadiazine 1·72 mg/kg.

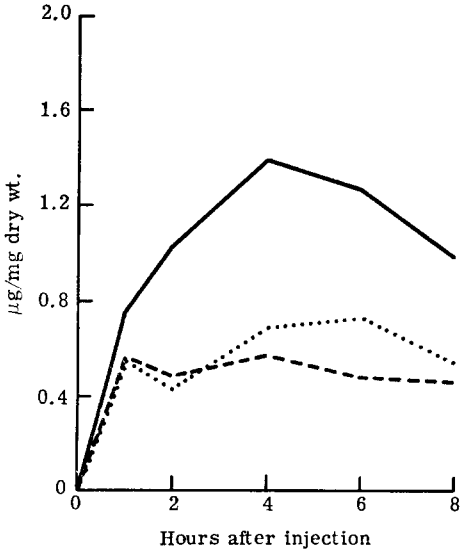


Fig. 4. Uptake of sulphadiazine by liver (– – – – – –), Walker tumour (————) and bone marrow (. . . . . .) after administration of 500 mg/kg sulphadiazine, i.p. in DMSO and measured by the method of Bratton and Marshall. Each point is the mean value for two animals.

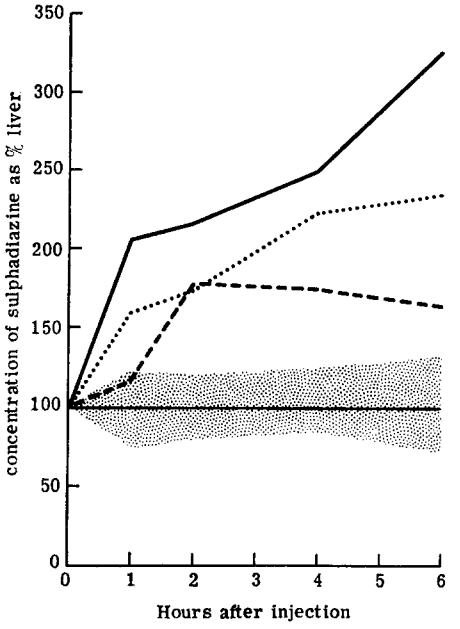


Fig. 5. Uptake of sulphadiazine by sarcoma 180 (————), NK lymphoma (. . . . . .) and PC6 plasma cell tumour (– – – – – –) as a percentage of liver. The stippled area is the range of values for liver obtained from all experiments. Each point is the mean value for at least two animals.

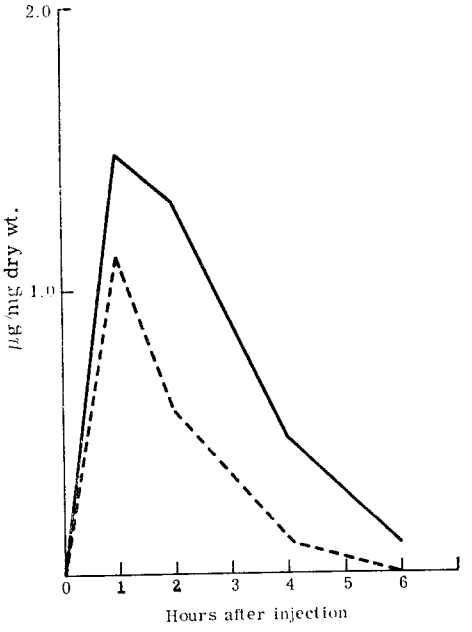


Fig. 6. Uptake of sulphathiazole by liver (————) and by Walker tumour (– – – – – –). Sulphathiazole was administered at 250 mg/kg, i.p. in DMSO and estimated by the method of Bratton and Marshall. Each point is the mean value for at least two animals.

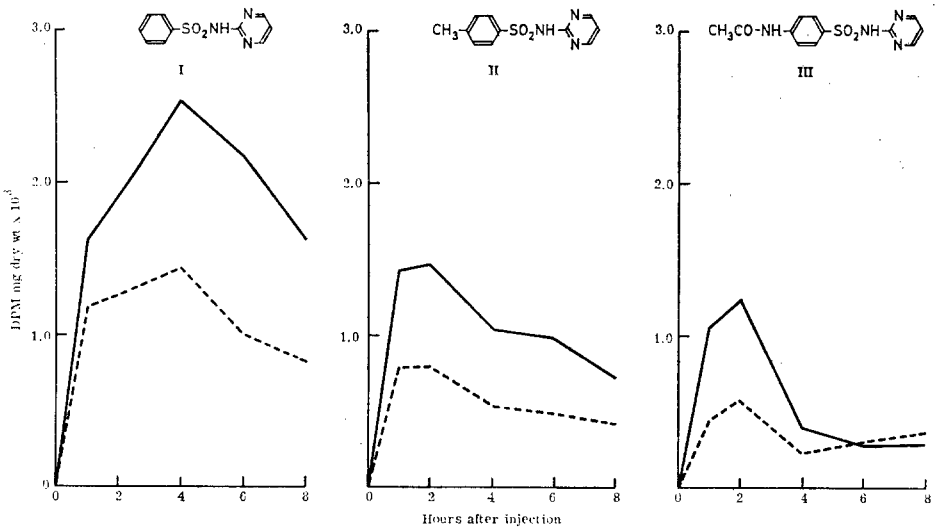


Fig. 7. Uptake of some 2-C^{14} labelled sulphapyrimidines related to sulphadiazine by liver (– – – – –) and by Walker tumour (————). All three compounds were administered i.p. in DMSO at doses between 1 and 2 mg/kg. Each point is the mean value for at least two animals.

in the Walker tumour to a much greater extent than in liver. However, a number of derivatives closely related to sulphadiazine still show a selective tumour concentration (Fig. 7).

DISCUSSION

The distribution pattern of sulphadiazine in liver and Walker tumour is the same whether it is measured by the Bratton–Marshall reaction after injection of high doses (250 mg/kg, i.p. in DMSO) or by scintillation counting following administration of small doses of the C^{14}-labelled material (1·7 mg/kg, i.p. in DMSO) (Fig. 1). The former method measures the primary aromatic amino group of the molecule and the latter method the pyrimidine moiety. Since the uptake patterns are almost identical, it is likely that both measurements are giving an accurate picture of the distribution of the intact molecule.

The pK_a (6·4) of the nitrogen mustard derivative of sulphadiazine is the same as sulphadiazine and it should, according to the theory, show a similarly high concentration in tumours of low pH. It is quite clear, however, from Fig. 2, that the mustard derivative is taken up only poorly by the Walker tumour. That the theory put forward to explain the affinity of certain sulphonamides for tumour tissue is not the correct one is further evident from a study of the distribution pattern of sulphadiazine after administration of low dose levels (Fig. 1). At a concentration of sulphadiazine that is less than the solubility of the drug at the pH of the tissue, the difference in concentration between liver and tumour would be expected to disappear, since there will be no differential precipitation of the material between the liver and the more acid tumour.

If selective accumulation of sulphadiazine occurs in a tumour at concentrations which are less than the solubility of the drug then it cannot be due to a pH dependent deposition of material in the tumour. Given that the ionized form of the drug is much more soluble than the unionised form, the effective solubility at various pHs can be calculated from the expression:

$$\text{Solubility (at given pH)} = \frac{100x}{\%\ \text{unionized at chosen pH}}\ \text{mg/100 ml } H_2O$$

where x = solubility of the unionized form in mg/100 ml H_2O.

Taking literature values for the solubility of sulphadiazine in water at pH 5·5 and 37°C as 14 mg/100 ml [7] and substituting in the equation one gets a value for the solubility of sulphadiazine at pH 6·5 (pH of the Walker tumour) of 26 mg/100 ml water. Differential concentration of sulphadiazine between liver and tumour is still found, however, after administration of 1·7 mg/kg of sulphadiazine when the amount in the tumour at maximum concentration is 2×10^3 d.p.m. per mg dry weight. This value is equivalent to only 0·25 mg/100 gm wet weight of tumour tissue and is therefore well within the solubility of the sulphonamide. Any selective accumulation at tumour concentrations below 26 mg/100 g wet weight must be due to a differential affinity for the tissue and not due to a pH dependent deposition.

Whatever the mechanism of selective deposition in tumours, however, there is no doubt that it is quite specifically related to structure. While sulphadiazine concentrates well in tumours, substitution of the aromatic amino group by a dichloropropyl moiety to make sulphadiazine mustard causes a complete loss of selectivity. It is not essential, however, to have a primary amino group since this may be replaced by methyl or acetylamino or even a hydrogen atom with retention of the selective affinity for the Walker tumour remaining (Fig. 7). The pyrimidine moiety can be substituted by a pyrazine group (to form sulphapyrazine) and activity remains, but not by a thiazole group since this structure does not concentrate selectively in the Walker tumour (Fig. 6).

Of particular importance was the finding that bone marrow did not concentrate sulphadiazine selectively (Fig. 4). It is the eventual aim to prepare cytotoxic derivatives of sulphadiazine with a selective affinity for tumour cells. In this respect the level of accumulation in liver is not particularly important since this organ is generally not sensitive to anti-tumour agents. Limiting toxicity in man following administration of anti-cancer agents usually involves the bone marrow. A cytotoxic derivative of sulphadiazine will only be expected to show an improvement over present day cytotoxic agents if the differential concentration is seen not only between the tumour and the liver, but also between the tumour and bone marrow.

A number of other derivatives of sulphadiazine are now under examination for their ability to accumulate selectively in various tumours. On the basis of these results, attempts will be made to design cytotoxic derivatives which have the appropriate structural requirements for selective concentration in tumours.

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The Liver Damaging Effect of L-Asparaginase—An experimental study of chronic toxicity

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Abstract—*Rats treated by subchronic administration of L-Asparaginase at various dosages did not usually show biochemical alterations. However, cytolysis, cellular infiltration, reticulum alterations and a clear increase of lipid content were present, and fine modifications of liver parenchymal cells could be seen by electron microscopy.*

L-ASPARAGINASE, an enzyme active against certain leukemias, has aroused much interest because its unique mechanism of action, namely a depletion of the amino-acid L-asparagine. It is thought that leukemic cells, especially lymphoblasts are unable to synthesize L-asparagine, possibly because of an acquired metabolic deletion, thus being brought to selective starvation by L-asparaginase. The normal cells, it was believed, would be able to synthesize asparagine and therefore would be unaffected by the drug. However, as more and more clinical trials were performed, a wide range of untoward effects was observed as result either of allergic reactions, or of damage to normal parenchymal cells, the latter being due, perhaps, to an inhibitory effect on the metabolism of cells undergoing rapid protein synthesis.

The more frequent form of toxicity was related to liver damage and, in patients treated with L-asparaginase, many biochemical and some histological findings demonstrating liver damage have been collected. In the first two weeks of treatment with L-asparaginase 75% of patients show an increase of serum transaminases, alkaline phosphatases and bilirubin level and some modifications of bromosulfonphthalein retention. In some cases hypoalbuminemia, hypoproteinemia, a decrease of serum triglycerides and cholesterol [1, 2] are present. The liver aspect, as shown by a few biopsies and by several p.m. examinations, seems to be mainly characterized by fatty metamorphosis, sometimes associated with parenchymal degeneration and intracanalicular cholestasis.

Oettgen *et al.* [1] noted that the severity of the hepatic fatty metamorphosis was unrelated to the L-asparaginase level. However, Pratt and Johnson [3] found that the fatty metamorphosis was of varying degrees of severity, with a relationship between this severity and the dosage of L-asparaginase; the hepatic abnormalities persisted up to 30 days after enzyme administration.

The fine pathology of liver damage is not known, nor is its relationship to the dose of drug administered and to the capacity of the liver to recover following withdrawal of the drug or during its continued administration.

Our object has been to follow the effects of L-asparaginase on the liver of rats treated with various dosages of the drug administered over a relatively long period. Both chemical and morphological findings are reported, with a particular emphasis on electron microscopy.

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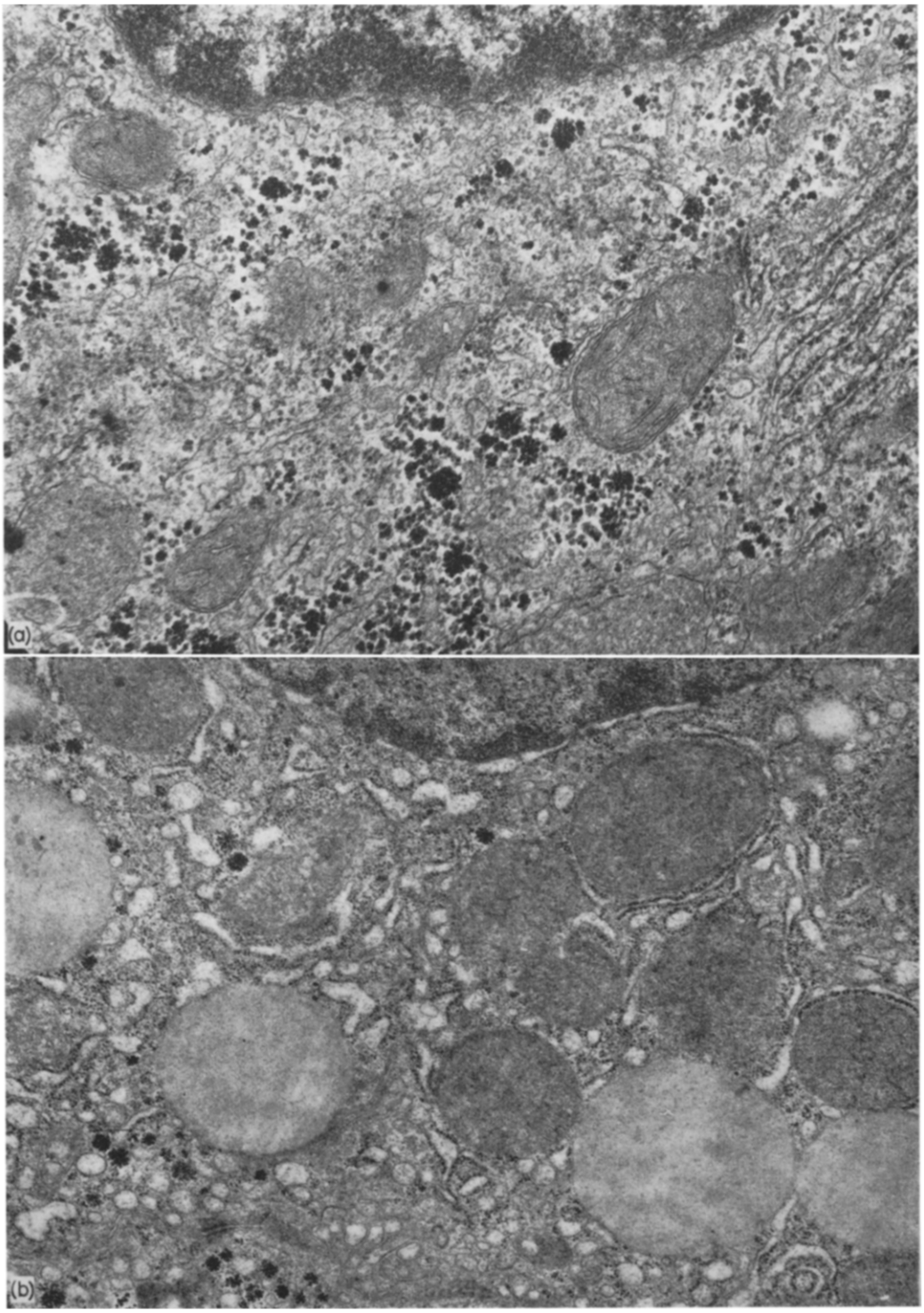

Fig. 3(*a*). *Decreased regularity of the rough endoplasmic reticulum in the liver cells of a female rat treated with* 200 *U/kg/daily of L-asparaginase for* 60 *days* (×40,000). (*b*) *Increase of the smooth endoplasmic reticulum in the liver cells of a male rat treated with* 200 *U/kg/daily of L-asparaginase for* 60 *days* (×27,000).

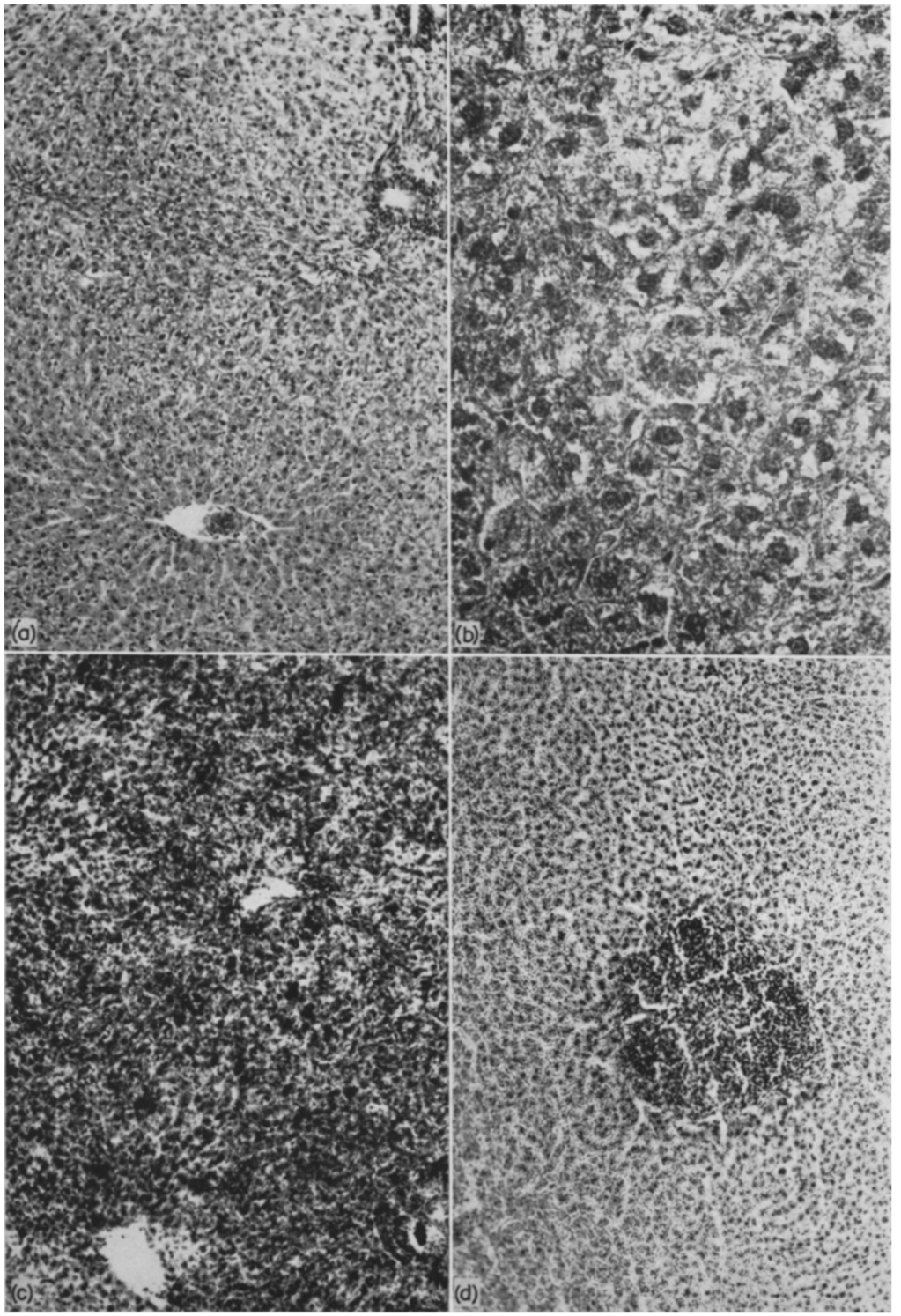

Fig. 2. (*a*) and (*b*). *Severe parenchymal necrosis of the liver in a male rat treated with* 3,200 *U/kg of L-asparaginase for* 15 *days* (*H and E*, × 100, × 400). (*c*) *Heavy increase of the lipid content in the liver of a rat treated with* 3,200 *U/kg of L-asparaginase for* 15 *days* (*Sudan*, × 100). (*d*) *Cellular infiltration in the liver of a rat treated with* 3,200 *U/kg of L-asparaginase for* 30 *days* (*H and E*, × 100).

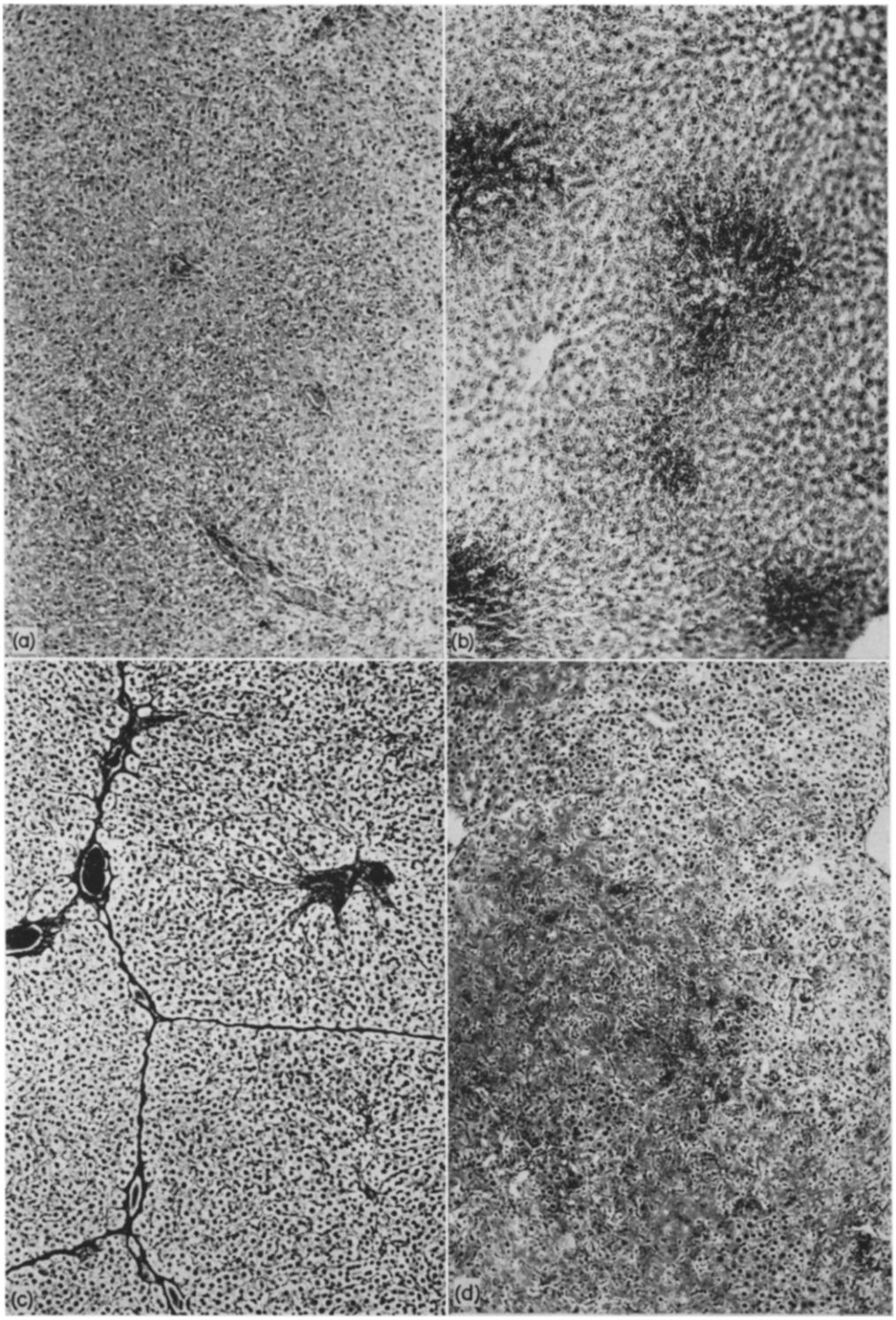

Fig. 1. (*a*) *Cytolysis and* (*b*) *clear increase of the lipid content in the liver of rats treated with* 200 *U/kg of L-asparaginase for* 45 *and* 60 *days respectively.* (*c*) *Alterations and* (*d*) *fragmentation of reticulum in rats treated with* 800 *U/kg of L-asparaginase for* 90 *days and with* 3,200 *U/kg for* 15 *days* (*Mallory, Sudan, H and E,* × 100).

(to face p. 56)

MATERIAL AND METHODS

Thirty-six rats, Wistar strain, were injected intraperitoneally with L-asparaginase, daily five times a week for various periods of time up to a maximum of 90 days. The L-asparaginase (Crasnitin) used was kindly supplied by Bayer. The animals were divided into three groups, each comprising 6 male and 6 female rats. The first group received daily 200 IU/kg b.w. of L-asparaginase, dissolved in 0·9% NaCl solution, the second 800 IU/kg b.w., and the third 3200 IU/kg b.w., again daily.

A fourth group (also 6 male and 6 female rats) was kept as control, receiving only the saline solution daily. Two animals (a male and a female) from each group were sacrificed every 15 days soon after withdrawal of blood. Activities of serum alkaline phosphatases and of SGOT and SGPT as well as bilirubin level were determined on the blood by the usual methods.

The laboratory's normal range for adult animals was from 16 to 36 U Bodansky for alk. phosphatases, up to 200 for SGOT, up to 52 for SGPT and up to 0·3 mg % for bilirubin. For the light microscope study, sections of the liver were made and its histology was studied by hematoxylin eosin staining and by lipid (Sudan, III) and reticulum (Gomory's method) staining. The presence of portal cellular infiltration, scattered cytolysis and massive necrosis was recorded; no attempt was made to perform a precise scoring of the lipid content but its increase or the presence of a true steatosis were noted. The electron microscopy study was limited to the group of animals receiving 200 IU/kg and to the pairs sacrificed every 30th day. A liver fragment was fixed for 3 hours in glutaraldehyde (2·5% in phosphate buffer 0·1%) and thereafter, following a 12 hr interval, postfixed in osmium tetraoxide (1% in phosphate buffer) for an hour. Dehydration, embedding and section were performed according to the usual techniques and staining with lead citrate by the method of Reynold. Observation and photography were made using an Elmiskop I (Siemens) electron microscope.

RESULTS

(*a*) General effects: the normal weight increase curve of animals was not affected by the treatment with L-asparaginase. No adverse effects were observed as far as the appetite and the general behaviour of the animals were concerned.

(*b*) The level of serum bilirubin, the activities of alkaline phosphatases and of SGOT and SGPT showed no significant differences between the control and the test animals throughout the whole period of treatment in all groups. Only the male rat of the highest dosage group, sacrificed 15 days after the beginning of the treatment showed a sharp increase of transaminase activity (SGOT 400 U., SGPT 500 U.).

(*c*) By conventional microscopy some histological alterations were observed in 16 of 36 treated rats. The more significant and consistent alterations were observed mainly at the first sampling 15 days after the beginning of the treatment, at every dose level of L-asparaginase.

Cytolysis, cellular infiltration, reticulum alterations and a clear increase of lipid content were prominent; a gross correlation between the histological alterations and the dose level administered was observed (Figs. 1 and 2).

The most severe parenchymal necrosis with reticulum fragmentation was found in the male rat receiving 3200 IU of L-asparaginase, showing the above reported increase of transaminases.

At the later times the histological alterations appeared to be less marked, with no relationship to the dose administered. The most noteworthy finding was a frequent increase of the lipid cellular content but some animals, irrespective of dosage, showed a mild hepatic cytolysis. Two control animals exhibited a mild cellular infiltration and a moderate lipid increase at the 60th day.

(*d*) No abnormal electron microscopy findings were present in the parenchymal cells of the liver of animals killed 30 days after the beginning of the treatment: however, in these animals, neither were histological alterations observed. The first significant changes were observed in the animals sacrificed after 60 days (Fig. 3). In the female rat droplets of lipids and glycogen blocks appeared to be increased, the rough reticulum was less regular, and the smooth reticulum increased. The ribosomes in the cytoplasm were less clearly defined and the reticular membranes dilated so that in some cases the cytoplasm assumed a vesicular aspect.

The cell contact decreased so that the cells separated from each other and an intercellular space appeared. Bile canaliculi seemed to be dilated and microvilli tended to be sparse and fragmented, sometimes missing. In the male rat the findings were similar although less sharp. Ninety days after the L-asparaginase treatment commenced the pattern was basically identical but for a further increase of the smooth reticulum and of the glycogen clumping.

DISCUSSION

The rats seem to tolerate quite well the chronic administration of L-asparaginase, at least for the observed period of time at the given dose level. In fact the general behaviour and aspect and the biochemical data remained unaltered. These findings are in accordance with that found by others [5]. Only when there was a massive hepatic necrosis did the transaminase level increase.

The hepatic morphological pattern seems to be more significant. Heavy liver steatosis and a slighter cell lysis, which sometimes is massive, are frequently present, fine cellular modifications may be seen by electron microscopy even in the absence of gross histological modifications. It should be emphasized that at first the degree of liver modifications is grossly proportional to the dosage administered but, as the drug administration is continued, the relationship seems to vanish and, on the whole, the modifications of the liver tissue become less and less evident. It seems that either the liver cells adapt themselves to the continuous deprivation of L-asparagine or the organism somehow manages to acquire resistance to the drug. This process could be achieved by an increase of intracellular asparagine synthetase and/or by development of immunological neutralizing factors. The electron microscopy findings, which show a late development of non-specific modifications, could be interpreted as the morphological expression of an adaptation process, at least as far as the increase of the smooth reticulum is concerned.

In the whole the results here presented seem to point to a gross dose-damage interrelationship, at least in the first phase of treatment as far as the histological pattern is concerned and to prove the capacity of the liver cells to recover from and to adapt to the L-asparaginase injury, despite the continued treatment with the drug.

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Studies on the Radiobiology of a Rat Sarcoma Treated *in situ* and Assayed *in vitro*

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Abstract—*Some of the characteristics of a rat sarcoma, induced by subcutaneous implantation of a plastic film, have been described. Cells from this tumour can be plated routinely into dishes with a plating efficiency of about 2·6% and survival can be assayed by a direct* in vitro *cloning technique. The cultured cells, when reinjected intradermally in rats, give rise to solid tumours similar in appearance and histology to the tumour of origin.*

Irradiation experiments have been performed by treating the tumour cells either in situ *or* in vitro *and by assaying the survival either* in vivo *or* in vitro. *The results indicated that the survival of tumour cells irradiated* in situ *was greater than that of cells irradiated* in vitro *independently from the assay being done either* in vivo *or* in vitro. *This difference could not be accounted for even if it was assumed the cells* in situ *were completely anoxic.*

Further experiments demonstrated that the cells in situ *are normally sensitive to the synchronizing (lethal and blocking) action of hydroxyurea and that hydroxyurea-synchronized populations exhibited an X-ray age-response similar to that of other systems in terms of the course of the survival variations during the cell cycle (but not in terms of radiation dose).*

FOLLOWING the introduction of quantitative methods for the estimation of the survival of individual cells *in vitro* by Puck *et al.* [1], and *in vivo*, by Hewitt and Wilson [2], it became a common assumption that all cells of mammalian origin, normal and malignant, in culture and *in situ*, have very similar radiosensitivities. Such an assumption is still accepted—after making allowances for extrinsic factors such as oxygen and intrinsic situations such as the position of the cell in the intermitotic cycle which are known to affect the radiation response.

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However, in 1964, only a few years after the first description of a dose-survival curve for cultured mammalian cells, a careful tabulation [3] of the survival parameters of all reported systems irradiated aerobically had already indicated a remarkable spread in D_o with values ranging from 35 to about 300 rads and in the extrapolation number with values varying from 1 to 100. These differences in radiosensitivity have been substantially confirmed in a recent review [4] concerning only *in vivo* cell systems, the spread in D_q—another parameter measuring the shoulder width [5]—being between 0 and 360 rads. Values of D_o higher than 300 rads are known for cells of solid tumours irradiated and assayed *in vivo* [6] but in these cases, the degree of oxygenation plays an important role in determining the radiation response.

We have already reported [7] D_o values for 15 primary cultures derived from biopsy specimens of benzpyrene-induced rat sarcomata ranging from 81 to 737 rads. Some preliminary work [8] indicated that cells of another rat sarcoma, induced by the subcutaneous implantation of plastic film, exhibited a normal radiosensitivity when irradiated *in vitro* but were extremely radioresistant when irradiated *in situ*, independently of the survival being assayed either *in vitro* or *in vivo*. Such a difference could not be accounted for even if it was assumed that the cells irradiated *in situ* were completely anoxic.

This rat sarcoma is of interest as it presents the possibility of being treated both *in vitro* and *in situ* and then assayed for survival either *in vitro* or *in vivo;* in this regard, it is similar to the rat rhabdomyosarcoma described in recent years by Reinhold, Barendsen, and coworkers [9, 10]. In addition it displays the anomalous radiation response mentioned above, which is of possible serious importance for the criteria of application to clinical radiotherapy of indications based on *in vitro* studies. For all these reasons, the aim of the present paper is to give a more detailed description of the radiation response of these tumour cells under various conditions of treatment and assay, and preliminary results of the application of some quantitative radiobiological techniques to the problem of their peculiar radioresistance. Unfortunately, the results of these investigations do not clarify the problem and leave the basic dilemma increased but they do have some intrinsic interest.

MATERIAL AND METHODS

The tumour

The rat sarcoma used in the experiments described in the present paper was induced by the subcutaneous insertion of a plastic film (Visking cellulose) in the back of a male F_1 hybrid rat (male Marshall and a female August cross). The sarcoma is routinely maintained by serial transplantation in F_1 hybrids.

Since this tumour was readily transplantable it was expected that cultured cells could be reinjected into host-strain animals with a large proportion of "takes" and that this property could be exploited to demonstrate that the cultured cells were indeed tumour cells.

In one series of experiments, 140 animals were injected intradermally with a suspension of cultured cells in 0·1 ml of medium and tumours developed, at the injection site, in each of the 140 animals. Tumours developed from as little as 10 cells and hence any selection of the cells injected must have been less than 1 in 10. When cells from tumours derived from cultured cells were returned into culture, the same characteristic compact colonies of oat-shaped cells were seen as in cultures from serially transplanted tumours. Sections of the original tumour were histologically indistinguishable from sections of tumours derived from cultured cells. It was concluded that Visking cells cultured *in vitro* were tumour cells and that tumours which developed after the injection of cultured cells were derived from these cells.

In some cases, when the tumour was excised from the animal, a peripheral zone 2 mm thick was stripped away from the inner fibrous kernal. The customary yield of about 10^6 cells per tumour was obtained from the peripheral zone while the cells obtained from the inner kernal were too few to count with a haemocytometer. Hence, it was concluded that when the entire tumour was trypsinized, the cell suspension obtained was derived almost entirely from the peripheral zone of the tumour which was adequately vascularised and the area of greatest proliferation as judged by the number of mitotic figures observed in histological sections.

Preparation of cell suspension from the tumour

Tumour tissue was minced with scissors into fragments about 1–2 mm^3. The fragments were suspended in 15–20 ml of 0·25% (w/v) trypsin solution in Hank's solution and transferred at 37°C into trypsinization tubes. The suspension was maintained for 30 min at this temperature and continuously stirred either by an electric shaker or by a finger pump connected to the trypsinization tube and driven by a variable speed motor (both apparatus gave the same results). After trypsinization, the contents of the tubes were left to stand for 1 min, to allow the large fragments to settle and the supernatant cell suspension was then collected. At this point a yield of 10^5–10^6 was obtained from tumours of about 1 cm diameter.

If a higher cell yield was desired, the trypsinization procedure was repeated a second time with the remaining fragments. Alternatively, the fragments were first treated with an 0·25% (w/v) collagenase solution in 199 medium (not all sources and batches of collagenase give comparable results and have to be tested individually) and then with trypsin. A secondary yield of at least half as many cells could be obtained. Our experience has indicated that

most of the cells obtainable by this method are to be found in the first two harvests which are comparable from the point of view of cell viability. The final pool of cell suspensions was centrifuged at 180 **g** for about 5 min, the supernatant trypsin solution discarded, and the cells resuspended in culture medium before plating.

Culture of cells

All cultures were maintained at 37°C in an incubator in an atmosphere of 5% CO_2 in air. The cells grew attached to the surface of glass bottles (for routine maintenance) or plastic dishes (for experimental purposes). The medium preferably used, according to preliminary experiments, was Eagle's Minimum Essential Medium (MEM). Similar results in terms of plating efficiency but not of colony compactness could be obtained with Medium 199 and HUT medium. The medium was supplemented with 15% calf serum as preliminary experiments showed this concentration gave the maximum plating efficiency. Rat serum or other sera gave less satisfactory results.

At about 10 days after plating, clones developed containing a few hundred to a few thousand cells. Clones containing 50 or more cells (a typical colony size distribution was observed) were considered to originate from a "clonogenic" tumour cell that retained the capacity for unlimited proliferation. The cells produced clones, under these conditions, with a plating efficiency of $2{\cdot}6 \pm 0{\cdot}8\%$ for unirradiated samples. This value was independent of a 20:1 variation in the number of cells plated and was not increased on the occasions when a feeder layer was employed. From the fact that a tumour developed from as few as 10 cells per animal, it is also clear that the "clonogenicity" in terms of tumour "take" is different from that in terms of plating efficiency. The value of 2·6% is somewhat low in comparison with the 35% for the rhabdomyosarcoma reported by Barendsen and Broerse [10] or the 65% for a C3H mouse fibrosarcoma reported by Malaise and Tubiana [11] but is similar or higher to that we have found for a variety of other human and rodent tumours. However, it must be kept in mind that no selection procedure *in vitro* was initially applied to the Visking sarcoma, and that the plating efficiency has been remarkably stable from 1964 to the present day.

Experimental procedures

The procedures depended on whether the cells were being treated *in vitro* or *in situ* and on whether the assay for survival was performed *in vitro* or *in vivo*.

(*a*) Irradiation *in vitro*, assay *in vitro* (*in vitro–in vitro* experiments). Cell suspensions were prepared from tumours (tumours approximately 1 cm in diameter) and plated into dishes. After incubation for 12–16 hr, in order for the cells to become firmly attached to the surface of the dishes and to enter the exponential phase of growth, the dishes were irradiated and then left for colony formation. Three to five dishes were used for each experimental point.

(*b*) Irradiation *in vitro*, assay *in vivo* (*in vitro–in vivo* experiments). Cells were irradiated in suspension and then a known number of cells in 0·1 ml of medium was injected intradermally in the center of the back into host animals (5 animals per experimental group). The number of viable cells in the innocula was estimated by means of a calibration curve from the time at which the resulting tumours first became palpable through the shaven skin, the animals being examined daily by the same operator. A calibration curve was previously determined by injecting varying numbers of cultured cells into groups of recipient animals and recording the times the tumours became palpable. At this stage, the tumours are of the order of 1 mm diameter or less; experience has shown that this is a more accurate method than recording the time to reach a measurable diameter as the variation of the growth rate of tumours between animals increases with time. The curve is shown in Fig. 1 (upper line, closed squares). When a "feeder layer" of approximately 10^5 cells (i.e. tumour cells which have received 10 krad of X-radiation) was included in each injection, the palpability time was decreased as shown in the lower curve (open squares) of Fig. 1. The influence of an addendum of heavily irradiated cells was initially described by Revesz [12].

(*c*) Treatment *in situ*, assay *in vitro* (*in vivo–in vitro* experiments). Tumours derived from cultured cells were irradiated *in situ* when approximately 1 cm in diameter in nembutalized animals that were shielded with 3 mm of lead, except for the dorsal, protruding tumour and breathing air; the arrangement of the shielding was such that it imposed no extra restriction on the blood supply to the tumour. The tumours were excised asceptically immediately after treatment and cell suspensions prepared as described above. The cells were plated into sets of 4–5 dishes per experimental point and incubated for colony formation.

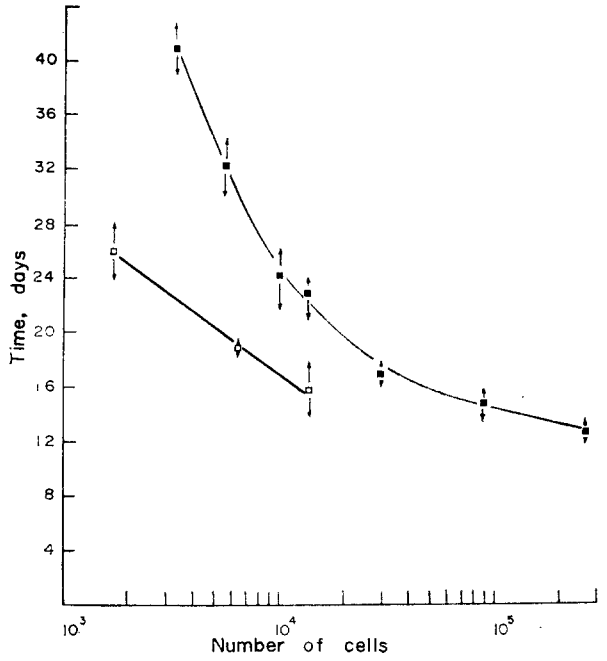

Fig. 1. *Calibration curve for use in* in vitro–in vivo *experiments. Time in days for a tumour first to be palpable plotted against number of tumour cells injected intradermally. Solid squares are when only viable cells injected, open squares when* 10^5 *heavily irradiated cells are added to the viable innoculum.*

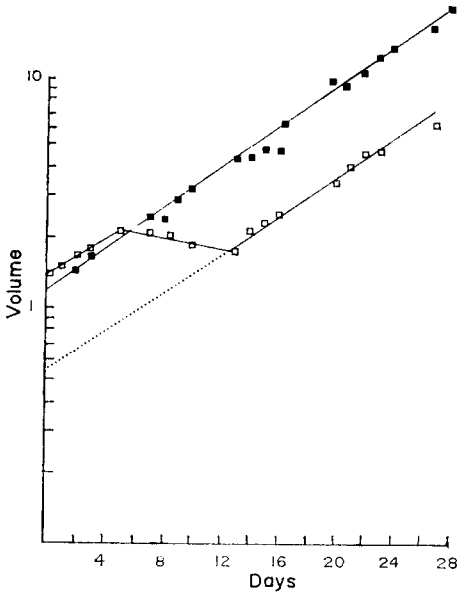

Fig. 2. *Volume of the Viskin sarcoma growing* in situ. *Volume calculated as the cube of the mean of two diameters measured with calipers. Closed squares are the mean volumes for a group of five control rats; open squares for a group given* 1000 *rad on day five.*

(*d*) Treatment *in situ*, assay *in vivo* (*in vivo–in vivo* experiments). An estimate of the cellular surviving fraction of tumours irradiated and left *in situ* was made from tumour growth curves similar to those shown in Fig. 2 (closed squares: unirradiated control; open squares: tumours given on day 5 a single dose of 1000 rads). Tumour volumes were estimated by measuring with Vernier calipers two diameters of the tumour through the shaven skin and are quoted as the cube of the mean of two diameters (the same operator made all the measurements). Over the size range used in constructing Fig. 2 these tumours grow very unifomly and have a very regular, near spherical shape. When the tumours become large, they tend to be irregular as they also do some time after a heavy dose of irradiation if this has caused tissue breakdown. For all points of the growth curves, volumes of at least five tumours were averaged; error bars are not indicated to avoid confusion (the standard error for each point is of the order of 10% of the value of the point). After irradiation tumours showed a period of reduced growth, dependent on the dose, and then resumed a growth curve parallel to the pre-irradiation curve. If this curve is extrapolated back to day zero the ratio of this volume to the initial volume is an estimate of the surviving fraction of the cells making the assumption that the volume of a tumour was a direct function of the number of dividing cells present. No allowances were made for the effect of division delay following irradiation since this would increase the estimate of the surviving fraction and thus favour (see the Results) the argument presented in the paper. It should be pointed out that, actually, the growth curve of the unirradiated tumour is only a simple exponential curve up to limited volume. The complete growth curve for this tumour can reasonably be described by a Gompertz function [13]. The typical experiments were started on day 15–17 after the injection, when the diameter of the tumours was of about 1 cm or slightly less and the growth curve can be described by a single exponential function for the next 30–35 days. Over this period the doubling time of the non-irradiated tumours was 7·5 days; this is somewhat long for a rodent tumour but may be due partially to the cells being injected intradermally.

Irradiation conditions

The irradiations were performed at room temperature. The X-ray machine was operated at 140 kV, 5mA, with no extra filtration. In the case of the tumours irradiated *in situ* the dose was measured with an ionisation chamber at the centre of the tumour using a wax phantom. For the *in vitro* radiation the dose was determined by the ferrous sulphate method duplicating the actual conditions when irradiating cells.

RESULTS

In vitro–in vivo experiments

The radiosensitivity of cells in culture is illustrated by the dose-survival curve shown in Fig. 3 which has a typical sigmoid shape. The D_o value is 155 rads, and the D_q value is 99 rads. This curve refers to cells freshly prepared from tumours. The dose-survival curves for cells "established" in culture some weeks prior to irradiation indicated some spread in radiosensitivity. The maximum and minimum values observed in different experiments for D_o were 165 and 136 rad and for D_q 114 and 67 rad. These differences in radiosensitivity may reflect a natural variability similar to the one described for primary cultures derived from the benzpyrene-induced rat sarcomata [7].

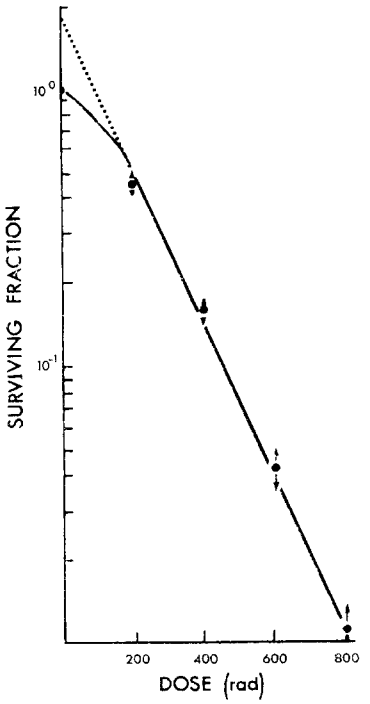

Fig. 3. *Dose survival curve for cultured cells irradiated and assayed* in vitro.

In vitro–in vivo experiments

Figure 4 shows the dose-survival curve for cells irradiated *in vitro* and assayed *in vivo*. The errors are fairly large but a D_o value of 180 rad and a D_q value of 165 rad can be assigned to the curve. If the irradiated cell population is injected together with a "feeder layer" of 10^5 inactivated cells, the D_o remains practically the same (171 rad) but the D_q decreases markedly approaching a no-shoulder situation, in agreement with the "Revesz effect" [12]

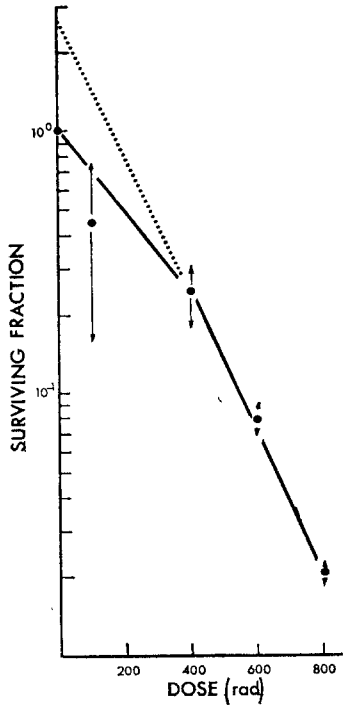

Fig. 4. *Dose survival curve for cells irradiated* in vitro *and assayed* in vivo *using the calibration curve of Fig.* 1. *The errors are much larger but the curve is very similar to that in Fig.* 3.

In vivo–in vitro experiments

The dose-survival curve for tumour cells irradiated *in situ* and assayed for viability by the plating technique is shown in Fig. 5. The exact shape of the curve can not be determined without going to lower survival values but Fig. 5 clearly shows the extreme radioresistance of

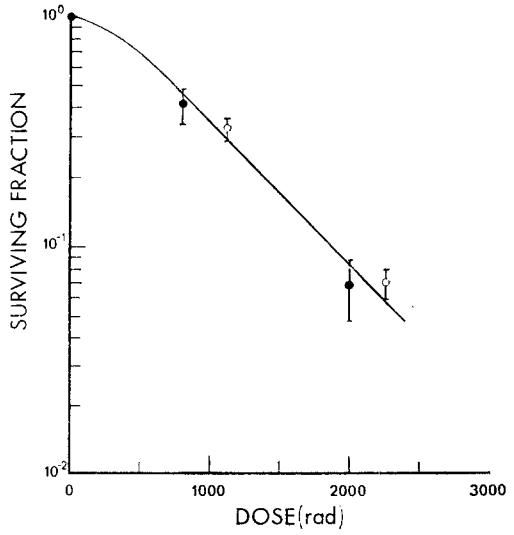

Fig. 5. *Dose survival curve for tumour cells irradiated* in situ *then put into culture and assayed by the plating technique. The* D_o *is* 709 *rad and the* D_q 302 *rad. Open and closed circles refer to experiments made at different times.*

the population under these conditions which will be particularly significant in comparison with the sort of dose used in conventional, fractionated radiotherapy. Assuming that there is not a protracted shoulder over several kilorads the survival parameters are a D_0 of 709 rad and a D_q of 302 rad. In one experiment, four tumours were given a dose 800 rad *in situ* (this dose corresponding to the first point on the survival curve) and then left intact for 24 hr before excision and assay for viability. The numbers of colonies in these four cases was as high as that in the controls. It is unlikely that this could be explained by repopulation of the tumour in the short time interval; it does suggest that an important recovery mechanism may operate when cells irradiated *in situ* are left undisturbed. This peculiarly high radioresistance of these tumours has remained practically unchanged for a span of over 7 years.

In vivo–in vivo experiments

An estimate of the surviving fraction of cells irradiated and left permanently *in situ* was made from tumour growth curves (Fig. 2) and is shown in the dose-survival curve of Fig. 6. The results are admittedly very approximate because of the method of estimation used. However, they indicate clearly that the D_0 is of the order of 1000 rad; the D_q would seem to be close to 0 rad but this may depend on the fact that the shoulder is not unmistakably delineated by points at higher doses; higher doses were not employed as these cause irregular growth and breakdown of the skin, making measurements of volume unreliable. These tumours may appear even more radioresistant than those described in the previous section but this may be a factor of the recovery phenomenon of cells left *in situ* and of the assumptions and errors in the assay.

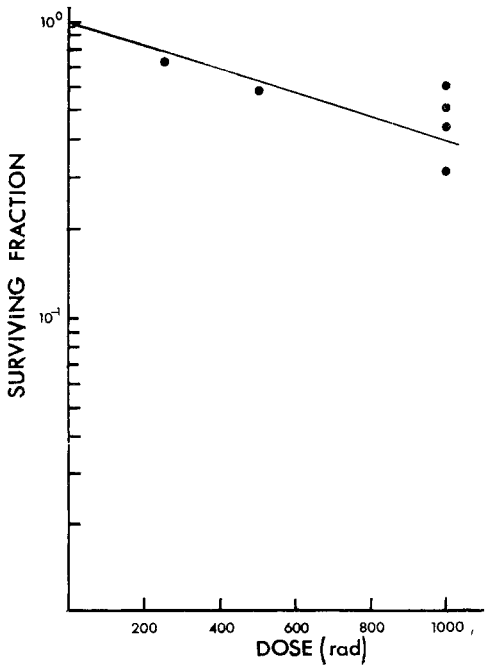

Fig. 6. Dose survival curve for cells irradiated and assayed in situ *by the method indicated in Fig. 2. The errors are large and there are assumptions in the calculation but the parameters of the curve are of the same order as in Fig. 5.*

Considerations apropos of the "oxygen effect"

Some of the difference between the *in vivo* and *in vitro* response to radiation could be due to differences in oxygenation. Cells in culture are well oxygenated; histological sections of the tumour show that the peripheral zone, which is the region of proliferating cells and the origin of the cells that are cultured in the *in vitro* assays, is well vascularized although it could still contain a proportion of cells that are anoxic in the radiobiological sense. In order to compare the survival of cells irradiated *in situ* with that of cells irradiated *in vitro* (for instance both determined in each case *in vitro*) the *in vivo–in vitro* survival curve and the *in vitro–in vitro* survival curve are replotted in Fig. 7 as curve D and curve A, respectively. No direct experiments on hypoxic cells in animals or in culture were undertaken but the other lines in Fig. 7

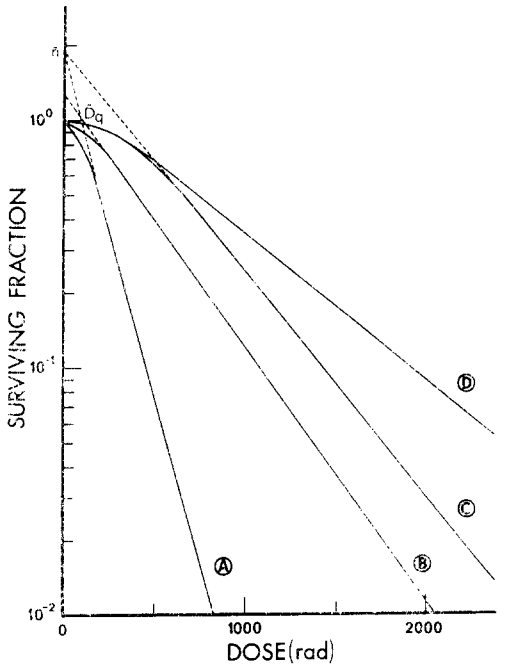

Fig. 7. Comparison of the sensitivity of cells irradiated in situ *and* in vitro. *Curve* A *is the* in vitro–in vitro *survival curve from Fig. 3. Curve* D *is the* in vivo–in vitro *survival from Fig. 5. Curve* B *is curve* A *replotted assuming that all the cells were anoxic and that there was an oxygen enhancement ratio of three and a constant value for* D:, *curve* C *is a similar re-plot but assuming a constant value for the extrapolation number. Thus it is not possible to explain the high resistance of cells irradiated* in situ *by a high content of anoxic cells.*

represent hypothetical curves for cells irradiated and assayed *in vitro* under anoxic conditions allowing either for a constant extrapolation number (curve C) or a constant value of D_q (curve B), based on an oxygen enhancement radio of 3, usually taken as the maximum for X-radiation. The 2000 rad point of curve D (one of the values used in the *in vivo–in vitro* experiment) lies well above the extreme case for anoxic cells *in vitro*. The point could be higher if the recovery phenomenon *in situ* or the *in vivo–in vivo* survival curve are considered. It must be concluded therefore that the survival of cells irradiated in a tumour *in situ* is greater than could be accounted for in terms of the survival of cells irradiated in culture if anoxia of the tumour cells was the only factor involved.

Effects of hydroxyurea on cells treated in vivo and assayed in vitro

Hydroxyurea is an inhibitor of DNA synthesis which kills cells in the S (DNA synthetic) phase [14] and blocks or at least slows down [15] the progression of cells from G_1 (pre-DNA synthetic phase) into S, and has been successfully employed as a synchronizing agent not only *in vitro* but also *in vivo* [16], [17]. It was thought of some interest to investigate the lethal and synchronizing effects of hydroxyurea on the Visking sarcoma cells in order to find out if the cells showed an anomalous resistant response to an agent other than radiation and to examine the age-response (fluctuations in survival throughout the intermitotic cycle) of these cells to X-rays and its possible relation to their peculiar radioresistance.

The doses of hydroxyurea needed to induce a synchronizing effect on the various tissues of both rats and mice show little variation, from 0·05 to 1·5 mg/g of body weight (in some cases the drug being administered in more than one fraction), and the actual concentrations of hydroxyurea in the blood are of the same order as those employed for obtaining the same effect on cultured cells [16].

The results of an experiment on the survival of tumour cells to hydroxyurea are shown in Fig. 8 (dotted line, open squares). The procedure is that of the *in vivo–in vitro* experiments, the animals being sacrificed at the times indicated. The drug, at a concentration of 0·5 mg/g of body weight was administered by intravenous injection in the tail vein at time zero. In some cases the dose was split into two equal fractions, given at time 0 and 1½ hr later. If the fractions were delivered as a single

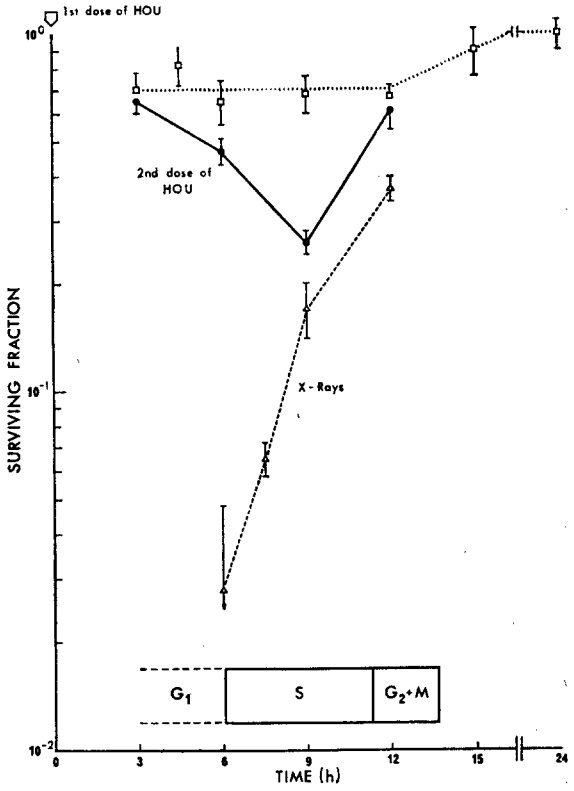

Fig. 8. Tumour cells synchronized in situ *by two i.v. doses of hydroxyurea. The open squares show the survival of the cells, as determined by the plating technique, from animals killed at various times after the second dose of hydroxyurea. The closed circles are for animals given a single dose of hydroxyurea at these times after the synchronizing treatment and then killed 3 hr later; a peak of drug effect can be seen in the S phase. The open triangles are from a similar experiment but instead of giving a dose of hydroxyurea the tumours were given a single dose of* 1500 *rad of X-rays, and the tumours then immediately removed for* in vitro *assay. The results indicate that the response to X-ray is decreasing from the time of the* G_1–S *boundary but whether or not this represents the maximum cannot be stated.*

dose, the results would not appreciably change in terms of survival, that is, in terms of killing of S phase cells, but they would in terms of induction of synchrony (inferred as response to a following agent).

The survival of the cells was reduced to about 70% of the control by 3 hr after the initial administration of the drug, and remained at this level until about 12 hr. If the survival assay is postponed for longer intervals of time, the cell survival reaches again a level of about 100%, presumably through division of the surviving, relatively synchronized cells [16]. The "clonogenic" Visking sarcoma cells appear to have a sensitivity to hydroxyurea similar to that of the other cell systems that have been studied. The results of another experiment are also shown in Fig. 8 (continuous line, closed circles). The cells surviving the first dose (in two fractions) of hydroxyurea were exposed to a

second, single dose (1·0 mg/g) at the times shown. The animals were sacrificed for the survival assay 3 hr after the second dose (that is, 3 hr after the times indicated by the points). A decrease in survival can be observed, with a trough at 9 hr, following by an increase in survival. These variations depict the parasynchronous progression of the cell population throughout the cycle after the disappearance of the hydroxyurea block. The sensitive region between 6 and 11 hr should correspond to the S phase, the length of which could be inferred as being about 5 hr (lower box in Fig. 8). The duration of the G_2+M period may also be inferred, assuming the final increase in survival to the first dose is indeed due to cell division and is of the order of 3 hr. Thus, whilst acknowledging that these estimations may be approximate, the length of the phases of the cycle of these cells appear to be similar to that usually observed for other systems. The duration of G_1—which may be of importance—cannot be inferred with this procedure but should be not unusually long considering that a synchronizing effect is induced by the treatment.

Age-response to X-rays after hydroxyurea synchronization

A similar experiment to investigate the age-response of the Visking sarcoma cells to X-rays is also included in Fig. 8 (open triangles, broken line). Tumours were irradiated (1500 rad) at various times after the initial (two fractions) dose of hydroxyurea and the animals were sacrificed for the *in vitro* survival assay immediately after the irradiation. The data shows a continuous increase in survival from 6 to 12 hr after administration of the drug. The variation could correspond to the increase in radioresistance during the S phase typical of the majority of mammalian cell systems *in vitro* [18] and *in vivo* [16]. In this respect, the results with X-rays are in agreement with those relative to the second treatment with hydroxyurea (closed circles, continuous line) indicating the middle of S phase at about 9 hr.

Finally, in Fig. 9 is shown a three-point survival curve for the time interval of 10 hr after the hydroxyurea treatment. The survival parameters for this relatively synchronized population are a D_o of 669 rad, and a D_q of 555 rad; therefore, this population (probably in late-S) has a radioresistance of the same order, possibly slightly greater, as the random population presented for the *in vivo–in vitro* experiments in a previous section. In conclusion, the radioresistance of these cells appears to be anomalously high, independently from the synchronous or asynchronous state of the experimental population and in spite of the fact that the modulation of the age-response seems to be of the usual type.

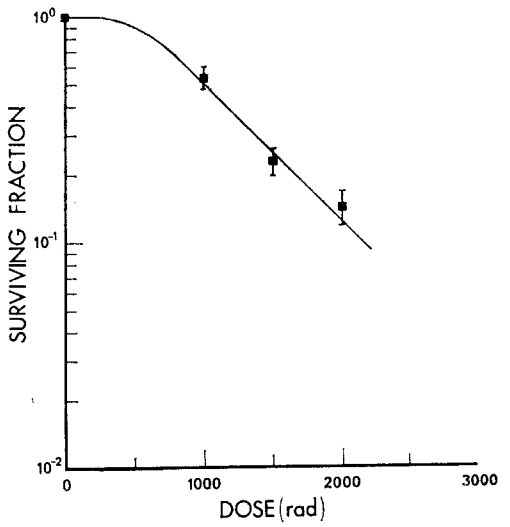

Fig. 9. *Tumour cells synchronized with hydroxyurea* in situ. *Ten hours after the drug treatment the tumours were irradiated (when presumably in the late S phase) and then removed for* in vitro *assay. The* D_0 *is* 669 *rad and so this para-synchroside population has the same sensitivity as the cells shown in Fig.* 5.

DISCUSSION

The experiments reported here indicate that there is a lack of correlation between the *in situ* and the *in vitro* estimates of the radiosensitivity of the Visking sarcoma cells. The different results obtained when cells are irradiated either *in situ* or *in vitro*, and assayed for survival either *in vivo* or *in vitro*, are summarized in Table 1. The response of the Visking rat sarcoma to single doses of X-radiation *in situ* could not therefore be accounted for in terms of the survival of the tumour cells irradiated *in vitro* at least as far as "clonogenic" (in the sense of colony-forming ability in dishes) sarcoma cells are concerned.

Errors or confidence limits have not been included in this table as the different assays involve different assumptions and approximations and any statistical test of significance might give a false sense of reality. For example, for the cells treated *in situ* and then put into suspension the plating efficiency is between 2 and 3% and so a selection principal may be included; in this respect we can only say that the plating efficiency is repeatable and that the survivors when reinjected into an animal, produce tumours which are apparently identical. It could well be that cells only "mildly" affected by irradiation *in situ* do not plate out.

Table 1. *Survival parameters of Visking sarcoma cells exposed to X-radiation according to the various experimental procedures reported*

Type of experiment		D_o (rad)	D_q (rad)
In vitro–in vitro	(fresh explant)	155	99
	("established" culture)	165–136	144–67
In vitro–in vivo	(without "feeder layer")	180	165
	(with "feeder layer")	171	~0
In vivo–in vitro	(random population)	709	302
	(cells around late-S)	669	555
In vivo–in vivo		~1000	?

However, such arguments do not invalidate any conclusion on the difficulties of interpreting data on tumours irradiated in the animal from data obtained with cultured cells.

The difference in responses and the anomalously high radioresistance of cells treated *in situ* cannot be explained even if:

(i) it is assumed that the cells *in situ* are completely anoxic;

(ii) it is assumed that the overall radioresistance is caused by an unusually modulated age-response.

Furthermore, cells treated *in situ* have been demonstrated to be: (i) normally sensitive to cytotoxic agents such as hydroxyurea (and, in unpublished experiments, ICRF 159, vinblastine and vincristine); (ii) normally responsive (in terms of shape but not of dose) to the cycle dependent action of X-rays; (iii) normally characterized as far as the cell cycle parameters and the growth kinetics, inferred in the present work, are concerned.

From the present results it is not possible to explain the anomalies observed, and unsupported speculations—such as the possibility that in the *in situ* situation greater or additional repair processes are going on and that we are still on the shoulder region of the survival curve—would be difficult to justify from the present data. However, considering the interest of such an *in vivo–in vitro* system and the possible importance for experimental radiotherapy, further work on the system is going on. In the rat the tumour has a volume doubling time of 7·5 days, the generation time of the cells in culture is 26 hr; kinetic experiments are under way in the attempt to identify the various subpopulations of the tumour *in situ* and to elucidate their characteristics as the reasons for these anomalies could well lie in this direction.

In conclusion, it must be emphasized that although the lack of correlation between *in situ* and *in vitro* estimates of radiosensitivity cannot be satisfactorily explained it implies that the X-ray response of a tumour *in situ* cannot be necessarily predicted by determinations *in vitro* of the radiosensitivity parameters of cells derived from biopsies of the tumour and that laboratory screenings based on this approach should be employed with extreme care.

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Recent Experience with Bacteremia in Patients Presenting Cancer

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Abstract—*In a hospital taking care of patients with cancer, 115 consecutive cases of bacteremia have been studied to define their characteristics and to correlate them to the prognosis.*

Infections caused by gram positive organisms were less lethal than those due to gram-negative bacilli. This was presumably due to the worse clinical condition of the patients presenting bacteremia caused by gram-negative rods and to the relative resistance of these organisms to the antibiotics used for initial therapy.

This report emphasizes the need for further investigation of empiric antimicrobial regimens to be used in patients presenting a serious underlying illness, such as cancer, and who are suspected of presenting a serious infection.

BACTERIAL infection is a major cause of morbidity and mortality in patients with cancer [1], but the clinical setting of bacteremia and the microorganisms responsible for it change as new medical techniques and new antibiotics come into use [2].

Previous studies of patients with gram-negative infections have shown the overwhelming importance, for the outcome, of the underlying disease of the patient [3, 4]. The review of a series of patients presenting similar underlying diseases and treated within a relatively short period of time within a single hospital provides a unique opportunity to outline the characteristics of severe infections and to study the role of various factors on the final outcome. Therefore we have reviewed our recent experience with bacteremia in a hospital devoted to modern chemotherapeutic and surgical therapy of neoplastic diseases.

METHODS

A retrospective analysis was made of the hospital charts of all patients with one or more blood cultures containing a potential pathogen, who were seen at the Institut Bordet between January 1970 and June 1972. Nearly all these patients were seen by the authors. Post mortem cultures were excluded from the analysis. Hospital records were examined for clinical, etiological and ecological parameters. In 14 instances (2 *E.coli*, 8 *Staph.albus*, 4 diphtheroids), the clinical courses were thought to be inconsistent with an infectious disease and these cases were rejected. This hospital specializes in therapy of cancer and allied diseases. It has no pediatric or obstetric patients, but a neurosurgical department and private wards do exist in the same building as the oncological section, which has about 120 beds.

Initial bacterial isolation was made in a bottle containing 90 cm^3 of brain-heart infusion (Difco) and 10 cm^3 of the patient's blood and in a paired bottle containing 90 cm^3 of fluid thioglycollate (Difco) and 10 cm^3 of blood. Characterization of bacteria was accomplished by standard routine methods; in particular for Enterobacteriacae the scheme of Ewing and Edwards was used [5]. The sensitivity of the isolates to commonly used antibiotics has been tested by the inocula-replicating method, as described by Steers *et al.* [6].

We evaluated 115 instances of bacteremia occurring in 109 patients, but on different admissions in all cases. Only deaths attributable to bacteremia and the pathophysiological

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alterations caused by it were included in the calculation of the case fatality ratio. When a patient was judged to have recovered from bacteremia and its manifestations, but died later during the same stay in the hospital, the death was not included in the case fatality ratio.

RESULTS

During the period of the study there were 16 episodes of bacteremia per 1,000 admissions to the oncological section of the hospital; 37 (32%) were caused by gram-positive micro-organisms and 74 (64%) by gram-negative bacilli (10·6 episodes per 1,000 admissions). In addition, we observed 4 instances of fungemia, all caused by *C. albicans*. As already mentioned, 14 instances of positive blood cultures without any clinical counterpart were observed: 2 (2·6% of all episodes caused by gram-negative rods) were associated with *E. coli* and 12 (24·4% of the episodes associated with gram-positive organisms) were caused by *Staph. albus* or diphtheroids. As pointed out by Myerowitz and co-workers the presence of gram-negative rods in a culture of the blood is almost always significant [7].

A total of 946 patients (13·6% of the patients admitted) had blood cultures performed during the 30-month period of the study, of which 12% were positive and considered as reflecting clinical illness. The mean number of paired aerobic and anaerobic blood cultures was 3·5 per patient.

The various micro-organisms responsible for infection in this series are indicated in Table 1. The mortality rate in gram-positive infections was 30% and that in gram-negative infections was 53% ($p < 0{\cdot}02$). Therefore, in the rest of this study, gram-positive and gram-negative infections will be considered separately with respect to the clinical outcome. Among the patients who died, the mean time of survival after the bacteriological diagnosis was 9·2 days for patients with gram-positive infections, 8·9 days and 4·0 days for patients with gram-negative bacillemia and fungemia respectively.

As in other series (3, 7) *E. coli* was the most frequently isolated single species, followed by Klebsiella–Enterobacter organisms and *Ps. aeruginosa*. However, if pooled together, the septicemias caused by the latter organisms outnumbered those due to *E. coli*; this is in accordance with observations made by Dupont and Spink who showed in their review from 1958–1966 that the incidence of bacteremia due to this group rose steadily over the years, whereas the incidence of bacillemia caused by *E. coli* remained constant [8].

Table 1. *Bacterial etiology of bacteremia and relationship between the type of micro-organism and the outcome*

Micro-organisms	Total		Died	
	No.	%	No.	%
Gram-positive	37	32	11	30§
Streptococci (α-hemolytic)	2	1·7	1	50
Streptococci (β-hemolytic)	5	4·3	1	20
Enterococci	6	5·2	1	16
Pneumococci	5	4·3	2	40
Staphylococci (coagulase+)	15	13·0	5	33
Staphylococci (coagulase−)	2	1·7	1	50
Listeria monocytogenes	1	0·8	0	0
Clostridium welchii	1	0·8	0	0
Gram-negative	74	64	39	53§
Escherichia coli	22	19·1	11	50
Citrobacter freundii	3	2·6	1	33
Klebsiella pneumoniae	13	11·3	7	54
Enterobacter cloacae	5	4·3	2	40
Proteus group*	7	6·0	5	71
Providence	2	1·7	2	100
Pseudomonas aeruginosa	11	9·5	7	63
Achromobacter	1	0·8	0	0
Salmonella†	5	4·3	2	40
Bacteroides	2	1·7	0	0
Multiple gram negative rods‡	3	2·6	2	66
Fungi	4	4	3	75

*Proteus group: 5 *P. mirabilis*; 2 *P. vulgaris*.
†*Salmonella*: 3 *S. typhimurium*; 1 *S. derby*.
‡*E. coli* + *P. mirabilis*; *Ps. aeruginosa* + *Kl. pneumoniae*, *E. coli* + *Kl. Pneumoniae*.
§$p < 0{\cdot}02$.

The mortality rate was similar in all groups of gram-negative bacilli for which adequate numbers of cases were available but was greater than that found in sepsis caused by gram-positive organisms, especially *Staph. aureus* (33%).

We have considered the source of infection as demonstrated when the same pathogen could be recovered from a clinically diseased site at the same time as from the blood culture. By this definition, no source for the bacteremia could be found in more than 50% of the patients in this series, most of whom were patients with leukemia or lymphoma in whom skin infections, gingivitis or pharyngitis was frequently present along with the bacteremia but without demonstrable pathogens elsewhere than in the bloodstream.

Gram-positive sepsis originated most often from respiratory or wound infections; in gram-negative infections, the surgical wounds, respiratory tract and urinary tract infections were equally important. In the entire series, the urinary tract accounted for 21% of the bacteremia and the respiratory tract for 45%. This can probably be explained by the high frequency in this series of upper and lower respiratory-tract neoplasms, of which the role in predisposing to serious pulmonary infection, especially gram-negative pneumonia, has been previously shown [9].

The source of infection did not influence significantly the mortality rate in this series. Other factors, such as age and sex of the patients, appeared also without a clear influence on the mortality rate.

Eleven (30%) of the 37 gram-positive bacteremias occurred during the first 7 days of the stay in the hospital and this happened for 18 (24%) of 74 gram-negative infections. These episodes could have been caused by microorganisms acquired outside the hospital. However, the separation between hospital-acquired and non-hospital-acquired pathogens is difficult here, since many patients had been followed in the clinic for several months and had been already hospitalized in the recent past. Most of the patients admitted for the first time had been hospitalized elsewhere prior to this admission. This might explain why no significant difference of the survival could be found between infections caused by "hospital" and "non-hospital" strains.

The role of the underlying illness for the outcome of gram-negative infections has been stressed in the past [3, 4]. Following the criteria described by McCabe and Jackson [3] only 13% of the patients in this series had "non-fatal" underlying diseases, i.e. a localized neoplasm well within the curative possibilities of radiation or surgical therapy. The mortality rate in this group was 21%. "Ultimately fatal" illnesses such as disseminated carcinoma, chronic leukemias and lymphoma had a similar mortality rate (about 50%) when gram-negative rods caused the infection; gram-positive sepsis caused the death of 55% of the patients with generalized solid tumours and its mortality in patients having lymphoma could hardly be evaluated, since only 4 patients were in this group, 3 of whom survived. These results are summarized in Table 2.

The mortality rate, however, was quite different among patients with acute leukemia depending on the type of bacterial sepsis: gram-positive infection was lethal in 1 out of

Table 2. Relationship between the underlying illness and the mortality rate

	Type of infection					
	Gram-positive		Gram-negative		Fungi	
Underlying illness	Total	Died	Total	Died	Total	Died
Acute leukemia	10	1 (10%)	11	9 (81%)	4	3 (75%)
Chronic leukemia, lymphoma	4	1 (25%)	17	8 (47%)	0	—
Disseminated tumour	17	9 (55%)	38	19 (50%)	0	—
Localized tumour	6	0 (0%)	8	3 (37%)	0	—

Table 3. Relationship between iatrogenic factors predisposing to infection and the mortality rate

	Type of infection			
	Gram-positive		Gram-negative	
	Total	Died	Total	Died
Radiation therapy	5 (13·5%)	1 (20%)	14 (18·9%)	8 (56%)
Major surgery	1 (2·7%)	1 (100%)	22 (29·7%)	8 (46%)
Chemotherapy	16 (42·3%)	2 (12%)	35 (47·2%)	24 (69%)
Corticoids	8 (21·6%)	2 (25%)	24 (32·4%)	17 (70%)
Prophylactic antibiotics	7 (18·9%)	2 (28%)	15 (20·2%)	11 (73%)
Therapy with antibiotics	16 (43·2%)	5 (31%)	39 (52·7%)	23 (59%)
Local antibiotics	7 (18·9%)	2 (28%)	19 (25·6%)	14 (43%)
IV catheter	23 (62·1%)	6 (26%)	55 (74·3%)	32 (58%)
Bladder catheter	14 (37.8%)	6 (42%)	39 (52·7%)	23 (59%)
Tracheostomy	3 (8·1%)	3 (100%)	7 (9·4%)	3 (42%)

10 patients, whereas gram-negative sepsis killed 9 (81 %) of 11 patients. The series is small but the difference is highly significant ($p < 0{\cdot}01$). The reasons for such a difference may depend on intrinsic differences between gram-positive and gram-negative sepsis since it has been found among patients with a similar underlying "rapidly fatal" disease. It should be pointed out, however, that the mean time of duration of the leukemia, after hematological diagnosis, was 3·4 months for patients presenting gram-positive infections and 9·3 months for those with gram-negative bacillemia. As could be expected, patients with acute leukemia and gram-negative bacillemia were in a worse clinical and hematological situation than those who developed gram-positive infections.

Many iatrogenic factors are considered to predispose to infection in patients with serious underlying disease. The role of several such factors was evaluated here and the observations are summarised in Table 3.

These factors were found with a roughly similar frequency in patients with gram-positive and gram-negative infections with the exception of major surgery that preceeded gram-negative infections in 30 % of the patients. A relatively high mortality was found in gram-positive infections among the patients who had an indwelling bladder catheter or a tracheotomy. Among those who presented gram-negative bacteremia, chemotherapy, treatment with corticoids, prophylactic antibiotics and topical antibiotics were associated with a relatively high mortality rate. It should be emphasized, however, that several predisposing factors were usually present in the same patient. This renders the present analysis more difficult.

Antimicrobial agents are probably essential for an adequate therapy of severe infections. In certain series, however, the role of the underlying disease in the outcome was so overwhelming that adequate antimicrobial therapy, i.e. as indicated by *in vitro* tests, could not always be correlated to an optimal issue [3, 4]. However, others have found that appropriate antibiotic therapy was associated with a decreased mortality in each category of underlying disease [7, 8]. In this series, appropriate antibiotics were given to 59 patients who presented gram-negative bacillemia with a resulting mortality of 50 %; inadequate therapy, as indicated by *in vitro* sensitivity tests, was given to 19 patients, 16 (84 %) of whom died. The difference cannot be explained by differences of underlying diseases and is highly significant

($p < 0{\cdot}01$). Fourteen patients were treated with gentamicin as the only agent to which the offending micro-organism was sensitive and 9 (64%) of them died.

Similar considerations are not obtainable for patients whose infection was caused by gram-positive organisms, since all these patients were treated with at least one antibiotic to which the micro-organism was sensitive *in vitro*.

In gram-positive and in gram-negative infections the mortality rate was similar whether one or several antibiotics were used. Synergistic combinations of antibiotics, however, were associated with a better clinical response than non-synergistic combinations, as could be expected from previous studies from this laboratory [10].

From the preceding considerations, it may be suspected that more resistant strains will be found in lethal episodes of gram-negative bacteremia.

The susceptibility to commonly used antibiotics of the micro-organisms responsible for fatal and non-fatal gram-negative infections is summarized in Table 4. These results are expressed as cumulative percentages of inhibited strains that have been pooled together since only a few strains were present in each group for a valid analysis. It can be seen that for the antibiotics tested, with the exception of gentamicin, the strains responsible for infections that were ultimately cured were more often sensitive. These results emphasize that before identification and testing of the isolates none of the agents tested can be considered alone as a reliable blind therapy for bacteremia in severely debilitated patients. They suggest also that gentamicin might play an important role as a part of an empiric regimen to be used under such conditions, because of its broad anti-gram-negative spectrum. It should be emphasized, however, that gentamicin alone, which is inactive against pneumococci, streptococci and other gram-positive organisms, has not always been found very effective in severe sepsis caused by *Ps. aeruginosa* [11] and that its clinical effectiveness in staphylococcal infections must be evaluated in large clinical trials, although preliminary experiments with gentamicin in that type of infection appeared promising [12].

The choice of optimal initial therapy for suspected infection in hospitalized patients is made even more difficult by the frequent impossibility of distinguishing, on clinical grounds alone, between gram-positive and gram-negative etiologies.

As indicated in Table 5, symptoms related to the cardio-vascular system were more frequent in patients infected with gram-negative rods. In addition to these signs, high fever (temperature > 40°C) was more frequently observed in patients with gram-negative infections. These various symptoms had different prognostic meanings whether in patients with gram-positive or gram-negative sepsis. In the former, cardiovascular signs such as tachycardia, hypotension and respiratory failure associated with vasomotor collapse or cardiac failure were associated with a high mortality rate. A high mortality was linked to these signs also in patients with gram-negative infections. In addition, high fever, gastro-intestinal symptoms and bleeding were associated with a very high mortality rate, suggesting perhaps the presence of endotoxemia. Abnormal laboratory values relative to liver, kidney, and bone marrow functions were found with a similar frequency in both groups of patients. A high mortality rate was associated, in gram-negative infections, with abnormal coagulation, which was suggestive of intravascular coagulation, with uremia (frequently associated with circulatory collapse) and with leucopenia. None of

Table 4. Cumulative percentages of inhibited strains causing bacteremia by commonly used antibiotics

	Cumulative percentage of inhibited strains							
	Fatal infections				Non-fatal infections			
Minimum inhibitory concentration (μg/ml)	0·7	3	12	100	0·7	3	12	100
Cephalothin	20	39	47	59	26	42	53	61
Ampicillin	9	18	23	26	19	22	46	50
Carbenicillin	6	23	34	55	26	61	61	80
Chloramphenicol	17	32	39	41	19	42	57	57
Tetracycline	14	28	34	41	37	38	57	65
Kanamycin	26	34	39	39	46	46	46	53
Gentamicin	68	84	97	100	72	88	96	100
Polymyxin B	6	23	34	55	26	61	61	80

Table 5. Signs and symptoms associated with bacteremia and their relationship to the outcome

	Type of infection							
	Gram-positive				Gram-negative			
	Total		Died		Total		Died	
	No.	%	No.	%	No.	%	No.	%
Chills	8	21	0	—	12	16	9	75
No fever	1	3	0	—	3	4	1	33
Temperature > 40°C	7	19	1	14	26	35	21	81
GI symptoms*	5	13	0	—	10	13	9	90
Tachycardia	2	6	2	100	28	38	22	78
Hypotension	5	13	4	80	27	36	23	85
Respiratory failure†	7	19	7	100	33	44	26	79
Bleeding	8	21	1	12	21	28	18	86
Jaundice	4	12	1	25	10	13	7	70
Abnormal liver function tests‡	7	19	2	28	16	21	11	68
Abnormal coagulation tests§	15	40	6	40	30	40	26	79
Elevated BUN	2	6	0	—	12	16	10	83
Anemia‖	16	43	1	6	41	55	28	68
Leucopenia (< 1000 gran/mm^3)	7	19	0	—	10	13	10	100
WCC < 10,000/mm^3	14	37	3	21	33	44	17	50
WCC > 20,000/mm^3	7	19	0	—	12	16	6	50
Thrombopenia (< 100,000/mm^3)	14	37	2	14	35	46	24	68
Therapy with corticoids	5	13	3	60	20	26	16	80
Therapy with vasopressors	4	12	1	25	17	23	16	94

*Nausea; vomiting; diarrhea; †associated with shock or cardiac failure; ‡abnormal bilirubin, alcaline phosphatase, GO or GP transaminases, prothrombin time; §fall in platelet count, fibrinogen, or rise in titer of products of degradation of fibrinogen; ‖males Hb < 14g%, females < 12g%.

the leucopenic patients with gram-positive infection died, but all the granulocytopenic patients presenting gram-negative infection did. Besides different characteristics of the patients and of the offending microorganisms in these groups, these findings suggest that granulocytes are a necessary adjunct to anti-gram-negative therapy. This may be due, in part at least, to different properties of some anti-gram-positive and anti-gram-negative antibiotics [13].

A high mortality rate was associated with the use of corticoids or vasopressors in addition to antibiotics. As in other retrospective studies, it is likely that these therapies were given to the sickest patients, especially those presenting a vasomotor collapse. It should be remembered here that prospective studies have shown that neither vasopressors nor corticoids were effective as an adjunct therapy to antibiotics in severe bacterial infections [14, 15].

DISCUSSION

This study analyses the natural history of severe infections in patients with cancer; in this type of patient, infection represents a major risk and very often, the bacteria enter the bloodstream. Gram-negative bacilli were the most frequent cause of bacteremia in this series (64%), and the attack ratio of bacillemia caused by gram-negative rods was higher than in previously reported series [3, 4, 8]; this ratio of gram-negative bacteremia (10·6‰ of admissions) was, however, very close to that reported by Myerowitz and co-workers in susceptible and extensively manipulated patients such as those with leukemia, carcinoma, or transplanted organs [7].

In this series, bacteremia caused by gram-negative rods was significantly more lethal than sepsis due to gram-positive micro-organisms; mortality rates were 53% and 30% respectively. This difference was mainly due to the high mortality rate found in leukemic patients who presented bacillemia caused by gram-negative rods. These patients had a much more advanced neoplastic disease than patients who had gram-positive infections. Moreover, the infections caused by gram-positive micro-organisms were always treated by an adequate antibiotic, as indicated by *in vitro* tests; whereas infections caused by gram-negative rods were due to more

resistant bacteria; and this explains why 19 patients with gram-negative rods bacteremia were not treated adequately in this series. The mortality rate in these patients was 84%, which is significantly higher than that found in adequately treated patients.

An essential step towards adequate treatment of serious infection in the compromized patient is the administration of an effective antimicrobial therapy [14]. Therefore, the search for an adequate empiric therapy in this type of patient is very important. Several different regimens have been advocated up to now [16, 17] and should be administered as soon as severe sepsis is suspected, since the day or two afforded by empiric therapy has been shown to be beneficial in patients presenting severe underlying illnesses [18].

Our studies showed also that infections caused by gram-negative rods represent a much greater burden to the cardiovascular system than gram-positive infections. Whether this is due to endotoxin [19] or to some other toxins such as myocardial depressant factor seen in a number of states of shock [20] has not been elucidated yet; nevertheless, it should be emphasized that the "toxic" properties of gram-negative bacilli make it mandatory to treat the infections caused by these bacteria, as soon as possible with effective antibiotics, since, once established, "toxemia" will cause irreversible lesions, that can prove to be fatal in spite of a late eradication of bacillemia. Corticoids, however, are not helpful as an adjunct therapy to antibiotics in severe infections [15].

High fever, prior to therapy with antibiotics, has been found associated with a high mortality rate in gram-negative infections. Whether hyperpyrexia is a trivial consequence of severe bacillemia or plays really a role in the mortality rate remains to be elucidated. It should be remembered, however, that experimental and clinical data suggest that suppression of fever can be useful in the management of severe infection [21]. The role of the control of fever under such circumstances is to be investigated in properly controlled studies.

Bleeding and laboratory evidence of intravascular coagulation has been found as frequently in gram-positive and in gram-negative infections. However, the mortality rate associated with troubles of the coagulation system was much higher in gram-negative infections. This suggests that intravascular coagulopathy might not be a major factor in the outcome of severe sepsis. Other investigators have shown that the correction of coagulation abnormalities by heparin does not prevent a high mortality in gram-negative infections [22], and our findings are in accordance with data published by Komp and Donaldson. These authors suggest that disseminated intravascular coagulation may contribute to shock in gram-negative septicemia, but that it is not the only cause of irreversible shock and that it cannot be triggered off by shock alone [23].

Several negative correlations as far as the outcome is concerned were shown in this series. Age, sex, the type of the offending bacteria, the length of stay within the hospital and the type of medical manipulation did not correlate clearly to the final issue of the bacteremia. Since the series presented here is relatively modest and because a serious underlying disease was present in almost all the patients, it would appear unwise to consider them as unimportant. However, our study emphasizes a preponderant role of adequate antimicrobial therapy for the survival of compromised patients who present severe infections. Further efforts should be made to evaluate suitable therapies with antibiotics under these circumstances.

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Letter to the Editor

Distributions of Cell Volume and DNA Content of Rhabdomyosarcoma Cells Growing *in vitro* and *in vivo* after Irradiation

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CELL VOLUME distributions obtained with electronic cell counters, e.g. the Coulter Counter, have been used in several types of investigations to analyze changes in volume distributions of cell populations resulting from various treatments which can cause partial synchrony [1–3]. Measurements of cell volumes may not provide an unambiguous correlation with cell age however, and the advent of impuls-cytophotometers, which enable the measurement of the amount of DNA per cell in large cell populations in suspension, may be expected to give more accurate information. It is of interest therefore to investigate the correlation of the two types of distributions, and to analyze with these techniques changes occurring after different radiation treatments in a tumour cell population which can be grown *in vivo* and *in vitro*.

The tumour cell system employed is derived from a rhabdomyosarcoma transplantable in an inbred strain of WAG/Rij rats and has been described in detail elsewhere [3–7]. R-1 cells from this tumour obtained by a cell dispersion technique can be grown *in vitro*, with a cell population doubling time of about 17 hr, whereby the fraction of proliferating cells in log phase growth is close to 1. Tumours developed from these cells, growing in the flanks of rats, have a volume doubling time of

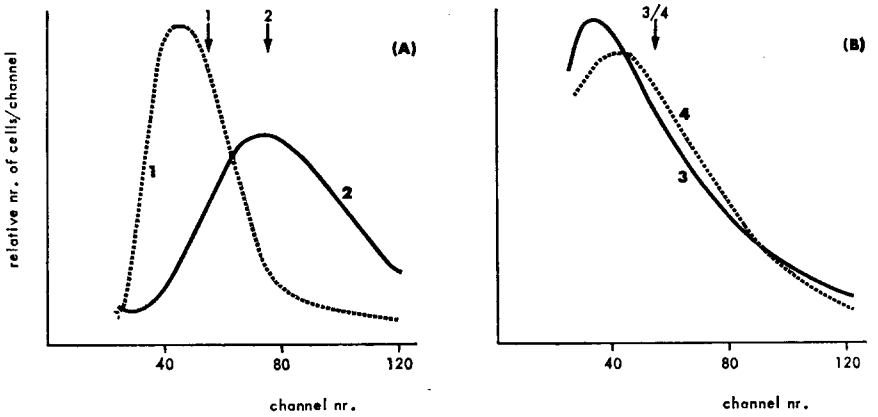

Fig. 1. *Cell volume distributions of rhabdomyosarcoma cells. Number of cells counted per channel are plotted on an arbitrary scale as a function of the channel number, which is proportional to the relative cell volume. At least* 50,000 *cells are counted for each curve. Arrows denote the mean cell volumes. (A) R–1 cells* in vitro *; curve* 1 *: control cells; curve* 2 *: R–1 cells* 16 *hr after a dose of* 2000 *rads of gamma-rays. (B) Cells from R–1 tumours; curve* 3 *: control tumours; curve* 4 *: tumour* 15 *hr after a dose of* 2000 *rads of X-rays.*

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about 4 days at a volume of 1 cm^3, the cell cycle time of the proliferating cells is about 20 hr and the growth fraction is about 0·4 [7]. Significant changes in cell volume distributions have been observed after irradiation of tumours with 4300 and 7000 rads of ^{137}Cs gamma-rays delivered at a relatively low dose-rate of 110 rads/hr [3]. The present report describes changes after acute irradiation with a dose of 2000 rads of ^{137}Cs gamma-rays administered in 16·4 min to R–1 cells *in vitro* and of 2000 rads of 300 kV X-rays administered in 6 min to the tumour in the anaesthetized animal.

In Fig. 1(*a*), cell volume distributions, obtained with a Coulter Counter, model B, interphased with a multichannel analyzer, are shown for cultured R–1 cells. Curve 1 represents the cell volume distribution of unirradiated R–1 cells, curve 2 represents the distribution of R–1 cells 16 hr after irradiation with 2000 rads of gamma-rays. The mean cell volume of irradiated cells is 1·4 time the mean cell volume of the control cells. In Fig. 1(*b*) cell volume distributions of cells are shown, derived directly from tumours by the dispersion technique. Curve 3 represents the volume distribution of cells from a control tumour, curve 4 the distribution of cells 15 hr after irradiation of a tumour *in vivo* with 2000 rads of X-rays. The mean cell volume of cells from the irradiated tumour is only 1·04 time the mean cell volume of cells from the unirradiated tumour. These shifts to larger cell volumes after single dose irradiation are presumably mainly due to blockade of cells in G_2 phase and slowing of cells in G_1 and S phase in their progression through the cell cycle. However, a contribution of other mechanisms, such as swelling of cells by leaking membranes cannot be excluded.

Measurements of changes in DNA content after irradiation of cells and tumours has now provided direct evidence about the phenomena causing shifts in cell volume distributions. With an impuls-cytophotometer (Phywe AG, Göttingen, Germany) distributions of DNA content have been obtained after fixation of cells in pure alcohol, incubation with RNAse and dying with the fluorescent ethidium bromide. In Fig. 2(*a*), curve 1 represents the DNA distribution of unirradiated R–1 cells in culture. The first peak represents cells in G_1 phase, the second peak cells in G_2+M phase. Cells in S phase are represented between these two peaks. Curve 2 of Fig. 2(*a*) represents the DNA distribution of cultured R–1 cells 16 hr after irradiation with an acute dose of 2000 rads of gamma-rays. From a comparison of these curves it can be concluded that the irradiated R–1 cells are accumulated or blocked in the G_2 phase. In Fig. 2(*b*), curve 3 represents the DNA distribution of unirradiated rhabdomyosarcoma cells of the solid tumour and curve 4 represents the distribution of DNA content in tumour cells 15 hr after irradiation of the tumour *in situ* with a dose of 2000 rads of X-rays. The first peak of curve 3 is due to leukocytes and normal cells of the connective tissues in the tumour. These cells cannot be entirely removed by the

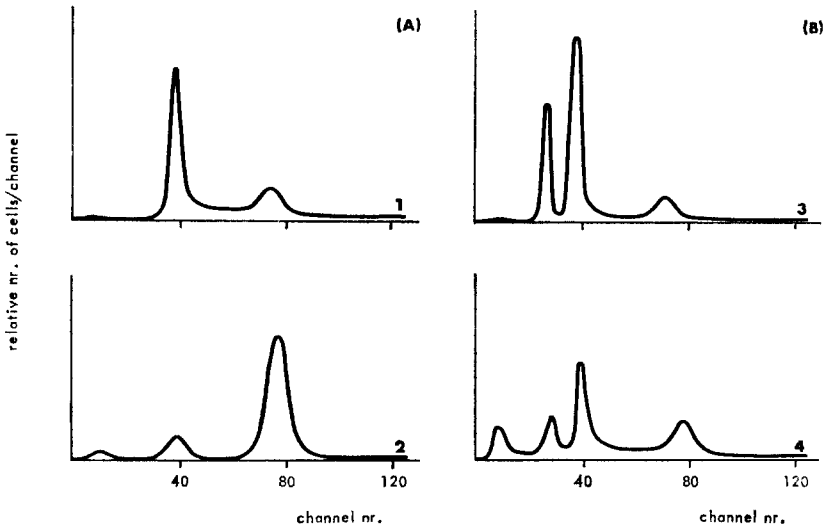

Fig. 2. Distributions of DNA content of rhabdomyosarcoma cells. Number of cells counted per channel are plotted on an arbitrary scale as a function of the channel number, which is proportional to the DNA content. For each curve 50,000 *cells are counted. (A) R–1 cells* in vitro*; curve 1: control cells; curve 2: R–1 cells 16 hr after a dose of* 2000 *rads of gamma rays. (B) Cells from R–1 tumours; curve 3: control tumour; curve 4: tumour 15 hr after a dose of* 2000 *rads of X-rays.*

trypsinization technique employed to obtain a monocellular cell suspension of tumour cells. The peaks in channels 5 to 15 are due to cell debris which are more pronounced after irradiation as shown in curves 2 and 4.

Comparison of the data presented in Figs. 1 and 2 shows clearly that, due to the irradiation dose of 2000 rads, proliferating cells in culture accumulate in G_2 phase. The fact that a considerable peak of cells with the DNA content of G_1 cells is still present for irradiated tumours after 15 hr, must be ascribed to the presence of about 60% non-cycling cells in a resting phase containing the same amount of DNA as G_1 phase cells. From this observation the conclusion can be drawn that, in this rhabdomyosarcoma, the non-proliferative cells are cells arrested in G_1 phase. The larger G_2+M peak and the smaller G_1 peak in curve 4 of Fig. 2 as compared with the corresponding peak in curve 3 of Fig. 2 indicate however, that proliferating cells in the tumour are effected in a similar way by irradiation as cells cultured *in vitro*. It can be concluded that comparison of distributions of DNA content and of cell volumes can provide a useful tool for investigating the influence of various types of agents on the kinetics of cells in tumours and in culture.

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Cell Kinetic Model of Tumour Growth and Regression for a Rhabdomyosarcoma in the Rat: Undisturbed Growth and Radiation Response to Large Single Doses

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Abstract—*A mathematical model of tumour growth and regression has been developed using experimental data on the cellular kinetics and radiation survival of cells of a rhabdomyosarcoma in the WAG/Rij rat. For the undisturbed tumour, the model assumes a time-dependent mean cell cycle time, cell proliferation parameter (1 + growth fraction) and cell-loss rate, consistent with available experimental data, to predict the (non-exponential) time variation of the tumour volume. To treat the response to radiation, it is assumed that the irradiated tumour is composed of two components: the "doomed" population, those cells which are incapable of unlimited proliferation but may undergo several divisions before dying, and the "surviving" population, those cells which may have received non-lethal damage but are capable of unlimited proliferation. Time dependence of the proliferating fraction, the cell density, and the reoxygenation rate are introduced to describe the regression and regrowth of the tumour after single massive radiation doses.*

The results show that reasonable time dependences of the parameters describe the response of the tumour to 2000 *and* 1000 *rads of X-rays. A long delay in tumour regrowth after* 600 *rads of fast neutrons, however, remains to be adequately explained in terms of the model in its present form.*

INTRODUCTION

THE RATE of growth of a tumour is determined to a first approximation by the rate of production of the proliferating cells and the rate of cell-loss due to death or removal. Consequently, in order to obtain insight into the growth of tumours and their response to various treatments, it is necessary to investigate quantitatively changes in cell proliferation, cell reproductive death, and cell elimination.

Data on the cell kinetics of various experimental tumours are still rather limited, especially with regard to changes after treatment with ionizing radiations and cytotoxic agents. In order to analyze the available data to the fullest extent possible and to obtain insights in the relative importance of various parameters, e.g. cell cycle time, growth fraction, cell-loss, and to predict results of treatments not yet investigated, it is important to attempt the development of a mathematical model consistent with the available data. In a series of experiments with a rhabdomyosarcoma transplantable in an inbred strain of WAG/Rij rats,

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data have already been obtained with respect to a variety of responses [1–5], and therefore it appeared of interest to investigate whether a model could be developed which would correctly describe these responses. The complexity of the model required a computer to handle the calculations sufficiently rapidly. In this paper the first results of this computer analysis will be presented.

A. GROWTH OF UNIRRADIATED TUMOURS

1. *The general model*

It is assumed that the rate of change in the number of tumour cells at a given time depends on the mean values of three cell kinetic parameters: the cell cycle time, a cell proliferation parameter related to the growth fraction, and the cell loss rate. These are related in the following equation:

$$\frac{dN}{dt} = \left[\frac{ln\alpha(t)}{T_c(t)} - k_L(t)\right] N(t) \qquad (1)$$

where dN/dt is the rate of change of cell number per unit of time, is a cell proliferation parameter and can be considered as the mean number of new proliferating cells produced per cell division, k_L is the cell loss rate, i.e. the fraction of cells lost per unit of time, and T_c is the mean cell cycle time.

The inclusion here of time-dependence for the parameters allows for the increase in cell population size in a non-exponential fashion. This formula is a generalization of some earlier considerations by Steel [6]. The time dependences of the parameters were chosen to be consistent with the experimental data obtained for the rhabdomyosarcoma tumour system [2].

The assumption is made that the cell density of the tumour (i.e. the number of cells per unit of volume) is constant throughout the period that the volume measurements are made. Thus, the total volume is directly proportional to the total number of cells.

2. *Time dependence of the kinetic parameters*

Since an adequate theory is not presently available to describe quantitatively the time dependence of the cell kinetic parameters, a simple functional dependence was arbitrarily chosen that was consistent with the experimental data and was computationally easy to handle. The expressions chosen for the time dependence of the cell proliferation parameter, α, and the cell-loss rate, k_L, involve integrated Gaussian functions as follows:

$$\begin{aligned} \alpha(t) &= 2 - H_\alpha(t) \\ k(t) &= K_L H_L(t) \end{aligned} \qquad (2)$$

where

$$H_\alpha(t) = \frac{1}{(2\pi)^{\frac{1}{2}}\sigma_\alpha} \int_o^t \exp\left[\frac{-(t'-T_\alpha)^2}{2\sigma_\alpha^2}\right] dt'$$

$$H_L(t) = \frac{1}{(2\pi)^{\frac{1}{2}}\sigma_L} \int_o^t \exp\left[\frac{-(t'-T_L)^2}{2\sigma_L^2}\right] dt' \qquad (3)$$

and K_L is a constant. The constants T_α and T_L denote the time-displacement of the centre of the Gaussians from $t' = 0$, and the σ_α and σ_L are the standard deviations of the functions.

The cell cycle time was given the time dependence:

$$T_c(t) = K_3\{1 - \exp[-(t+K_1)/K_2]\} \qquad (4)$$

where K_1, K_2, and K_3 are constants. This function implies that the cell cycle time is approaching a constant (K_3) as time increases. The speed with which it approaches this constant is controlled by the values of K_1 and K_2.

It is hoped that the arbitrary time-dependence of these parameters will be replaced as more is learned about the mechanisms that control the changes in these parameters.

The computer program allows variation of T_α, T_L, σ_α, and σ_L in order to minimize by a least-squares analysis the deviation of the curve from the experimental data.

B. VOLUME CHANGES OF IRRADIATED TUMOURS

1. *The general model*

After a dose of radiation, many factors influence the complex response of the tumour. It is convenient to group them such that the same cell kinetic parameters developed for the unirradiated tumour can be used to describe the volume changes in the irradiated tumour. Again, in this case, the time variation of the parameters is arbitrary and will undoubtedly be replaced by more soundly based functions when the mechanisms causing the time dependence have been identified. The basic assumption of the model for the irradiated tumour is that the cell population after irradiation can be considered as consisting of two components: the "doomed" fraction and the "surviving" fraction. The "doomed" cells, N_I, are those which sustain the kind of damage that cause

them to die even though they may pass through several divisions first, i.e. there will be no survivors among their descendents. The "surviving" cells, N_{II}, are those which recover and, although perhaps sustaining some non-lethal damage, will be capable of unlimited proliferation. It is the latter component which will cause the regrowth of the tumour. The simple addition of these two components, with proper account taken for the changing cell density, is assumed to account for the observed changes in tumour volume:

$$V(t) = \frac{N_I(t) + N_{II}(t)}{\rho(t)}$$

where $V(t)$ is total volume at time t, N_I and N_{II} are the number of cells in the two populations and $\rho(t)$ is the average density of cells.

2. *The surviving fraction of cells*

The surviving fraction consists of those cells which are capable of unlimited proliferation after irradiation. It is initially assumed that the unirradiated tumour comprises two populations of cells with distinctly different radiosensitivities, representing the oxygenated and hypoxic fractions [3]. The fraction of each population surviving a radiation dose is obtained from a survival expression for that population. Therefore, survival curves for oxygenated cells and for hypoxic cells must be obtained or deduced for the type of radiation used for the tumour volume studies. The resulting surviving fraction in the tumour is the surviving oxygenated fraction multiplied by the initial oxygenated fraction plus the surviving hypoxic fraction multiplied by the initial hypoxic fraction. In this way the two fractions after irradiation can be kept separated.

It is assumed that the hypoxic fraction does not proliferate *in vivo*, but can be either oxygenated (reoxygenation) or lost by normal cell-loss processes. After an initial mitotic delay, the oxygenated fraction is assumed to have a rather large growth fraction and to lead ultimately to the regrowth of the tumour. When the oxygenated fraction of the surviving population reaches its initial value before irradiation, the survivors take on the characteristics of an unirradiated tumour with modified cell kinetic parameters.

The assumed cell kinetic parameters for the "surviving" tumour cells are: (*a*) Cell cycle time: The same as for the "doomed" population (see below). (*b*) Cell proliferation parameter: The experimental data, indicating a longer doubling time for the regrowing tumour, can be interpreted by assuming that α is dose- (or damage-) dependent as well as time-dependent, i.e. the lower the initial survival the lower α is. (*c*) Cell loss rate: The experimental data are consistent with a value of k_L equal to the initial value (before irradiation) plus the time-dependent value calculated for an unirradiated tumour of the same size.

With these kinetic parameters, the "surviving" tumour cells fill up the tumour volume, increasing the cell density to its pre-irradiated value, but *not* increasing the volume until the cell density has almost reached its final value. This assumption was made so that the cell number, cell density, and tumour volume would not all be varying independently. Undoubtedly, it is true only approximately.

3. *The doomed fraction of cells*

The doomed fraction consists of those cells which have sustained lethal damage, causing either immediate death, or mitotic delay and eventual death of descendents. These cells may undergo several mitoses before dying, but they are distinct from the surviving fraction in that they cannot proliferate indefinitely, and all are eventually eliminated from the tumour. The cell kinetic parameters for the doomed fraction are obtained from the assumption that, after a dose-dependent mitotic delay of 1·5 hr/100 rads of X-rays and 4·5 hr/100 rads of neutrons, these parameters change in an approximately linear mode from their pre-irradiation values to the values determined experimentally at 4 days post-irradiation. No experimental data are available between 0 and 4 days after irradiation. The proliferation parameter, α, was assumed to vary linearly from a value halfway between unity and its initial value to the experimentally determined value of 1·24 after four days [2]. Again, integrated Gaussian functions are used for the changes of the mean cell cycle time and the cell-loss rate. The change of cell-loss rate after irradiation is assumed to be linearly dependent on the dose.

The density of cells is assumed to decrease slightly immediately after irradiation, reflecting an increasing number of enlarged cells in G_2, due to a mitotic block.

RESULTS

(*a*) *Unirradiated tumours*

The experimental data on tumour volume variations as a function of time, determined by Hermens and Barendsen [2], was compared with the volume–time relationship calculated

by the cell kinetic model described above. The kinetic parameters were obtained in that study for the tumour at two volumes, 0·3 cm^3 (the "small" tumour) and 1·5 cm^3 (the "large" tumour).

The computed tumour volume vs. time curve using equations 1–4 is compared with the experimental results in Fig. 1. The time dependence of T_c, α, and k_L are also shown. In Table 1 the cell kinetic parameters measured by Hermens and Barendsen [2] for the "small" and "large" tumours are compared with the values calculated from the model. It can be concluded that a good fit is obtained to the experimental data for volume variations as a function of time by means of a cell kinetic model of tumour growth using functions for the cell kinetic parameters, which for the two volumes at which experimental data are available, are close to the observed values.

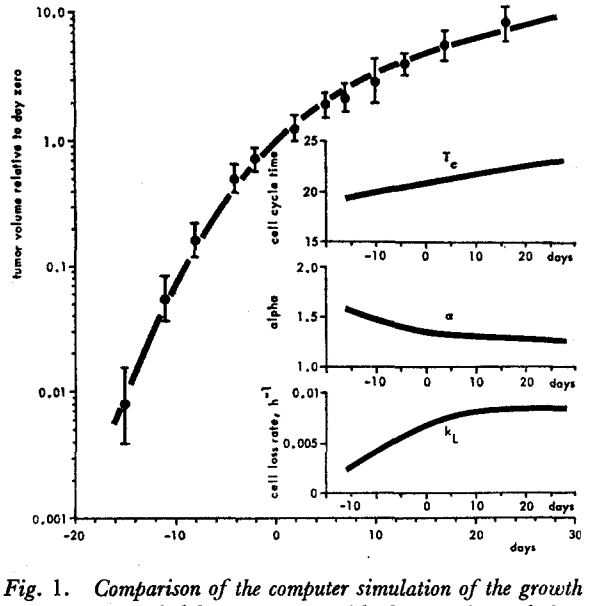

Fig. 1. Comparison of the computer simulation of the growth curve of a rat rhabdomyosarcoma with the experimental data of Hermens and Barendsen [2]. The time dependence of the cell cycle time, T_c, the cell proliferation parameter, α, and the cell-loss factor, k_L, used for the calculation of the growth curve are also shown in the inserted graphs. The tumour volume is 1·5 cm^3 at t = 0, the day of irradiation in subsequent figures.

(b) Irradiated tumours

1. *Initial surviving fraction.* The surviving fractions of cells in the hypoxic and oxygenated states immediately following irradiation were taken from the work of Barendsen and Broerse [3]. The hypoxic data were obtained from animals killed 10 min before irradiation, and the oxygenated data were obtained from irradiations performed *in vitro*. In each case, the data were analyzed assuming a two-mechanism inactivation process consisting of a single-hit single-target component, and a multi-target component. Analytic expressions of the form:

$$S = \exp(-D/D_1)\{1-[1-\exp(-D/D_2)]^n\} \quad (5)$$

were assumed,* and the best values for the three parameters, D_1, D_2, and n were obtained using all the available experimental data on this system. This was done for the oxygenated and hypoxic data separately for both X-ray and neutron irradiations. The procedure yielded for the oxygenated case: $D_1 = 400$ rads, $D_2 = 170$ rads, $n = 15$ for X-rays, and $D_1 = 160$ rads, $D_2 = 180$ rads, $n = 6$ for neutrons; for the hypoxic case: $D_1 = 920$ rads, $D_2 = 455$ rads, $n = 12$ for X-rays and $D_1 = 220$ rads, $D_2 = 270$ rads, $n = 5$ for neutrons.

*This formula fits the data within experimental errors. However, it should not be inferred that we subscribe to the multi-target model as the mechanism for cell inactivation.

Table 1. Comparison of experimental and model cell kinetic parameters

	Small tumour volume 0·3 cm^3		Large tumour volume 1·5 cm^3	
	Experimental	Calculated	Experimental	Calculated
Cell cycle time (hr), T_c	20·4	20·3	18·5 (periph.) 25·5 (centre)	21·0†
Cell proliferation parameter, α	1·42	1·424	1·29	1·349
Cell loss rate, k_L	0·0058*	0·0051	0·0079*	0·0070
Instantaneous doubling time (hr)	60*	56·1	144*	94·9

*These experimental values result from quantities obtained by eye from the growth curve, taking into account the local variation of experimental data. The calculated values were obtained assuming monotonically varying cell kinetic parameters and overall best fit to the entire growth curve.

†It has been assumed as a first approximation that a single value for the cell cycle time in the "large" tumour would adequately describe the proliferation of cells.

The results were combined in the form

$$S_T = f_H S_H + (1 - f_H) S_0$$

to obtain the total surviving fraction. Here S_H is the fractional survival of the hypoxic cells, S_0 is the fractional survival of the oxygenated cells, and f_H is the assumed fraction of hypoxic cells at the time of irradiation. The resulting curves with $f_H = 0{\cdot}15$ adequately fit the survival data obtained from the tumour cells irradiated *in vivo* [3].

It is well-known that, in a variety of animal tumours, reoxygenation of hypoxic cells occurs after irradiation with large single doses. No definite experimental data are available which yield information about the rate of oxygenation of hypoxic cells after irradiation of the R–1 rhabdomyosarcoma with large single doses. Data obtained for fractionated treatments with 300 rads per day indicate that some reoxygenation occurs [4]. Furthermore results of experiments on the oxygenation status of R–1 rhabdomyosarcomas after treatments with gamma-rays at low dose rates of 100 rads/hr for 20 hr indicate that considerable reoxygenation occurs within 20 hr [7]. Accordingly, in the model, we have assumed that reoxygenation also occurs after single large doses, and a rate of reoxygenation during 24 hr after irradiation has been assumed, which is consistent with the available data on the rate of regrowth of the tumour and the rate of increase of the number of clonogenic cells after irradiation. This assumption is recognized to be arbitrary, however, and this demonstrates the importance of obtaining experimental data about the rate of reoxygenation and about its mechanism.

Using the model outlined above, curves of the total volume, "surviving" fraction and volume of "doomed" cells were calculated. In Fig. 2 the results are presented for 2000 rads of 300 kV X-rays. Also shown are the experimental tumour volumes (circles) and a shaded area indicating the estimated range of the fractions of clonogenic cells (relative to the number of cells at the time of irradiation) produced by the surviving cells, as deduced from the experimental data of Barendsen and Broerse [3].

Studies by Hermens and Barendsen have shown that, at a four-day post-irradiation, the mean cell cycle time, T_c, has decreased from 20 to 12 hours, the cell proliferation parameter, α, for the "doomed" cells has changed from 1·29 to 1·24, the cell-loss rate, k_L, has increased from 0·006 to 0·0252 hr^{-1} and the relative cell density has decreased from 1·0 to 0·5. The parameters used in the model are fitted to the

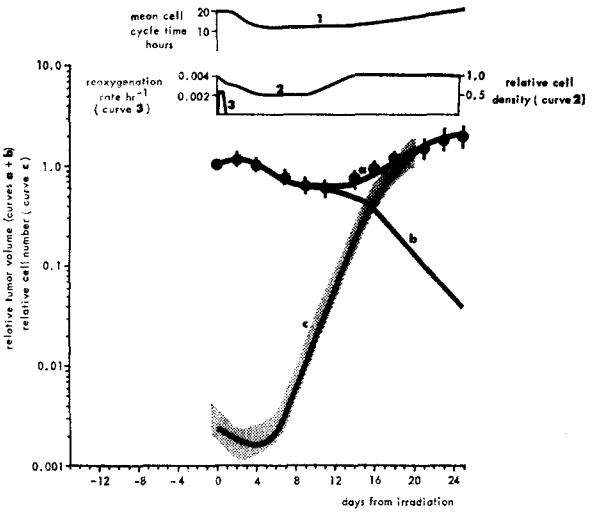

Fig. 2. Results for 2000 *rads of* 300 kV *X-rays. Tumour volume relative to that at the day of irradiation is plotted as a function of time after irradiation at* t = 0. *The curves give the results of the calculations and are (a) the total tumour volume, (b) the volume of the "doomed" population, and (c) the "surviving" cell fraction as described in the text. The circles are the experimental points of the tumour volume as measured after irradiation. The shaded area covers the region of experimental data for the clonogenic offspring produced by cells surviving the irradiation. Also shown are:* (1) *the mean cell cycle time,* (2) *relative cell density, and* (3) *reoxygenation rate assumed in the model, all plotted as a function of time.*

experimentally determined values for these quantities at the two time intervals $t = 0$ and $t = 4$ [2]. In Fig. 2 the time dependence of the mean cell cycle time, the relative cell density, and the reoxygenation rate of the surviving cells are shown, as calculated by the model.

In Fig. 3, we present the case for 1000 rads of 300 kV X-rays, showing the same quantities

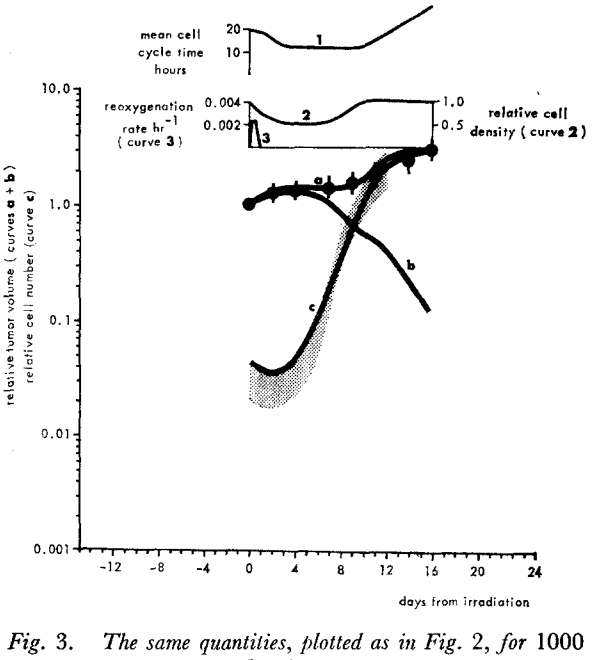

Fig. 3. The same quantities, plotted as in Fig. 2, *for* 1000 *rads of X-rays.*

as in Fig. 2, compared with the experimental data. In this case, the cell kinetic parameters are not available, and a time dependence had to be assumed. Both the cell-loss factor and the proliferation parameter for the surviving population were made dose-dependent, as well as the initial value of the surviving fraction through the survival curve expressions indicated above.

In Fig. 4 we present the results of the model for 600 rads of 15 MeV neutrons, compared with the experimental results. Here again, cell kinetic data are not available, and assumptions similar to those for 2000 rads of X-rays had to be made with regard to their time dependence.

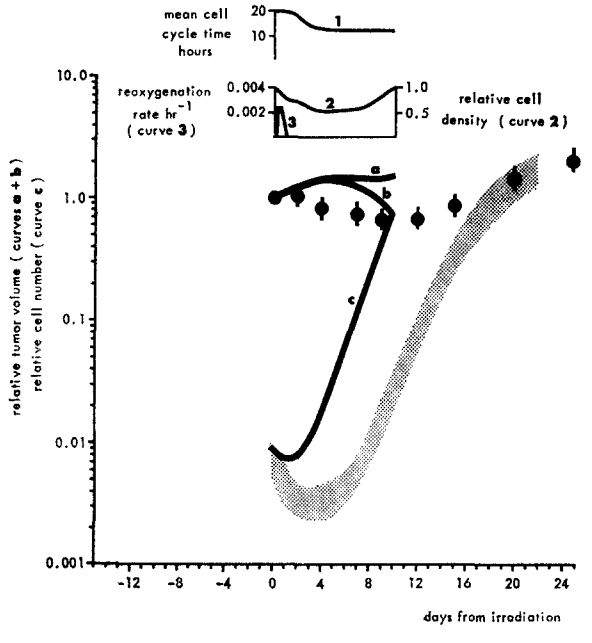

Fig. 4. The same quantities, plotted as in Fig. 2, for 600 *rads of* 15 *MeV neutrons. The early increase in the calculated surviving fraction is due to the larger fraction of oxygenated surviving cells present after neutron irradiation, as discussed in the text.*

DISCUSSION

Although the model described in this paper adequately fits the available experimental data for unirradiated tumours and tumours irradiated with 1000 and 2000 rads of X-rays, it will be clear that several assumptions about changes of parameters with time still lack a firm basis. The possibility cannot be excluded that other factors, which have not been taken into account, e.g. the adequacy of nutrient supply, play an important role. This becomes especially apparent in the wide discrepancy between the calculated and experimental results for the time dependence of the surviving fraction after neutron irradiation, shown in Fig. 4. The reason for this discrepancy is quite straight-forward. As a consequence of the low OER for fast neutron irradiation, relatively more oxygenated cells survive after neutron irradiation than survive an X-ray irradiation producing the same overall survival level. Therefore, since tumour regrowth is assumed in the model to be due to proliferation of oxygenated cells, the neutron-irradiated tumour is calculated to regrow with less delay than the X-irradiated tumour. The observed long delay is unlikely to be due to a very large RBE for mitotic delay. It has already been assumed in calculating the time-dependence of the surviving fraction in Fig. 4 that the mitotic delay is three times that expected from the same dose of X-rays: 27 hr for a neutron irradiation of 600 rads.

The long regrowth delay observed experimentally for the 600 rads of neutrons was reflected in a slightly larger RBE for tumour regrowth (3·3) than for other end-points as reported by Barendsen and Broerse [3]. The present analysis indicates clearly that this larger RBE is due to the unexplained delay occurring in the regrowth of the surviving fraction after the neutron irradiation. It is important to note that the calculated curves for the time dependence of the surviving fraction show that after 600 rads of neutrons this fraction returns to the pre-irradiation value of 1·0 at the same time as calculated for 1000 rads of X-rays, whereas the experimentally observed data indicate that this return occurs much later, at about the same time as observed after 2000 rads of X-rays. The initial values of the surviving fractions are 3·6, 0·27 and 0·70% after doses of 1000 and 2000 rads of X-rays and 600 rads of neutrons respectively, i.e. the value for 600 rads of neutrons is closest to that for 2000 rads of X-rays. The calculated curve *c* for 2000 rads of X-rays could be made to fall closer to the curve *c* for 600 rads of neutrons by increasing the rate of reoxygenation. However, then the agreement for X-rays with the experimental data would be lost. Consequently, another as yet undefined mechanism common to both X-ray and neutron irradiation would have to be invoked to account for the delay in the rise of the curves *c* after both doses of X-rays and the dose of neutrons. Another possibility is to assume that an LET dependent effect exists which overshadows the known cellular mitotic delay. A satisfactory description of all observed data obviously must await future experimentation about the rate of reoxygenation, the rate of repopulation and their underlying mechanisms.

CONCLUSION

A model of tumour growth and regression after irradiation has been developed, in which experimentally determined cell kinetic parameters describable by assumed time-dependent functions are utilized in calculating the changes in tumour volume as a function of time before and after irradiation. A rhabdomyosarcoma in the rat was used in this study because of the wealth of data available for this system. The general features, including the underlying mathematical structure, may well be common to other solid tumours. An interesting delay in the regrowth of a tumour after neutron irradiation is noted; the length of the delay cannot be explained by the theory in its present form.

It can be concluded that analysis of tumour growth and regression by means of a mathematical model can yield insight into the relative importance of such known factors as the cell kinetic parameters, cell reproductive death, the hypoxic fraction, and the reoxygenation rate in determining the radiation response of tumours. In addition, and perhaps more importantly, it can demonstrate which factors have yet to be studied in more detail.

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Comparative Antitumour and Haematological Effects of Some Bifunctional Alkylating Agents Containing Mixed Functional Groups

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Abstract—*Some bifunctional alkylating agents having groups reactive to both myeloid and lymphatic tissue have been prepared in an attempt to produce a truly "radiomimetic" compound with respect to its effect on the blood cells. 4-bromobutyl methanesulphonate, a compound possessing a therapeutic index on the Walker carcinoma three times as great as that of the clinically active 1,4-dimethanesulphonoxybutane (busulphan), satisfied this criterion. In most cases the anti-tumour activity of the compounds was greater than the corresponding derivatives in which the two alkylating functions were the same. Structure–activity relationships, using the rate of reaction with nucleophiles and the ether-water partition coefficients as parameters, are discussed.*

INTRODUCTION

IN GENERAL monofunctional alkylating agents are inactive as tumour growth inhibitors [1] difunctionality being a necessary criterion for this type of biological activity. The biological effects produced by the alkylating agents often so closely resemble those induced by radiation that they were called "radiomimetic" [2], although this term has recently been criticized [3]. The type of biological activity shown often depends on the nature of the functional group. Thus bis-methanesulphonates are more specific inhibitors of the growth of myeloid tissue [4] whereas *bis*-2-chloroethylamines are more specific for lymphatic tissues [5]. A combination of busulphan, an example of the former type, and chlorambucil, an example of the latter, produces an haematological response very similar to that observed with X-radiation [6].

In the present work, bifunctional alkylating agents having groups specific to both myeloid and lymphatic tissue have been prepared. Since these two groups produce their cytotoxic effect at different phases of the cell cycle [7], drugs of this type will depend on at least two variables, and theoretically the selectivity should be increased [8]. Also, by suitable combination of groups, it was hoped to produce a complete "radiomimetic action" as far as the blood elements are concerned.

Very few compounds with mixed alkylating functions have been synthesized. A nicotinic acid derivative containing a 2-chloroethylamino and ethyleneimide group has been prepared as a potential irreversible antagonist of NAD [9], and (2S,3S)-1,2-epoxy-3,4-butanediol 4-methanesulphonate has been prepared from (2S,3S)-threitol 1,4-*bis*-methanesulphonate by the addition of 50% of the theoretical amount of alkali [10].

In the present work, the following combination of groups have been prepared:

(*a*) alkanesulphonoxy—epoxy;
(*b*) alkyl bromide—epoxy;
(*c*) alkyl bromide—alkanesulphonoxy.

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MATERIAL AND METHODS

Infra-red (i.r.) spectra refer to the title compound and were taken on a Perkin Elmer 257 Grating Infra-red Spectrophotometer using sodium chloride discs, either as the oil, or as a mull of a solid in Nujol. Nuclear magnetic resonance (nmr) spectra were taken on a Perkin Elmer R 10 spectrometer, operating at 60 Mc/s, in chloroform solvent, with T.M.S. as reference standard. Melting points (mp) were determined on a Kofler hot-stage apparatus and are corrected. Thin layer chromatography (t.l.c.) was conducted on glass microscope slides coated with Kieselgel G (Merck 7731). Spots were detected by exposure to iodine vapour unless otherwise stated. Preparative column chromatography was performed using Kieselgel G (Merck 7734).

4-*Methanesulphonoxy-but*-1-*ene*

1-Buten-4-ol [13] (14·4 g; 0·2 mole; dried over anhydrous K_2CO_3) was dissolved in pyridine (31·7 g; 0·2 mole; KOH dried and redistilled). The solution was cooled to $-10°C$ when methanesulphonyl chloride (22·9 g; 0·2 mole; redistilled) was added dropwise with stirring, keeping the temperature of the reactants below 0°. When the addition was completed, the solution was kept at 0° overnight and then treated with "ice-cold" dilute hydrochloric acid until acidic, when the ester was precipitated as an oil; the pyridine hydrochloride dissolving completely. The oil was extracted with chloroform, dried (anhydrous Na_2SO_4) and distilled under reduced pressure in the presence of a little K_2CO_3, which prevented decomposition [14]. This yielded the title compound as a colourless oil bp 62°/0·15 mm; yield 24·5 g (85%) $n_D^{22·5}$ 1·4439 (Found: C, 40·4; H, 6·8; S, 21·2%. $C_5H_{10}O_3S$ requires C, 40·0; H, 6·7; S, 21·3%).

ν_{max}^{film} 1650 cm^{-1}, 1350 cm^{-1} and 1040 cm^{-1}.

The following compounds were prepared in essentially the same manner using the appropriate sulphonyl chloride. Propane-2-sulphonyl chloride was prepared by the method of Johnson and Sprague [15].

4-*Ethanesulphonoxy-but*-1-*ene*

bp 93–94°C/0·3 mm; yield73 % n_D^{18} 1·4485 (Found: C, 44·1; H, 7·4; S, 19·6%. $C_6H_{12}O_3S$ requires C, 43·9; N, 7·3; S, 19·5%).

4-(*Propane*-2-*sulphonoxy*)*but*-1-*ene*

bp 78–80°C/0·45mm. $n_D^{26·5}$ 1·4559. (Found: C, 47·4; H, 7·8; S, 18·5%. $C_7H_{14}O_3S$ requires C, 47·2; H, 7·9; S, 18·0%).

PREPARATION OF EPOXIDES

1,2-*Epoxy*-4-*methanesulphonoxybutane*

To an ice-cold solution of perbenzoic acid [16] (19·95 g; 0·1445 mole) in chloroform (300 ml) was added 4-methanesulphonoxy-but-1-ene (19·5 g; 0·13 mole) in chloroform (100 ml) and the mixture was kept at 5°C for 21 days, after which 83% reaction had taken place (determined by iodometric titration of the perbenzoic acid). The solution was washed three times with sodium carbonate solution (10%), dried (K_2CO_3) and evaporated. T.l.c. on the oily residue in benzene gave R_f 0·2 (benzoyl peroxide) and 0·05 (title compound). The oil dissolved in benzene (AnalaR) was applied to a column (3·5 × 24 cm) of Kieselgel (100 g) and eluted with the above solvent. Yield: 14·2 g (66%), $n_D^{21·5}$ 1·4542. (Found: C, 36·2; H, 6·2; S, 19·1%. $C_5H_{10}O_4S$ requires C, 36·2; H, 6·1; S, 19·3%).

ν_{max}^{film} 1350 cm^{-1}, 1260 cm^{-1}, 1060 cm^{-1} 880 cm^{-1} and 820 cm^{-1}.

τ5·65 (triplet, $J_{3,4}$ 6·0 H_z, H−4), 6·95 (singlet, OSO_2Me), ~6·90 (sextet, H−2), 7.20 (triplet, $J_{1,2} = J_{1,1'}$ 4·5 H_z, H−1), 7·45 (quartet, $J_{1,1'} = J_{1'2}$; 4.5 H_z, H−1′), 8·05 (sextet, $J_{3,4}$ 6·0, $J_{2,3}$ = 12·5 H_z, H−3).

The following compounds were prepared by essentially the same method:

1,2-*Epoxy*-4-*ethanesulphonoxybutane* was purified by chromatography using diethyl ether—light petroleum (bp 40–60°C) (1:1) n_D^{20} 1·4540. (Found: C, 39·8; H, 6·6; S, 18·0%. $C_6H_{12}O_4S$ requires C, 40·0; H, 6·7; S, 17·8%).

1,2-*Epoxy*-4(*propane*-2-*sulphonoxy*)*butane*. bp 116°C/0·4 mm, $n_D^{22·5}$ 1·4523. (Found: C, 43·4; H, 7·1; S, 16·4% $C_7H_{14}O_4S$ requires C, 43·4; H, 7·2; S, 16·5%).

1,2-*Epoxy*-4-*bromobutane*. bp 26–27°C/0·4 mm, n_D^{27} 1·4748. (Found: C, 32·0; H, 4·6; Br, 52·9%. C_4H_7BrO requires C, 31·8; H, 4·6; Br, 53·0%).

1,2 *Anhydro*-6-O-*methanesulphonyl*-D-*mannitol* mp 80–81°C [11]. $[\alpha]_D^{32} + 67·5°$ (c = 1, water).

1,2-*Epoxy*-3-*methanesulphonoxypropane*. Glycerol 1,3-dimethanesulphonate [17] (4·89 g; 0·0197 mole) in water (10 ml) and acetone (10 ml) containing phenolphthalein (2 drops) was treated dropwise with sodium hydroxide (19·7 ml N) at 35–40°C with stirring. The rate of addition of alkali was regulated so that there

was never more than one drop in excess and the solution never became completely colourless. The solution was then added dropwise to a rapidly stirred suspension of sodium carbonate (30 g) in ethyl acetate (150 ml) over 10 min. Stirring was continued for a further 5 min. The solid was removed by filtration and washed with ethyl acetate (40 ml). The filtrate was evaporated under reduced pressure at 40° and distilled in the presence of a little K_2CO_3 bp 90°C/0·25 mm (Lit[18] bp 78–79°/0·2 mm). Yield 2·5 g (83%). $n_D^{20·5}$ 1·4524 (Found: C, 31·5; H, 5·4; S, 20·9%. $C_4H_8O_4S$ requires C, 31·6; H, 5·2; S, 21·0%). τ 5·43 (quartet, $J_{3,3'}$ 6·0, $J_{2,3}$ 12·0 H_z, H−3), 5·91 (quartet, $J_{3,3'}$ 6·0, $J_{2,3'}$ 12·0 H_z, H−3′) ~6·82 (sixteen lines $J_{2,3}$ 12·0, $J_{1,2}$ 5·0 H_z H−2), 6·90 (singlet OSO_2Me), 7·10 (triplet $J_{1,1'}$ 5·0, $J_{1',2}$ 5·0 H_z H−1′), 7·29 (quartet $J_{1,1'}$ 5·0, $J_{1,2}$ 3·0 H_z H−1).

4-*Bromobutyl methanesulphonate* had bp 112–114°C/0·5 mm; n_D^{20} 1·4832 (Lit [12] bp 117–120°C/0·6 mm n_D^{20} 1·4812).

ESTIMATION OF RELATIVE ALKYLATING ABILITY OF OXIRANE AND ALKANE-SULPHONATE FUNCTIONS

The compound (0·001 mole) in dry acetone (5 ml) was added to 0·1N sodium thiosulphate (40 ml) and acetone (35 ml) containing phenolphthalein, which had previously been brought to neutrality. The solution was kept at 25°C and the alkalinity developed was titrated continuously with 0·176N acetic acid. After a sufficient period of time, distilled water (50 ml) was added and the thiosulphate remaining was estimated by titration with 0·1N iodine (starch indicator). This procedure was repeated several times. The solution was stirred mechanically during the addition of acetic acid to ensure rapid mixing.

DETERMINATION OF ETHER-WATER PARTITION COEFFICIENTS

The ether-water partition coefficients were measured by assaying the ethereal solution (5 mmole in 100 ml ether) of the compound before and after shaking with water for 5 min. For the epoxy compounds the assay reaction used was the determination of the amount of hydroxyl ion liberated in 30 min when an aliquot was added to an excess of thiosulphate in 50% aqueous acetone. An allowance was made for the contraction in volume of the ether solution on shaking with water.

An aliquot of the original ethereal solution was evaporated with 50% aqueous acetone, and the hydroxyl ion liberated in 30 min in the presence of thiosulphate was determined. A similar aliquot was shaken with 2·5 times its volume of water for 5 min, the ether was evaporated off and the hydroxyl ion released in 30 min was determined as before.

For compounds without the oxirane function the reaction was taken to be the consumption of thiosulphate (determined by iodometric titration) in a given time interval, and the partition coefficient was determined as before.

Anti-neoplastic assay

The protocol for testing compounds for inhibitory action against the Walker rat carcinoma is described by Connors *et al.* [19]. Compounds were administered in 10% acetone in arachis oil by a single intraperitoneal injection on the day following implantation.

Haematological effects

The procedure for determining haematological effects of drugs is described by Elson *et al.* [20].

Determination of hydrolysis rates

Hydrolysis rates were determined using a "Radiometer titrator" (TTTI) as previously described [21].

RESULTS AND DISCUSSION

Figure 1 shows that replacement of one or both mesyl groups in busulphan by oxirane groups leads to a change of the typical "Myleran blood response pattern" [4] towards the typical "mustard type" response pattern. The steady prolonged fall in neutrophils and similar but very much less intense fall in lymphocytes shown in the response to busulphan is replaced by a rapid fall in both neutrophils and lymphocytes, the depression of neutrophils however being more severe than that of lymphocytes. The neutrophil depression is followed by rapid recovery and a period of very marked transient neutrophilia which is characteristic of a "mustard response pattern" [5]. Platelets do not show the initial rapid moderate fall typical of many "nitrogen mustard type" compounds,

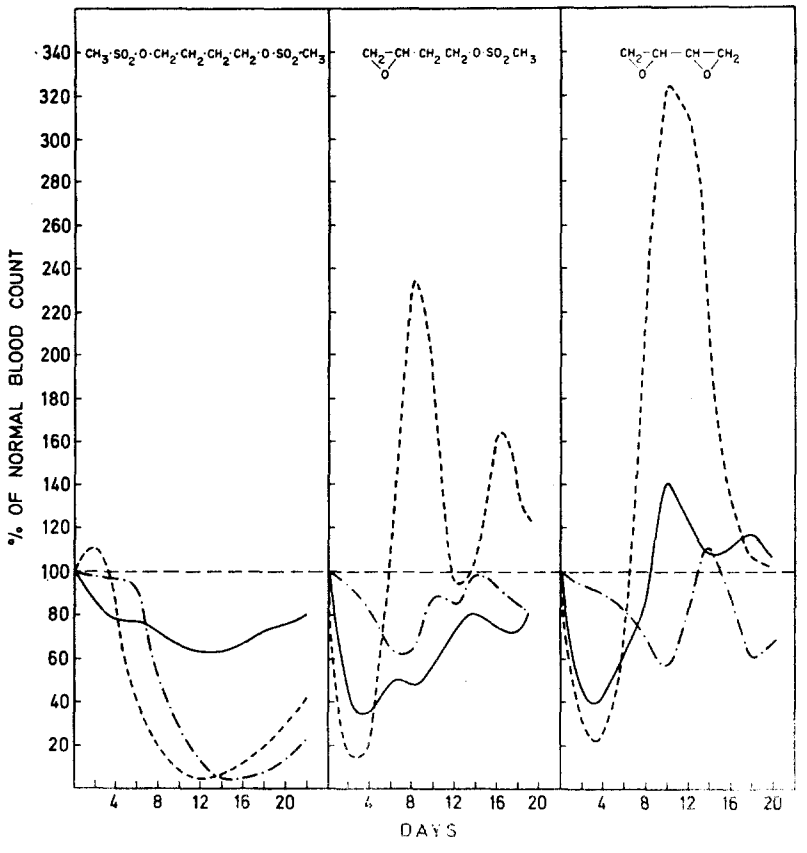


Fig. 1. *Effect on the haematological response in the rat of replacement of the mesyl groups of busulphan (Myleran®) by oxirane groups. Busulphan* 15 *mg/kg* (I); 1,2-*epoxy*-4-*methanesulphonoxybutane* 500 *mg/kg* (II); 1,2-3,4-*Diepoxybutane* 50 *mg/kg* (V).

Key for Figs. 1–4:
– – – – – neutrophils
——— lymphocytes
–·–·–·– platelets

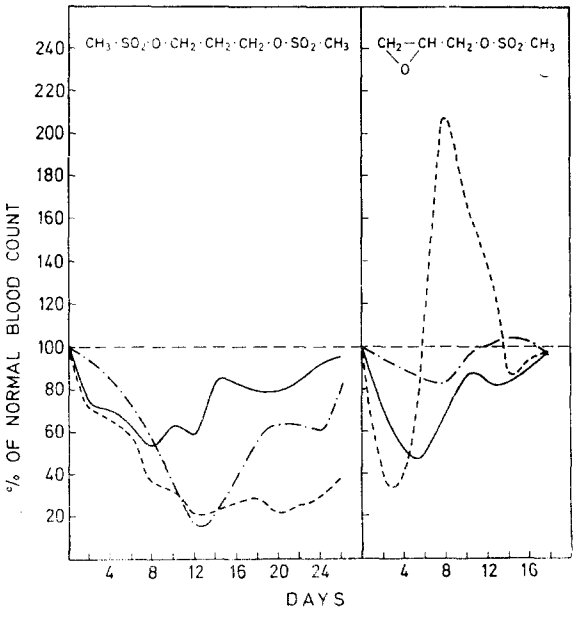


Fig. 2. *Effect of replacement of one mesyl group in* 1,3-*dimethanesulphonoxypropane by an oxirane group.* 1,3-*Dimethanesulphonoxypropane* 40 *mg/kg* (VI); 1,2-*epoxy*-3-*methanesulphonoxypropane* 500 *mg/kg* (VII).

but show a slight and less steep fall, followed by a fairly rapid recovery to normal numbers. Both epoxy compounds are very much less toxic than busulphan and much less pharmacologically active—doses greater than 500 mg/kg are required to produce a similar depression of blood neutrophils to that produced by 15 mg/kg of busulphan.

Figure 2 shows a similar haematological response for the corresponding three carbon homologues. The effect is the same as for busulphan (cf. Fig. 1), i.e. a loss of "Myleran like" character of blood response pattern and development of the characteristics of a "mustard type" pattern, which, however, shows greater depression of neutrophils than of lymphocytes. Again, the epoxy compound (VII) shows much reduced toxicity and pharmacological activity, a dose of 500 mg/kg being required to produce neutrophil depression comparable to that produced by 40 mg/kg of (VI).

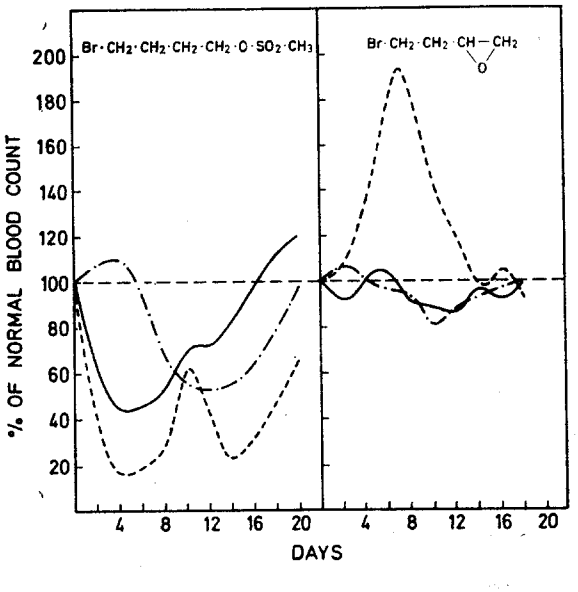


Fig. 3. Effect of replacement of one mesyl group of busulphan by bromine, and of replacement of one mesyl group by bromine and the other by an oxirane group. 4-Bromobutylmethanesulphonate 300 *mg/kg* (VIII); 1,2-*Epoxy*-3-*bromobutane* 250 *mg/kg* (IX).

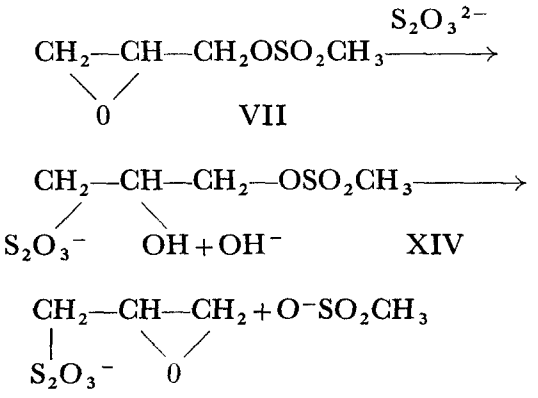
With compound (VIII) in Fig. 3, the "Myleran like" nature of the blood response pattern is largely retained, but modified in that a more rapid fall in neutrophils and a more rapid and rather more severe fall in lymphocytes occurs. A partial, but abortive recovery of neutrophils occurs at about the time of the marked neutrophilia shown in the typical "nitrogen mustard type" blood response pattern. Platelets show the same type of response pattern as for busulphan, but the severity of the fall is much less. The dose required to produce an equivalent neutrophil fall (300 mg/kg) is considerably greater than for busulphan (15 mg/kg), but much less than for dibromomannitol (900 mg/kg). The haematological response produced by (VIII) is similar to that shown by X-radiation [22]. As shown by the lack of effect of the bromo-epoxide (IX) replacement of both mesyl groups of busulphan leads to loss of pharmacological activity on both the haemopoietic system and on the Walker tumour (see Table 2).

The rather complex blood response pattern of mannitol myleran (X) may be explained by its partial conversion *in vivo* to the diepoxy derivative, 1:2,5:6-dianhydro-*D*-mannitol (XII) [20], both mesyl groups having been replaced by epoxy groups. This replacement *in vivo* could be a stepwise procedure, and part of the pharmacological effects of mannitol Myleran (X) could be due to the mono-epoxy compound (XI). As might be expected (XI) shows an intermediate type of blood response pattern between that of (X) and (XII) (Fig. 4). It shows a rather stronger relative depressive action on lymphocytes which are depressed to nearly the same extent as neutrophils. Recovery of neutrophils is more rapid than recovery of lymphocytes, but leads to only a very slight degree of transient neutrophilia. It has only a slight depressing action on platelets. Its leukocyte depressing activity is considerably greater than that of (X) but less than that of (XII).

Compound (XI) gives a good therapeutic index [16] against the Walker rat carcinoma (see Table 2). Its blood effects are relatively slight—70% depression of leukocytes and 20% depression of platelets at a dose of 150 mg/kg which is very nearly the LD_{50} (160 mg/kg). It could be considered a candidate for chemotherapy of solid tumours.

An attempt was made to estimate the relative alkylating abilities of the epoxy and mesyl groups, by a comparison of their reactivities towards a typical nucleophile (thiosulphate). The results are shown in Table 1. In all cases the velocity constant $k_g^{S_2O_3^{2-}}$ was obtained by a first order plot, i.e. plotting $\log_{10}$ (a–x) as ordinate and time (t) as abscissa, where the symbols have their usual significance. The slope of this line gives $-k/2{\cdot}303$.

With compounds (VII) and (XI) a competitive reaction commences when the concentration of intermediate (XIV) reaches a certain level, and a simple first order plot is not obeyed over the whole of the reaction.



The compounds (II, III and IV) in which the two alkylating centres are similar have epoxide —alkanesulphonoxy ratios which are very close, as might be expected. For these three compounds the ratio of the reactivity of the two ends is about 2:1, i.e. the epoxide grouping would be expected to react twice as rapidly with thiol in biological systems, as would the alkanesulphonate group. It is not surprising therefore, that (II) exhibits a predominantly "epoxide" effect on the blood elements (Fig. 1). Compounds (III) and (IV) show a similar haematological response to compound (II) and so are not illustrated in Fig. 1. Similarly with compounds (VII) and (XI) the epoxide group is more reactive towards nucleophiles than the methanesulphonoxy group and the overall "epoxide" response in Figs. 2 and 4 for these compounds could be explained by this enhanced reactivity.

Quantitative correlation is obtained between the lymphocyte to neutrophil depression for the compound (L/N) and log of the ratio of the reactivities of the two alkylating functions

$$= k_{\mathbf{g}}^{'S_2O_3^{2-}}/k_{OSO_2R}^{'S_2O_3^{2-}}$$

The regression equation is of the form:

$$L/N = 0{\cdot}37 \log k^{'S_2O_3^{2-}}/k_{OSO_2R}^{'S_2O_3^{2-}} + 0{\cdot}56$$
$$^{n}4\ ^{r}0{\cdot}959\ ^{s}0{\cdot}107$$

where n is the number of data points,
r is the correlation coefficient and
s is the standard deviation

neglecting compound (IV) which shows the biggest deviation from the line. Thus the overall haematological response would seem to be very dependent on the relative alkylating ability of the two functional groups.

For 4-bromobutyl methanesulphonate (VIII) calculation [23] shows that for sulphur containing nucleophiles $k_N^{Br}/k_N^{OSO_2CH_3} = 100$, while for nitrogen-containing nucleophiles $k_N^{Br}/k_N^{OSO_2CH_3} = 10$. Thus the alkyl bromide part of (VIII) might be expected to react with protein SH compounds while the mesyl end could react with nucleotide bases leading to an enhanced protein—DNA cross linking reaction [24] over that anticipated with busulphan. This may also explain the higher therapeutic index of (VIII) (Table 2) over

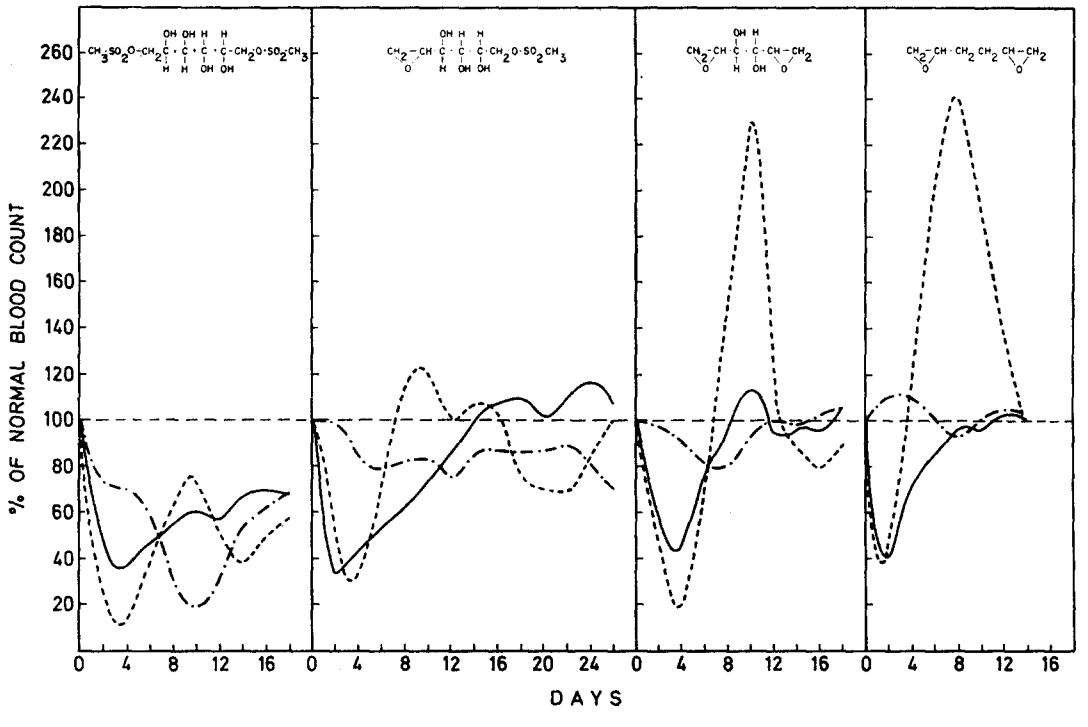
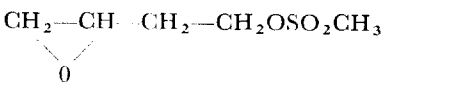
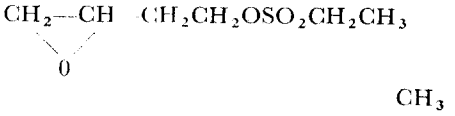
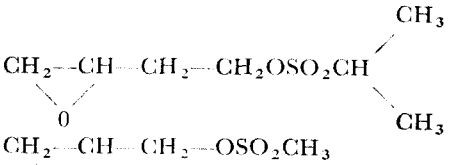
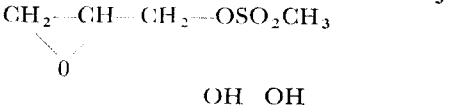
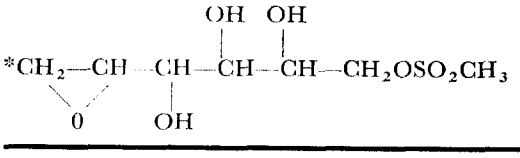


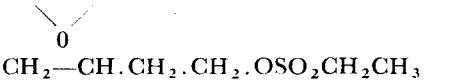
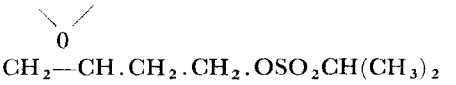
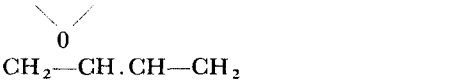
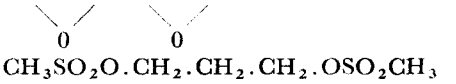
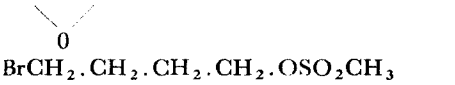
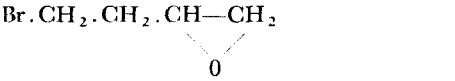
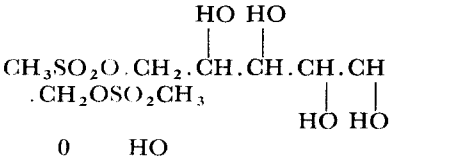
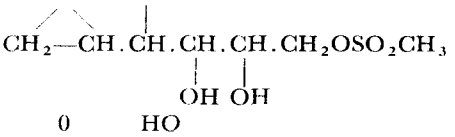
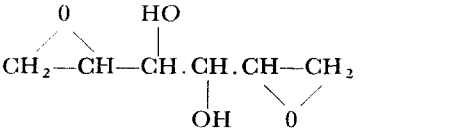
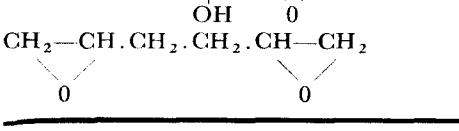
Fig. 4. Effect of replacement of the mesyl groups of mannitol Myleran by oxirane groups. Mannitol Myleran 250 *mg/kg* + *glucose* (X); 1, 2-*anhydro*-6-0-*methanesulphonoxy*-D-*mannitol* 150 *mg/kg* (XI); 1,2:5,6-*dianhydro*-D-*mannitol* 8 *mg/kg* (XII); 1,2:5,6-*diepoxyhexane* 90 *mg/kg* (XIII).

Table 1. *Velocity constants* $k'_{S_2O_3^{2-}}$ *and* k'_{OSO_2R} *towards thiosulphate at* 215°

Compound	No.	$k'_{S_2O_3^{2-}}$ $min^{-1} \times 10^3$	k'_{OSO_2R} $min^{-1} \times 10^3$	$\frac{k'_{S_2O_3^{2-}}}{k^1_{OSO_2R}}$
	II	14·4	6·42	2·24
	III	13·6	6·88	1·98
	IV	15·3	6·55	2·34
	VII	13·7	3·07	4·46
	XI	23·9	1·72	13·9

*Determined in purely aqueous solution.

Table 2. *Tumour inhibition tests against the Walker* 256 *rat carcinoma, ether-water partition coefficients and hydrolysis rates at* 60° *pH* 7

Compound	No.	LD_{50} (mg/kg)	ED_{90}† (mg/kg)	T.I.‡	PC^{Ether/H_2O}	$10^3\ k_{H_2O}$ min^{-1}
$CH_3SO_2OCH_2CH_2CH_2CH_2OSO_2CH_3$	I	20	18	1·1		7·36*
	II	640	304	2·1	1·6	2·59
	III	710	620	1·1	1·7	1·85
	IV	865	580	1·5	2·3	1·71
	V	70	35	2·0	1·0	—
$CH_3SO_2O.CH_2.CH_2.CH_2.OSO_2CH_3$	VI	70	−ve	—	—	—
	VII	420	−ve	—	6·8	1·99
$BrCH_2.CH_2.CH_2.CH_2.OSO_2CH_3$	VIII	350	100	3·5	42·8	5·21*
	IX	500	−ve	—	309	0·94
	X	2000	200	10	—	—
	XI	160	10	16	0·04	15·42
	XII	14	3	4·7	0·02	—
	XIII	100	75	1·3	—	—

*$k_2/k_1 > 100$.

† −ve, not active at tolerated dose.

‡T.I. = therapeutic index (LD_{50}/ED_{90}).

busulphan, as well as the slightly different haematological response shown in Fig. 3.

Although the overall picture is complex, there is an indication that the "mustard-like" haematological effects are due to reaction with cellular thiol groups and that the "busulphan like" effect may be due to reaction with oxygen and nitrogen nucleophiles in biological systems.

The tumour inhibition tests against the Walker 256 rat carcinoma are shown in Table 2, together with the ether-water partition coefficients and hydrolysis rates of the alkanesulphonates and bromides at 60°C.

The anti-tumour effects of compounds (I) to (V) are of a low order and relatively little difference is made by replacing mesyl by oxirane. These compounds have 4-carbon atoms in the difunctional alkylating chain whereas the inactive compounds (VI) and (VII) only have 3-carbon atoms in the chain. This suggests that either cycloalkylation or cross-linking of groups 5–7°A apart could be responsible for the carcinostatic effect. The relatively high therapeutic index of compound (VIII) and the inactivity of (IX)—the compounds have comparable host toxicity—is unexpected. Compounds (X), (XI) and (XII) all show appreciable carcinostatic activity with good therapeutic indices. There is a considerable increase in potency on introducing an oxirane group.

The relatively high indices observed for these compounds appear to be related to their high water solubility. Also compound (IX) is 7·2 times more soluble in ether than compound (VIII) and this could account for the difference in their activity.

Although no quantitative correlation exists between activity and partition coefficient, a good relationship does exist between the ED_{90} and the velocity coefficient for hydrolysis of the alkanesulphonate. For alkanesulphonates with 4 or more carbon atoms separating the alkylating centres, the regression equation of log $^1/ED_{90} \times 10^4$ on log $k^{H_2O}_{OSO_2R} \times 10^3$ is

$$\log {}^1/ED_{90} = 2{\cdot}22 \log k - 0{\cdot}1\ {}^n6\ {}^r0{\cdot}955\ {}^s0{\cdot}109$$

which accounts for 95% of the variance. Thus no compound with a log k less than 0·08 (i.e. $k = 1{\cdot}2 \times 10^3\ min^{-1}$) will show total inhibition of the Walker tumour. The bromo-epoxide (IX) with $k = 0{\cdot}94 \times 10^3\ min^{-1}$ would not be expected to show activity which correlates with the observed result (Table 2). The regression equation also shows the importance of di-alkylation for effective tumour inhibition. Thus, although the oxirane group of (IX) is as reactive towards nucleophiles as that of a comparable agent which shows activity, e.g. (XI) [25], the low reactivity of the second functional group as measured by its hydrolysis rate must account for its failure to effectively inhibit the growth of the Walker tumour.

4-Bromobutyl methanesulphonate (VIII) appears to be potentially useful as a cancer chemotherapeutic agent showing a therapeutic index on the Walker tumour three times that of the clinically useful busulphan (I), with less depression on platelets (Fig. 3). It could be of use in chronic myeloid leukemia in patients showing diminishing response to busulphan, but in whom further increase in dosage of busulphan is precluded because of already low platelet count.

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Plasma Prolactin in Breast Cancer

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Abstract—*Plasma prolactin levels have been measured in normal women and in women with benign and malignant breast disease. No difference in circulating prolactin levels has been demonstrated between any of these groups of patients.*

INTRODUCTION

IT HAS been recognized for a number of years that pituitary prolactin has a powerful stimulating effect on the breast and on some types of breast cancer in experimental animals [1–4].

The demonstration of prolactin in cultured human and monkey pituitary glands by both Pasteels [5] and also by Friesen and his colleagues [6] allowed the development of specific radioimmunoassays for the hormone in human plasma [7, 8].

This communication describes the measurement of human prolactin in the plasma of patients with breast disease by means of a heterologous radioimmunoassay system.

MATERIAL AND METHODS

1. *The assay*

Human prolactin was determined by a heterologous double antibody radioimmunoassay. Standard human pituitary prolactin (71/222) and a human reference plasma (71/167) containing a high concentration of prolactin were obtained from the WHO International Laboratory for Biological Standards. Rabbit antiserum to human prolactin (AHPrS-R) and ovine pituitary prolactin (OPr) (NIH-P-S8) were gifts from Dr. H. G. Friesen (Montreal) and Dr. V. C. Stevens (Columbus, Ohio) respectively. Antiserum to rabbit globulins (ARGS-S) was raised in sheep.

A series of standard solutions were prepared by diluting human prolactin (71/222) in 0·05 M-phosphate buffer pH 7·5, containing 5 mg/ml human albumin and 0·01% merthiolate (HSA buffer). AHPrS-R was diluted (1/400) in 0·05 M-phosphate buffer pH 7·5, containing 0·05 M-EDTA. ^{131}I-labelled OPr was prepared by a modification of the method of Greenwood *et al.* [9], and diluted in HSA buffer to a concentration of 5 ng/ml. ARGS-S was diluted (1/9) in HSA buffer.

The assay protocol was as follows. One hundred μl of each solution of standard human prolactin (71/222) was treated with 200 μl AHPrS-R (1/400) and 100 μl HSA buffer. The solutions were mixed and incubated at 4°C for 2 days. Then 100 μl ^{131}I-labelled OPr was added, mixed and incubated at 4°C for a further two days. Finally, 200 μl ARGS-S (1/9) was added. After mixing, the solutions were left overnight at 4°C. They were then centrifuged at 1500 g for 30 min at 4°C, the supernatants were decanted and the tubes counted in an auto-γ-counter. Radioactivity precipitated by antibody was expressed as a percentage of that bound in the absence of added standard prolactin. The results were plotted on linear-linear or on probability-log scales. The latter procedure usually gave a straight line relationship. When plasma samples were assayed, standard solutions of prolactin were replaced by 100 μl of the unknown plasma and plasma (100 μl)

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from a hypophysectomized patient was included in the standard curve. Concentrations of prolactin were expressed in terms of milliampoules of standard.

The mean standard curve for ten assays is shown in Fig. 1. Human growth hormone, human follicle stimulating hormone, human luteinizing hormone, human thyroid stimulating hormone and human chorionic somatomammotrophin did not interfere with the assay system. The addition of plasma from an hypophysectomized patient reduced binding of ^{131}I-labelled OPr from 35 to 28% of the total counts added in the absence of standard prolactin. Reference plasma (71/167), which contained a high concentration of prolactin, inhibited binding of ^{131}I-labelled OPr to antibody. Serial dilutions of this plasma in hypox. plasma gave a line parallel to that of the standard (Fig. 2).

Human amniotic fluid contains high concentrations of prolactin [7]. Human mid-term amniotic fluid was extracted for prolactin by a modification of the method of Simkin and Goodhart [10]. Serial dilutions in buffer of this extract and a prolactin-rich fraction of human pituitary glands gave a curve parallel to that of the standard (Fig. 3).

2. *Clinical study*

Plasma prolactin was estimated in 90 women of whom 38 were in Cardiff and 52 in Edinburgh. Ten patients had been admitted to hospital for the treatment of conditions other than breast disease; 12 had benign disease of the breast; 27 had primary breast cancer and 41 had metastatic breast cancer, of whom 29 had localised disease and 12 generalised disease as defined previously by us [11]. Twenty-four were pre-menopausal (having regular periods or up to 2 years from their last menstrual period); 17 were menopausal (2–5 years since their last menstrual period) and 48 were post-menopausal (more than 5 years from their last menstrual period).

In most cases, blood was collected by venepuncture at random times after admission to hospital but in a few patients samples were collected as outpatients. In patients admitted for breast biopsy or mastectomy collections of blood were made before operation. In patients with advanced disease, blood samples were collected before any form of treatment was initiated. As far as could be ascertained, no patient was receiving phenothiazines, L-dopa, monoamine oxidase inhibitors or other drugs known to affect the secretion of prolactin.

Following collection, venous blood was placed in heparinized containers which were immediately refrigerated, centrifuged and the plasma stored at −30°C. All samples were stored under identical conditions but the time of storage varied, the maximum period being 20 months.

RESULTS

As the circulating levels appeared to have a log normal distribution, the logarithms of the measured values in milliampoules/ml of WHO 71/222 reference standard were used in the analysis. No differences were noted between the hospital controls, the patients with benign breast disease or the patients with primary or advanced breast cancer (Fig. 4, Table 1).

There was no significant difference in the ages of the patients studied, except for those with benign disease who were younger than the other groups.

The patients with breast cancer were divided into pre-menopausal, menopausal and post-menopausal groups. The mean levels of prolactin were significantly lower in the post-menopausal group, compared with the combined pre-menopausal and menopausal groups ($P < 0{\cdot}05$, Table 3).

DISCUSSION

These preliminary results indicate that women with breast cancer do not show any striking differences in circulating prolactin levels compared with a small group of hospital control women. They also show that women with advanced breast cancer of either localized or generalized type do not have different levels from those with primary disease. Nor was any difference noted in the circulating levels of prolactin in women with benign disease compared with any of the other groups. Women with benign disease were significantly younger than either the hospital controls or breast cancer patients. Yet, when they were compared with women of similar menstrual status with breast cancer, no difference was seen.

There is a need to define more clearly the relationship between age, menstrual status and plasma prolactin in normal women, before accepting unequivocally that women with breast disease have normal levels for their age. This we are currently doing.

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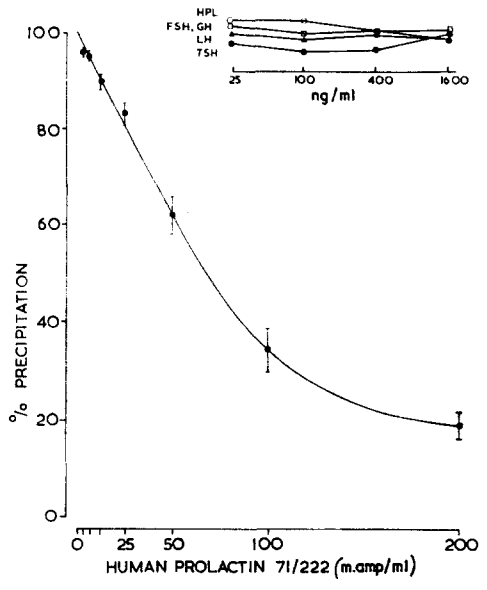

Fig. 1. *Standard curve for Human Prolactin* (71/222) *diluted in HSA buffer. Mean + SEM (10 assays) HPL—Human Chorionic Somatomammotrophin (WHO* 70/194).

HGH—Human Growth Hormone (1*st IRP HGH*).
FSH—Human Follicle Stimulating Hormone(CPD/8 Butt).
LH—Human Luteinizing Hormone (*IRC-2-Hartree* (10.12. 64)).
TSH—Human Thyroid Stimulating Hormone (*DE* 32.2 *Hartree* (28.6.69)).

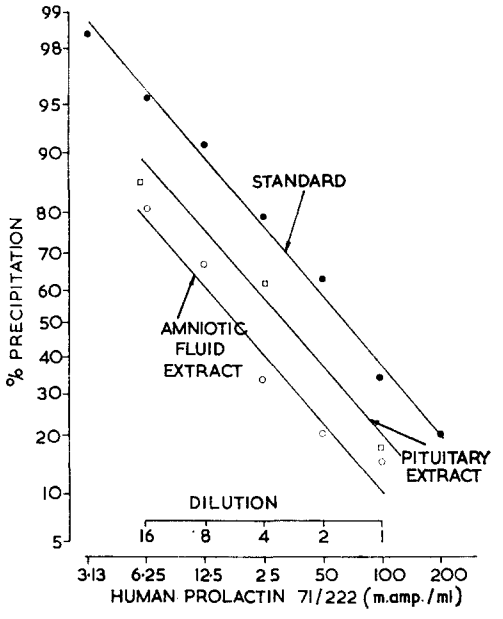

Fig. 3. *Dilution curves in buffer of Human Prolactin* (71/222), *pituitary extract and amniotic fluid extract.*

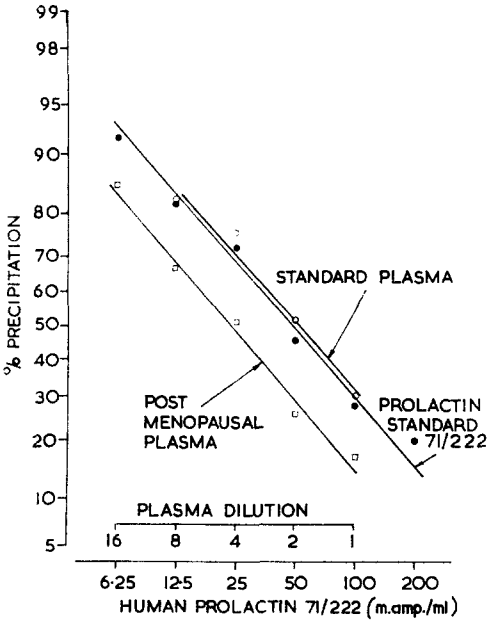

Fig. 2. *Standard curve for Human Prolactin* (71/222) *diluted in plasma from a hypophysectomized patient. Serum Standard Plasma* 71/167 *and post-menopausal plasma also diluted in hypox. plasma.*

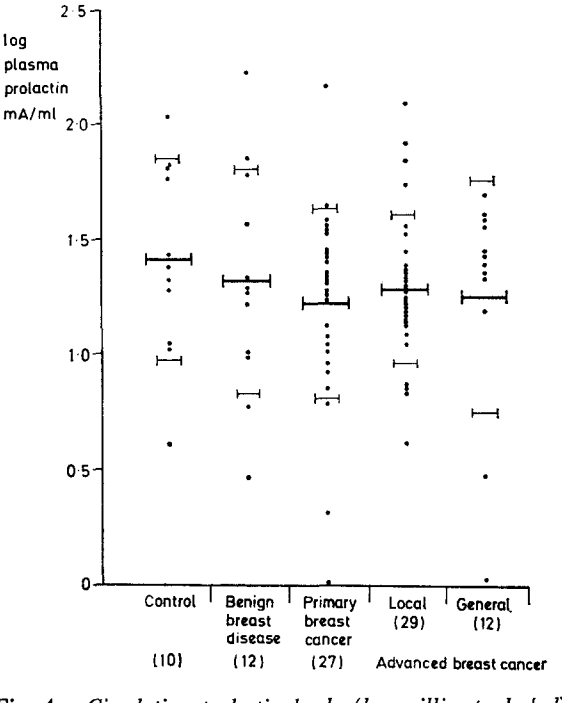

Fig. 4. *Circulating prolactin levels* (*log milliampoules/ml*) *in patients with breast cancer, benign breast disease and hospital controls.*

Table 1.

	No. in Group	Mean age ± S.D.	Mean log Prolactin ± S.D.
Controls	10	56·0 ± 14·3	1·411 ± 0·44
Benign breast disease	12	38·8 ± 8·6	1·313 ± 0·48
Primary breast cancer	27	58·8 ± 12·2	1·214 ± 0·42
Localised advanced breast cancer	29	60·8 ± 12·9	1·279 ± 0·32
Generalised advanced breast cancer	12	53·8 ± 10·1	1·245 ± 0·50

Mean age and circulating prolactin levels (log Milliampoules/ml) in groups of patients studied.

Table 2.

	No. in group	Mean log prolactin ± S.D.
Benign breast disease	11	1·316 ± 0·51
Breast cancer	11	1·259 ± 0·48

Circulating prolactin levels (log Milliampoules/ml) in pre-menopausal patients with benign breast disease and breast cancer.

Table 3.

	No. in group	Mean log prolactin ± S.D.
Post-menopausal	42	1·171 ± 0·37
Pre + menopausal	25	1·384 ± 0·39

Circulating prolactin levels (log Milliampoules/ml) according to menstrual status in patients with breast cancer.

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The Biochemical Mechanism of Selective Heat Sensitivity of Cancer Cells—IV. Inhibition of RNA Synthesis

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Abstract—*The mechanism by which exposure of ascites tumour cells to supranormal temperatures causes an irreversible inhibition of uridine incorporation into RNA was investigated. Heat-treated cells were still able to incorporate labeled nucleotides, and even nucleosides, into RNA, if these precursors were added at sufficiently high concentrations.*

The passive permeability of the cell membrane increased exponentially with temperature, but this increase was fully reversible. At variance with the results obtained with agents which increase cell permeability, or which inhibit nucleoside permeation, heat treatment of Ehrlich ascites cells did not modify the ability of labeled uridine to be metabolized by these cells. The inhibition of uridine incorporation following heat treatment cannot therefore be attributed to a damage at the cell membrane level; the experimental data can be tentatively accounted for by the hypothesis of a block in the maturation of pre-rRNA into rRNA, with reutilization of the pre-rRNA degradation products.

INTRODUCTION

PREVIOUS work from this laboratory had shown that exposure to 42–44°C of cells prepared from various malignant tumours resulted in an irreversible biochemical damage [1, 2, 3]. The heat sensitivity of tumour cells, although with some variation depending on the type of tumour, appeared to be a widespread, if not general, character of either experimental or spontaneous mammalian neoplasms; regenerating liver cells, used as controls, remained unaffected in the same experimental conditions. These findings, which have been confirmed by other authors [4, 5, 6], have found application, with satisfactory results, in the therapy of solid tumours in experimental animals [6, 7] as well as in human subjects [1, 8, 9, 10].

The inhibitory effect of supranormal temperatures on some biochemical parameters of tumour cells disappeared in cell-free preparations, and showed some similarities to the inhibition caused by agents which increase membrane permeability [2, 3, 11]. Other experiments, indicating that the "heat treatment" brought about an early and irreversible alteration of transcellular migration of glutamate through tumour cells [12], and possibly also a modification of the immunogenicity of these cells [13], have seemed to substantiate the hypothesis of a damage at the level of the cell membrane [9, 14]. Since uridine incorporation into RNA is one of the biochemical parameters most severely affected [3, 15], it seemed of interest to ascertain which step of this biosynthetic pathway was irreversibly modified by exposure of the cells to a high temperature, and whether the inhibition observed could be related to alterations of the diffusion and/or transport properties of the cell membrane. In order to deal with cells having a surely intact membrane, ascites tumour cells were used. Although a temperature of 42°C, or even of 41°C, was already able to produce a significant degree

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of inhibition, in the present work a temperature of 44°C was generally chosen, so as to make the phenomenon more evident.

MATERIAL AND METHODS

Ehrlich and Yoshida ascites tumour cells were harvested 6 to 8 days after i.p. transplantation of 4–7 million cells in male Swiss mice or Wistar rats, respectively. Sarcoma I was obtained in the ascites form through the courtesy of Dr. Nathan Kaliss, The Jackson Laboratory Bar Harbor, Maine, and maintained by i.p. inoculation in female (C57BL/6J × A/J)F_1 mice. Cell viability was checked by the dye exclusion test, using trypan blue, 1 mg/ml in isotonic NaCl [16]. Only non-hemorrhagic cell samples containing more than 90% viable cells were used. In most experiments the cells were washed and resuspended in Krebs–Ringer bicarbonate solution; alternatively, medium 199, Eagle's minimal essential medium n° 109 or Hank's balanced salt solution were also used.

Chemicals were usually purchased from E. Merck, Darmstadt, Germany, or from British Drug Houses, Poole, England. Nucleotides from Boehringer, Mannheim, Germany. Radiochemicals from the Radiochemical Centre, Amersham, England. Culture media from Grand Island Biological Co., Grand Island N.Y., or from Difco Laboratories, Detroit, Mich., U.S.A.; fetal calf serum from Microbiological Associates, Bethesda, Md. Lucensomycin was a gift of Farmitalia, Milan, Italy, and persantin of C. H. Boehringer u. Sohn, Ingelheim, Germany.

Radioactive samples were dissolved in 1 ml of soluene (purchased from Packard Instrument Co., Downers Grove, Ill., U.S.A.) and counted, in a standard toluene-PPO-POPOP solution, on a Packard Tri-Carb model 3380 liquid scintillation counter.

When dealing with intact cells, RNA biosynthesis was simply evaluated by adding ^{3}H- or ^{14}C-labelled uridine to a suspension of 2–5 million cells/ml, and by following, through repetitive sampling, the increase in time of the label in the acid-insoluble material. In the conventional assay, uridine-5-^{3}H (spec. act. = 5 Ci/mmole) was added, at 0·4 μM final concentration, to the cell suspension at 30°C; under these conditions, the incorporation was, after a 3–4 min delay, linear with time for more than 20 min [17].

The incorporation of labeled nucleoside or nucleotide in the RNA of lucensomycin-treated cells was estimated as previously described [17], i.e. in Krebs–Ringer bicarbonate

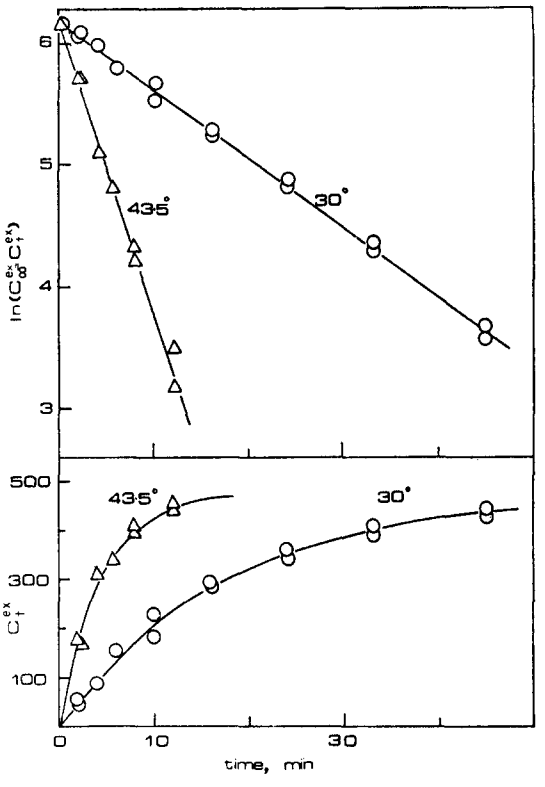

Fig. 1. Fluorescein efflux from ascites cells. Fluorescein efflux, from a suspension of Ehrlich ascites cells preloaded with the dye, was followed by repetitive sampling and centrifugation at 1000 × **g** *during an incubation at* 30°C *and* 43·5°C, *in Krebs–Ringer bicarbonate solution. Cells concentration:* 6×10^6/ml. *The concentration of fluorescein in the psernatant,* C_t^{ex}, *is expressed as absorbance (at* 490 *nm)* $\times 10^3$. *The data are either shown as such (below) or after conversion to a semi-logarithmic plot (above).*

containing 3·7 mM ATP, 2·2 mM GTP, 0·3 mM CTP, 50 mM mercaptoethanol, 3mM $MnCl_2$, 60 mM Tris-HCl buffer (final pH 7·6), and appropriate amounts of labeled uridine, UMP or UTP (see Table 2). The lucensomycin to cell ratio was 5 μg of polyene/million cells, so as to ensure full permeabilization of the cell membranes. Previous experiments had shown [17] that, under such conditions, the labeled precursor is incorporated into RNA, and that the rate of incorporation remains constant for at least 15 min at 30°C.

For the evaluation of the relative amounts of uracil, uridine, and uridine nucleotides in the acid-soluble material, the cells were taken at various times after addition of the label and lysed, without separating them from the incubation medium, with Triton X-100 (1%, v/v, final concentration); after addition of 0·4 M $HClO_4$, the supernatant was subjected to descending chromatography on Whatman 1 paper, using water-saturated *n*-butanol + 15 N ammonia (100:1, v/v) as developing solvent [18]. The chromatograms were then analyzed with a radiochromatogram scanner Packard model

7201, and the various components estimated from the areas of their peaks. Under normal conditions, the labeled acid-soluble material was distributed in three spots, corresponding to uracil (Rf = 0·33), uridine (Rf = 0·10) and uridine nucleotides (Rf = 0·02).

Cell permeability was evaluated by following, through repetitive sampling, the efflux of fluorescein from cells which had been loaded with the dye by previous incubation with fluorescein diacetate, as described by Celada and Rotman [19]. As shown in Fig. 1, fluorescein efflux followed an exponential law: if we indicate by C_t^{ex} the concentration of fluorescein in the extracellular fluid at a given time t and by C_∞^{ex} its concentration after at least 60 min at 45°C (i.e. after an "infinite" time), a plot of $\ln (C_\infty^{ex}-C_t^{ex})$ *versus* time yields a straight line, the slope of which may be considered the rate constant of the exit process.

RESULTS

Effect of "high" temperature on nucleoside incorporation into RNA

Figure 2 shows the effect of a relatively short preincubation at different temperatures on the ability of Ehrlich ascites cells to incorporate, during a subsequent assay at 30°C, labeled uridine into RNA. The results obtained with two different batches of Ehrlich cells, suspended in Krebs–Ringer bicarbonate, are illustrated. It can be seen that exposure to 44°C causes a dramatic inhibition of RNA synthesis, the rate of uridine incorporation decreasing apparently as an exponential function of the preincubation time. In some cases (Fig. 2*b*), a certain degree of inhibition was already produced by preincubation *in vitro* at a physiological temperature (37–38°C); this behaviour was, however, not constant, being exhibited only by certain batches of cells, and being much reduced by the use of richer media, such as Eagle's 109 or medium 199 (Table 1). The inhibitory effect of exposure to a supranormal temperature was instead more constant, and was not affected by use of richer media. On the other hand, dye exclusion tests did not allow to attribute this effect to mere loss of cell viability, more than 80% of the cells remaining "viable" after 1 hr at 44°C. Sarcoma I and Yoshida ascites cells gave, as a rule, results similar to those of Fig. 2(*a*).

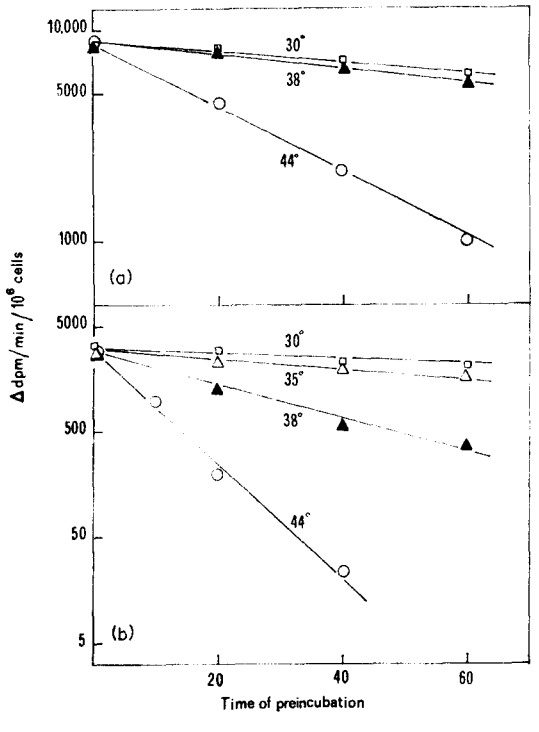

Fig. 2. Effect of preincubation at different temperatures on the ability of Ehrlich ascites cells to incorporate, at 30°, uridine into RNA.

The cells, 1.6 × 10⁶/ml, were suspended in Krebs–Ringer bicarbonate; incorporation was started, after equilibration at 30°, by addition of 2 μCi/ml of uridine—5—³H (spec. act. = 5 Ci/mmole). (a) and (b) refer to two different batches of cells.

Evaluation of the RNA polymerase activity, and dependence of heat inhibitory effect upon precursors concentration

Addition of the polyenic antibiotic lucensomycin to cell suspensions had been found [17] to increase the permeability of the cell membrane, so that the RNA polymerase activity could be directly evaluated from the ability of lucensomycin-treated cells to incorporate the uridylic moiety of labeled UTP into RNA. As shown in Fig. 3, by such an assay the difference between untreated and heat-treated cells was of moderate entity.

Since lucensomycin-treated cells are still able to utilize labeled UMP or even labeled uridine for RNA synthesis, provided that other nucleotides be present and that the assay be performed with a high (≧ 0·8 mM) concentration of the labeled nucleoside or nucleotide [17], the effect of supranormal temperatures on these parameters was also investigated. As indicated in Table 2, in all cases exposure to heat had only minor effects. In fact, it could be shown that, even in the absence of lucensomycin, the use of a high uridine concentration relieved the inhibition by heat (Table 2, line 2).

Uridine metabolism in Ehrlich ascites cells

The data so far obtained seemed to support the hypothesis of an inability of heat-treated cells to utilize uridine for the synthesis of RNA,

Table 1. *Effect of the composition of the suspension medium on the heat sensitivity of Ehrlich ascites cells and of Sarcoma I ascites cells*

Type of cells	Suspension medium during preincubation	Temperature of preincubation	dpm/min/10^6 cells
Ehrlich	Krebs–Ringer bicarbonate	30°	8280
		37°	6120
		44°	728
Ehrlich	199	30°	10150
		37°	9980
		44°	1020
Sarcoma I	Krebs–Ringer bicarbonate	30°	10230
		37°	10100
		44°	2880
Sarcoma I	199	30°	10760
		37°	11530
		44°	3270

The cells were suspended in Krebs–Ringer bicarbonate or medium 199 at a concentration of 35×10^6 cells/ml, then diluted ten-fold with either Krebs–Ringer bicarbonate or medium 199, and preincubated for 30 min at 30°, 37° or 44°. Each stock of cells was then subjected to a two-fold dilution with either medium 199 or Krebs–Ringer bicarbonate, so that the final incorporation medium was the same for all cell samples, i.e. contained equal amount of Krebs–Ringer bicarbonate and medium 199. After addition of 2·5 μCi of ^{3}H-uridine/ml, the incorporation of the radioactive label into acid-insoluble material was followed by repetitive sampling.

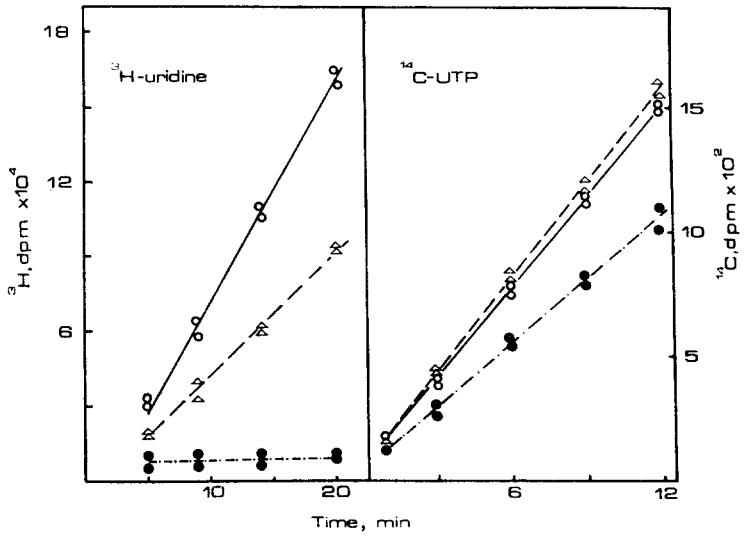

Fig. 3. *Temperature dependence of uridine or UTP incorporation into RNA of Ehrlich ascites cells.*

A suspension of 6×10^7 *cells/ml in Krebs–Ringer bicarbonate was preincubated at* 30° (○————○), 38° (△ – – – – △) *or* 44° (●–·–·–●) *for* 20 *min, then equilibrated at* 30° *for* 5 *min. The ability to incorporate uridine was then checked by diluting the suspension* 1:20 *in Krebs–Ringer bicarbonate containing* 5μ*Ci* ^{3}*H-uridine (spec. act. =* 5 *Ci/mmole)/ml; samples of* 0·6 *ml were taken in duplicate. The ability to incorporate UTP was checked by diluting the suspension* 1:10 *in a Krebs–Ringer bicarbonate solution additionned with CTP, ATP, GTP and cofactors (see Methods) and containing* 30μ*g lucensomycin/ml and* 1·5 μ*Ci.* ^{14}*C-uridine (spec. act. =* 53 *mCi/mmole)/ml; samples of* 0·5 *ml were taken. Data are expressed as dpm, of* ^{3}*H and* ^{14}*C respectively, in the acid-insoluble material from each sample.*

Table 2. Effect of nucleoside or nucleotide concentration on the heat sensitivity of intact or lucensomycin-treated Ehrlich ascites cells

Labeled substrate	Conc. of substrate μM	μg lucensomycin per million cells	pmoles of substrate incorp./min/10^6 cells, after 20 min preincubation at 30°C	38°C	44°C
uridine	1	—	0·82	0·61	0·04
uridine	800	—	3·62	3·56	2·21
uridine	800	5	0·63	0·54	0·38
UMP	51	5	0·20	0·22	0·14
UTP	28	5	0·21	0·25	0·17

Incorporation was performed at 30°. The medium was either Krebs–Ringer bicarbonate alone (for experiments in the absence of lucensomycin) or Krebs–Ringer bicarbonate with added nucleotides and cofactors, as indicated under Methods.

unless the nucleoside was in very relevant amounts. In order to ascertain whether or not uridine entered the cells, and could then be metabolized for further utilization, the distribution of the label in the acid-soluble fraction was investigated. The validity of this approach was checked by examining the effects of a cell-permeabilizing agent, lucensomycin, and of a substance which selectively inhibits nucleoside uptake [20], persantin. The results are illustrated in Table 3. It can be seen that, as it had been shown by Jacquez [21], only a small amount of uridine was, under normal conditions, phosphorylated, most of it being instead converted to uracil. Uracil could not be incorporated into RNA, nor had any effect on uridine incorporation into RNA. Persantin blocked both pathways, while in the presence of lucensomycin uridine was still degraded to uracil, but could not—at the concentrations used—be phosphorylated. In heat-treated cells uridine was still able to follow both pathways, indicating that neither its entrance nor its phosphorylation are affected; the amounts of uridine phosphates did not differ significantly from those found in untreated cells.

Temperature dependence of cell membrane permeability

Cell membrane permeability, as indicated by the rate $(-k)$ of efflux of fluorescein from dye-loaded cells, showed, in both Ehrlich and Yoshida ascites tumour cells, an exponential dependence from temperature (Fig. 4). An Arrhenius plot of $\ln(-k)$ vs. $1/T$ showed no discontinuity in the 24–46°C range, the activation energy being approximately 20 kcal/mole (Fig. 5).

The values of $(-k)$ were, for a given temperature, independent from the cell number (between 10^5 and 10^7 cells/ml), from the fluorescein concentration within the cells, and from the nature of the medium in which the cells had been suspended (Krebs–Ringer bicar-

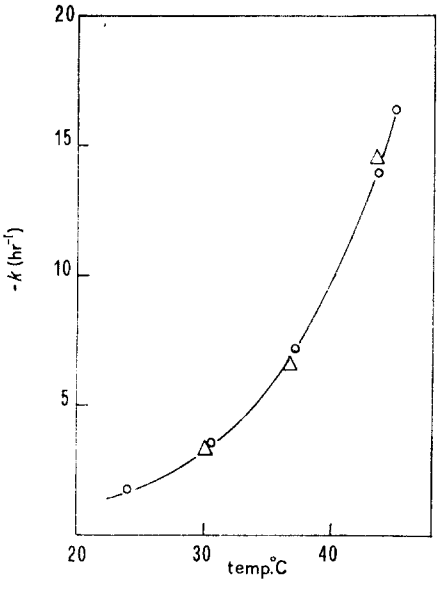

Fig. 4. Dependence of the rate (−k) of fluorescein efflux upon temperature, in Ehrlich (○) or Yoshida (△) ascites cells.

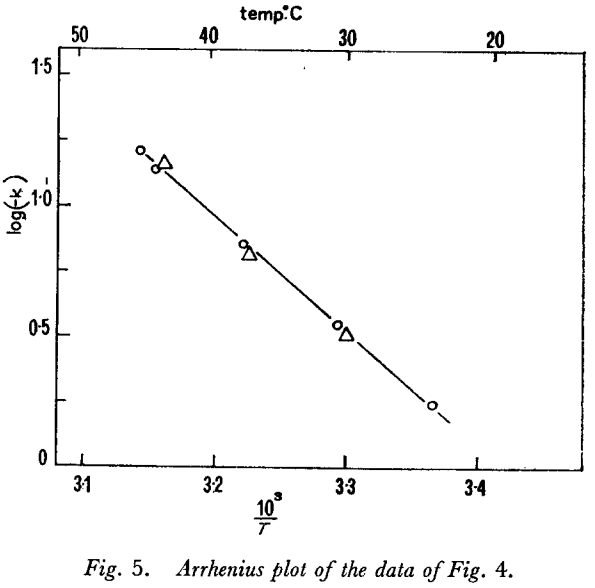

Fig. 5. Arrhenius plot of the data of Fig. 4.

Table 3. Effect of heat-treatment, lucensomycin and persantin on the fate of labeled uridine in Ehrlich ascites cells

		Exp. 1			Exp. 2		
		30 min preinc. at 30°C	30 min preinc. at 38°C	30 min preinc. at 44°C	No addition	+lucensomycin, 2 μg/10^6 cells	+persantin, 50 μM
Rate of incorporation into RNA, dpm/min/10^6 cells		471	480	184	800	10	10
Percentage distribution in acid-soluble material	labeled nucleotides	20; 21; 23	18; 30; 32	19; 30; 32	25; 36; 40	2; ~0; ~0	5; 4; 6
	uridine	48; 35; 14	51; 20; 7	47; 23; 10	63; 40; 25	77; 62; 52	89; 90; 84
	uracil	32; 44; 63	31; 50; 61	34; 47; 58	12; 24; 35	21; 38; 48	6; 6; 10

In both experiments, the suspension contained 7×10^6 cells/ml in Krebs–Ringer bicarbonate; incorporation, at 30°C, was started by addition of 0·75 μCi ^{14}C-uridine/ml (spec. act. = 58 mCi/mmole). The relative amounts of the various labeled components of the acid-soluble material are expressed as per cent of the total radioactivity in the supernatant after addition of Triton X-100 and $HClO_4$ (see Methods). The three numbers under each heading indicate the values after 5, 15 and 25 min, respectively, of incorporation. Incorporation into RNA was linear in the 5–25 min interval.

Table 4. Reversibility of the temperature-dependent variations of passive permeability

	Temperature and time of preincubation	Temperature of assay of cell permeability	$-k$ (hr^{-1})
Exp. 1	controls kept at 4°	30°	3·51
	20°, 30 min	30°	3·44
	37°, 30 min	30°	3·47
	42·5°, 30 min	30°	3·53
Exp. 2	—	42·5°	12·54
	42·5°, 6 min	30°	3·41

Exp. 1: Yoshida ascites cells, suspended in Krebs–Ringer bicarbonate, were kept for 30 min at 20°, 37° or 42·5°, then loaded with fluorescein diacetate, washed, resuspended in Krebs–Ringer bicarbonate at $1{\cdot}5 \times 10^7$ cells/ml, and the rate of fluorescein efflux determined at 30°.

Exp. 2: 10 ml of fluorescein-loaded Ehrlich ascites cells at 2×10^8 cells/ml were added to 40 ml of Krebs–Ringer bicarbonate equilibrated at 42·5°, and fluorescein efflux determined by sampling every 90 sec. After 6 min, 10 ml of the remaining cell suspension were transferred in 40 ml of Krebs–Ringer bicarbonate equilibrated at 30°, and the new rate of fluorescein efflux determined.

bonate, Hank's balanced salt solution, Eagle's minimal essential medium with or without fetal calf serum). The value of $(-k)$ was dependent only upon the temperature at which fluorescein efflux was measured, and not upon previous exposure of the cells to different temperatures (Table 4).

In previous work [14], an increased permeability to Trypan blue upon exposure to 42°C of Moloney-virus induced lymphoma ascites cells had been demonstrated. The phenomenon required a rather long lag-time (1 hr for the immunosensitive strain, almost 3 hr for the immunoresistant one), but it could be shown that the lesion had already occurred after 40 min at 42°C. Similar results had also been obtained by Giovanella *et al.* [5]. With Ehrlich or Yoshida ascites cells, suspended in Eagle's minimal essential medium + 10% fetal calf serum, this was not the case, the fraction of Trypan blue permeated cells not exceeding 10% even after 20 hr at 43°C. Only upon incubation in saline alone was the decline in cell viability significant ($\sim$20%) after 2–3 hr at 43°C.

DISCUSSION

The restoration, partial if not complete, of heat-inhibited RNA synthesis by high nucleoside concentration, together with the finding that the path leading from UMP to RNA was scarcely affected by heat treatment, was at first considered as indicating that the lesion caused by exposure of the cells to supranormal temperatures was mainly in the steps converting extracellular uridine to intracellular UMP. Plagemann and coworkers [22, 23] have shown that in Novikoff hepatoma cells growing in suspension culture the permeation of uridine is the rate-limiting step for its incorporation into the RNA of either the cells themselves or of mengoviruses infecting them. According to Jacquez [21], uridine permeation in Ehrlich ascites cells can occur either by a facilitated transport mechanism or by simple diffusion, the former mechanism prevailing at extracellular uridine concentrations below 0·8–1 mM, and the latter becoming significant only at [uridine] $\geqq$ 1 mM. The reversal of the heat-dependent inhibition of uridine incorporation into RNA by high nucleoside concentrations could be attributed to an inactivation of the facilitated transport system, so that only in the presence of high extracellular concentrations could uridine reach intracellular values high enough to allow phosphorylation and subsequent incorporation into RNA. Alternatively, the cell membrane could have become so "leaky" that uridine intracellular concentration might not be maintained unless the extracellular one be high enough.

The data on the efflux of fluorescein after loading with fluorescein diacetate allow a direct estimate of the temperature dependence of the diffusion constant. In fact, according to Fick's first law of diffusion [24],

$$\frac{dn}{dt} = D \times a \times \frac{\Delta c}{\delta}$$

where dn/dt is the number of molecules dn crossing an area a of the cell membrane in the time interval dt when a concentration difference Δc is applied over the membrane thickness δ. Since, in our experimental conditions, the volume V_{ex} of the extracellular fluid exceeded the packed cell volume by two or even three orders of magnitude, Δc was, almost until the end of the diffusion process, practically equal to the intracellular fluorescein concentration. For each cell of inner volume V_i, surface area a, membrane thickness δ and containing, at time t, n_t^i moles of fluorescein, Fick's law can therefore be simplified as:

$$\frac{dn}{dt} = -D \times a \times \frac{n_t^i}{V_i \times \delta}$$

By integration, assuming that V_i, a, δ and D remain invariant in the course of the assay,

$$ln\, n_t^i = -\frac{D \times a}{V_i \times \delta} \times t + ln\, n_o^i$$

The values of n_t^i and n_o^i can easily be calculated if the extracellular fluorescein concentrations C_∞^{ex} and C_t^{ex}, the number of cells N and the total volume V_{ex} are known:

$$n_o^i = C_\infty^{ex} \times \frac{V_{ex}}{N}$$

and

$$n_t^i = (C_\infty^{ex} - C_t^{ex}) \times \frac{V_{ex}}{N}$$

Simple mathematical passages show that, for a diffusion process,

$$\ln (C_\infty^{ex} - C_t^{ex}) = -\frac{D \times a}{V_i \times \delta} \times t + \ln C_\infty^{ex}$$

Plot of $\ln(C_\infty^{ex} - C_t^{ex})$ vs. time yields a straight line, with a slope $k = -(D \times a)/(V_i \times \delta)$ independent from N and from V_{ex}—as it is the case for fluorescein efflux.

Since the cell size undergoes little variation in the temperature range investigated, the data of Figs. 4 and 5 reflect mainly the temperature dependence of the diffusion coefficient D. It can be seen that the typical Arrhenius law is followed:

$$D = D_o \times e^{-A/RT}$$

the activation energy A having values around 20 kcal/mole. No change of activation energy, indicating a phase transition, could be detected at any temperature in the 24–46°C range. The temperature-dependent increase of permeability was moreover fully reversible. The physical properties of the cell membrane are not therefore irreversibly modified, as far as passive permeability is concerned, by exposure to "high permeability is concerned, by exposure to "high" temperature.

A specific transport system could, however, have been inhibited. Analysis of the acid-soluble material, showing that the heat-inhibited cells fully maintained their ability both to degrade uridine to uracil and to phosphorylate it, failed to support the hypothesis of a damage at the level of nucleoside entry into the cells.

It has been shown by Warocquier and Scherrer [25] that, in HeLa cells growing *in vitro*, exposure to 42° results in a block, at the nucleolar level, of the maturation of pre-rRNA into functional ribosomal RNA. The inhibition of uridine uptake into RNA, which is fully and rapidly reversible in HeLa cells under the experimental conditions used by these authors, would be due to the availability in the cells of an increased ribonucleotide pool originating from the degradation of the pre-rRNA which cannot be transformed into rRNA. A nucleolar damage has also been shown to occur, in a reversible fashion, upon exposure to supra-normal temperatures of BHK cells [26, 27].

Also the results illustrated in the present work could be accounted for by a similar mechanism. Preliminary experiments with α-amanitin, both in intact and in lucensomycin-permeabilized cells, indicate that over 70% of the RNA synthesis is catalyzed by an α-amanitin insensitive enzyme, and occurs therefore [28, 29] at the nucleolar level. Nucleotides newly formed from the labeled uridine would be competed for, as far as their incorporation into RNA is concerned, by the intracellular ribonucleotide precursor pool, which increases upon heat-treatment because of the block in the maturation of pre-rRNA. Such a dilution, by the cold nucleotides, of the precursors originated from the labeled nucleoside would be lessened by an increase of the label itself. Similar results would also be expected when, in lucensomycin-permeabilized cells, the rate of incorporation of labeled nucleotides is measured. The possibility that intracellular activation of lysosomal hydrolases be responsible of the degradation of newly formed RNA should also be considered [7], although previous investigation [30] has failed to substantiate it.

Some aspects of the inhibition by high temperature on tumour cells require, however, further investigation. It should be considered

that the inhibition we have observed is, differently from that measured by Warocquier and Scherrer [25] and by Simard *et al.* [26, 27], irreversible. Moreover, a damage at the level of the maturation of pre-rRNA does not explain why, as shown in previous work [3], thymidine incorporation into DNA is also severely, and irreversibly, inhibited by heat treatment of the neoplastic cells.

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Kinetic Differences of Autotransfused ^{3}H-Cytidine Labeled Blood Lymphocytes in Leukemic and non-Leukemic Lymphoma Patients*

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After in vitro *incubation with ^{3}H-cytidine, labeled blood lymphocytes were autotransfused in* 2 *hematologically normal persons,* 6 *patients with chronic lymphocytic leukemia (CLL) and* 3 *non-leukemic patients with malignant lymphomas,* 2 *with lymphosarcoma (LSA) and* 1 *with reticulum cell sarcoma (RSA).* Five minutes *after completion of autotransfusion labeling indices were found to be* 3–5% *in the CLL-patients,* 3% *in the RSA-patient,* 2% *in the* 2 *hematologically normal patients and about* 1% *in both patients with LSA. From these indices and their extrapolation to zero time it is deduced that the lymphocytes distributed themselves within minutes into a "space"* 1·7 *to* 8 *times larger than the "circulating lymphocyte pool (CLP)" calculated on the basis of blood lymphocyte concentration multiplied with the blood volume.*

During the first hour *after autotransfusion, the labeling index of the blood lymphocytes decreased until a plateau was reached. The level of this plateau was found to be between* 0·2% and 0·6% *in the non-leukemic and between* 1·8% and 3·6% *in the leukemic patients. At this time labeled lymphocytes were found in lymph node sections and bone marrow smears presumably outside the blood vessels. Therefore, the autotransfused lymphocytes distributed themselves within* 1 *to* 2 *hr into a "rapidly exchangeable lymphocyte pool (RELP)" containing intra- and extravascular subpools. The RELP/CLP ratio ranged between* 2·5 *and* 5·7 *for the* 6 *CLL-patients. It was* 6·2 *and* 19 *for the* 2 *hematologically normal patients,* 14 *for the RSA-patient and* 46 *and* 51 *for the* 2 *LSA-patients.*

The findings indicate that the method of autotransfusion of ^{3}H-cytidine labeled lymphocytes is suitable to determine the ratio between a circulating and a rapidly exchangeable lymphocyte pool. They suggest that lymphocytes from CLL-patients exchange only poorly with extravascular lymphocyte pools, while this exchange seems normal or even increased in patients with the diagnosis of LSA and RSA.

INTRODUCTION

RESEARCH on the functional properties of lymphocytes in patients with lymphomas, mainly in those with lymphocytic lymphomas [1, 2] indicates important differences in comparison with lymphocytes from healthy individuals. Thus, in patients with chronic lymphocytic leukemia (CLL) decreased rates of lymphocyte proliferation [3–6], a delayed and diminished responsiveness of the blood lymphocytes to *in vitro* stimulation with phytohaemagglutinin (PHA) [7] and a predominance of lymphocytes with B-cell characteristics in the peripheral blood [8] were found. As hypo-

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thesized by Vincent and Gunz [9] a fundamental defect of CLL-lymphocytes may be alterations of their cell surface resulting in abnormal functional behaviour. Therefore CLL-lymphocytes may show abnormalities as far as their circulation and recirculation is concerned. Recent studies support such a suggestion [10–11].

It is the purpose of this paper to report investigations in the distribution pattern of lymphocytes in 9 patients with different forms of lymphoma and in 2 hematologically normal patients. Using the method of retransfusing autologous blood lymphocytes after *in vitro* labeling with ^{3}H-cytidine [12–15] differences have been observed in the exchange of blood lymphocytes with intravascular and extravascular lymphocyte pools in 6 patients with CLL as compared with a group of 3 patients with nonleukemic lymphomas and 2 patients with normal hemopoiesis.

MATERIAL AND METHODS

Patients‡

Relevant clinical and haematological data of the patients are given in Table 1. The 11 patients are grouped according to their hematological diagnosis and listed in order of their blood lymphocyte concentration. None of the patients received any treatment related to their hematological disease before or during this study.

Eight of the 9 lymphoma patients had general enlargement of the lymph nodes. The other one, patient M.G., had at the time of this study pronounced enlargements of cervical nodes only. Within the following 6 months this patient developed also a generalized lymphadenopathy. Before or during this study from each of these 9 lymphoma patients a lymph node was excised. Patient M.G. was diagnosed histologically as having reticulum cell sarcoma (RSA) and the other 8 were considered to have lymphocytic lymphomas (1, 2): Patients H.N. and A.U. presented, however, a nonleukemic blood picture, normal serum levels of γ-globulins and normal lymphocyte responsiveness to *in vitro* stimulation with PHA, leading to the clinical diagnosis of lymphosarcoma (LSA) (1–2, 16–17); the remaining 6 lymphoma patients showed increased numbers of small lymphocytes in the blood, low γ-globulin-concentrations and a delayed and diminished PHA-responsiveness, consistent with the diagnosis of CLL [7]: patients M.R. and M.S. showed several months later an increased blood lymphocytosis up to 20,000 and 50,000 cells per mm^3 respectively.

Lymphocyte labeling and autotransfusion

From each patient we collected 500–600 ml of venous blood in a plastic bag (Fenwal Transfer Pack, TA–4, Travenol International GmbH, München, Germany), containing 75 ml of ACD (ACD-Stabilisator USP XVII A Biotest-Serum-Institut, Frankfurt/Main, Germany). 1·0 mcCi ^{3}H-cytidine (spec. act. 4·5 Ci/mM, Schwarz Bioresearch Inc., Orangeburg, N.Y., USA) or 2·0 mCi (spec. act. 2·0 Ci/mM, Radiochemical Center Amersham, England) were then added. After an *in vitro* incubation period of 1, 2, or 4 hours of 21°C or 37°C (Table 2), a 1000-fold amount of nonradioactive ("cold") cytidine was injected into the bag. Autotransfusion was then completed within 10 to 20 min.

^{3}H-cytidine was used because of its high efficiency in labeling human blood lymphocytes, mainly in their RNA [14]. Raising the incubation temperature to 37°C resulted in a higher rate of ^{3}H-cytidine uptake [18], and prolonging the incubation time to 2 and 4 hr (Table 2) gave a more stable labeling of lymphocyte-RNA [15]. These variations in the labeling conditions do not have an obvious influence on the distribution pattern of the autotransfused lymphocytes as studied in this paper.

Sampling of blood

During autotransfusion, blood samples of 2–4 ml were taken three times from the blood bag. From the controlateral arm of the patient, blood samples were withdrawn immediately after autotransfusion and thereafter at frequent intervals during the first day. In general, 4 samples were taken on the second day, 3 on day 3–5, 2 daily until day 7–10, once a day for the following week, and thereafter at less frequent intervals. The blood was mixed immediately with 1 ml of a EDTA-Plasmagel (Plasmagel$^{(R)}$, Roger Bellon, Neuilly, Hauts-de-Seine, France) mixture to prepare leuko-

‡The patients studied were under the care of the Department of Hematology (Prof. Dr. H. Heimpel), Center of Internal Medicine and Pediatrics, University of Ulm. We are highly indebted to Prof. Dr. Heimpel as well as the physicians in charge: Drs. W. Adam, H. Burkhardt, M. Dietrich, H. Feyen, Dr. Keefe, N. Konietzko, B. Kubanek, K. Lossnitzer and E. Marcard for their cooperation in the performance of these studies.

Table 1. *Clinical and hematological data of autotransfused patients*

Group of patients	Patients	Age	Sex	Height (m)	Weight (kg)	Lymphocyte count per mm^3 blood	Duration of symptoms (month)	Enlargement of			Lymphoid infiltration of marrow (%)
								Spleen*	Liver*	Lymph nodes†	
I	Haematologically normal										
	V.P.	59 yr	m	1,70	73,5	1 500	—	—	—	0	
	F.S.	63 yr	m	1,60	61	2 000	—	—	—	0	
II	Non-leukemic lymphoma‡										
	M.G.	58 yr	m	1,70	58	2 000	6	—	—	+	35
	H.N.	71 yr	f	1,68	63	2 000	12	6	2	++	45
	A.U.	63 yr	m	1,70	58	4 500	6	—	—	+++	70
III	Chronic lymphocytic leukemia										
	M.R.	63 yr	f	1,59	73	8 500	6	—	—	+	95
	M.S.	47 yr	f	1,65	55	12 000	24	—	2–3	+	90
	K.H.	61 yr	m	1,68	72	25 500	6	—	3	++	75
	P.K.	70 yr	m	1,78	69	91 000	96	5	—	++	90
	E.K.	53 yr	m	1,74	66,4	200 000	24	5	4	+++	95
	A.O.	58 yr	m	1,64	55	420 000	6	15	5	+++	90

*cm below costal margin
†From 0 to +++.
‡Patient M.G. had reticulum cell sarcoma, H.N. and A.U. had lymphosarcoma.

cyte concentrate smears after erythrocyte sedimentation (20–30 min) [14], which were then processed for autoradiography.

Blood volume determination

The volume of blood given back was determined from the difference in the weight of the bag before and after autotransfusion, using 1·050 as the specific gravity of the blood [19]. The total blood volume of the patients was derived from their body weight, height, age and sex (19–20) with corrections for enlargement of the liver and spleen [21]. Blood volumes from other lymphoma patients derived in this way correlated well with *in vivo* measurement using the ^{51}Cr-erythrocyte or the ^{131}I-albumin dilution method (unpublished observations).

Tissue specimens

Between 1·5 and 4·5 hr after autotransfusion lymph nodes were excised from 7 patients (M.G., A.U., M.R., M.S., P.K., E.K. and A.U.) and marrow was aspirated from 3 patients (P.K., E.K. and A.O.). Sections of the lymph nodes and smears of marrow particles were processed for autoradiography.

Table 2. *Conditions of* in vitro *labeling of lymphocytes in the blood bag with* ^{3}*H-cytidine*

Group of patients	Patients	Spec. Act. of ^{3}H-cytidine (Ci/mM)	mCi (added to blood bag)	*In vitro* maintenance	Incubation temperature	Autoradiographic exposure time (days)	Percentage of small lymphocytes labeled in the blood bag*
I	Hematologically normal						
	V.P.	4,5	1	45 min.	21°C	308	62,7%
	F.S.	4,5	2	4 hr	21°C	167	89,0%
II	Non-leukemic lymphoma						
	M.G.	2,0	2	4 hr	37°C	169	82,0%
	H.N.	2,1	2	60 min	37°C	215	100,0%
	A.U.	2,0	2	4 hr	37°C	82	98,0%
III	Chronic lymphocytic leukemia						
	M.R.	4,5	1	4 hr	21°C	172	84,3%
	M.S.	4,5	1	4 hr	21°C	61	80,0%
	K.H.	4,5	2	2 hr	21°C	127	99,5%
	P.K.	4,5	1	2 hr	37°C	155	99,0%
	E.K.	4,5	1	4 hr	21°C	200	94,0%
	A.O.	2,0	2	4 hr	37°C	70	95,0%

*Label limit 10 grains.

Autoradiography

Standard methods of autoradiography were used (22). Thin paraffin sections of the lymph nodes were dipped in Kodak L 4 liquid emulsion. Kodak AR 10 stripping film was applied to the methanol-fixed blood and marrow smears after they had been washed in tap water for 30–60 min to remove any unbound radioactive material. Sets of slides from each patient representing all available samples of blood, marrow and lymph node were exposed at 4°C for the same periods of time, ranging from 61 to 308 days (Table 2). After photographic processing the cells were stained through the emulsion with dilute Giesma stain at ph 5·75 [14].

Evaluation of autoradiographs

The labeling index (LI), defined as the labeled proportion of small lymphocytes, was determined in each blood smear by examining at least 2000 lymphocytes with diameters less than 8·5 μm. Cells showing 10 grains or more were scored as labeled; control smears processed with the others did not show more than 10 grains over such lymphocytes. An entire section of each lymph node was searched for the presence of labeled lymphocytes, amounting to an examination of at least 10^5 lymph node cells. Smears of marrow particles were also scanned for labeled cells.

RESULTS

Pattern of labeling indices in the blood during the first 24 hr after autotransfusion

In 2 patients with normal hemopoiesis (Fig. 1), the LI was about 2 % 5 min after completion of autotransfusion. This value decreased rapidly within the first hour, leveled off at about 0·6 % and remained at this level for several hours, indicating the establishment of an equilibrium of lymphocytes leaving and entering the vascular system. Similar observations were obtained in 3 patients with non-leukemic lymphomas diagnosed as LSA or RSA (Fig. 2); the LI was 1–3 % 5 min after autotransfusion, declined during the first hour, and thereafter the LIs fluctuated at a level of about 0·5 % for several hours.

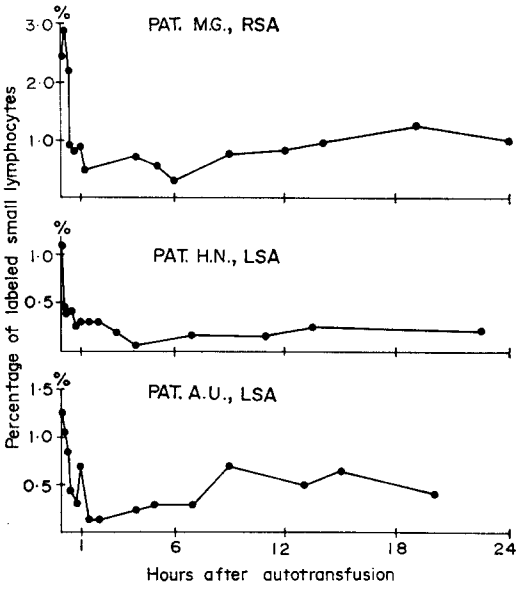

Fig. 2. Changes in labeling indices of small lymphocytes in the blood during the first 24 hr after autotransfusion in three non-leukemic patients with malignant lymphomas, diagnosed as lymphosarcoma (LSA) or reticulum cell sarcoma (RSA).

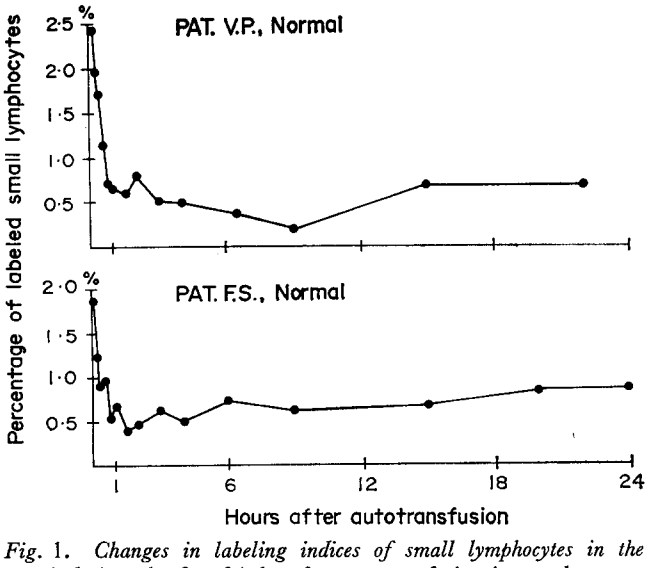

Fig. 1. Changes in labeling indices of small lymphocytes in the blood during the first 24 hr after autotransfusion in two haematologically normal patients.

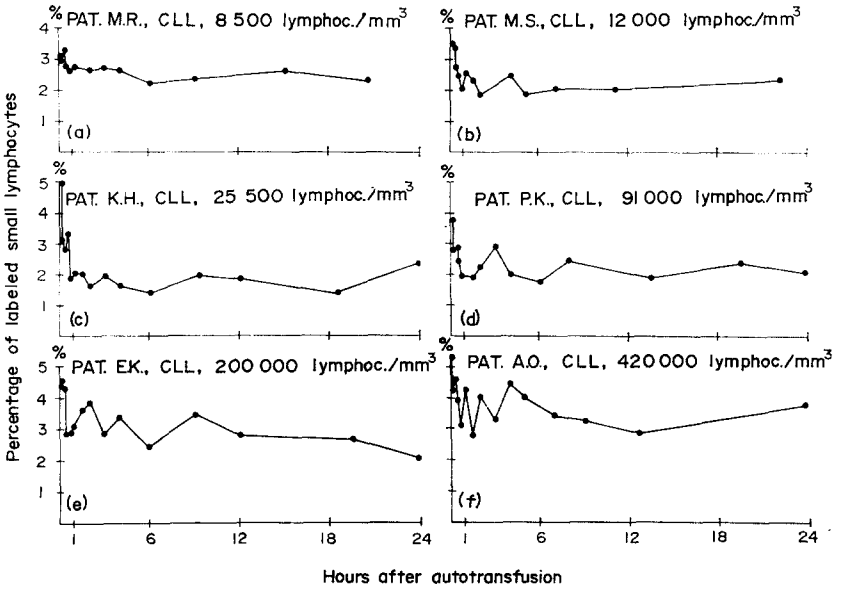

Fig. 3. Changes in labeling indices of small lymphocytes in the blood during the first 24 hr after autotransfusion in six patients with chronic lymphocytic leukemia (CLL). The patients are grouped according to the number of lymphocytes in the blood.

Figure 3 shows no correlation between the LI and the degree of lymphocytosis in the 6 CLL-patients. The 5-min LI varied between 3 and 5% in these patients. Again, after an initial decrease the LI leveled off. However, this level was between 1·8 and 3·6%, distinctly higher than that for non-leukemic patients.

Calculations of sizes of lymphocyte pools

In all patients studied the flat part of the LI curve that was reached about 1 hour after autotransfusion is taken to indicate the establishment of an equilibrium in the distribution of the labeled lymphocytes within a "rapidly exchangeable lymphocyte pool (RELP)". For reasons to be discussed later, in each patient, the mean LI from 1 to 4 hr after autotransfusion was used for calculations (Table 3).

Part of this RELP is the "circulating lymphocyte pool (CLP)". It is defined as the product of lymphocyte count per unit volume of blood and the patient's blood volume. For each patient the size of the RELP was found to be larger than of the CLP (Table 3). The ratio of the RELP to the CLP varies in the patients with normal hemopoesis between 6 and 19; in the patients with non-leukemic lymphomas between 14 and 51; and in the 6 patients with CLL between 2·5 and 6 (Table 3). Although the number of patients is still too small to allow a statistical analysis on the differences between these groups, the data indicate that the ratio for the 6 CLL-patients is well below that for the other 5 non-leukemic patients.

Calculation of the portion of autotransfused lymphocytes in circulating blood

In addition, this flat part of LI curve can be used to calculate the number of labeled lymphocytes present in the CLP during the period 1–4 hr after autotransfusion (Table 3). By dividing this number by the total number of autotransfused labeled lymphocytes we can show (Table 3, last column) that in the 5 non-leukemic patients only 2–10% of the autotransfused lymphocytes were recovered in the CLP, compared to 18–33% in the 6 CLL-patients. This indicates that in these leukemic patients a smaller percentage of the autotransfused lymphocytes had left the circulation.

Extravascular appearance of autotransfused labeled lymphocytes

All lymph node biopsies and marrow aspirations, performed 1·5–4·5 hr after autotransfusion, showed labeled lymphocytes. Ratios for the CLL-patients were determined to be one labeled lymphocyte per 5,000 to 7,000 lymph node cells and one per 5,000 bone marrow cells. In the lymph nodes from non-leukemic lymphoma patients A.U. and M. G. the ratio was one to about 10,000 and 50,000 lymph node cells respectively. In the lymph node sections from the CLL-patients about half of the labeled lymphocytes appeared to be extravascular, in the 2 non-leukemic patients even more lymphocytes appeared to be extravascular. These findings are taken to indicate that labeled, autotransfused lymphocytes leave

Table 3. Data for calculation of rapidly exchangeable lymphocyte pool and of intravascular "retention" of autotransfused, labeled lymphocytes

Group of patients	Patients	Lymphocyte count per mm^3 blood	Blood volume (ml)	Circulating lymphocyte pool (CLP)	Labeled lymphocytes autotransfused	Labeling index in blood*	Rapidly exchangeable lymphocyte pool (RELP)	Ratio of RELP to CLP	Labeled lymphocytes in circulating blood	Portion of autotransfused labeled lymphocytes in circulating blood*
I	Hematologically normal									
	V.P.	1 500	5 000	$0{,}75 \times 10^{10}$	$0{,}49 \times 10^{9}$	0,66%	$4{,}66 \times 10^{10}$	6,2	$0{,}050 \times 10^{9}$	10,0%
	F.S.	2 000	4 400	$0{,}88 \times 10^{10}$	$1{,}02 \times 10^{9}$	0,55%	$16{,}50 \times 10^{10}$	19	$0{,}048 \times 10^{9}$	4,8%
II	Non-leukemic lymphoma									
	M.G.	2 000	4 400	$0{,}88 \times 10^{10}$	$0{,}86 \times 10^{9}$	0,57%	$12{,}37 \times 10^{10}$	14	$0{,}050 \times 10^{9}$	5,8%
	H.N.	2 000	4 700	$0{,}94 \times 10^{10}$	$1{,}11 \times 10^{9}$	0,23%	$48{,}20 \times 10^{10}$	51	$0{,}022 \times 10^{9}$	2,0%
	A.U.	4 500	4 300	$1{,}94 \times 10^{10}$	$2{,}54 \times 10^{9}$	0,28%	$88{,}90 \times 10^{10}$	46	$0{,}054 \times 10^{9}$	2,1%
III	Chronic lymphocytic leukemia									
	M.R.	8 500	4 500	$3{,}82 \times 10^{10}$	$3{,}98 \times 10^{9}$	2,69%	$12{,}48 \times 10^{10}$	3,3	$1{,}028 \times 10^{9}$	25,8%
	M.S.	12 000	4 000	$4{,}80 \times 10^{10}$	$5{,}34 \times 10^{9}$	2,33%	$18{,}31 \times 10^{10}$	3,8	$1{,}118 \times 10^{9}$	21,0%
	K.H.	25 500	5 000	$12{,}75 \times 10^{10}$	$13{,}70 \times 10^{9}$	1,86%	$73{,}00 \times 10^{10}$	5,7	$2{,}480 \times 10^{9}$	18,2%
	P.K.	91 000	5 300	$48{,}25 \times 10^{10}$	$51{,}40 \times 10^{9}$	2,28%	$222{,}7 \times 10^{10}$	4,6	$11{,}100 \times 10^{9}$	21,6%
	E.K.	200 000	5 300	$106{,}0 \times 10^{10}$	$108{,}00 \times 10^{9}$	3,35%	$302{,}5 \times 10^{10}$	2,8	$35{,}500 \times 10^{9}$	32,9%
	A.O.	420 000	4 700	$196{,}5 \times 10^{10}$	$228{,}00 \times 10^{9}$	3,80%	$570{,}0 \times 10^{10}$	2,5	$74{,}700 \times 10^{9}$	32,8%

*Mean for the period 1–4 hr after autotransfusion.

the blood, since the labeled cells were found in the marrow in dense particle smears and in the lymph nodes in tissue areas not showing red cells.

Pattern of the labeling indices in the blood during the first few weeks after autotransfusion

Figures 4–6 show for each patient the changes in the LI during the first 2–4 weeks after autotransfusion. Although the labeling intensity of the autotransfused lymphocytes decreased after reinfusion, as would be expected as a result of their nucleic acid metabolism [15], in all patients some labeled lymphocytes were found 10 to 30 days after autotransfusion.

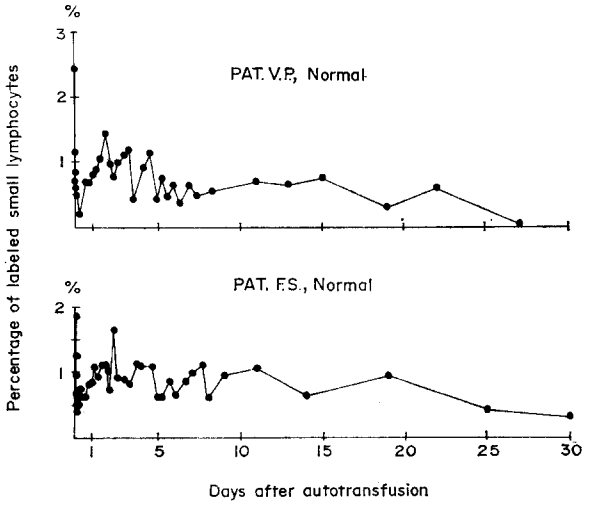

Fig 4. Changes of the percentage of ^{3}H-cytidine labeled small blood lymphocytes in two hematologically normal persons during 30 days after autotransfusion.

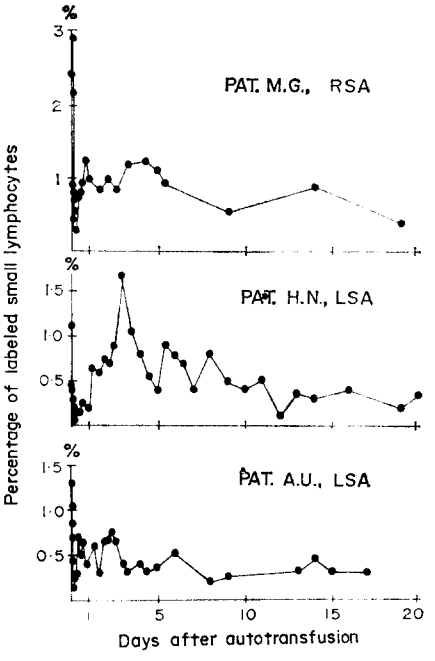

Fig. 5. Changes of the percentage of ^{3}H-cytidine labeled small blood lymphocytes in patients with reticulum cell sarcoma (RSA) or lymphosarcoma (LSA) during 20 days after autotransfusion.

In Fig. 4 the LI curves in the 2 hematologically normal persons definitely rose between 12 hr after autotransfusion and the fifth day, and thereafter they fell off slowly. All 3 patients with non-leukemic lymphoma, after stabilizing at a low level in the first six hours, had a moderate to marked increase in the LI between the first and the fifth day (Fig. 5). In the next two

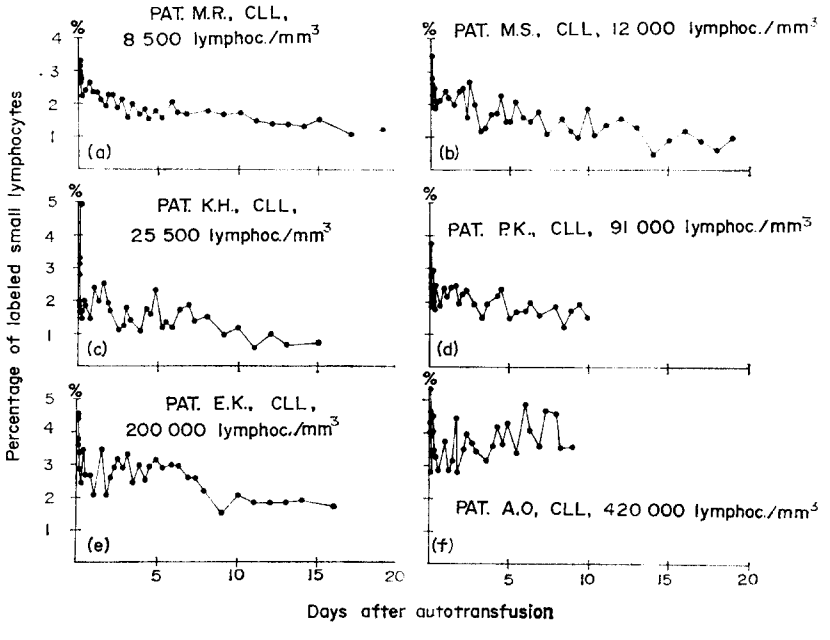

Fig. 6. Changes of the percentage of ^{3}H-cytidine labeled small blood lymphocytes in six patients with chronic lymphocytic leukemia (CLL) during 20 days after autotransfusion. The patients are grouped according to the numbers of lymphocytes in the blood.

weeks the percentage of labeled lymphocytes decreased slowly to the early low levels. Figure 6 shows that none of the CLL-patients studied had a consistent increase in the LI after autotransfusion. Instead, the LI decreased very slowly, similar to the later phases of the other patients, but at a higher level. This gradual decrease was not demonstrated during the 10 days of observation of patient A.O.

DISCUSSION

Although the leukemic patients showed consistently a higher LI at all times, the data presented indicate that the patterns of disappearance of autotransfused, labeled lymphocytes are similar in the leukemic and non-leukemic lymphoma patients and in the 2 hematologically normal patients, and can be considered as representing a series of catenated phases.

Phase 1*: Intravascular distribution of lymphocytes*

Immediately after completion of autotransfusion, initial LIs of 7–13 % would be expected on the basis of the size of each patient's CLP and of the corresponding number of labeled lymphocytes autotransfused. By extrapolating the slope of the initial decrease of the LIs to zero time (the completion of autotransfusion) LIs of 3·2 to 6·5 % were calculated in the 6 CLL-patients, whereas in the 3 non-leukemic lymphoma patients the calculated values varied between 1·6 to 3·0 % and were 2·4 and 3·1 % in the 2 hematologically normal persons. Therefore it must be concluded that during the period of reinfusion, the autotransfused lymphocytes were distributed rapidly in a cell pool that is larger than the CLP. Thus, for the CLL-patients this cell pool of initial distribution was calculated to be 1·7 to 3·2 times larger than the CLP. In the RSA-patient and in the 2 normal persons this pool was 2–5, and in the 2 LSA-patients about 8 times the size of the CLP.

Similar ratios between 2 and 6 have been found for CLL-lymphocytes in other studies [12, 13, 23–26], for normal lymphocytes [27, 28], for monocytes [29] and for leukemic blast cells [30–32]. An analogous factor of 2 is reported for normal granulocytes [33, 34]. In analogy to the "marginal granulocyte pool" presumed to be an intravascular pool [33, 34], one may assume that this lymphocyte pool of initial distribution in addition to the CLP is also located intravascularly and should then be called "marginal lymphocyte pool (MLP)". Although French *et al.* [35] could not ascertain that the cells of the MLP were localized in the blood near the walls of the vessels, it has been shown that such lymphocytes can be mobilized into the CLP within minutes by injecting epinephrine and to some extent the MLP has been localized in the spleen [28]. Furthermore Stryckmans *et al.* [25], after a rapid retransfusion of autologous lymphocytes within 2 min, observed an initial LI equal to that expected, whereas after a retransfusion lasting 30 min only half of the expected LI was found. Thus, CLP and MLP can be considered as subcompartments of the entire intravascular blood lymphocyte pool.

Phase 2*: Initial decrease of labeling index*

The second phase is characterized by a decline of the LI within the first hour after autotransfusion. Several factors should be considered in the interpretation of this observation: because the labeling intensity (number of grains per lymphocyte) decreases only slowly during many days of observation [15, 36], this initial decrease of LI cannot be a consequence of loss of label due to nucleic acid metabolism, but represents a disappearance of labeled cells from the circulation and their replacement by unlabeled cells. The data presented in Table 2 and Figs. 1–3 show that *in vitro* incubation of lymphocytes for 4 hr at 37°C does not result in an obvious change in the pattern of disappearance of autotransfused lymphocytes; if this procedure would have impaired the functional properties of the lymphocytes, a very rapid removal could have been expected as studied by others [10, 27]. That these lymphocytes are unchanged in their viability is supported by a constant rate of RNA synthesis by lymphocytes during and after a 4-hr *in vitro* period [14] and by the fact that the trypan blue dye exclusion test showed a viability of more than 95 %. Thus the variations in the labeling techniques do not seem to affect the pattern of disappearance of labeled lymphocytes indicating that the observed decrease of the LI is due to a progressive distribution of labeled viable lymphocytes probably into extravascular pools. Evidence for such an extravascular migration of autotransfused lymphocytes is obtained by the observation of labeled lymphocytes in the parenchyma of the lymph nodes and in smears of marrow particles 1·5 to 4·5 hr after autotransfusion and by the appearance of recirculating, labeled lymphocytes in the thoracic duct lymph [11, 37–39].

Phase 3: Extravascular exchange of lymphocytes

Phase 3 is represented by the plateau in the LI-pattern which lasts for several hours, indicating the establishment of an equilibrium between the efflux and influx of labeled lymphocytes. We suggest that the level of this plateau is correlated with the quantity of autotransfused lymphocytes, which exchanged with extravascular tissue lymphocyte pools. Thus, in the group of non-leukemic patients, which had low LIs during this phase, the number of autotransfused lymphocytes leaving the vessels is relatively great. These lymphocytes seem to recirculate normally, as demonstrated directly by a thoracic duct drainage study in patient A.U. [11, 37]. But in the CLL-patients, the high levels of this plateau indicate that only a small fraction of labeled blood lymphocytes exchanged with extravascular pools and a comparatively large fraction remained intravascularly. In addition, we could determine that a greatly diminished fraction of autotransfused blood lymphocytes of these CLL-patients recirculated via thoracic duct lymph [11, 37]. Thus, these differences in the LI-levels of the plateau may reflect a functional impairment of CLL lymphocytes. These findings support the differences we found in the relative sizes of the RELP in the leukemic and non-leukemic patients. Furthermore, the above differences in the fractions of blood lymphocytes leaving the circulation may explain the differences observed in the LIs 5 min after autotransfusion. In a number of studies, spleen, lymph nodes, marrow and liver have been found to be preferential sites for the extravascularization of lymphocytes [10, 23, 25, 27, 28]. Similar differences in the lymphocyte kinetics between patients with CLL and haematologically normal patients have been reported in recent autotransfusion studies by Hersey [10] using ^{51}Cr-labeled lymphocytes. For an analysis of differences between the kinetics of autotransfused lymphocytes in haematologically normal persons and in non-leukemic lymphoma patients, a greater number of patients is required.

Phase 4: Late persistence of lymphocytes (life span)

During the weeks after autotransfusion, in each patient a slow decrease of LIs was observed, compatible with a life expectancy of these labeled blood lymphocytes in the non-leukemic as well as in the leukemic patients in the order of several weeks or more [3–5, 40]. However, a quantitation of the lymphocyte life span and mode of removal cannot be derived from these autotransfusion data because of loss of the label due to nucleic acid metabolism and the relatively short observation period of 10–30 days. Similar considerations should also be taken into account in autotransfusion studies using ^{51}Cr-labeled lymphocytes [10, 23, 27].

The interpretation of the transient increase in the blood lymphocyte LI in all non-leukemic patients between day 1 and day 5 (Figs. 4 and 5) is difficult. Because the lymphocytes of these patients exchanged readily with extravascular spaces, such an increase of the LI may occur by the selective return of recirculating lymphocytes and the release of transiently trapped labeled lymphocytes. However, autotransfusion studies using ^{51}Cr-labeled lymphocytes failed to show such a transient rise in haematologically normal persons and in CLL-patients [10, 23, 27].

Alternatively, we found evidence for the *in vivo* labeling of additional cells by "free" tritiated material (not bound to cells) that was infused with the contents of each blood bag, despite the addition of an excess of "cold" cytidine. By autoradiography and by scintillation counting of biochemically extracted nucleic acids we found that this increase in the LI can be completely avoided by washing the labeled lymphocytes twice before autotransfusion to reduce the unbound radioactivity to less than 10% of the original dose injected into the bag (unpublished observations in non-leukemic patients). However, there is no explanation at present, why the cold cytidine should not have affected the specific activity in such a way, that additional *in vivo* cell labeling would be prevented.

After an intravenous injection of 0·5 mCi [12] or 1·0 mCi ^{3}H-cytidine to 2 patients with CLL there were no labeled lymphocytes even after autoradiographic exposures up to 200 days [6, 36], as might be expected from the low rate of lymphocyte proliferation in CLL-patients [3–6]. Haematologically normal patients and patients with LSA and RSA have higher mitotic and ^{3}H-thymidine labeling indices of the lymphoid cells [13, 40–43]. Thus, in these patients, it appears possible that the presence of free tritiated material resulted in the *in vivo* labeling of lymphocytes, appearing in the circulation beginning about 4 hr after autotransfusion, as shown by studies of lymphocyte kinetics with ^{3}H-thymidine [4–5, 13]. Therefore, the RELP was calculated on the basis of the mean of the LIs during the period 1–4 hr after autotransfusion.

The observed differences in the extent of disappearance and exchange of autotransfused, labeled lymphocytes between the patients

studied, encourage further investigations on functional and kinetic aspects of the lymphocyte populations of patients with various lymphatic disorders.

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Combinations of the Effect of Radiotherapy, Alkylating Agents and Phytohemagglutinin on Sarcoma 180 in Swiss Mice

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Abstract—*Mice with Sarcoma* 180 *were treated with irradiation, chemotherapy and PHA alone and in various combinations. There was no significant difference between the results for mice treated by a single form of therapy and the nontreated control. The combination of irradiation with chemotherapy was more effective than each one of these treatments alone. The addition of PHA to the two methods of treatment resulted in most cases in a greater reduction in tumor volume and prolongation of survival time. In order to obtain the best results the sequence of treatments was found to be important.*

INTRODUCTION

RADIOTHERAPY and chemotherapy are of great value in reducing tumor volume both in animal and human tumors. The effect of alkylating agents resembles that of ionizing radiation, and they are sometimes classified as radiomimetic agents. The radiomimetic agents are mutagenic, interfere with mitosis, inhibit the immunization process and block enzyme activity [1].

The use of combinations of radiotherapy and chemotherapy has been frequently described. Discussing the results of some clinical trials, Newton claims: "It seems reasonable to anticipate that the chemotherapeutic effect will be at least additive to radiotherapy" [2]. Some authors suggest that under certain circumstances a therapeutic synergism develops between the local effect of irradiation and the systemic effects of the chemotherapy resulting in prolongation of the host survival time [3].

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Phytohemagglutinin (PHA) has been shown to reduce tumor volume in mice with an ascites tumor and in hamsters with a sarcoma [4, 5].

The purpose of this study was to investigate whether the combination of radiotherapy, alkylating agents and PHA administration would be more effective than treatment with only one or two of these methods.

MATERIAL AND METHODS

Mice

Male, Swiss albino random bred mice aged 8–10 weeks and weighing 20 to 22 g were used in the experiments. (The mice were raised by Mr. A. Loebenstein, Yokneam, Israel.) They were kept in cages (15 animals per cage) and were given food and water ad libitum.

Tumor

Sarcoma 180, which has been passaged by trocar for many generations, was kindly given to us by Dr. B. Shohat, Department of Pharmacology, The Rogoff Medical Research Institute, Beilinson Medical Center, Petach Tikva, Israel. This spontaneously arising sarcoma was brought to Israel in 1965 from the Sloan Kettering

Institute of Cancer Research, New York. The tumours have been maintained since then by serial subcutaneous transplantation of a single piece of the tumor (approximately 1 mm^3 and containing $2 \cdot 10^5$ to $5 \cdot 10^5$ cells) by a trocar.

Treatment

Irradiation of the tumor area was performed with the Picker Vanguard X-ray machine. The dose was calculated in roentgens (r) per mouse in air and was given at 126 KV and 5 MA with HVL of 0·6 mm Al.

The mice were anesthetized by ether and were given a single dose of 2000 r in 3·9 min. The output was 515 r/min. The animals were irradiated at a 19-cm skin target distance.

The following drugs were administered intraperitoneally:

1. ThioTEPA (Triethylene thiophosphoramide), Lederle Laboratories Division, American Cyanamide Company, 0·015 mg/mouse.*
2. Cytophosphan—Cyclophosphamide—Taro Pharmaceutical Industries Ltd., Haifa, Israel, 1 mg/mouse [6].

"Wellcome" phytohemagglutinin was used throughout (Wellcome Research Laboratories, Beckenham, England). The freeze dried material containing 50 mg/vial was stored at 4°C. Before using, it was diluted with 5 ml of sterile saline and 1 mg was injected intraperitoneally. This amount has been shown to reduce the tumor volume in mice bearing an ascites tumor [4, 5].

STATISTICAL ANALYSIS

The statistical significance of the difference in mean tumor growth and in mean survival time between different groups was tested by the *t*-test, using either the standard *t*-test, or when the variances in the groups compared were significantly different, Bailey's modification [7]. For tumor growth comparison, the average tumor volume in the period from +25 days to +30 days was divided by the average tumor volume in the period from +8 days to +13 days to give a value that represents the tumor growth for each animal. Differences between groups were considered significant only when the *p* values of comparison were 0·05 or smaller.

EXPERIMENTAL PROCEDURE

Two sets of experiments, each using 225 mice, were performed. The mice were divided into 15 groups of 15 mice each. All were implanted subcutaneously with the tumor in the inguinal region on day 0, and were treated when tumors were palpable as follows:

Group 1. Served as non-treated control.
Group 2. Chemotherapy† on the 10th day after tumor implantation.
Group 3. Chemotherapy on the 10th day and irradiation‡ on the 14th day.
Group 4. Irradiation on the 10th day and chemotherapy on 14th day.
Group 5. PHA on the 10th day and chemotherapy on the 14th day.
Group 6. Chemotherapy on the 10th day and PHA on the 17th day.
Group 7. Chemotherapy on the 10th day, irradiation on the 14th day and PHA on the 17th day.
Group 8. PHA on the 10th day, chemotherapy on 14th and irradiation on the 17th day.
Group 9. PHA on the 10th day, chemotherapy on 14th, irradiation on 17th and PHA on the 20th day.
Group 10. Irradiation on the 10th day, PHA on 14th and chemotherapy on the 17th day.
Group 11. PHA on the 10th day, irradiation on 14th and chemotherapy on the 17th day.
Group 12. Irradiation on the 10th day.
Group 13. PHA on the 10th day.
Group 14. PHA on the 10th day and irradiation on the 14th day.
Group 15. Irradiation on the 10th day and PHA on the 14th day.

The tumors were measured with a caliper every third day from the day of appearance of palpable tumors. The effect of the treatment was determined by prolongation of the survival time and by decrease in the tumor volume. The tumor volume was calculated according to the formula $0 \cdot 4\ a\ b^2$ where *a* is the major diameter and *b* is the minor diameter [8].

The survivors were killed on the 65th day when the experiment was terminated.

RESULTS

No significant difference was found between the mice treated with a single form of therapy such as X-rays, chemotherapy or PHA and the control, when either survival time or volume was used as a measure of effectiveness.

*The mouse dose was calculated on a weight basis from the human dose.

†A single dose of either thioTEPA or cyclophosphamide was injected intraperitoneally.

‡Irradiation was given to the tumor area only.

The results of the two treatments (Table 1) show that X-rays and thioTEPA or thioTEPA and X-rays prolonged significantly the host-survival and reduced significantly the tumor volume, as compared to control or to those treated with X-rays or thioTEPA alone. The treatment with X-rays first and thioTEPA afterwards gave better results than thioTEPA first and X-rays afterwards. There was no significant difference from the control in the survival time and tumor volume of mice treated with X-rays and PHA, PHA and X-rays, thioTEPA and PHA, and PHA and thioTEPA.

The combination of cytophosphan followed by X-rays resulted in a significant prolongation of the survival time when compared with the control or with animals treated singly with cytophosphan, PHA or X-rays (Table 1). There was no significant difference from the control in the survival and tumor volume of the mice treated with cytophosphan and PHA or PHA and cytophosphan.

The effects of three treatments on the survival and tumor volume of the mice are summarized in Tables 2 and 3. Statistical analysis (Table 4) of the survival times gave the following results:

Table 1. *The effect of combining two treatments, such (PHA, chemotherapy and X-rays) on the survival time and tumor volume of Swiss mice implanted with S-*180

Treatment	Median survival time in days	% of survivors at the end of experiment	Survival significantly longer than corresponding control	Tumor volume smaller than corresponding control	Survival significantly better than single treatment	Tumor volume significantly smaller than single treatment
ThioTEPA, X-rays	*	66	+		thioTEPA	
X-rays, thioTEPA	*	80	+	*	thioTEPA, PHA, X-rays	X-rays
PHA, thioTEPA	32	40				
ThioTEPA, PHA	44	40				
PHA, X-rays	47	40				
X-rays, PHA	38	40				
Control	40	32				
Cytophosphan, X-rays	*	74	+			PHA
X-rays, cytophosphan	*	54				
Cytophosphan, PHA						
PHA, cytophosphan						
Control	40	32				

* More than 50% of the animals survived up to the end of the experiment.

Table 2. *The effect of combining three treatments (PHA, chemotherapy and X-rays) on the survival time and tumor volume of Swiss mice implanted with S-*180

Treatment	Median survival time (in days)	% of survivors at the end of the experiment	Survival significantly longer than corresponding control	Tumor volume significantly smaller than corresponding control
ThioTEPA, X-rays, PHA	*	66	+	+
PHA, thioTEPA, X-rays	52	45	+	+
X-rays, PHA, thioTEPA	*	86	+	+
PHA, X-rays, thioTEPA	*	60	+	+
PHA, thioTEPA, X-rays, PHA	*	68	+	
Control	40	32		
Cytophosphan, X-rays, PHA	*	54	+	+
PHA, cytophosphan, X-rays	62	47	+	+
X-rays, PHA, cytophosphan	*	66	+	+
PHA, X-rays, cytophosphan	*	66	+	+
PHA, cytophosphan, X-rays, PHA	*	54		+
Control	40	32		

*More than 50% of the animals survived up to the end of the experiment.

Table 3. *The effect of combining three treatments compared to one treatment and two treatments on the survival time and tumor volume of Swiss mice implanted with S-*180

Treatment	Median survival time (in days)	% of survivors at the end of experiment	Tumor volume on the 30th day
Single treatment			
PHA	32	32	0·892
ThioTEPA	41	32	0·705
Cytophosphan	53	47	1·311
X-rays	48	40	0·455
Control	40	32	1·782
Combined treatments			
X-rays, thioTEPA	*	80	0·120
ThioTEPA, X-rays	*	66	0·372
PHA, X-rays, thioTEPA	*	60	0·120
PHA, thioTEPA, X-rays	52	46	0·271
X-rays, PHA, thioTEPA	*	86	0·081
ThioTEPA, X-rays, PHA	*	66	0·481
X-rays, cytophosphan	*	54	0·502
Cytophosphan, X-rays	*	74	0·511
PHA, X-rays, cytophosphan	*	66	0·272
PHA, cytophosphan, X-rays	62	46	0·613
X-rays, PHA, cytophosphan	*	66	0·234
Cytophosphan, X-rays, PHA	*	54	0·912

*More than 50% of the animals survived up to the end of the experiment.

Table 4. *Statistical analysis of the results obtained by three treatments*

Treatment	Survival significantly longer than corresponding controls	Tumor volume significantly lower than corresponding controls
X-rays, PHA, thioTEPA	X-rays, PHA, thioTEPA	X-rays X-rays, thioTEPA thioTEPA, X-rays
ThioTEPA, X-rays, PHA	PHA PHA, X-rays PHA, thioTEPA	
PHA, thioTEPA, X-rays	X-rays, PHA	X-rays, PHA
PHA, thioTEPA, X-rays, PHA	PHA, thioTEPA PHA, X-rays	
PHA, X-rays, cytophosphan	PHA cytophosphan, PHA PHA, X-rays	cytophosphan cytophosphan, X-rays
PHA, cytophosphan, X-rays	PHA	
Cytophosphan, X-rays, PHA	PHA cytophosphan, PHA	
X-rays, PHA, cytophosphan		X-rays cytophosphan, X-rays
PHA, cytophosphan, X-rays, PHA		X-rays

ThioTEPA, X-rays and PHA in various sequences are significantly better than control, better than the one treatment and better than the two treatments. There was no statistically significant difference in the survival between the groups treated with X-rays and thioTEPA and those treated with X-rays, PHA and thioTEPA, but the tumor volume was significantly less in the last group.

Using three treatments the combination of X-rays, PHA and thioTEPA was the best in reducing tumor volume and in prolonging survival.

Treatment with cytophosphan, X-rays and PHA in various sequences prolonged significantly the life of the mice compared with control (Table 2). All the five combination sequences of three treatments reduced significantly the tumor volume as compared with the control (Table 2) or with those treated with cytophosphan followed by X-rays or those treated with PHA or cytophosphan alone (Tables 3, 4).

It should be pointed out that in most cases of the three methods of treatment more than 50% of the animals survived up to the end of the experiment.

DISCUSSION

In the present work we have found that survival times and tumor volumes obtained by a single form of treatment did not differ significantly from their corresponding controls. However, no treatment caused death before control, which suggests that the treatment was neither lethal nor toxic to the animals.

With the combination of X-ray and chemotherapy, or chemotherapy followed by X-rays, less than 50% of the animals had died at the end of the experiment. The combination of thioTEPA followed by X-rays was significantly better than control and then thioTEPA alone as measured by prolongation of survival time. When X-rays were given before thioTEPA, the mice survived significantly longer than control and all the other forms of single treatment. The same combination decreased significantly the tumor volume of the treated mice compared to their corresponding control and the irradiated mice. The finding that irradiation with chemotherapy was more effective than irradiation with PHA or chemotherapy with PHA was not surprising, as it is known that these two together with surgery are the best cancer methods available today. Chemotherapy with irradiation, has been shown to be effective in the treatment of human cancer [9, 10] as well as against experimental animal tumors [11, 12]. It has been suggested that many chemicals are capable of increasing the radiation induced regression of the tumor and prolonging the lives of the tumor-bearing hosts [3].

The question that we were interested in was whether the addition of PHA to irradiation and chemotherapy would improve the results. In 9 out of 10 combinations the three treatments prolonged significantly the survival time of the mice, and in 5 out of 10 cases a significant decrease in tumor volume was achieved. It is evident that in most cases the triple treatment resulted in a significant prolongation of survival time and in cures of tumor bearing animals compared to mice given a single treatment or a combination of two treatments.

In two cases the addition of PHA to radiotherapy and cytophosphan decreased significantly the tumor volume compared to the results obtained by combination of these two only:

1. when PHA was the first treatment, X-rays the second and cytophosphan later;
2. when PHA and cytophosphan followed the X-rays.

These two sequences were found to be more effective than the others. The timing and dosage of chemotherapeutic agents, the route of administration, the time interval between the various treatments and sequence of treatments are of great importance in achieving successful therapy in malignancy [13].

There was no correlation between the sets of treatments which caused the best survival and those which resulted in the smallest tumor volume. This lack of correlation is also encountered clinically.

There are many reports in the literature concerning the effects of PHA on the immune response. Some investigators claim that PHA suppresses antibody production [14, 15], while others suggest that it enhances it [16, 17]. Jassin and Ziff [18] contend that different lymphoid cell populations are of differing sensitivity which could account either for suppression or stimulation. If this is true, activation of T cells by PHA could have acted as a non specific stimulator for the immunologically competent cells [19, 20]. However, most authors agree that changing the dose, time and route of PHA administration may alter its effect.

The role of the reticuloendothelial system in the host defense mechanism against cancer has been described [21].

A wide spectrum of agents like BCG, MER, and bacterial endotoxins are known to act as non specific stimulators of the immune system, and, in some cases, to retard tumor growth [22]. These findings have led to the combination of Zymosan with surgery and radiotherapy in the treatment of murine mammary tumors [23], or bacterial toxins and surgery and radiotherapy in the treatment of reticulum cell sarcoma of bone in humans [24]. Killed cultures of *C. parvum* have also been used with anti-tumor globulin [25].

PHA has been found to reduce tumor volume in mice with an ascites tumor and hamsters with a sarcoma [4, 5]. In the present work PHA alone was not effective. One of the reasons for that could be that in the previous work PHA was given before tumor challenge, whereas in the present study it was given afterwards. Another explanation could be the difference between the ascites form and the solid tumor.

Our results show clearly that combined treatment of PHA with chemotherapy was less effective than radiotherapy with chemotherapy. It is clear therefore that PHA cannot replace radiotherapy or chemotherapy.

From the three combination experiments we see that the addition of PHA in certain sequences improved the results over those achieved with radiotherapy and chemotherapy. The possibility that other combinations may give better results is at present under investigation.

In preliminary experiments—using other chemotherapeutic drugs—we have found that a different sequence gave the most effective treatment. It is therefore to be expected that the optimal treatment sequence varies for different chemotherapeutic agents and for different tumors.

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Further Studies on the Self-Limitation of Growth of JB-1 Ascites Tumours

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Abstract—*When cell-free ascitic fluid obtained from JB-1 ascites tumours at the plateau is injected into exponentially growing JB-1 ascites tumours, the tumour cells are specifically arrested in the G-1 and the G-2 phases of the cell cycle. By means of ultrafiltration it was demonstrated that the inhibition of cells in G-1 is due to a substance or substances with a molecular weight between 10,000 and 50,000 while substances with molecular weights between 1,000 and 10,000 are responsible for the G-2 inhibition.*

INTRODUCTION

IT IS GENERALLY agreed that the decelerating growth rate of the ageing ascites tumour is mainly due to increasing generation time and to a varying degree a decreasing growth fraction, while the cell loss is constant during most of the growth [1–5].

It was previously demonstrated that with the age of the JB-1 ascites tumour an increasing part of the cell population is accumulated in the G-1 and the G-2 phases of the cell cycle [6, 7], and when cell-free ascitic fluid obtained from advanced ascites tumours is injected into rapidly growing regenerating ascites tumours, the tumour cells are specifically arrested in both the G-1 and the G-2 phases [8].

In the study presented here it was investigated whether the inhibition of the tumour cells in the G-1 and the G-2 phase is due to one or more components of the JB-1 ascitic fluid.

MATERIAL AND METHODS

The JB-1 ascites tumour is a transplantable plasmacytoma maintained syngeneically in our inbred AK strain of mice by intraperitoneal passages of 5×10^6 ascites tumour cells every 9th or 10th day.

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Ascitic fluid was obtained by aseptic aspiration of the ascites tumour from mice 11 days after intraperitoneal inoculation of 5×10^6 tumour cells and subsequent centrifugation at 1500 **g** for 10 min at 0°C.

Ultrafiltration of the acellular ascitic fluid was performed by means of an Amicon ultrafiltration cell and the Diaflo(R) membranes XM 50, PM 10 and UM 2. According to the manufacturers, these filters have cut-off levels at molecular weights of 50,000, 10,000 and 1,000, respectively, which means that substances with molecular weights below these levels and solvents will pass. Owing to the nature of molecular filtration the cut-off level is not a precise point, but an approximation. The protein concentration in the filtrate was determined according to the method of Lowry *et al.* [9].

Immediately after centrifugation the acellular ascitic fluid was placed in the ultrafiltration cell. The filtration was carried out at 0°C, using nitrogen at a pressure of about 25 p.s.i.

The test mice were inoculated with $2{\cdot}5 \times 10^6$ JB-1 ascites tumour cells, and 10 days later as much as possible of the tumour was aspirated. Twenty-four hours after the aspiration, in the recurrent phase of growth, the mice received an intraperitoneal injection of 2 ml of one of the various fractions of the ascitic fluid to be examined.

In order to investigate whether the inhibitory effect is dependent on a substance or substances of protein nature hydrolysis of the proteins in the ascitic fluid was performed by means of the insoluble enzyme "Enzite-Agarose-Protease(R)" (Miles-Seravac), which has a very broad specificity attacking almost all types of peptide linkages.

The accumulation of tumour cells in mitosis was followed by means of Colcemid(R), 1 mg/kg of body weight, given immediately after the injection of the derivatives of the ascitic fluid. Four hours after the addition of Colcemid(R), the mice were given tritiated thymidine, 1 μC/g of body weight, and 15 min later smears were prepared for autoradiography.

For 4 hr after injection of Colcemid(R) only, no change was observed in the labelling index of the JB-1 ascites tumours in recurrent growth, which is in accordance with the previously calculated 8-hr duration of G-1 in these tumours [7].

For further details as to the Colcemid technique and autoradiography, see ref. No. 7.

The mice were kept on Althromin(R) and tap water *ad lib.*

RESULTS

As previously demonstrated, injection of 2 ml of cell-free ascitic fluid obtained from mice bearing ascites tumours in the plateau phase of growth is followed by a break in the accumulation curve 2–3 hr after the injection, indicating a block of the cells in the G-2 phase [8].

It appears from Fig. 1 and Table 1 that injection of 2 ml of the fraction of acellular ascitic fluid which has passed the filters XM 50 and PM 10 is followed by inhibition of the cells in G-2, whereas the UM 2 filtrate has largely lost this capacity. This means that the arrest of the cells in the G-2 phase is probably caused by a substance with a molecular weight between 1,000 and 10,000.

Table 2 shows the labelling index of JB-1 ascites tumour cells in recurrent growth at 4 hr after the injection of 2 ml of the filtrates XM 50, PM 10 and UM 2. Usually, injection of 2 ml of cell-free ascitic fluid from mice bearing ascites tumours at the plateau is followed 2–5 hr after the injection by a decrease in the labelling index indicating a block in the presynthetic phase. It appears from Table 2

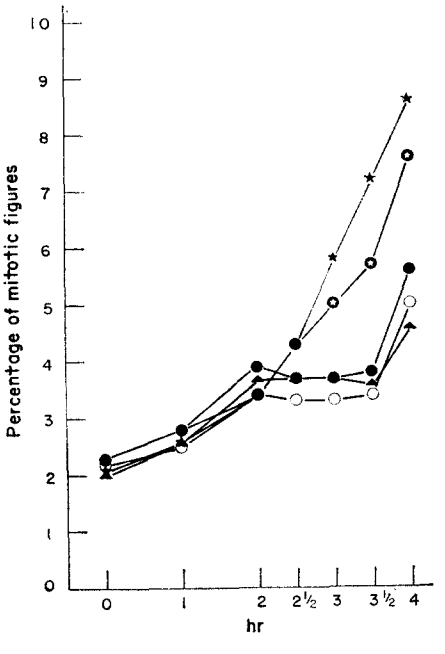

Fig. 1. *Accumulation of cells in mitosis of the JB-1 ascites tumour in recurrent growth following injection at zero hour of Colcemid(R) and various fractions (see text) of the cell-free ascites fluid obtained from JB-1 ascites tumours at the plateau. Symbols as in Table* 1.

Table 1. *Accumulation of cells in mitosis ± S.D. of JB-1 ascites tumour in recurrent growth following injection at zero hour of Colcemid(R) and various fractions (see text) of the cell-free ascites fluid obtained from JB-1 ascites tumours at the plateau (see Fig.* 1)

No. of animals		Hours after injection of Colcemid(R)						
		0 hr	1 hr	2 hr	2½ hr	3 hr	3½ hr	4 hr
★-★	controls (recurrent growth)	2·1 ± 0·2	2·6 ± 0·1	3·4 ± 0·3	4·3 ± 0·3	5·8 ± 0·4	7·2 ± 0·7	8·6 ± 0·7
▲-▲	addition of 2 ml of cell-free ascites	2·0 ± 0·3	2·6 ± 0·3	3·7 ± 0·4	3·7 ± 0·3	3·7 ± 0·1	3·6 ± 0·2	4·6 ± 1·2
●-●	addition of 2 ml of XM 50 filtrate	2·3 ± 0·1	2·8 ± 0·2	2·9 ± 0·2	3·7 ± 0·3	3·7 ± 0·2	3·8 ± 0·3	5·6 ± 1·2
○-○	addition of 2 ml of PM 10 filtrate	2·2 ± 0·1	2·5 ± 0·4	3·4 ± 0·6	3·3 ± 0·4	3·3 ± 0·2	3·4 ± 0·3	5·0 ± 0·5
✪-✪	addition of 2 ml of UM 2 filtrate	2·3 ± 0·2	2·5 ± 0·2	3·4 ± 0·3	4·3 ± 0·2	5·0 ± 0·3	5·7 ± 0·6	7·6 ± 1·0

S.D. = Standard deviation.

Table 2. Labelling index (L.I.) ±*S.D. of JB-1 ascites tumours in recurrent growth at 4 hours after injection of 2 ml of the various fractions (see text) of the cell-free ascites fluid. (Two different experiments—symbols as in Table 1—in brackets number of animals*

Symbols	L.I. ± S.D.	L.I. ± S.D.
★-★	59 ± 1 (4)	60 ± 1 (4)
▲-▲	42 ± 2 (5)	51 ± 3 (5)
●-●	43 ± 3 (12)	44 ± 5 (8)
○-○	58 ± 3 (11)	60 ± 3 (8)
✪-✪	58 ± 3 (8)	60 ± 2 (5)

S.D. = Standard deviation.

that the filtrate XM 50 still possesses this ability, but that the labelling index of the tumour cells remains unchanged after the injections of the filtrates PM 10 and UM 2.

Thus, the inhibition of cells in the presynthetic phase or G-1 seems to be due to a substance with a molecular weight between 10,000 and 50,000.

Determined by the method of Lowry *et al.* [9], the protein concentration in the XM 50 filtrate was 100 μg/ml. Since the XM 50 filtrate of the cell-free ascitic fluid was found to contain the inhibitor of both G-1 and G-2, hydrolysis of the proteins by means of the protease was carried out using the XM 50 filtrate instead of whole acellular ascitic fluid in order to reduce the amount of protein to be hydrolysed.

Having washed the enzyme "Enzite-Agarose-Protease(R)" three times in an excess of isotonic NaCl, 5 ml of the enzyme were added for 30 min to 20 ml of the filtrate XM 50 at 25°C and pH 7·5. The enzyme was removed by centrifugation at 1500 g for 10 min, and 2 ml of the hydrolysed XM 50 were injected intraperitoneally into each of 10 mice bearing JB-1 ascites tumour in recurrent growth.

The accumulation of cells in mitosis and the labelling index were determined as usual by means of Colcemid(R) and tritiated thymidine.

No decrease in the labelling index and no break in the mitotic accumulation curve was observed after this treatment, while XM 50 filtrate after identical treatment but without the addition of the enzyme was found to arrest the tumour cells as usual in both the G-1 and the G-2 phase.

DISCUSSION

It is generally agreed that a decreasing growth rate with increasing tumour size is a characteristic feature of most ascites tumours (10–15).

It was first demonstrated by Lala and Patt [1] that the decreasing growth rate of the Ehrlich ascites tumour with increasing tumour mass is due to a gradual prolongation of the mitotic cycle and a progressive decline in the growth fraction, while the rate of cell loss does not change appreciably during most of the growth.

Malaise and Tubiana [16] showed that neither the well-known lack of glucose, nor the low oxygen tension in the ascites tumour can explain the prolongation of the generation times. In accordance with these observations, two different ascites tumours grew almost independently to their respective maximum levels when inoculated simultaneously into the same mouse [17, 18].

In previous experiments it was demonstrated that when cell-free ascitic fluid obtained from JB-1 ascites tumours in their plateau phase of growth is injected into mice bearing JB-1 ascites tumours in recurrent growth, the tumour cells are specifically arrested in both the G-1 and the G-2 phases of the cell cycle [18].

In accordance with this observation an increasing portion of the tumour cells was found to accumulate in the G-1 and the G-2 phase with increasing age of the JB-1 ascites tumour [6, 7].

DeCosse and Gelfant [19] demonstrated that in the Ehrlich ascites tumour immune inhibition may restrain ascites tumour cells from cycling and block them in G-2, and apparently this block was neutralized by antilymphocyte sera.

The low molecular weights of the inhibitors in the ascitic fluid and the production of ascitic fluid with inhibitory properties in mice treated with sublethal irradiation [20] seem, however, to exclude the possibility that immune reactions could be responsible for the G-1 and the G-2 inhibition of the cell cycle in the JB-1 ascites tumour.

On the basis of the present and previous investigations [6, 7, 8, 18, 21, 22] on the JB-1 ascites tumour it is suggested that the growth of this tumour is controlled by a negative feedback mechanism similar to the growth regulation of some normal tissues. It is proposed that the control is brought about by humoral inhibitors produced by the tumour cells and specifically restraining these cells from proliferation by arresting them in the G-1 and the G-2 phases of the cell cycle.

In several normal tissues such as epidermis [23], granulocytes [24, 25], lymphocytes [26–28], skin melanocytes [29], liver [30–32], lung [33] and kidney [30, 34] humoral physiological

substances have been demonstrated which appear to act as the chemical messenger in a negative feedback mechanism controlling the growth of these tissues.

From studies on epidermis, the inhibitor, originally believed to be a single substance, was named chalone by Bullough [35].

The chalone was thought to be a humoral tissue-specific but species-unspecific mitotic inhibitor synthesized in the mature cells of a tissue and inhibiting the proliferation of the progenitors of the same tissue.

In all probability, the delicate growth control of a tissue is maintained by a number of inhibitory and stimulatory influences, and recently it has been demonstrated that the epidermal chalone is probably composed of at least two different substances inhibiting the cells in both the G-1 and the G-2 phases of the cell cycle [36–39].

Nobody has so far succeeded in purifying chalone, and since some laboratories are investigating the G-1 inhibitory effect and other laboratories the inhibition in G-2, it remains unknown whether the term chalone in future will stand as the name of a single substance or be reserved for a concept of the negative feedback autoregulation of normal growth.

The increasing growth inhibition of the ageing JB-1 ascites tumour seems in principle to be in accordance with a feed back phenomenon similar to the situation in normal tissues.

In some malignant tumours, e.g. the rabbit epidermal carcinoma V × 2 [40] and a rat granulocytic leukaemia [41], the cells apparently continued to synthesize the chalone of their tissue of origin, but it was found that the intracellular concentration of chalone was reduced to 1/10–1/40 of normal, probably because of increased permeability of the cell membrane, but still the tumour cells responded with mitotic inhibition if the concentration of the chalone extracted from the tissue of origin was artificially raised.

Thus, a seriously reduced intracellular concentration of chalone of the tissue of origin referable to increased loss through the cell membrane has been suggested to be a common characteristic of malignant tumours [42].

With an increasing number of tumour cells the extracellular concentration of chalone will increase and possibly, provided the host lives long enough, reach a level sufficiently high to produce a new balance between cell gain and cell loss. In this respect, malignant growth has been compared with a hypertrofied normal tissue [42].

However, it is a question whether the balanced growth of a tumour at the increased level is, as suggested, a condition of "hypertrophy" due to "simple" loss of chalone through the cell membrane, or whether the plateau observed in the ascites tumour represents an altered, but natural and genetically determined physiological upper limit [43] of the size of this new tissue.

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The Action of the Walker 256 Carcinoma and Toxohormone on Amino Acid Incorporation into Diaphragm Protein*

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Abstract—*In rats bearing the Walker* 256 *carcinoma, the diaphragm was markedly depleted of protein, the proteins of the contractile elements being depleted to a greater extent than sarcoplasmic or extracellular proteins. Under* in vitro *conditions the incorporation of* ^{3}H-4, 5-*lysine into the myofibrillar but not the sarcoplasmic proteins of diaphragm was significantly reduced. Toxohormone, prepared from the Walker* 256 *carcinoma, caused a marked inhibition of the* in vitro *incorporation of* ^{3}H-*lysine into diaphragm proteins both when the preparation was injected into the animal and also when it was added to the incubation medium. The inhibitory effect of toxohormone was directed at incorporation into myofibrillar proteins.*

INTRODUCTION

MANY OF the systemic effects of tumours on their hosts have been reproduced in normal animals by the injection of purified extracts of tumour tissue which were given the name "toxohormone" by Nakahara and Fukuoka [1]. Thus increases in liver weight, adrenal weight, liver protoporphyrin, plasma unesterified iron binding capacity, and decreases in spleen weight, thymus weight, liver catalase, kidney catalase, blood haemoglobin, plasma bound iron, liver ferritin, nicotinamide dinucleotide synthesis and liver absorbic acid, all characteristic findings in tumour-bearing animals have also been observed in rats injected with toxohormone [2, 3, 4].

Depletion of muscle protein is quantitatively the most important cause of tissue protein loss in tumour-bearing animals [5]. In the present work therefore a study was made of the effect of a toxohormone preparation on amino acid incorporation into protein of isolated diaphragm since previous work had shown depletion of muscle protein was associated with an impairment of protein synthesis [6].

MATERIAL AND METHODS

Treatment of animals

Male Wistar rats of fasting body weight 175–225 g were injected subcutaneously in the dorsal region with a suspension of Walker 256 carcinoma cells and paired with control rats on the basis of initial body weight. Rats were housed individually and fed daily 14 g finely ground rat cake (North Eastern Agricultural Cooperative Ltd.), made into a paste with water. Both groups of rats consumed completely all food offered during the experimental period. After 10 to 14 days, when tumours had grown to between 20–40 g, tumour-bearing animals and their controls were killed and the diaphragm was removed, either maintained on its

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rib cage ("intact diaphragm" preparation) as described by Kipnis and Cori [7], or with central tendon lateral and dorsal slips dissected away and the remaining muscle bisected ("hemi-diaphragm preparation") [8].

On two successive days, 8 normal animals maintained as described in the preceding paragraph were injected intraperitoneally with 150 μg purified toxohormone dissolved in 1 ml distilled water and 4 others were injected with distilled water alone. Three hours after the second injection, 4 of the rats given toxohormone and 2 of the control rats were killed and the remaining animals were killed after a further 24 hr. Hemidiaphragm preparations were made from these animals.

Fractionation and estimation of diaphragm protein

Sarcoplasmic, myofibrillar and extra-cellular proteins of hemi-diaphragms were fractionated as described by Dickerson [9]. Protein content of the sarcoplasmic and myofibrillar fractions was estimated by the method of Lowry *et al.* [10] and that of the extracellular fraction by a micro-kjeldahl nitrogen determination [11].

In vitro *incorporation of ^{3}H-lysine into diaphragm protein*

Intact diaphragms or hemi-diaphragms were incubated in stoppered erlenmeyer flasks in Krebs–Henseleit buffer, pH 7·4 (Krebs and Henseleit, 1932) gassed with O_2:CO_2 (95: 5v/v) and containing 0·3 or 3 μCi per ml. L-^{3}H-4, 5-lysine (5·5 Ci per m mole Radiochemical Centre, Amersham). The total volume of the incubation medium was 25 ml with intact diaphragms and 5 ml in the case of hemi-diaphragms. In some experiments unlabelled L-lysine was added to the incubation medium to give a final concentration of 5 mM and in others, the medium supplemented with an amino acid mixture patterned on the proportions of amino acids found in rat serum [13, 14], the final concentrations of amino acids being—L-alanine, 0·80 mM, L-arginine, 0·44 mM, L-asparagine, 0·08 mM, L-aspartic acid 0·08 mM, L-cysteine, 0·88 mM, L-glutamic acid 0·38 mM, L-glutamine, 0·38 mM, glycine, 0·43 mM L-histidine, 0·18 mM, L-isoleucine, 0·19 mM, L-leucine, 0·34 mM, L-lysine, 0·96 mM, L-methionine, 0·14 mM, L-phenylalanine, 0·16 mM, L-proline, 0·48 mM, L-serine, 0·58 mM, L-threonine, 0·58 mM, L-tryptophan, 0·14 mM, L-tyrosine, 0·18 mM and L-valine, 0·40 mM. In experiments to study the *in vitro* effect of toxohormone on ^{3}H-lysine incorporation by hemi-diaphragms, 30 μg toxohormone was added per ml of incubation medium which was supplemented with the amino acid mixture.

The flasks were shaken at 110 oscillations per minute in a Gallenkamp Metabolic shaker. At the end of the incubation period tissues were removed from the flask, blotted and hemi-diaphragms frozen immediately in liquid nitrogen. The intact diaphragms were dissected from the rib cage, dorsal and lateral slips and central tendons removed and the remainder frozen in liquid nitrogen. When incorporation into total intracellular protein was being studied, the frozen muscles were minced finely with scissors and homogenized ia 0·3 N NaOH at 0° in a Potter–Elvehjem homogenizer [15]. The homogenate was centrifuged at 600 g for 15 min at 2°C. The precipitate was re-homogenized twice in 0·3N NaOH and centrifuged after each homogenization. The original supernatent and washings were collected and protein precipitated by the addition of 0·5 vol. 30% (w/v) trichloracetic acid, centrifuging between each washing. The protein was then washed twice with ethanol, once with ether and dried under vacuum. When incorporation into myofibril and sarcoplasmic proteins was studied these fractions were separated from the frozen muscle according to Dickerson [9] and each fraction treated as described for total intracellular protein.

The dried proteins were milled for 10 minutes in an agate ball-mill (RIIC Ltd London) and counted using NE210 scintillator gel (Nuclear Enterprises, G.B. Ltd). The specific activity of samples was found to be independent of milling times longer than 7 minutes and amounts of protein less than 10 mg.

Preparation of toxohormone

The method followed was essentially that of Yunoki and Griffin [16]. Walker 256 caricomata were extracted with a mixture of acetone and glacial acetic acid [32:1). Ether was added to the extract and the precipitate was separated by centrifugation and dried. 2 g of the precipitate were suspended in 200 ml 0·1M glycine-NaOH buffer, pH 9·0 and any material which did not dissolve was removed by centrifugation. The supernatant was applied to a 2·5 × 40 cm column of amberlite CR-50 (200 mesh) previously equilibrated with the glycine-NaOH buffer. The column was developed with the same buffer at a flow rate of 300 ml/hr and the eluate monitored at 283 mμ using an LKB Uvicord mark II. After the first fraction was eluted, the pH of the eluant was increased gradually by the addition of a 0·1M glycine buffer pH 11·0, to the pH 9·0

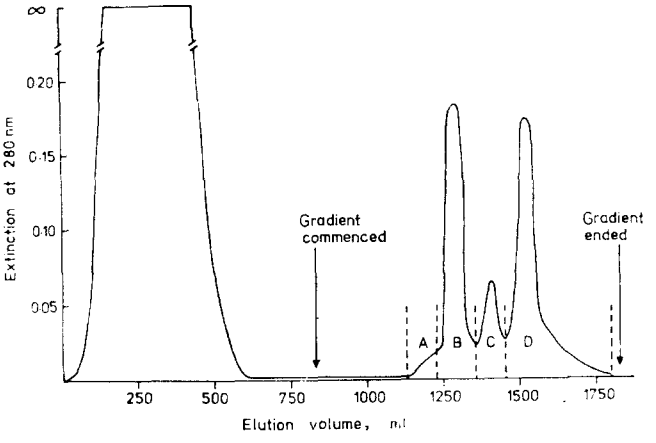

Fig. 1. Elution pattern of crude toxohormone from amberlite CR 50 *column. The starting buffer was* 0·1M *glycine*—NaOH, *pH* 9·00, *and the gradient was produced by the addition of* 500 *ml.* 0·1M *glycine*—NaOH *to* 500 *ml of the starting buffer through a* 500 *ml. mixing chamber. The flow rate was* 300 *ml/hr.*

buffer using a 500 ml mixing chamber. The elution pattern is shown in Fig. 1. Fractions A-D were lyophilized, dissolved in the minimum amount of distilled water and passed through a 1·5 × 90 cm column of Sephadex G-15 to remove glycine and Na^+. The material eluted with the void volume when each of the 4 fractions was passed through the Sephadex column caused depressions of more than 30% and 50% in plasma iron when injected into mice at levels of 5 and 25 ug per mouse respectively. The large molecular weight fraction from the 4 sephadex separations were therefore combined, lyophilized and used as a source of toxohormone in the experiments with incubated diaphragms. The amount of material present in the fractions and in the final preparation was estimated using the micro-biuret method of Itzhaki and Gill [17] with crystalline bovine plasma albumin (Sigma) as standard.

Studies on the Spinco Model E ultracentrifuge using the meniscus depletion equilibrium method [18] indicated that the final preparation contained two components with molecular weights in the region of 8,000 and 14,000 respectively.

Assay of toxohormone activity

The activity of toxohormone preparations was assayed by the extent to which they lowered plasma Fe levels in mice. Groups of 6 mice weighing 20–25 g were injected intra-peritoneally, between 21·00 and 22·00 hr, with toxohormone dissolved in distilled water or with distilled water alone. After 12 hr the mice were anaethestised with an intraperitoneal injection of 0·15 ml 6% (w/v) sodium pentobarbitone and blood removed from the heart using a finely-drawn heparinized Pasteur pipette. Plasma Fe was estimated essentially as described by Sanford [19]. Figure 2 shows the dose-response curve of the final purified toxohormone preparation used in the incubation experiments.

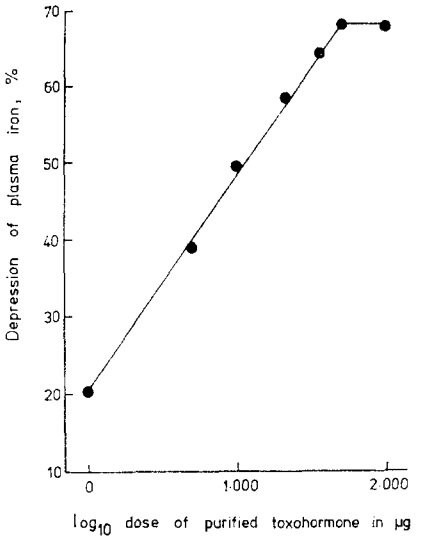

Fig. 2. The effect of intraperitoneal injection of increasing amounts of purified toxohormone on mouse plasma iron.

RESULTS

Alterations in protein content of diaphragm in rats bearing the Walker 256 *carcinoma*

Previous work with rats bearing the Walker 256 carcinoma showed that the effect of the tumour on skeletal muscle protein varied in different types of muscle [20]. Table 1 shows that the Walker 256 carcinoma caused a marked loss in weight and protein content of the diaphragm. From the change in percentage

Table 1. *The effect of the Walker* 256 *carcinoma on diaphragm weight and total protein*

	Control	Tumour-bearing	*P**
Initial body weight (g)	195 ± 5	204 ± 3	—
Final body weight (g)	219 ± 9	189 ± 2†	< 0·01
Tumour weight (g)	—	25 ± 2	—
Diaphragm weight per 100 g initial body weight (g)	0·308 ± 0·015	0·234 ± 0·015	< 0·01
Total diaphragm protein per 100 g initial body weight (mg)	59·40 ± 3·20	44·08 ± 3·45	< 0·01

Results are expressed as the mean ± S.E. of 6 rats.

*In this and the following 2 tables, *P* refers to the statistical significance of the differences between control and tumour-bearing rats by Students *t*-test.

†Weight of tumour excluded.

Table 2. *The effect of the Walker* 256 *carcinoma upon the relative amounts of myofibrillar, sarcoplasmic and extra-cellular proteins of the diaphragm*

	mg protein per 100 mg total protein		
	Sarcoplasmic	Myofibrillar	Extra-cellular
Control	25·27 ± 0·75*	64·89 ± 0·40	10·30 ± 0·34
Tumour-bearing	27·48 ± 0·42	61·51 ± 0·58	10·68 ± 0·30
P	< 0·05	< 0·01	N.S.

*Results are expressed as the mean ± S.E. of 6 rats.

Table 3. *The effect of the Walker* 256 *carcinoma upon the* in vitro *incorporation of* ^{3}*H-lysine into protein by intact and hemi-diaphragms*

	Diaphragm preparation	Additions to incubation medium: 5 mM lysine	Additions to incubation medium: Amino acid mixture*	c.p.m. per mg protein	*P*
Control	Intact†			501 ± 11	
Tumour-bearing	diaphragm	−	−	254 ± 8	< 0·001
Control	Hemi-†	−	−	447 ± 17	
Tumour-bearing	diaphragm			234 ± 12	< 0·001
Control	Hemi-‡	+	−	972 ± 22	
Tumour-bearing	diaphragm			555 ± 26	< 0·001
Control	Hemi-‡	−	+	962 ± 45	
Tumour-bearing	diaphragm			452 ± 14	< 0·001

Results are expressed as the mean ± S.E. of 5 rats

*See "Experimental" section.

†0·3 μC_i ^{3}H-lysine per ml incubation medium.

‡3·0 μC_i ^{3}H-lysine per ml incubation medium.

distribution of diaphragm proteins in tumour-bearing rats (Table 2), it is evident that a disproportionate depletion of myofibrillar proteins had occurred, although sarcoplasmic and extra-cellular proteins were also decreased in total amount.

The in vitro *incorporation of ^{3}H-lysine into proteins of diaphragm of normal and tumour-bearing rats*

The *in vitro* incorporation of ^{3}H-lysine into total protein was significantly decreased in intact diaphragms or hemi-diaphragms from tumour-bearing rats (Table 3). The possibility that the effect of the tumour on lysine incorporation into protein was secondary to changes in lysine precurser pools appeared to be excluded by the finding that the effect of the tumour still persisted when a large excess of unlabelled lysine was added to the medium.

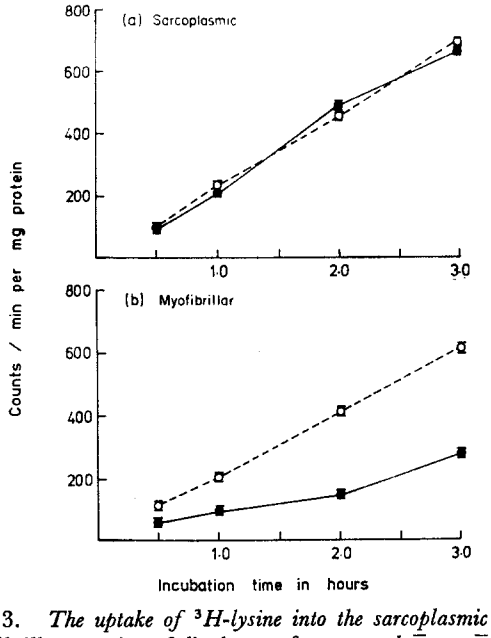

Fig. 3. *The uptake of ^{3}H-lysine into the sarcoplasmic and myofibrillar proteins of diaphragm from normal ○ . . . ○ and tumour-bearing rats ● – ●. The specific activity of the medium was initially* 0·3 *μCi per ml.*

The addition to the medium of an adequate supplement of amino acids at approximately twice the concentration at which they occur in normal rat serum also did not alter the relative incorporation rates of diaphragms from control and tumour-bearing rats, suggesting that the defective incorporation by the latter was probably not due to one or more amino acids being present in limiting amounts. Figure 3 shows the incorporation of ^{3}H-lysine into myofibrillar and sarcoplasmic proteins of hemidiaphragms from control and tumour-bearing rats with increasing time of incubation. With sarcoplasmic proteins there was no significant difference in uptake between the two preparations, whereas incorporation into myofibrillar proteins was reduced in muscles from tumour-bearing rats at all times studied

The effect of toxohormone administered intraperitoneally on the ability of hemidiaphragms to incorporate ^{3}H-lysine into protein

The injection of 150 μg toxohormone into rats on two successive days resulted in a significant decrease in the ability of the hemidiaphragms isolated from these animals to incorporate ^{3}H-lysine into protein (Table 4).

Table 4. *The effect of the administration of* 300 *μg toxohormone to rats on the* in vitro *ability of their diaphragms to incorporate ^{3}H-lysine into protein*

	c.p.m. per mg protein*	*P*‡
Control rats	486 ± 9	—
3 hr after second† toxohormone injection	394 ± 13	< 0·005
24 hr after second toxohormone injection	143 ± 7	< 0·001

Results are the mean ± S.E. of 4 rats.
*The specific activity of the medium was 0·3 μC_1 per ml.
†See "Experimental" section.
‡In this and the following table *P* refers to the statistical significance of the differences between control and treated rats by Student's *t*-test.

This effect was found to be significantly greater at 24 hr than at 3 hr after the last injection ($P < 0·001$). During the period of the experiment the animals given toxohormone consumed all food offered and showed a similar weight gain to that of the control rats.

When toxohormone was added to the incubation medium containing hemidiaphragms from control rats it was found to decrease significantly the incorporation of lysine into myofibrillar proteins but to have little effect on incorporation into sarcoplasmic proteins (Table 5). The differential effect of the preparation on protein synthesis in muscle is thus similar to that shown by the tumour itself.

Table 5. *The* in vitro *effect of toxohormone on the ability of isolated rat diaphragms to incorporate ^{3}H-lysine into myofibrillar and sarcoplasmic protein*,†*

	c.p.m. per mg protein‡	
	Sarcoplasmic	Myofibrillar
Control	1517 ± 79	681 ± 89
Toxohormone‡	1319 ± 70	329 ± 35
P	N.S.	< 0·005

*Incubation medium supplemented with amino acid mixture (see "Experimental").
†Specific activity of medium was 3 μCi per ml.
‡30 μg per ml incubation medium.

DISCUSSION

From the results in Table 1 it is evident that the diaphragm of the rat is a muscle which undergoes marked wasting due to the Walker 256 carcinoma. The extent of weight and protein loss was more extensive than that observed previously in the gastrocnemius and soleus in rats bearing the same tumour at a similar stage of growth [20]. The protein loss in the gastrocnemius and soleus was restricted to the contractile elements whereas the loss in the diaphragm appeared more general.

The difference in response of the diaphragm and soleus to the tumour is of particular interest, since in the rat both have a predominance of red fibres and have similar, and, for skeletal muscles, relatively rapid rates of protein synthesis [21]. There is however evidence that the red fibres in the diaphragm have a relatively rapid rate of contraction in contrast to the rate found in red fibres in a typical tonic muscle such as the soleus [22]. Thus the evidence available would suggest that the depleting action of Walker 256 carcinoma is more marked in muscle fibres with a rapid rate of contraction.

The Walker carcinoma inhibits the ability of polyribosomes from the gastrocnemius to incorporate amino acids into protein [6]. From the decrease in the incorporation of ^{3}H-lysine observed in diaphragms of tumour-bearing rats it is seen that the tumour causes an impairment in the protein synthesis in this muscle also (Table 3). The possibility that the tumour causes an increased catabolism of protein in the diaphragm is not excluded in the present work. Indeed, the fact that in tumour-bearing rats there was no alteration in the rate of incorporation of ^{3}H-lysine into sarcoplasmic proteins although these were depleted (Tables 1 and 2) would appear to indicate that this was occurring.

The mechanism whereby the tumour brings about changes in the tissue of the host remains unknown. The theories of Mider *et al.* [23] and of Wiseman and Ghadially [24] which postulated that the tumour deprives the tissues of essential nutrients and thus causes them to stop synthesis are inadequate to explain all facts of tumour-host relationships [25]. The idea that a humoral substance is secreted by the tumour and acts on the host tissues was proposed by Greenstein [26] in 1943. In the present work, a fraction was isolated from the Walker 256 carcinoma comprising 2 components which were of the same order of molecular size as that of the most active toxohormone preparations of other workers [27, 28]. The findings that this preparation, either when administered to intact animals or when added to the isolated diaphragm, caused a decrease in the ability of the muscle to incorporate amino acids into protein lends support to the view that the effect of the tumour may be mediated by the secretion of such factors. The fact that both the toxohormone and the action of the tumour specifically affected the synthesis of myofibrillar protein is of significance in this respect. The presence of such substances in the blood of the tumour-bearing host, however, remains to be established.

The observation that the *in vitro* effect of the preparation on the ability of the diaphragm to incorporate amino acids was more pronounced at 24 hr than at 3 hr after the last injection of toxohormone (Table 4) suggests that the initial action of the preparation on protein synthesis may be permanent and that the effect of toxohormone on fundamental aspects such as RNA synthesis should be investigated.

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1-(2-Chloroethyl)-3-Cyclohexyl-1-Nitrosourea (CCNU, NSC-79037): A Comparison of Drug Administration at Four-week and Six-week Intervals

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Abstract—1-(2-*Chloroethyl*)-3-*cyclohexyl*-1-*nitrosourea* (*CCNU, NSC*-79037) *is a new oral nitrosourea compound, which had demonstrated antitumor activity both in animal tumor models and in man. The clinical efficacy of the compound as well as the toxicity were evaluated in* 50 *adult patients with a variety of malignant tumors on two different dosage schedules:* 130 *mg/m*^{2} *either every* 4 *weeks or every* 6 *weeks. Responses were observed in patients with bronchogenic carcinoma, glioblastoma, adenocarcinoma of the colon, and pancreatic carcinoma. The main dose-limiting toxicity was delayed thrombocytopenia and leukopenia occurring* 4–6 *weeks, respectively, after drug administration. Patients receiving the every* 4 *week schedule tolerated at least* 3 *courses of therapy without severe dose-limiting toxicity.*

INTRODUCTION

THE NITROSOUREA derivatives are a group of recently developed investigational chemotherapeutic agents which have shown a high degree of efficacy in representative animal tumor systems [1]. The first of the nitrosoureas to receive extensive clinical trials was 1,3-*bis*-(2-chloroethyl)-1-nitrosourea (BCNU, NSC-409962), which was found to be effective against a wide spectrum of malignant neoplasms. Subsequently, other related nitrosourea compounds have been synthesized and tested in animal tumor systems. One of these is 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU, NSC-79037). CCNU differs from BCNU structurally by having one of the chloroethyl groups replaced by a cyclohexyl moiety, as shown in Figure 1. This compound has also demonstrated superiority over standard agents such as 5-fluorouracil, vincristine, methotrexate, or cyclophosphamide in experimental tumors such as Walker carcinosarcoma 256, leukemia L1210, melanoma B16, and intracerebrally implanted gliomas [1].

Other features make CCNU a desirable compound for use in clinical trials. In contrast to BCNU, CCNU is readily absorbed from the gastro-intestinal tract and can be given orally. Also, the lipid solubility of CCNU (Pi value 2·5) is about double that of BCNU (Pi value 1·39) [1].

Initial clinical investigations of CCNU in a limited number of patients have previously been reported by us [2]. Activity was observed

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against several human solid tumors and has been confirmed by other reports [1]. Furthermore, CCNU's superiority over BCNU in the treatment of advanced Hodgkin's disease has been established [3].

At the recommended moderately toxic dose of 130 mg/m^2 in patients with solid tumors, the main dose-limiting toxicity was delayed hematological toxicity, with the nadir of thrombocytopenia and leukopenia occurring at 4–6 weeks respectively after administration of the drug [2]. In the initial clinical investigations, the interval between doses had been 8 weeks and thereafter was shortened to 6 weeks when it became apparent that the latter schedule was well tolerated. Since then we have explored a dose of 130 mg/m^2 every 4 weeks and compared it to the q 6-week schedule for drug tolerance. The purpose was to evaluate the possibility of increasing the total amount of drug given in a fixed period of time without affecting host toxicity, thereby increasing the therapeutic index of the compound.

This report describes our experience with CCNU at two different dose schedules of administration, the q 4-week and the q 6-week schedules. It also includes a review of the current status of the clinical efficacy of the compound.

MATERIAL AND METHODS

Fifty patients (48 males and 2 females) with histologically confirmed diagnosis of cancer have been adequately evaluated. The ages of the patients ranged from 21 to 74 years, with a median age of 51 years. Twenty-five patients had received previous chemotherapy or radiotherapy, or both. Normal white blood count (above 5000/mm^3) and platelet count (above 250,000/mm^3) were prerequisites for entering the study.

Drug and mode of administration

CCNU (a yellow powder supplied in 10, 40 and 100 mg capsules by the Cancer Therapy Evaluation Branch of the National Cancer Institute) was given orally at a dose of 130 mg/m^2 on two different dosage schedules. On one schedule, the drug was given on an every 4-week basis, independent of subsequent hematological parameters while the other schedule was q 6-weeks, again given independent of

Table 1. Effect of CCNU in adults with solid tumors.

	130 mg/M^2 q 6 weeks			130 mg/M^2 q 4 weeks		
Tumor type	CR†	PR‡	NC-PROG§	CR†	PR‡	NC-PROG§
Bronchogenic carcinoma						
Adeno		1 (36)*	4			
Large-cell anap		1 (28)*	1			1
Small	1 (12)*		2			
Squamous			1			
Esophageal						4
Stomach						3
Colon-rectum			2	1 (10)*	1 (8)*	3
Pancreas						
Adeno.					1 (30)*	
Anap.			1			
Melanoma			4			
Reticulum cell carcinoma				1 (41+)*		
Myeloma			1			
Cancer of oral cavity			3			2
Glioblastoma		2 (10) (50)*	4		1 (6)*	
Others:						
Testicular embryonal			1			
Hypernephroma			1			
Prostate carcinoma						1
Chordoma						1
Totals	1	4	25	2	3	15

* Numbers in parenthesis denote weeks in remission or response.
† CR = complete response, defined as complete regression of all measurable tumors.
‡ PR = partial response, defined as partial regression of more than 50% of objectively measurable tumors, using the product of the longest and widest perpendicular diameter.
§ NC – Prog = no change or progression.

subsequent hematological parameters. Following the initial dosage of drug, subsequent courses were given on the same schedule as long as the patient was free of progressive disease or did not develop pronounced toxicity.

Assessment of results

The criteria of drug response were those of objective regression of neoplastic lesions as measured by physical examination, or by *x*-ray films. A complete response (CR) was defined as complete regression of all measurable tumors while a partial response (PR) was a more than 50 per cent regression of objectively measurable tumors, using the product of the longest and widest perpendicular diameter. A third category, no change or progression (NC-PROG), indicated that the disease either remained static or progressed during therapy. Patients with glioblastoma, for which the usual criteria of objective response in most cases could not be applied, were classified as having a partial response when an improvement in neurological signs and/or symptoms and/or decrease of more than 50% in brain scans lasting more than one month was observed. Routine blood counts, electrolytes, and liver function studies were done for evaluation of toxicity.

RESULTS

Clinical response

Of the 50 patients receiving CCNU, 30 were given the drug every 6 weeks, while 20 patients received CCNU every 4 weeks (Table 1). Objective improvement occurred in 3 of 11 patients with bronchogenic carcinoma and 2 of 6 glioblastoma patients when an every-6-week schedule was utilized. Responses seen on the every-4-week schedule were observed in 2 of 5 patients with adenocarcinoma of the colon, 1 of 2 with pancreatic adenocarcinoma, 1 with reticulum cell sarcoma, and 1 with glioblastoma. The duration of response ranged from 10 to 50+ weeks on the 6-week schedule, and from 6 to 41+ weeks on the 4-week schedule of drug administration.

Clinical toxicity

Of the 136 courses of therapy in the 50 patients, 79 courses were on the q 6-week schedule, while 57 were on the q 4-week schedule. Nausea and/or vomiting, usually occurring 4–8 hours after drug intake, was observed in almost all patients. Definite drug related hepatic or renal toxicity was not observed in any patients.

The delayed hematological toxicity of thrombocytopenia and leukopenia occurring 4–6 weeks respectively after CCNU has been previously noted and reported elsewhere [2]. Hematological toxicity in this report has been evaluated by observing the nadir of the hematocrit, white blood count, and platelets following each dose of CCNU. Nadirs of counts were evaluated separately for successive courses of therapy and listed for the individual courses. Delayed toxicity after 3 courses of therapy could not be determined with accuracy on the q 4-week schedule, since successive nadirs often overlapped additional courses of therapy. The differences in hematologic toxicity between the two schedules is shown in Table 2. It can be appreciated that at least three courses of CCNU can be given with either schedule before decrease occurs in median nadirs of white blood count, while thrombocytopenia occurs faster on the 4-week schedule. The duration of thrombocytopenia and leukopenia on the q 6-week schedule was similar to the data previously reported [2]. Four patients received CCNU q 4 weeks for more than 6 months without signs of cumulative toxicity. In one of these, however, the drug dose was decreased to 75 mg/m^2 after 11 courses because of thrombocytopenia below 30,000 and leukopenia below 1000/mm^3. No consistent change was noted in the hematocrit or reticulocyte count.

Table 2. Leukopenia and thrombocytopenia following single and repeated doses of CCNU Q 6 weeks in 30 patients

	Leukopenia nadir Counts × 10^3		Thrombocytopenia nadir Counts × 10^3	
Course	Median	Range	Median	Range
1	3·0	1·4–5·8	67·0	20–186
2	3·4	1·3–4·5	83·0	16–192
3	3·5	1·1–4·3	94·0	40–174
Leukopenia and thrombocytopenia following single and repeated doses of CCNU Q 4 weeks in 20 patients				
1	3·5	2·3–6·3	135·0	49–188
2	3·0	1·1–6·9	132·0	58–189
3	2·5	1·0–3·8	62·0	11–170

DISCUSSION

Clinical experience with CCNU is increasing rapidily. The drug is proving to be as active an agent as BCNU in some tumor types, and more active than BCNU in others. CCNU also has the advantage of oral administration.

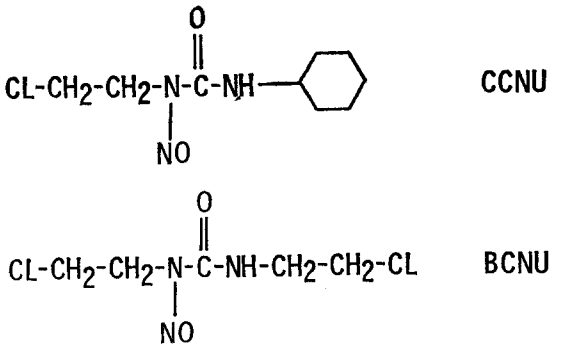

Fig. 1. *The structure of* 1-(2-*chloroethyl*)-3-*cyclohexyl*-1-*nitrosourea* (*CCNU, NSC*–79037) *and* 1, 3-*bis* (2-*chloroethyl*)-1-*nitrosourea* (*BCNU, NSC*–409962).

The results indicate that, when CCNU is given alone, repeat courses can be given with relative safety for at least three 4-week courses of therapy. We have, however, extended therapy in some patients beyond twelve weeks and found the drug to be tolerated well without dangerous thrombocytopenia. Leukopenia does not present the same problem, although some cumulative toxicity is also noted after the third course of therapy.

The data accumulated from the q 4-week schedule suggest that the same amount of drug can be administered in a shorter period of time than when the q 6-week schedule is used. In the twelve week period of q 4-week administration, a total dosage of 390 mg/m^2 can be given, while only 260 mg/m^2 on the q 6-week schedule has been given during the same time period. Additional drug administration in a shorter period of time would be particularly advantageous in patients with rapidly progressing disease.

Preclinical data from animal tumor models have demonstrated that nitrosoureas, when used in combination therapy with antimetabolites and alkylating agents, exhibit a potentiating effect. Specifically, in murine leukemias such as L1210, the combination of CCNU with either cytosine arabinoside or hydroxyurea is potentiating in terms of cytotoxic effects in that tumor system [1, 4]. Also, both BCNU and methyl-CCNU have demonstrated potentiating cytotoxic effects in the L1210 tumor system and in the Lewis lung tumor system when used in combination with cyclophosphamide [1, 5, 6, 7]. With the ability of CCNU to be given every 4 weeks, concomitant administration with cyclophosphamide may be accomplished with the two agents.

The biochemical effects of the nitrosoureas have been studied primarily with BCNU. It is generally agreed that this class of compounds exhibits its oncolytic effect by inhibiting the synthesis of DNA and RNA. Several mechanisms of action for this inhibition have been postulated: alkylation following the formation of alkyl diazohydroxides and/or 2-chloroethylamine [8]; selective interference with the utilization of histidine in 1-carbon metabolism through the inhibition of forminotransferase [9]; increased NADase activity and decrease of the concentration of NAD + in tumor cells [10]; and decrease of DNA nucleotidyltransferase activity [11]. Further studies by Cheng *et al.* seem to indicate a dual role of CCNU in the interaction with molecular products within the cell [12]. Their experimental results suggest that CCNU, perhaps via cyclohexylisocyanate, modifies proteins mainly by cyclohexylcarbamoylation of lysine residues and that CCNU modifies nucleic acids via alkylation [13]. Probably due to the effects of RNA, DNA and Protein synthesis, the compound has not been shown to be a cell-cycle specific agent but appears to work in all phases of the cell-cycle. Nevertheless, specific effects during the *S*-phases of the cell-cycle have been observed. Bray *et al.*, studying the cell-cycle effects produced by CCNU and its decomposition products in L1210 ascites leukemia, demonstrated a prolongation of *S*-phase to twice normal when CCNU, BCNU, and cyclohexylisocyanate were used, while no prolongation was noted when cyclohexylamine, NN'dicyclohexylurea or cyclophosphamide were utilized [14]. Thus, cyclohexylisocyanate appears to be responsible, at least in part, for the prolongation of the duration of the *S*-phase noted after treatment with CCNU.

Table 3. *Current status of CCNU in adults with solid tumors.*

Tumor	No. of patients responding	Objective response rate	Ref.
Glioblastoma	8/16	50%	15
Bronchogenic carcinoma	3/12	25%	
Gastrointestinal carcinoma	9/63	14%	16
Hodgkin's disease	14/19	74%	3
Renal cell carcinoma	2/12	17%	19

Other data suggest a high degree of activity of the compound in the treatment of brain tumors with 3 of 7 patients responding to therapy. Other clinical data also point to the drug usefulness in the treatment of primary brain tumors [15]. The rationale for its use in brain tumors is predicated on the drug high

degree of lipid solubility and lack of ionization at physiological pH, thus making passage across the blood-brain barrier possible. Furthermore, pharmacological studies have confirmed the drug's passage into the cerebrospinal fluid. The physiologic distribution of radioactive CCNU was studied in 13 patients with malignancy by Oliverio *et al.* [17]. Plasma levels of the isotope label were prolonged following oral administration at doses ranging from 30 mg/m^2 to 100 mg/m^2; the half-life in the plasma ranged from 16 hours to 2 days. Serial cerebrospinal fluid determinations showed concentrations which were about 50% of concurrent plasma ^{14}C levels of labeled CCNU. Various tissues taken at about 15 minutes after administration contained ^{14}C concentrations comparable to that of plasma.

Walker *et al.* have studied the drug in 18 patients with malignant gliomas at a dose of 130–150 mg/m^2 every 2 months and observed 5 complete or partial remissions [15]. Other workers have confirmed these findings [1]. The usefulness of a nitrosourea (BCNU) in combination with radiotherapy has also been demonstrated in a prospective controlled randomized group [18]. In that study, the combination of BCNU and radiotherapy in patients with glioblastoma yielded a significant prolongation of survival over groups of patients receiving only supportive therapy, or BCNU alone.

In the lymphomas, the superiority of CCNU over BCNU has recently been reported [3]. Thirty-eight patients with advanced Hodgkin's disease, resistant to commonly used agents, were randomized between comparable dosages of BCNU and CCNU. An objective response of more than 50 was seen in 14 of 19 (74%) patients on CCNU and 5 of 19 (26%), patients on BCNU, including 4 and 1 complete remissions, respectively. The difference was statistically significant at the $p = 0{\cdot}01$ level. No cross-resistance was noted with alkylating agents such as mechlorethamine and cyclophosphamide.

Carcinoma of the lung has, in the past, been relatively insensitive to standard chemotherapeutic agents. Hence the preliminary response in 3 of 12 patients reported here are of considerable interest.

The activity observed in gastrointestinal carcinoma has been confirmed by other investigators. Moertel *et al.* [16] have indicated a 12% objective response rate in a variety of gastrointestinal malignancies, primarily adenocarcinoma of the colon including patients resistance to other chemotherapeutic agents. Antitumor effect has also been seen in 2 of 12 patients with metastatic renal cell carcinoma of more than 6 months duration [19].

In summary, CCNU has demonstrated definite antitumor effect against a variety of solid tumors when used as a single agent (Table 3). The lack of cross resistance to other classes of anti-neoplastic agents, oral administration, high degree of lipid solubility, and predictable toxicity add to its clinical usefulness. Furthermore, the demonstration of the synergistic effect of nitrosoureas with antimetabolites and alkylating agents in preclinical models suggest the importance of further clinical studies combining CCNU with these agents.

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Letter to the Editor

Metastasis and Triton WR 1339

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FRANCHI *et al.* [1] find that large doses of Triton WR 1339 (200 and 500 mg/kg), given intraperitoneally within 3 days of implantation of the Lewis lung carcinoma (3LL), prevent spontaneous pulmonary metastases from this tumour in about half their mice. The animals with metastases had a reduction of approximately 85% in the number of pulmonary secondaries as compared with controls. Franchi *et al.* feel, therefore, that Triton WR 1339 may be of therapeutic interest in the treatment of human cancer.

Using the same tumour, and essentially the same method of assessment [2], we gave Triton WR 1339 (courtesy of Dr. J. A. Stock, Chester Beatty Research Institute) at 500 mg/kg and at 50 mg/kg i.p., from day 3 to day 20, following implantation of the 3LL. We also gave 500 mg/kp i.p. from day 14 to day 20. Each group consisted of 6 mice. All the lungs of all mice had metastases, and all except three had large numbers of metastases. The three exceptions were mice treated with the highest dose of Triton WR 1339 from day 3 onwards and they had only a few metastases in their lungs.

Carter *et al.* [3] using a spontaneously disseminating hamster lymphoma to assess the inhibitory effects of Triton WR 1339 found that this substance actually facilitated the spread of the lymphoma.

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In view of these differing results, it would seem to be premature and perhaps inappropriate to consider Triton WR 1339 as a candidate for clinical trials at the present time.

With partially effective agents, it is necessary to define quantitative criteria (e.g. degree of inhibition of metastases) and set levels of activity which must be reached before substances are likely to be of clinical interest. We have as yet no such criteria. They do exist, however, for anticancer agents of potential clinical interest in treatment of solid tumours and leukaemias [4]. A compound consistently unable to give more than 45% inhibition of transplantable solid tumours, or less than 25% increase in Median Survival Time of mice with leukaemia L1210 would not be considered of biological and much less of clinical interest, unless as in the case of asparaginase, there were more cogent reasons for supposing it to be useful.

No similar difficulty would arise in the case of an agent which is completely successful in inhibiting metastases [2, 5, 6] even in one tumour, but there must be considerable reservations about an agent that, at best, can only reduce numbers of metastases and that can, even with one tumour, facilitate metastatic spread.

Although the observations of Franchi *et al.* are undoubtedly of considerable experimental interest, we feel it necessary to make the above points, so that adequate consideration can be given to them in any assessment of Triton WR 1339 for clinical trials.

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Letter to the Editor

A Radioimmunoassay for MTV-Antigens

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THE NEED for a sensitive and accurate method for the quantitative screening of the different antigens of MTV (Mammary Tumour Virus) in either peripheral blood and/or tissue extract led us to the development of a radioimmunoassay for the antigenic components of mouse MTV.

We approached the problem from two angles:

1. Trying to isolate the antigens first and label the purified components separately.
2. A short-cut method: iodinating—with special precautions—a complete virus preparation obtained from MTV-positive mouse mammary tumours [1] and separate the relevant material from the other labelled substances afterwards.

The second method of approach resulted in a useful assay by which at least two different sets of antigens can be distinguished. Figure 1 represents regression lines obtained with respectively 4 plasma samples from female tumour-bearing mice: (C3H × 020) F1, GR and Balb/c foster C3H, one sample of MTV-positive milk (Balb/c foster C3H), and a mammary tumour extract from (C3H × 020)F1. Plasma and milk samples from Balb/c as well as a RLV (Rauscher Leukemia Virus) preparation extract show no inhibition of binding (average 56% bound at all dilutions). As can readily be seen all the plasma and milk samples from MTV-positive mice show parallel inhibition of binding of the labelled antigen, whereas samples from MTV-negative sources show no inhibition at all. The non-parallelity of the regression line obtained with the tumour extract indicates that at least partial degradation of one or more antigenic determinant sites has occurred during the extraction procedure and this material is therefore unsuitable for an internal laboratory reference preparation.

Figure 2 represents regression lines obtained with the same biological specimens as employed in the experiments of Fig. 1, however, now in the assay set-up for the detection of the other group of antigens. The average value for the MTV-negative samples (Balb/c plasma and milk, and RLV) is 25% bound.

The non-parallelity of the regression lines obtained with the diverse samples may be due to:

(a) the presence of antibodies in the biological material [2];

(b) an immunological difference between the antigenic determinants of the MTV's derived from the various inbred strains of mice;

(c) enzymatic degradation during storage or extraction of tumour material is suggested by the much flatter slope of the regression line of the (C3H × 020)F1 tumour extract compared to the one obtained with (C3H × 020)F1 plasma (see Fig. 1).

From preliminary experiments we have the impression that the radioimmunoassay represented in Fig. 1 measures mainly viral coat antigen, whereas the other assay represented in Fig. 2 is mainly measuring viral nucleoid antigens.

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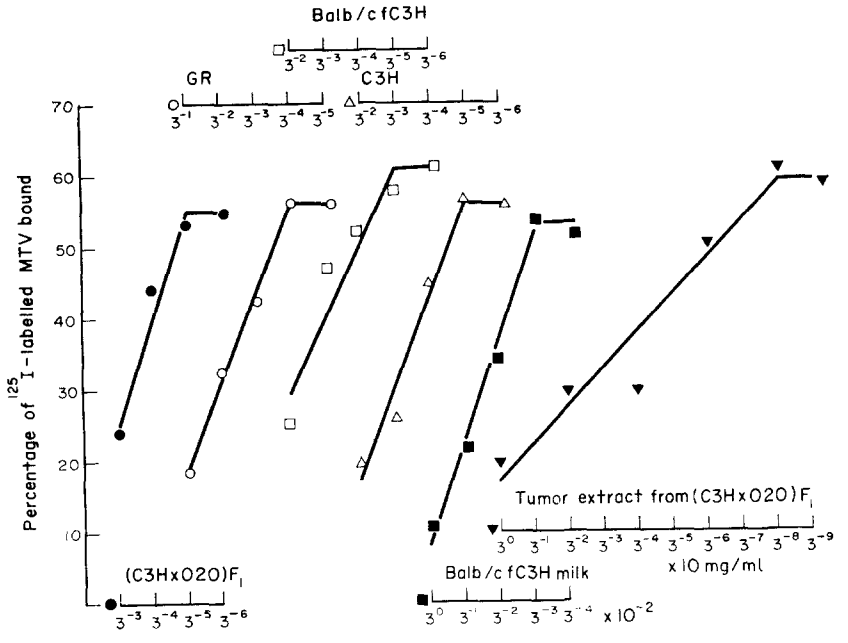

Fig. 1. *Inhibition of the binding of* ^{125}*I-MTV to rabbit-anti-MTV-antiserum by respectively* 4 *plasma samples, one milk sample and a mammary tumour extract. Plasma and milk samples from Balb/c as well as a RLV-preparation show no inhibition of binding (average* 57% *bound).*

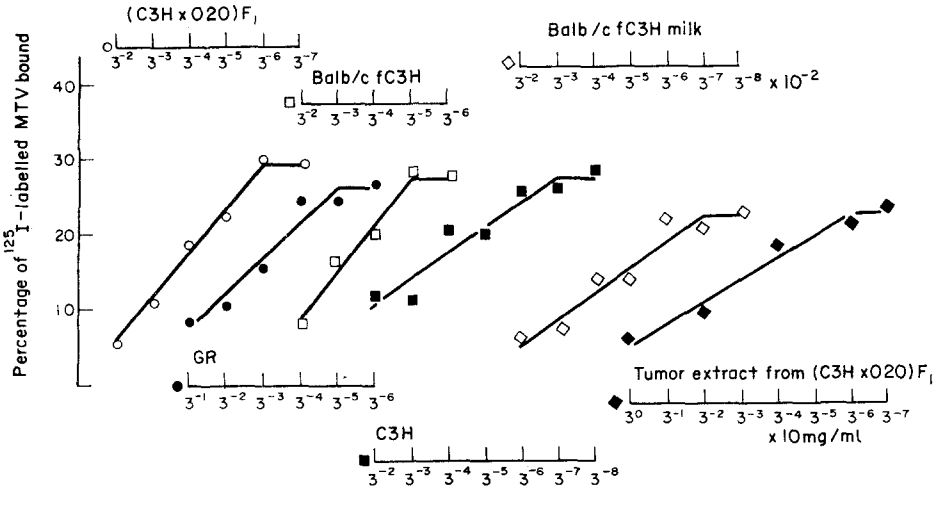

Fig. 2. *Inhibition of the binding of* ^{125}*I-MTV to rabbit-anti-MTV-antiserum by the same biological samples as employed in the experiments of Fig.* 1, *however, now in the assay set-up for the detection of the other group of antigens. Also here plasma and milk from Balb/c as well as a RLV-preparation show no inhibition of binding (average* 25% *bound).*

Experiments are in progress to elucidate the relationship of the two labelled preparations to the five different MTV-antigens described by Nowinski *et al.* [3].

From the assay results of Balb/c MTV-positive milk it can readily be deduced that for detection of MTV-antigens the first radio-immunoassay system is some 10–20 times as sensitive as the micro-Ouchterlony double-diffusion technique [4], and the second one 150–300 times.

Experiments are now in progress along two lines:

(a) Comparison of sensitivity and specificity with other immunological detection methods, e.g. immunofluorescence and immunofluorescence adsorption, etc.

(b) To check the claims for the occurrence in human plasma of MTV-related antigens [5] and antibodies to MTV-related antigens [6].

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Cellular Recovery in Human Skin Reactions: Application to Dose Fraction Number Overall Time Relationship in Radiotherapy

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Abstract—*The aim of this study is to investigate on human skin the role of the cellular recovery, progression in the cell cycle and repopulation during a fractionated irradiation.*

We have determined the dose increment $D_r = 2\ D_i - D_s$ *to be added when a single dose* D_s *is replaced by two equal doses* D_i *at an interval* i, *for doses ranging from* 2×150 *rads to* 2×850 *rads and time intervals ranging from* 1 *hr to* 4 *days.*

For i = 6 *hr,* D_r *is less than* 300 *rads for* $D_i = 850$ *rads; it diminishes with* D_i *and becomes very small for doses* D_i *below* 200 *rads. Assuming that for this time interval,* D_r *is mainly related to* cellular recovery *from sublethal injuries (Elkind recovery), this latter mechanism seems to become important only for large doses of several hundred rads.*

The experimental data are only compatible with a two-component cell model in which a great part of the cell killing is due to lethal events; cell killing by accumulation of sublethal events seems to be of minor importance for the usual doses per fraction in radiotherapy.

The variation of D_r *for intervals ranging from* 6 *hr to* 4 *days is attributed to the variation of sensitivity to* progression in cell cycle. *For* $D_i = 850$ *rads,* D_r *reaches a maximum* (400 *rads*) *at* 48 *hr.*

From the experimental data one can compute the dose increase associated with increasing number of fraction. By comparison with the usual iso effect relationship (Strandqvist's *type*) *one can derive the role of* repopulation *associated to the overall time of treatment repopulation rate has been found equivalent to* 30–40 *rads/day.*

It is concluded that, under usual regimes of radiotherapy, when the number of fractions and overall time are simultaneously increased, the dose increase is mainly associated to cellular recovery *if the number of fractions is small (i.e. if the dose per fraction is large), and to* repopulation *when the dose per fraction becomes small, i.e. practically when the number of fractions exceeds* 15.

INTRODUCTION

RADIOBIOLOGICAL experiments and clinical observations show that the total dose required to produce the same biological effect increases with the number of fractions and with the overall time of the irradiation.

This increase is ascribed to cellular recovery from sublethal injuries (Elkind), cell proliferation, and some variation of radiosensitivity during the course of the treatment.

The relative importance of these mechanisms has not been definitely established, particularly in the range of rather small doses per fraction and of large overall times which are usual in radiotherapy. More precise data on these mechanisms for normal and malignant tissues

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would be very useful to define an irradiation regime leading to a maximum differential effect between the two tissues.

The aim of this study is to obtain some data on *human skin* by assessing the amount of recovery as a function of the dose per fraction and the kinetics of the recovery following an irradiation. The results are compared with a cell model and we discuss their application to the dose fraction number/overall time relationships.

MATERIAL AND METHODS

The data are obtained by comparison of skin reactions observed on the same patient on two fields irradiated with two different protocols.

The number of patients entering this study is limited by the usual restrictions for any clinical investigation.

There must be a medical indication for the irradiation; the treatments have been curative and prophylactic irradiations of neck and groin lymph nodes.

The therapeutic efficiency of the experimental plans of irradiation must be competitive with any other plan; our clinical experience of concentrated irradiation has proved this kind of treatment to be satisfactory as full treatment or as part of a treatment.

The intensity of the reactions must not exceed a tolerance limit and on the other hand must be distinctly observable; these requirements keep the acceptable doses within a narrow range of variation.

On account of these limitations the number of patients elected for this study has been only 64 in 2 years and 7 out of these observations have been discarded because the degree of the reactions was too small.

Irradiations were performed with constant potential 250 kV X-rays of HVL: 1, 35 mm Cu and dose rate 80 rads/min at 50 cm SSD.

The two fields were bilateral with the same shape and the same area (7×7 to 10×10 cm^2).

The reaction considered for the biological test is skin desquamation because it has some significance related to cell survival. It has been scored as described in Table 1.

Table 1. *Skin reaction score*

0·5	Desquamation threshold
1	Light dry desquamation over the entire field
1·5	Brisk dry desquamation on a part of the field
2	Brisk dry desquamation over the entire field
3	Moist desquamation on a part of the field
4	Moist desquamation over the entire field.

Erythema and pigmentation have also been coded but have not been used in this study.

More important than the score itself is the judgment of a *clear difference* between the two fields; the reactions are coded "equal" when no difference is observed. There has never been any disagreement between the two observers on this judgment.

These judgments were made both on direct observations of skin reactions and on photographs; photographs tend to diminish the difference and thus to decrease the accuracy of the judgment.

The principle of the study is to compare the reaction R (s) produced by a single dose D_s with the reaction R (d) produced by two equal fractions D_i at an interval i, i.e. a split dose $2D_i$.

The result of the comparison is expressed in terms of "recovered dose" (D_r) which is considered here as the increment $2D_i - D_s$ required to obtain with 2 equal fractions D_i the same biological effect as with the single dose D_s. Inequality in reactions on the two irradiated fields results of course in an inequality between D_r and $2D_i - D_s$:

$$R(s) \left(\begin{matrix} > \\ = \\ < \end{matrix}\right) R(d) \text{ results in } D_r \left(\begin{matrix} > \\ = \\ < \end{matrix}\right) 2D_i - D_s$$

On the graphs (Figs. 2, 3, 4) the conditions of irradiation of each patient are defined by a point the coordinates of which are given by the doses used D_i (abscissae) and $2D_i - D_s$ (ordinate). The direction of the arrow shows the direction of the inequality observed between D_r and $2D_i - D_s$.

For doses D_i lower than 700 rads the reaction obtained with one split irradiation ($2 \times D_i$) is not sufficient. The irradiations are resumed at an interval of two days, until a reasonable reaction is reached. As the dose D_i is decreased the number of irradiations is naturally increased. Table 2 shows some examples of treatment plans.

RESULTS

1. *General survey of the skin reactions*

(*a*) *Maximum reactions as a function of the dose for a single irradiation.* As indicated in Table 3 the maximum reaction is observed at about 30 days after the irradiation.

Figure 1 shows the distribution of the observed reactions for 44 patients receiving a

Table 2. Examples of treatment plans

Number of sittings	Overall time* (days)	Dose per sitting (rads)		
		Single dose D_s	Split dose† $2D_i$	$2D_i - D_s$
1	—	1500	2 × 850	200
		1300	2 × 750	200
2	2	800	2 × 550	300
3	4	600	2 × 400	200
8	16	350	2 × 200	50

*Interval between first and last sitting.
†Interval for the split dose: i = 6 hr.

single X-ray dose ranging from 1100 to 1500 rads.

The kinetics of the skin reaction was not significantly different for the fractionated irradiations. This is likely due to the fact that

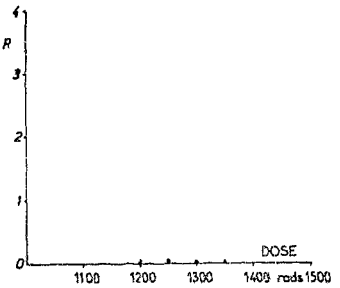

Fig. 1. Reaction intensity (scored as indicated on Table 1) after a single dose—44 patients.

the first sittings have the largest weight in the kinetics of the reaction.

It can be pointed out that in all cases the kinetics was similar on the two compared areas and that the maximum occurred at the same time. Thus the statement of inequality—or equality—of reaction was made on simultaneous observations.

The experimental points are too few and too scattered to show any precise relationship between the reaction and the dose. However, one can notice that no reaction higher than light desquamation (score 1) appears for a dose lower than 1200 rads and that above 1350 rads there is always some observable reaction.

The influence of individual variations of radiosensitivity should be cancelled out by the method of comparing skin reactions on the same patient.

(*b*) *Kinetics of the skin reactions.* Table 3 shows: the time of appearance of the first sign of desquamation (score: 0·5); the time when the reaction reaches a maximum; the time when a clear regression is observed (the difference between the two last times is the duration of the reaction at its maximum).

Observations have been pooled, on the Table, according to the maximum reached during the course of the follow-up.

2. *Recovered dose as a function of* D_i

(*a*) *Recovered dose at* i = 6 *hr:* (Fig. 2). The limited number of experimental points does not allow any accurate determination of D_r. On the other hand there are a few inconsistancies in the direction of the arrows particularly for the highest values of D_i, which may

Table 3. Kinetics of skin reactions after a concentrated irradiation

Maximum reaction	Number of cases	Average time (days) after the irradiation		
		For score 0·5	Maximum	Regression to lower score
Score 0·5	10	32	—	—
1	10	24	32	39
2	18	21	30	36

*Single dose (1100–1450 rads) or two doses (2 × 700–2 × 850 rads) at less than 1 day interval.

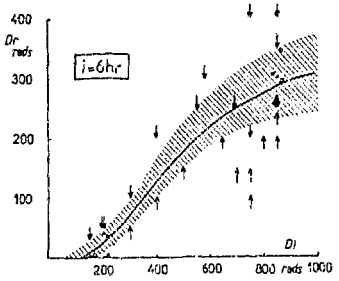

Fig. 2. Recovered dose D_r for a split irradiation $2 \times D_i$ as a function of the dose per fraction D_i; interval between the 2 fractions: i = 6 hr. Representative point for each observation is the head of the arrow.

be attributed to some individual variation of D_r from one patient to another.

However, the two sets of arrows pointing upward and downward delineate a region (hatched area) which gives with an acceptable confidence the upper and lower limits of the D_r values. These limits stay at $\pm 25\%$ from the solid line drawn through the hatched area.

The most important conclusions are: the "recovered dose" D_r is very small for doses D_i less than 200 rads: it is less than 50 rads, and from the available observations the actual value is likely to be closer to 0 than to 50 rads; D_r increases rapidly with D_i in the range D_i = 200–600 rads and tends to level off for larger doses D_i.

(*b*) *Recovered dose at* i = 24 *hr* (Fig. 3). The recovered dose at i = 24 hr has been investigated for

(i) large doses—2 × 850 rads vs. single dose;

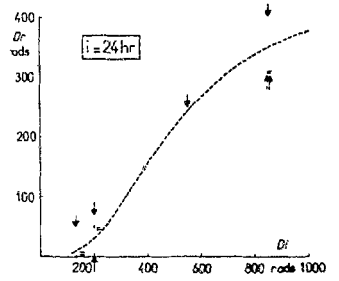

Fig. 3. Recovered dose D_r for a split irradiation $2 \times D_i$ as a function of the dose per fraction D_i; interval between the 2 fractions: i = 24 hr. *The dotted line is derived from the solid line on Fig. 2 by multiplying the ordinates by a factor* 1·25 (*see text*).

(ii) medium doses—4 daily doses D_i of 500 rads vs. 2 doses D_r at 2 days interval;

(iii) small doses—daily doses D_i of 200 or 150 rads (6 times a week for 3 weeks) vs. doses D_r every other day (3 times a week).

Results are shown on Fig. 3.

For D_i = 850 rads, $D_r = 350 \pm 50$ rads, i.e. an increase of 25% with respect to the mean value of D_r (280 rads) observed at i = 6 hr (Fig. 2). The curve on Fig. 3 has been derived from the curve on Fig. 2 assuming the same relative increase at any dose D_i. This curve is compatible with the observed results.

3. *Kinetics of early recovery*

(*a*) *Recovered dose for* D_i = 750 *rads as a function of* i (0–6 *hr*). This series of observations was aimed at investigating the kinetics of recovery during the first hours following the irradiation.

Results are shown by the arrows on Fig. 4. They are consistent with an exponential function of time (full lines in Fig. 4) with the usually accepted half time (1–1·5 hr) for cellular recovery [1].

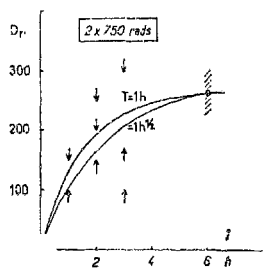

Fig. 4. Recovered dose for split irradiation 2×750 rads as a function of the interval i *between the 2 fractions. The arrows show clinical observations and the full lines are calculated "recovery curves" passing through* D_r = 260 *rads at* 6 hr—*value taken on the solid line Fig. 2—with different assumed values of the half-time of recovery.*

Some additional observations have been made for an interval i = 2 hr and doses D_i = 400 and 500 rads (Table 4). They too are consistent with the D_r values at i = 6 hr (Fig. 2) and a half time T = 1·5 hr for cellular recovery.

(*b*) *Recovered dose for* D_i = 850 *rads as a function of* i (6 *hr–4 days*). The reactions obtained with D_i = 750 rads are too small when the interval i exceeds 6 hr. Thus for investigating the recovery for larger intervals the dose D_i has been increased to 850 rads.

Table 4. Recovered dose at i = 2 hr (4 patients)

D_i	D_r at i = 6 hr*	Calculated D_r at 2 hr†	Observed D_r at 2 hr
400 rads	130 rads	85 rads	D_r < 100 rads
400 rads	130 rads	85 rads	D_r < 100 rads
500 rads	180 rads	120 rads	D_r < 150 rads
500 rads	180 rads	120 rads	D_r = 150 rads

*Taken on Fig. 2—midline of hatched area.

†Calculated from D_r at 6 hr (column 2) assuming a recovery half time T = 1·5 hr, i.e. $\frac{D_r(2\text{ hr})}{D_r(6\text{ hr})} = 0{\cdot}65$.

Figure 5 suggests a variation of D_r in the time interval 6 hr–4 days with a significant increase between 6 and 48 hr and a possible slight decrease for larger intervals.

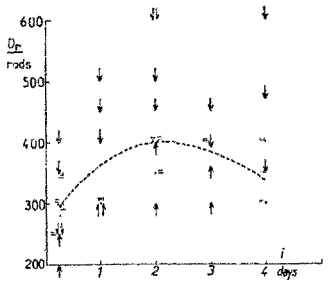

Fig. 5. Recovered dose for split irradiation 2 × 850 rads as a function of the time interval i between the fractions.

DISCUSSION

1. *Significance of* D_r

The additional dose ($D_r = 2D_i - D_s$) to be added when a single dose D_s is replaced by 2 equal doses D_i at an interval i is associated to three main mechanisms, namely: cellular recovery of sublethal injuries, redistribution of the cell population in the cycle phases, and repopulation.

For time intervals which do not exceed a few days *repopulation* is likely to be negligible.

Cellular recovery of sublethal injuries should be complete at 6 hr if one assumes that this recovery obeys an exponential function of time with a half time of the order of 1 hr. Thus, the D_r value for i = 6 hr (Fig. 2) should correspond mainly to the mechanism of *cellular recovery*. However, *progression in the cell cycle* may not be negligible for an interval of 6 hr [2] and D_r values may be partly related to this mechanism.

Progression in the cycle is likely to be the only mechanism responsible for the variation of D_r between 6 hr and larger intervals (Fig. 3–5) up to a few days.

The variation of D_r suggested on Fig. 5 does not show the large cyclic variations which could be expected from the progression in the cell cycle [3]; but the accuracy of the clinical method which has been used is probably not sufficient for investigating this mechanism. Further, the cell cycle time in skin might be rather long (several days), so that rapid fluctuations would not be seen.

2. *Comparison of* D_r *to data from the literature*

Values of the "recovered dose" can be found or can be calculated from the experimental data (Fig. 6), given in the literature. However, there is a lack of data for the relatively low doses per fraction which have been investigated in this paper.

For D_i = 850 rads, our D_r value (for i = 6 hr) stands at the lower limit of the published data. It must be pointed out that for most other experiments the time interval is relatively larger than in our experiments. Furthermore, while our values of D_r have been determined for equal dose fractions, most of the listed experiments have been carried out with a constant large conditioning dose followed by a variable second dose, i.e. unequal dose fractions; the large size of these conditioning doses is particularly important.

3. *Cell model*

The sigmoid shape of the experimental curve D_r (D_i) is not compatible with the classical multitarget single hit cell model.

An agreement can be obtained with the values calculated for a two component model consisting of n sublethal targets with a radiosensitivity parameter D_n plus one lethal target

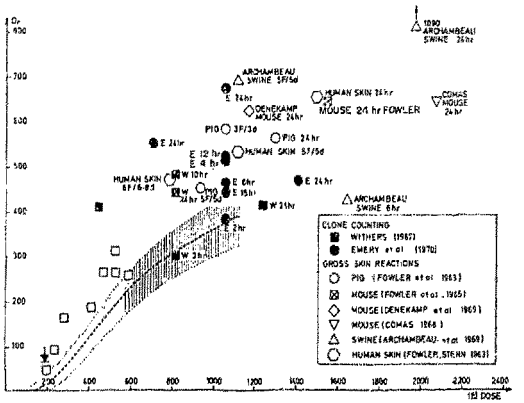

Fig. 6. Recovered dose as a function of the first dose for a split irradiation. Comparison of our results on human skin (hatched area) to literature data [3–13].

In order to allow the comparison of data obtained with different radiation qualities the doses have been converted to the expected values for ^{60}Co γ-rays, assuming an RBE value of 0·85 for high energy radiation with reference to conventional X-rays.

Open squares correspond to gross skin reactions on Mouse—Dutreix et al. [14].

The arrow at D_1 = 190 rads corresponds to an unpublished clinical trial in which the effects on the oral mucosa as well as on the tumour of the tonsil were found more important for 14 sessions of 2 × 190 rads (at 2 hr interval) than for sessions of 330 rads, i.e. D_r < 50 rads.

with a parameter D_1 (the final slope of the cell survival being $(1/D_o) = (1/D_1) + (1/D_n)$.

An acceptable fit is obtained for $n = 15$ and $D_1 = 2D_n$ in a large range of D_o values (100–160 rads).

Thus the experimental values of D_r (D_1) are compatible with Cohen's data [15]:

D_o = 145 rads, D_1 = 455 rads, D_o = 208 rads n = 15.

4. *Use of D_r for total dose/number of fractions relationship*

The total dose $N.D_N$ (N fractions of D_N rads) required to cause the same survival as a single dose D_s can be derived from D_r (D) assuming no cell multiplication which will be considered later.

If a single dose D_s is replaced by a split irradiation in two fractions (N = 2) the dose D_2 per fraction is given by

$$D_s = 2D_2 - D_r(D_2)$$

If each fraction D_2 is replaced by two fractions (N = 4) the dose D_4 per fraction is given by

$$D_2 = 2D_4 - D_r(D_4)$$

and so on for N = 8, 16, 32 . . . fractions:

$$D_{N/2} = 2D_N - D_r(D)_N$$

We have seen (Figs. 2 and 3) that D_r becomes very small when the dose per fraction D_N becomes smaller than 200 rads; then the total dose $N.D_N$ should practically have reached a maximum and *should not increase* when the number of fractions increases further.

The usual "iso effect relationships" express the variation of the total dose D_t as a function of overall time t; the number of fractions N is usually related to t ($N = 5t/7$ or $6t/7$ for 5 or 6 treatments per week respectively). D_t keeps increasing at large values of t, i.e. at large values of N and also small values of the dose per fraction D_N. The difference between D_t and $N.D_N$ can then be reasonably attributed to repopulation during the time t. We shall compare $N.D_N$ calculated from experimental D_r data with some usual iso effect relationships.

(*a*) *Power law iso effect relationship.* Since the original work of [16] the variation of the total dose D_t as a function of overall time t is most often expressed in a power law form:

$$D_t = k \cdot t^p$$

The value of the exponent p varies from p = 0·22 (Strandqvist) to p = 0·33 [17].

The comparison of $N.D_N$ with D_t has been made assuming a 5 fractions a week schedule and treatment starting on Monday (Fig. 7) for a

nominal single dose 2000 rads which leads in Strandqvist graph for $t = 1$ day, i.e. $N = 2$ fractions to $D_t = 2550$ rads (2×1275 rads).

$N.D_N$ has been computed from D_t values at 24 hr interval (Fig. 3) which is relevant to "daily" fractionation. Variation of D_t as a function of t has been computed for $p = 0{\cdot}22$, $0{\cdot}25$ and $0{\cdot}33$.

The difference between D_t and $N.D_N$ can reasonably be attributed to repopulation; the difference increases linearly with time at a rate of 30 rads/day for $p = 0{\cdot}22$ and 37 rads/day for $p = 0{\cdot}25$. The main difference associated to the value of the parameter p is the lag before the "start of repopulation": 20 days for $p = 0{\cdot}22$ and 5 days for $p = 0{\cdot}25$.

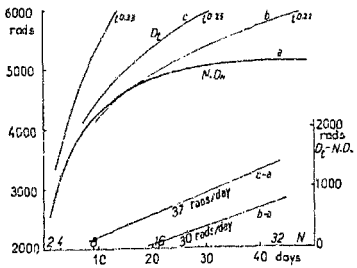

Fig. 7. Iso effect total dose $N.D_N$ computed from D_t values (Fig. 3) show the dose increase expected from cellular recovery when the number of fractions N is increased.

It is compared to Strandqvist's type iso effect total dose $D_t = kt^p$ as a function of time. The correspondence between N and t is made for 5 fractions a week and t = 1 day for N = 2.

Different values of exponent p have been considered.

The difference $D_t - N.D_N$ (right hand ordinates) is assumed due to cell repopulation.

Starting point for the comparison is 2×1275 rads (Strandqvist's nominal single dose: 2000 rads).

The value $p = 0{\cdot}33$ leads to an unrealistic repopulation rate (80 rads/day). The values 30 rads/day and 37 rads/day are within (but at the upper limit of) the range of the experimental data published in the literature [18].

It appears from this graph that: for a small number of fractions the increase of total dose D_t is mainly due to cellular *recovery* plus eventually *some progression in the cell cycle*; when N exceeds 15 fractions, the increase of D_t is practically due only to *cell multiplication*.

In other words when the dose per fraction is smaller than 300 rads, i.e. under *usual conditions of radiotherapy*, cellular recovery is of little importance compared to repopulation and the total dose increase is related mainly to *overall time*, while the *number of fractions* is an accessory factor.

(*b*) *Comparison with Fowler's experiments on pig skin* [7–9, 19] In these experiments Fowler has determined the doses causing the same desquamation for 5, 9 and 21 fractions given in the same overall time of 27 days. Table 5 compares these results with calculation based on D_t (Fig. 2)

The total doses obtained by experiment (for 21 fractions) and calculation (for 20 fractions) do not seem incompatible. Slight difference in D_t between human and pig skin could account for the difference. On the other hand, by considering that in these experiments with the same overall time, the only factor responsible for the dose increase *is the number of fractions*, it is assumed that the repopulation rate is the same after the small and the large fractions, and this assumption may not be strictly valid. Fowler's experiments show that the total dose must be increased from 3600 to 4200 rads when 5 fractions are given in 28 days instead of 4 days. Thus as stated by Fowler the increase of the total dose when changing from 5 fractions/4 days

Table 5

N Fractions	Fowler's data		Our calculations	
			Based on D_t at 24 hr	
	D_N	$N.D_N$	D_N	$N.D_N$
5	840	4200	840*	4200*
9	565	5100		
10			540 ($D_t = 240$)	5400
20			315 ($D_t = 90$)	6300
21	265	5600		

*Starting values for calculations.

to 21 fractions/27 days is mainly associated to the increase in number of fractions, i.e. cellular recovery—and only slightly associated to the increase in overall time—i.e. cell multiplication. However, this statement does not imply that cellular recovery is still an important factor for 21 fractions, and cannot be opposed to Cohen's model [20].

(*c*) *Comparison to Ellis* [21] *formula* (*NSD*). It seems from our experiments that the role of the number of fractions (N) is overestimated in Ellis formula and correlatively that the role of overall time (t) is *underestimated* for a large number of small fractions. This may have no practical importance on the calculations when the two parameters are linked as in usual regimes of treatment ($N = 5/7\ t$) but should have some influence for unusual regimes with small number of fractions and large overall times.

The usual expression of the repopulation rate in terms of equivalent dose per day, corresponds to dD/dt, where dD is the dose increment to be added for an increment dt in the overall time.

Table 6. *Repopulation rate*

t days	$p = 0{\cdot}11$ (Ellis)	$p = 0{\cdot}22$
	rads/day	
10	56·3	96·4
20	30·3	56·1
30	21·1	40·9
40	16·4	32·7
50	13·4	27·5
60	11·4	23·8

Irradiation equivalent to 6000 rads/42 days.

The number of fractions is assumed constant (or without importance for the total dose—cf. * a).

$$D = k.t^p \qquad \frac{dD}{dt} = k\,p\,t^{p-1} \qquad k = \frac{6000}{42^p}$$

If the total dose varies as a function of t (for a definite number N of fractions) according to a power law $D = k\,t^p$, the repopulation rate is given by $dD/dt = k\,p\,t^{p-1}$. The corresponding values are given in Table 6 for different values of p; the end point of the irradiation corresponding to $D = 6000$ rads for $t = 42$ days, i.e. $k = (6000/42^p)$.

The power law leads to a continuous decrease of the repopulation rate (dD/dt) when t increases. A variation of this kind may actually correspond to the biological reality. However, the magnitude of the variation given by the power law seems to be unrealistic as well as the values obtained for t less than 10 days.

Thus the power law does not seem to be suitable and its *practical use* should be restricted to limited ranges of t, with increasing values of the exponent when t increases.

It seems from this calculation that for overall times larger than 20 days the exponent 0·11 of parameter t is too small and that a better agreement with the experimental data (30–40 rads/day—cf. § a) would be obtained with some larger value close to 0·22.

In agreement with this conclusion Liversage [22] has already shown that exponent of parameter t is too small when overall time exceeds 7 days. However, this question is still controversial since Fowler [23] and Field [24] are led to the opposite conclusion that the exponent of t should possibly be smaller.

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Basal Prolactin Blood Levels in Three Strains of Rat with Differing Incidence of 7,12-Dimethylbenz(a)anthracene Induced Mammary Tumours

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Abstract—*The incidence of mammary tumours induced in three strains of rat is different. Plasma prolactin levels have been measured in these strains of rat during the dioestrous phase of the oestrous cycle. The strain showing the highest incidence of induced mammary tumours also showed the highest circulating prolactin levels.*

INTRODUCTION

THE INCIDENCE of rat mammary tumours induced by 7,12-dimethylbenz(a)anthracene (DMBA) is variable. As tumour growth is dependent upon serum prolactin levels [1, 2] we have tried to decide if a relationship exists between circulating prolactin levels and tumour incidence. In this study we have measured plasma prolactin in strains of rats with differing incidence of tumour induction.

METHOD

Animals—three strains of rats were studied. (i). A strain of Sprague–Dawley originally obtained from Oxfordshire Laboratories, but bred for the past 6 years in the small animal quarters of the Department of Clinical Surgery, University of Edinburgh. (ii). A strain of Sprague–Dawley obtained from the Animal Diseases Research Association (ADRA), Edinburgh. (iii). A strain of Long–Evans, a gift from Dr. C. B. Huggins of the Ben May Research Laboratory, Chicago.

Animals were housed at a temperature of 68°F ± 2°F with light provided from 0600 to 2000 hr daily.

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Vaginal smears were performed at 50 days and 33 animals in the dioestrous phase of the oestrous cycle were selected. Blood was obtained between 0900 and 1100 hr by cardiac puncture after the animal had been anaesthetized with ether. The blood was centrifuged, the serum separated and stored at −20°C until assayed, the assay being performed within three weeks.

Measurement of plasma prolactin

Immunoreactive prolactin was measured in serum samples by double antibody radioimmunoassay using a kit distributed by the National Institute for Arthritis and Metabolic Diseases (NIAMD), Bethesda, Md., U.S.A. NIAMD-Rat prolactin I-1 was labelled with (^{131}I) iodine (Radiochemical Centre, Amersham) by the method of Greenwood, Hunter and Glover [3] and separated from iodide on columns of Biogel P-60 (Bio-Rad Laboratories). The labelled hormone was diluted in assay buffer (50 mM-phosphate buffer, pH 7·3, containing 0·01% merthiolate and 0·5% serum albumin) to give a concentration of approximately 20,000 counts/min/100 μl (5 ng/ml). Antiserum to rat prolactin (NIAMD-anti-rat prolactin S-1) was diluted (1/100,000) in 50 mM-phosphate buffer containing 50 mM-EDTA and 1/400-non-immune rabbit serum. Standard rat prolactin (NIAMD-rat prolactin RP-1) was diluted in assay buffer to give a series of stan-

dard solutions in the range 1–125 ng/ml. Antiserum to rabbit serum globulins was raised in sheep by the intramuscular injection of the antigen emulsified in Freund's complete adjuvant and was diluted (1/10) in assay buffer. Standard curves were prepared as follows: 100 μl of prolactin standard was mixed with 200 μl of antibody to prolactin, 100 μl plasma from a hypophysectomized male rat and 300 μl assay buffer. After incubation for 1 day at 4°C, 100 μl ^{131}I-labelled prolactin was added and the solution incubated for 1 day at 4°C. Finally, 200 μl sheep-anti-rabbit globulin serum was added and the solution incubated overnight at 4°C. The tubes were centrifuged at 1500 **g** for 30 min at 4°C and the supernatants decanted. After draining, the tubes were counted in a Nuclear Chicago gamma counter. When serum samples were assayed, 100 μl samples replaced the standard prolactin solution and 100 μl assay buffer replaced plasma from a hypophysectomized male rat. Assays were carried out in duplicate, the radioactivity in each tube being expressed as a percentage of the radioactivity present in the tubes containing the zero standard. Standard curves were linearized by plotting on probability-log paper.

Tumour induction

A special 15% fat emulsion with 7,12-dimethylbenz(a)-anthracene, 5 mg/G (supplied by Dr. P. Schurr, Upjohn Company, Kalamazoo), was used to induce mammary tumours. Three groups of rats aged 50 days ± 1 day were given 5 mg of DMBA in this fat emulsion by injection into a dorsal tail vein. Thereafter, the rats were examined regularly and tumour incidence noted. At 250 days of age, a post-mortem examination was performed and a portion of each mammary tumour taken for histological examination.

RESULTS

The incidence of mammary tumour is shown in Table 1. There was a much higher incidence of tumours in the Clinical Surgery Sprague–Dawley strain than in the Long–Evans and ADRA Sprague–Dawley strains. Histological confirmation was obtained that every tumour was an adenocarcinoma.

Table 1. Incidence of mammary tumours induced by 7,12-DMBA in different rat strains

Strain	No. of rats	No. of rats with mammary carcinoma
Clinical Surgery Sprague–Dawley	04	39
ADRA Sprague–Dawley	27	3
Long–Evans	30	0

The values of circulating prolactin are shown in Fig. 1. The concentration of prolactin in the serum of Clinical Surgery Sprague–Dawley rats were significantly higher than those of the other groups ($p < 0{\cdot}05$, Kruskal–Wallis one-way analysis of variance).

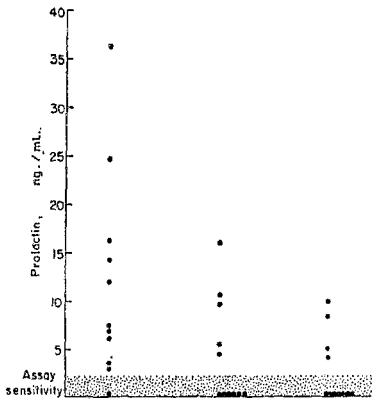

Fig. 1. Plasma prolactin levels in different rat strains in the dioestrous phase of the oestrous cycle.

DISCUSSION

Serum immunoreactive prolactin in the rat undergoes considerable fluctuations during the oestrous cycle, but the lowest concentrations are reached during dioestrous [4]. It is likely, therefore, that the rats in our study were bled at a time when serum prolactin levels were relatively steady. Stress produced by ether anaesthesia may induce release of prolactin from the pituitary [5]. In view of this, the serum prolactin concentrations that are reported here, cannot be regarded as representative of the lowest levels of the circulating hormone reached during dioestrus.

However, the strain of rats which showed the highest serum prolactin concentration after ether stress also showed the highest incidence of mammary tumours. The data suggest that the high incidence of tumours in Clinical Surgery Sprague–Dawley rats and the low incidence of tumours in Long–Evans and ADRA rats may be related to different concentrations of circulating pituitary prolactin in these strains.

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Murine Reticulum Cell Neoplasms Type B (Hodgkin's-like Lesions) Induced in BALB/c Mice with Field Isolates of Murine Leukemia Virus

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Abstract—*Twenty-four per cent of our specific pathogen-free BALB/c female control mice and* 9% *of control males developed lymphoid neoplasias. Three fourth of the neoplasms were reticulosarcomas type B (Hodgkin's-like lesions).*

Without change in the proportion of reticulosarcomas the tumor incidence was tripled by infecting newborn BALB/c mice with 0·05 *ml* 5 *times concentrated subcellular extracts of reticulosarcomas, doubled by injecting* 10^3 *XC-units of either of two leukemia-type viruses recently isolated from BALB/c mice, and tripled by grafting* 10^4 *cells from leukemia virus-infected syngeneic secondary embryonic fibroblast cultures. Intrathymic inoculation gave the same result as subcutaneous administration. A four log reduction in virus given dose s.c. only lowered the tumor incidence slightly.*

The mean latent period was 13 *months following inoculation of cell-free virus preparations and* 8 *months after injection of infected tissue culture fibroblasts. Adult mice receiving virus did not show enhanced tumor incidence.*

INTRODUCTION

RETICULOSARCOMA type B is the mouse tumor resembling human Hodgkin's disease [1] and the most common lymphoid tumor in BALB/c mice [2]. In continuation of previous work with cell-free transfer in BALB/c mice of such tumors [3] we here report *in vitro* and *in vivo* characteristics of field isolates of mouse leukemia virus inducing reticulosarcoma type B.

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MATERIAL AND METHODS

Animals

BALB/c mice derived by Caesarian section from our conventional colony and since kept under specific pathogen-free (SPF) conditions were used. Sex segregation was carried out at weanling.

Source of virus was tumor tissue from 5 old BALB/c mice with reticulosarcoma type B (3), a BALB/c mouse without tumor [4] and two macroglobulin-producing (CBA × DBA/2)F$_1$ leukemias [5].

Isolation of leukemia virus was performed by infecting both BALB/c and NIH-Swiss secondary mouse embryo fibroblasts with 10% tissue extracts. After three weeks the medium

and cells were harvested, sonicated and cleared by centrifugation [4]. In addition, 10% tumor extract was tested directly for virus content in the XC plaque test.

Subcellular extracts were prepared from tumor tissue of conventionally reared BALB/c mice with reticulosarcoma type B [3] by differential centrifugation [6] and passed through a 450 mμ Millipore filter and given a final volume of one fifth the volume of the tissue (five equivalent).

Virus preparations to be used for infection were harvested from a third passage culture, and contained 2×10^6 XC plaque forming units (PFU)/ml. Intact infected cells were also obtained by trypsinization from the third passage. B-tropic virus and B-virus-infected cells were harvested from a BALB/c culture, whereas F-virus was from a culture of NIH-Swiss cells. F_1-virus-infected cells to be inoculated were from a BALB/c culture.

In vitro titration of murine leukemia virus was carried out with the XC-test of Rowe *et al.* [7].

Neutralization of the virus was done by incubating virus and antiserum [8] diluted 1:20, 1:40 and 1:60 at 37°C for 30 minutes prior to infecting in the XC-test. Neutralization was defined as a reduction in the number of plaques to one third or less. In this test the source of virus was the same 10% extract of the tissue from which the virus was isolated except B_2-virus, which had been in tissue culture before testing.

Test for helper activity of the leukemia viruses was performed with Moloney's sarcoma virus [9].

MSV-pseudotypes were prepared by infecting mixed cultures of HT-1 hamster tumor cells containing the MSV-genome [10].

Infection of mice

Newborn BALB/c mice received either 0·05 ml subcellular extract or 0·05 ml virus suspension containing 10^5 PFU (10^{-0}) or 10^4 infected secondary embryo fibroblasts i.p. Adult mice received 10^6 XC-units virus subcutaneously. Controls received 0·05 ml 5 equivalent extract of tissue from 2-month-old non-diseased BALB/c mice or 0·05 ml of harvest from non-infected cultures of BALB/c or NIH-Swiss cultures.

Electrophoresis of serum [11] was performed on cellulose acetate strips.

RESULTS

In vitro *characterization of virus*

From tumor tissue of five out of six old BALB/c mice with reticulosarcoma type B virus was isolated by infecting BALB/c tissue culture, whereas no virus was recovered from infected NIH-culture. One virus was chosen for further characterization (B_1-virus] along with a BALB-tropic virus from a non-diseased BALB/c mouse (B_2-virus).

From the two (CBA × DBA/2)F_1 leukemias virus (F_1 and F_2) was recovered on NIH culture cells and not on BALB/c cultures.

That we were not dealing with a mixture of leukemia virus was indicated by an unchanged ratio of titer in BALB/c to titer in NIH cells after a tissue culture passage in both cell strains [12] (Table 1).

Both the B- and F-viruses were neutralized by Gross passage A antiserum but not by Moloney antiserum. The Gross serum used neutralizes Gross passage A virus and all isolates from naturally infected mice thus far tested [4]. By testing with AKR antiserum the B- and F-viruses could be distinguished; AKR antiserum neutralized the F-viruses but not the B-viruses. Isolates from naturally infected BALB/c and C_{57}Bl mice have been found to fall into two serological sub-groups: Those which are neutralized by both Gross and AKR antisera and those which are neutralized only by the Gross antiserum. In contrast, all isolates from other inbred strains thus far tested have been neutralized by both antisera (Hartley *et al.*, unpublished data). The two B-viruses also were strictly BALB-tropic, whereas the F-viruses were N-tropic. All viruses could function as helper viruses with defective MSV [9] and infection of hamster tumor HT-1 yielded leukemia virus and sarcoma virus that could induce leg tumors in newborn mice [10].

Leukemia incidences

Newborn BALB/c mice receiving subcellular extracts of normal tissue or uninfected tissue culture or uninfected cells exhibited the same tumor incidence as untreated controls (24% in females and 9% in males when all control groups are combined) (Table 2). Extract of reticulosarcoma type B and both tissue culture harvested B_1- and B_2-virus all enhanced the tumor incidence (41–80% in females and 33–57% in males). The F_1- and F_2-viruses, however, were without effect.

Intrathymic inoculation of B-virus gave the same result as subcutaneous inoculation.

Table 1

	Virus neutralized by antiserum to				Growth in BALB/c NIH cells	MSV rescue	Sarcoma Pseudotype
	MSV	Moloney	AKR	Gross			
B_1-virus (tumor mouse)	—	—	—	(+)	10^4/1	+	+
B_2-virus (normal mouse)	—	—	—	+	10^3/1	+	+
F_1-virus	(+)		+	+	1/20	+	+
F_2-virus	—		+	+	1/400	+	+

Infected syngeneic secondary fibroblasts were very effective in inducing reticular neoplasms (91% in females and 52% in males). Even BALB/c cells carrying F_1-virus enhanced the tumor incidence slightly. Adults receiving B_1- and B_2-virus did not differ from control mice in tumor frequency.

In vivo *dose dependence*

Using one log dilutions of the B_1-virus we found tumor incidence to decrease only slightly with decreasing dose (text—Fig. 1).

Mean survival time

The mean survival time of 164 control females without tumor was 17 months and that of 238 males 15 months. Forty seven female mice with spontaneous reticulosarcoma type B had a mean survival time of 17 months and 20 males with spontaneous reticulosarcoma 14 months. The latent period for virus-induced reticulosarcoma type B was 14–16 months both for females and males, and the mean survival time was not significantly different in any groups receiving cell-free virus,

Table 2. *Incidence of reticulosarcoma type B and lymphatic leukemias in specified pathogen-free BALB/c mice infected with field isolates of murine leukemia virus*

	Females				Males			
	Number of mice	Lymphatic leukemia	Reticulo* sarcoma	Total tumor incidence	Number of mice	Lymphatic leukemia	Reticulo* sarcoma	Total tumor incidence
Newborns								
Untreated	106	7	23	29%	126	2	12	11%
Tissue extract from normal young mouse s.c.	14	0	4	28%	35	0	0	0%
Non-infected tissue culture s.c.	59	3	9	20%	55	0	3	5%
Non-infected tissue culture intrathymic	6	0	0	0%	14	0	2	14%
Non-infected syngeneic cells i.p.	6	0	0	0%	8	0	1	12%
Extract of reticulosarcoma sc.	33	2	22	80%	33	3	11	42%
B_1-virus s.c.	21	2	13	66%	19	3	9	52%
B_2-virus s.c.	14	3	3	41%	9	0	3	33%
B_1-virus intrathymic	11	0	5	45%	7	0	4	57%
F_1- and F_2-virus s.c.	23	1	6	30%	19	0	2	11%
B_1-infected BALB/c cells	22	6	14	91%	21	5	6	52%
F_1-infected BALB/c cells	7	1	5	86%	10	1	2	30%
Adults								
B_1- and B_2-virus s.c.	21	3	5	37%	20	0	2	10%

*Reticulosarcoma type B except for 6 intermediaries between reticulosarcoma and lymphocytic leukemia and 7 reticulosarcomas type A.

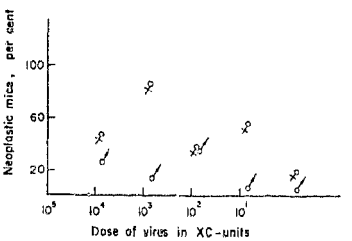

Fig. 1. Incidence of neoplasms of the lymphoid tissue in relation to dose of murine leukemia virus B-1. Each dose was administered to 30 to 40 BALB/C mice less than 24 hr old.

including those receiving log dilutions of virus. Mice, males as well as females, that developed reticulosarcoma type B subsequent to grafting of infected tissue culture fibroblasts had a mean survival time of 8 months.

B_1-virus was furthermore given s.c. to 10 to 15 newborns of each of the following strains, CBA, DBA, C3H and AKR. A similar number of mice of each strain received non-infected tissue culture material. No influence on tumor development was observed.

Types of neoplasm found

In all groups of BALB/c mice, 3/4 of the lymphoid tumors were found, macroscopically and microscopically as well, to be reticulosarcomas type B according to the classification of Dunn [1] (Figs. 1–2).

Paraproteinemia of γg type was found in 8 of the 154 mice with reticulosarcoma type B. The plasmocytic differentiation of these 8 and of 6 other reticulosarcomas was graded II–III according to the classification of Rask-Nielsen and Gormsen [13]. Seven reticulosarcomas were of type A [1]. The remaining were of grade IV. These tumors occurred in both virus-inoculated and non-infected mice.

In six cases, neoplasms that histologically were intermediaries between reticulosarcoma and lymphatic leukemia were found. The remaining 42 neoplasms were lymphocytic leukemias.

Transplantation experiments

Minced tumor tissue was grafted from 21 experimental and 13 control mice with reticular neoplasms. Each tumor was transferred to all members of one litter. Of 21 litters having received grafts from experimental mice 12 litters revealed neoplasm in at least one mouse after 12 months and another 9 litters had neoplasms after 17 months. Of the 13 litters receiving tumors from control mice 5 litters developed neoplasms within 12 months and after 17 months 11 litters showed neoplasms.

The twelve-month observation period is given since spontaneous reticulosarcomas nearly exclusively occur in mice older than 14 months [3], and reticulosarcomas induced by transplanting spleen cells from old non-tumorous BALB/c mice develop in mice after a latent period exceeding 12 months [14].

Amyloid was present in the spleens of about half of the males and a few females of all groups.

DISCUSSION

Reticulosarcoma type B [1] is the mouse tumor resembling Hodgkin's disease in man.

The histologic resemblance is often striking, although fibrosis is seldom pronounced in mice [1, 15]. The slow clinical progression adds to the similarity. In man, however, Hodgkin's disease is not uncommon in young adults, whereas reticulosarcoma type B is a disease of the old mouse. In view of recent work pointing to a possible etiologic role of herpes type (Epstein–Barr) virus in Hodgkin's disease of man [16, 17, 18] it is noteworthy that leukemia viruses can induce Hodgkin's-like lesions.

Our present experiments add to earlier observations [3, 19] on an enhanced incidence of both reticulosarcoma type B and lymphatic leukemia when newborn BALB/c mice are inoculated with subcellular extracts of reticulosarcomas from F_1 and BALB/c mice. As the same result was obtained with two leukemia viruses isolated from the BALB/c mice the results with the subcellular extracts must be attributed to such viruses. Since the incidence of lymphatic leukemia was also raised after virus inoculation in all groups treated, including those which received what appeared to be only one tissue-culture-grown virus, the two types of neoplasm may have a common etiology. Observation of a few neoplasms that histologically seemed to be intermediaries between the two tumor types support that notion. Transitional forms and a change of a Hodgkin's-like picture to a leukemia picture have also been reported in humans [20, 21].

The lack of tumor induction by the subcellular extract of tissue from normal young mice is consistent with failure to isolate virus from young BALB/c mice [4]

The preferential development in virus-treated mice, whether inoculated s.c. or in the thymus, of the tumor type most common in untreated animals gives indirect evidence for the etiologic role of C-type virus for spontaneous tumors of the reticuloendothelial system.

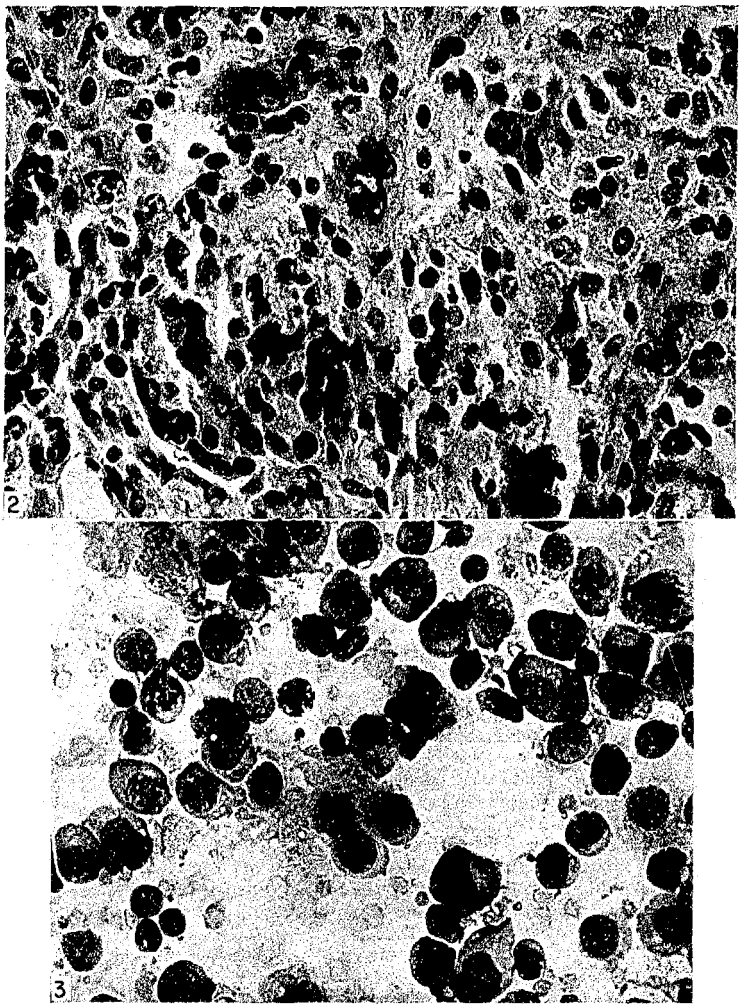

Fig. 2. Section of spleen from 18-month-old BALB/c mouse with reticulosarcoma type B. Large, irregular, pale cells interspersed with small lymphocytes and plasma cells. Periodic acid–Schiff × 840.

Fig. 3. Imprint of the spleen shown in Fig. 1. Large, pale cells including a binucleated one resembling a Sternberg–Reed cell. Intermingled with these are lymphocytes and plasma cells. Giemsa × 840.

(to face p. 176)

That host factors influence the histology of the tumor is illustrated by the development of reticulosarcomas instead of lymphatic leukemia in thymectomized mice receiving Moloney leukemia virus [22]. A virus-determined preference for certain target cells, probably due to the cell membrane coating the virus receives when budding, however, also seems to occur [23].

The B_1- and B_2-viruses isolated from BALB/c mice are B-tropic and the F_1- and F_2-viruses N-tropic [12] as shown by our *in vitro* and *in vivo* tests. Only the B-tropic virus induced reticulosarcomas in our BALB/c mice, and the B_1-virus did not induce tumors in adult mice of 4 strains having the Fv-1^n allele and being relatively resistant to B-tropic virus [24, 25].

That even groups receiving BALB/tropic cell-free B_1- and B_2-virus did not obtain a high incidence of neoplasm may be characteristic of many field isolates of leukemia virus [26].

From the nearly identical effect on incidence and survival time of giving newborn BALB/c mice 10^5 or 10^4 PFU of B-tropic virus we must assume that no threshold dose exists for newborns, whereas the results clearly indicate a state of resistance in adults.

Most likely a tolerant infection exists in those infected as newborns [27].

The identity of latent period with different virus doses with that of tumor development in untreated mice shows that a host factor governs the latent period. From experiments on grafting of young BALB/c mice with syngeneic spleen cells from non-diseased old and young donors it appears that a non-immunologic age-dependent mechanism greatly influences the tendency for lymphoid cells to undergo malignant transformation [14]. This host mechanism probably is overrun when more potent viruses, such as the Friend Moloney Rauscher viruses, are used. In that case an inverse relationship between the dose of virus inoculated and the median time-to-death is found [6].

Injecting syngeneic infected secondary embryo fibroblasts was the most potent tumor inducer used here. As the usual reticulum cell tumors developed and non-bound virus is totally inactivated by the trypsinization [28] we must assume an oncogenic effect through shedding of virus. The effect of syngeneic cells infected with the F_1-virus in contrast to the lack of effect of cell-free F_1-virus preparations indicates that inoculation of infected tissue culture cells may be of value when transfer of virus against host-range restrictions [12, 24] is desired. In our experiments these restrictions were apparent both in the attempts to infect BALB/c mice (Fv-1^b) with F_1-virus (N-tropic), and CBA, DBA, C3H and AKR mice (Fv-^{n}) with B-tropic viruses.

The transplantation experiments asserted the neoplastic nature of the lesions observed and again [3] showed that a long observation period is needed for deciding whether the graft is successful. That we are dealing with growth of grafted cells is inferred from our previous observation of identity of type of paraprotein in donor and recipient mice when paraprotein-producing reticulosarcomas are grafted [29]. The virus-induced neoplasms seemed to be no more or less transplantable than spontaneous tumors from control animals.

The use of Caesarian derived SPF mice in the present experiment instead of conventionally reared animals [3] has increased the mean survival time of control males and greatly reduced the incidence of amyloidosis which was considered a main cause of death in conventional males [29]. Also the amyloid-accelerating effect of subcellular leukemogenic extract [30] is no longer detectable. This might be due only to loss of an amyloid-inducing factor in the subcellular extract. Subcellular leukemic extracts used in the present experiments, however, were derived from the conventionally reared mice used in prior experiments. Infection with murine leukemia virus furthermore has been shown under certain conditions to accelerate the amyloid development occurring in mice being immunized with casein [31]. We therefore assume that in establishing the SPF mice we lost a micro-organism different from the leukemia virus that enhanced amyloid development, something which prevented leukemia virus from influencing amyloid development.

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Initial Observations on Instrumental Differential Blood Leukocyte Counts during Chemotherapy of Patients with Leukemia

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Abstract—*Differences in fluorescence emission of blood leukocytes supravitally stained with acridine orange can be measured at two different wave lengths by a commercial flow-through cytofluorimeter, and used to distinguish at least three classes of cells. Instrumental differential leukocyte counts were performed in this way on a group of leukemic patients during one or more courses of chemotherapy. The counts obtained correlated well with manual counts, and with the clinical status of the patients, despite wide variations in total white cell count. While these are preliminary observations on a relatively small number of patients, they warrant further study of instrumental differential leukocyte counts as a possible additional means of monitoring chemotherapy of leukemia.*

In the course of studies on photometric properties of cells, we have examined the fluorescence characteristics of blood leukocytes supravitally stained with acridine orange. When the cells are suspended in dilute, isotonic solution of the dye, excitation by blue light will elicit green fluorescence from the nuclei and red fluorescence from granules in the cytoplasm [1, 2]. While the intensity of fluorescence per nucleus is relatively uniform for all normal mature leukocytes, there are quantitative differences in the red cytoplasmic fluorescence that distinguish at least three different classes of cells [3]. This has been proposed as a basis for automated differential leukocyte counts [4].

Patients with leukemia may have abnormal distributions of cells among these classes, and in some of the acute leukemias we have seen cells with increased nuclear fluorescence [5]. During chemotherapy changes are produced in the distribution of leukocytes characterized according to their acridine orange fluorescence. Those changes were followed in this study during the course of treatment of a number of leukemic patients, and correlated with manual counts and clinical status.

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MATERIAL AND METHODS

Specimen preparation

Samples of blood are collected by venipuncture in E.D.T.A. (ethylenediamine tetra acetic acid), or obtained without anticoagulant by finger puncture. The whole blood is then stained by diluting one volume with twenty-five volumes of isotonic saline containing acridine orange at a concentration of 10^{-6} g/ml, phosphate buffered at pH 7·4. With very high white blood cell counts proportionally greater dilutions are used. Measurements are made after incubating for 8 min at room temperature, at which time leukocyte stain uptake is near maximum. Red blood cells do not take up perceptible amounts of the fluorescent dye, and hemolysis is not necessary.

Instrumentation

We have used a commercial cytofluorimeter, the Cytofluorograf†, to obtain simultaneous two color measurements of cellular fluorescence. The stained leukocytes, in liquid suspension, are transported through the instrument at rates near one thousand cells per second. Fluorescence is generated as the cells pass in single file fashion through an elliptically-focused 488 mμ wavelength laser beam. The red and green components are separated optically and recorded simultaneously by two photomultiplier tubes. The signals so generated determine the coordinates of a flashed dot on the face of a cathode ray tube. Each dot represents a single cell; amplitude of nuclear green and cytoplasmic red fluorescence are indicated respectively by ordinate and abscissa. A photographic record of the light dots for a given sample of cells forms a scatter diagram that we have called a cytofluorogram, in this study each comprising measurements on 5000 cells.

Manner of presenting data

Figure 1 is an illustration of the cytofluorogram for normal blood leukocytes supravitally stained with acridine orange. The amplitude of green fluorescence of nucleus is uniform for all normal leukocytes, possibly reflecting their uniform DNA content. Differences in red emission distinguish at least three separate classes of cells. There is good evidence from direct observations on the stained cells [1, 2] from cytofluorimetry of selected, individual leukocytes [6] and from correlative counts of Wright–Giemsa stained smears [4] that Population I corresponds to lymphocytes, Population II to monocytes (and perhaps some large lymphocytes) and Population III to granulocytes. Chronic lymphocytic leukemia is characterized by a predominance of cells in Group I, chronic myelogenous leukemia by cells in Group III with a wider than normal distribution of measurements for cytoplasmic (red) fluorescence, and acute leukemias are characterized by cells in or near Group I with little or no red fluorescence (Fig. 2). In some acute leukemias cells are present with increased nuclear fluorescence, presumably blast cells with elevated nuclear DNA [5].

RESULTS

Sequential examinations of blood were carried out on 13 patients with leukemia during the course of their therapy. Eight patients had acute myelogenous or myelomonocytic leukemia, two had acute lymphoblastic leukemia, two had chronic myelogenous leukemia and one had leukolymphosarcoma. More than 200 examinations were performed on these 13 patients, the fewest for any individual being six during a five week period and the greatest being 31 over 10 week's time. The cytofluorograms obtained for any particular patient at any time were reproducible, and sometimes quite distinctive (Fig. 3). Changes that occurred during the course of disease and treatment can be illustrated by the following cases.

R.F.: Fig. 4 shows the sequence of changes in peripheral blood of a 27-year-old man with acute myelomonocytic leukemia. He had been diagnosed six months earlier and was in remission after treatment with several courses of arabinosylcytosine and 6-thioguanine. At our first examination (Fig. 4a) he had a pancytopenia (WBC = 1400/mm^3) with relative lymphocytosis (82% lymphocytes, 6% monocytes, 12% granulocytes) and a hypocellular marrow, attributed to the chemotherapy. Cytofluorographic counts were 83% Population I, 13% Population II and 4% Population III. Drugs had been discontinued 16 days earlier. The patient was transfused and watched. Five days later (Fig. 4b) there was a slight increase in the white blood count (2000/mm^3), with a distinct increase in granulocytes (47% lymphocytes, 11% monocytes, 30% granulocytes and 12% immature or unclassified cells). The cytofluorogram showed 46% cells in Population I, 22% in Population II and 32% in Population III. After two weeks more he had a white blood count of 2200/mm^3 with an essentially normal differential (35% lymphocytes, 6% monocytes and 59% granulocytes) and a normal cytofluorogram (46% Population I, 12% Population II and 42% Population III) (Fig. 4c). Arabinosylcytosine and thioguanine were begun again, but discontinued in six days because of bone marrow hypocellularity. His peripheral white count was then 2800/mm^3, the middle population had disappeared in the cytofluorogram (39% Population I, 0·5% Population II, 60·5% Population III) (Fig. 4d), and the manual differential count was 30% lymphocytes, 2% monocytes and 68% granulocytes. Within the next few days the patient developed a severe, acute hepatitis with fever to 105°F, serum transaminase (SGOT) over 12,000 and bilirubin over 10 mg%. His peripheral white blood count was 4300/mm^3 with 84% lymphocytes

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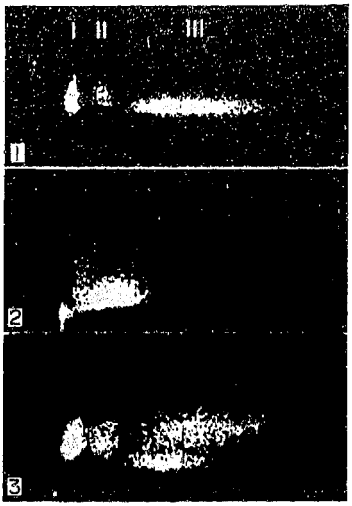

Fig. 1. *Scatter diagram of fluorescence intensities of peripheral blood leukocytes from a normal individual, supravitally stained with acridine orange. Each dot indicates simultaneous measurements of red and green fluorescence from one cell; the intensity of green fluorescence is plotted on the ordinate, and red fluorescence on the abscissa. Population I corresponds to lymphocytes, Population III to granulocytes and Population II to monocytes with probably some large lymphocytes.*

Fig. 2. *Acute myelomonocytic leukemia with a white blood count of* 69,000/mm^3 *and* 90% *blasts or immature monocytoid cells. Instead of the three populations seen normally, most of the cells fall into a single group with low red fluorescence, characteristic of the acute leukemias generally. Note also that there are cells with increased green fluorescence, presumably due to DNA alteration or synthesis.*

Fig. 3. *R.R., a* 25*-year-old Puerto Rican man with acute (?myeloblastic) leukemia of* 6 *month's duration, now in clinical remission. At this time, he had a white count of* 6400/mm^3 *with normal differential except for an eosinophilia of* 15%. *It is thought that the extra population, below Population III, probably represents eosinophils. We have no explanation for the lowered green fluorescence of these cells, but have noted it in other patients with eosinophilia. The cytofluorogram in some cases, as in this one, is sufficiently unique to be readily recognized as from a particular patient.*

(to face p. 182)

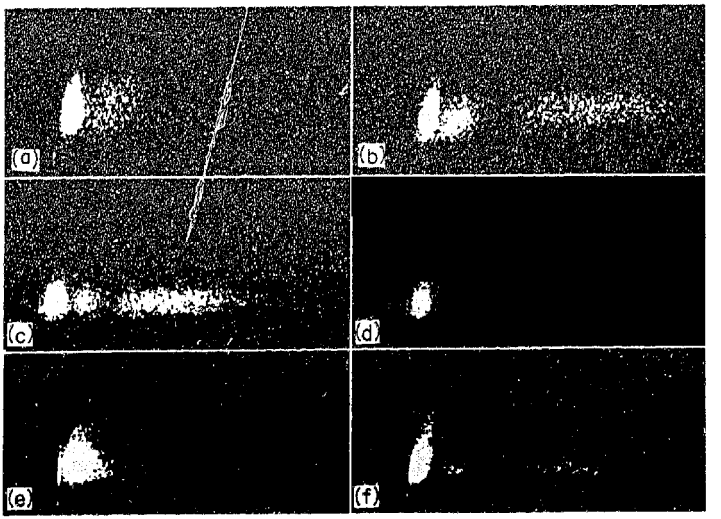

Fig. 4. A sequence of cytofluorograms from a patient with myelomonocytic leukemia during recovery from a drug induced granulocytopenia (a, b, c), followed by another course of chemotherapy (d) complicated by acute hepatitis with lymphocytosis (e), and beginning recovery (f). See text for full description.

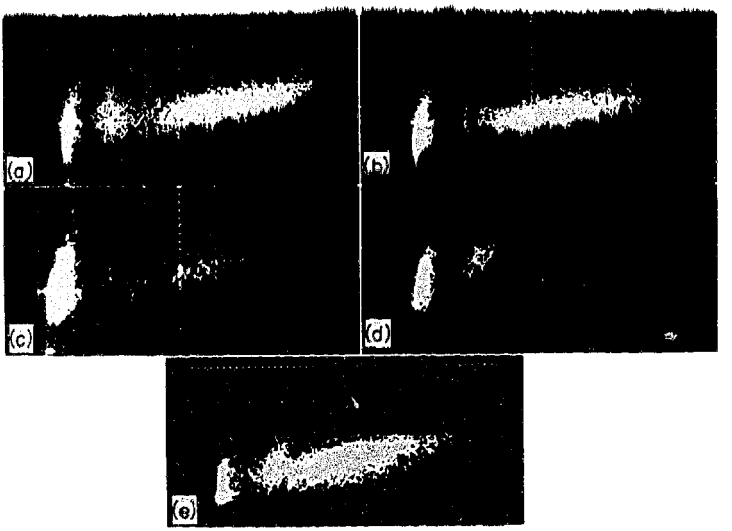

Fig. 5 Effect of chemotherapy in a patient with acute myelogenous leukemia. This man had been responding well to drugs, and at the completion of therapy had a normal differential white blood count and cytofluorogram (a). He subsequently developed a transient granulocytopenia (b, c, d), and then recovered (e). See text for full description. This was an early case studied by us and the populations are less compact because of technical factors in the instrumentation.

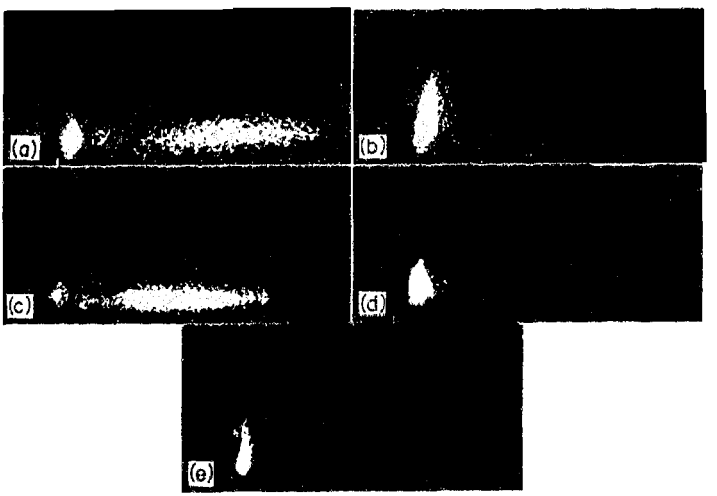

Fig. 6. Lenkolymphosarcoma in a 40-year-old man who initially had a nearly normal cytofluorogram while under treatment (a), then became leukopenic and granulocytopenic (b), and finally developed an infection with granulocytosis (c). He was taken off chemotherapy, and treated successfully with antibiotics. Later, chemotherapy was re-instituted and he again became transiently leukopenic (d), but was then refractory to the drugs and terminally had a rising white blood count. The final cytofluorogram (e) was taken shortly before death when he had a total white count of 75,500/mm³ with 80% lymphoblasts and 10% lymphocytes in peripheral blood. See text for details.

and 12% monocytes; the cytofluorogram showed a merging of the first two populations which together accounted for 94% of leukocytes (Fig. 4e). Improvement was prompt. One month later the patient felt well and was afebrile; bilirubin was 1·0 mg.% and SGOT only slightly elevated (74 units). Peripheral white blood count then was 4700/mm^3 with a modest lymphocytosis (78% lymphocytes, 5% monocytes and 17% mature neutrophils); the cytofluorographic count was 74% lymphocytes, 1·5% monocytes and 24·5% granulocytes (Fig. 4f). Marrow showed only 1% blasts.

R.N.: Fig. 5 is the series of cytofluorograms obtained from peripheral blood of an adult man with acute myelogenous leukemia. He had just completed a nine day course of arabinosylcytosine and 6-thioguanine, and his initial cytofluorogram was normal (Fig. 5a). He then had only 2% myeloblasts in the marrow, and an essentially normal differential peripheral white blood count with 3400 cells/mm^3. Four days later, a further effect of the drugs is apparent in that the middle population of cells has disappeared (Fig. 5b), and the total white cell count dropped to 2700/mm^3. By the seventh day (Fig. 5c) the white cell count was 2400/mm^3, and Wright–Giemsa stained smears of the peripheral blood showed 95% lymphocytes. On the 16th day after chemotherapy was discontinued, the peripheral blood showed a reappearance of the middle population (Fig. 5d). At this time, the total white cell count was still only 2200/mm^3, but there were 84% lymphocytes, 8% monocytes and 8% granulocytes in a differential count of 50 cells. The final examination, two months after the first, shows a cytofluorogram that is normal except for some merging between the 2nd and 3rd populations (Fig. 5e). The patient was then in remission with a total white cell count of 4800 cell/mm^3 and an essentially normal differential count.

L.M.: Figure 6 is from a 40-year-old man with leukolymphosarcoma and generalized lymphadenopathy who had had several courses of chemotherapy and was responding well. His peripheral white blood count was 3500/mm^3 with a normal differential at the time of the first cytofluorogram (Fig. 6a), which also was essentially normal though there were few cells in Population II. Bone marrow showed 7% blasts, however, and Daunomycin was begun. One week later he became leukopenic (WBC = 400/mm^3) and febrile (103·4°F). A manual differential count could not be done because of the leukopenia, and only a few lymphocytes and monocytes were found in the blood smear. The cytofluorogram indicated 94% lymphocytes, 2% monocytes and 4% granulocytes (Fig. 6b). Daunomycin was discontinued and antibiotics begun. Within a few days the patient improved, and eight days later he was afebrile with a white blood count of 8600/mm^3 and marked granulocytosis (5% lymphocytes, 1% monocytes and 94% granulocytes including 3 stabs). A corresponding granulocytosis was evident in the cytofluorogram (Fig. 6c) (7·5% lymphocytes, 3% monocytes and 89·5% granulocytes). The patient did well for about one month, and then went into relapse. Bone marrows showed from 20 to 60% blasts. He became transiently leukopenic on drugs (1800/mm^3) with a relative lymphocytosis (Fig. 6d) (76% lymphocytes, 8% monocytes and 16% granulocytes), but failed to go into remission. Terminally his white count rose to over 75,000 with 80% lymphoblasts and 12% lymphocytes, both cell types represented in Population I of the cytofluorogram (Fig. 6e).

DISCUSSION

While the feasibility of automated differential leucocyte counts is evident in prior studies [4, 5], clinical usefulness at this relatively early stage of development remains to be established. Initially it is likely that the greatest value will be for patients who require frequent blood counts, offering hope of improving reproducibility and providing new ways to follow cell changes. For that reason, this first attempt was made to describe the sequence of changes in a series of patients with leukemia, and to correlate them with clinical data. Patients with leukemia were also of interest for this study because wide variation in blood counts could be expected during the course of chemotherapy. Unfortunately, none of the patients were untreated when first seen by us, though we were able to follow several during exacerbations and remissions of their disease.

Good correlation was found between the instrumental counts, manual counts and clinical status of the patients. Variations in total white blood count did not interfere with the instrumental differential counts. In fact, reproducible differential white counts could be obtained with the instrument when the total white cell count was too low for reliable manual differential counts.

Experience with the instrumented technique still is very limited, and much of what has been observed remains to be explored. For example, in several cases we noted that Population II was the first to disappear when patients

receiving drugs became leukopenic, and the first to reappear as they recovered. We also noted that each of the three populations varied in number from time to time and from case to case according to the kind of illness and type of treatment, but were unable to explain a variation in the distribution of cells within each population. It is likely that these intra-group variations represent further heterogeneities that may be correlated with clinical or hematological findings. It is also possible that a sub-classification of the cell populations can be brought out by modifications in the staining technique, or by introducing new parameters. Further work along these lines is needed, and is in progress.

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Radioimmunoassay of Mouse Prolactin

Prolactin Levels in Isograft-Bearing Orchidectomized Mice

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Abstract—*Two groups of* 24 (C57BL X CBA)F_1 *mice were orchidectomized at the age of* 6–8 *weeks and received a hypophyseal isograft under the kidney capsule. One group received progesterone pellets implanted subcutaneously. Each group was divided into four sub-groups, which received oestrone in the drinking water at four dose levels: respectively* 1 *mg/l*, 250 *μg/l*, 12·5 *μg/l* and 1·25 *μg/l. Plasma samples were taken from each mouse by puncture of the retrobulbar venous sinus on days* 7, 21, 190, 270, 360 *and* 450 (*day of operation = day* 0). *To obtain statistically reliable estimates of tumour data* 290 *mice of the same hybrid combination were divided into two similarly treated groups. Each group was divided only in three sub-groups, which received respectively the three higher oestrone dose levels.*

The assay results in the group of mice receiving no implants of progesterone pellets were suggestive of a dose–response relationship between oestrone and plasma prolactin values extending over the whole range of the oestrone dose levels employed. The higher levels of prolactin observed in the 1 *mg/l sub-group compared to those in the* 250 *μg/l sub-group, however, could be ascribed to the contribution by the* in situ *glands, which enlarged tumourously only in all the mice in this sole sub-group. A real oestrone dose-related secretory response of the isografts was observed in the three sub-groups respectively on* 250 *μg/l*, 12·5 *μg/l and* 1·25 *μg/l. This oestrone-dependent response of the isograft was also reflected in the estimates of the degree of enlargement of the isografts in the respective sub-groups.*

In contrast only suggestive evidence was obtained for a dose–response relationship between either oestrone or plasma prolactin levels induced by this steroid on the one hand and mammary tumour incidence or the average age attained by the tumour-bearing mice in the various sub-groups on the other hand. The results indicated that continuously increased rather than "pharmacologically" high prolactin levels maintained in the circulation were mandatory for the induction of mammary tumours. Progesterone was found to have a clear-cut synergistic effect on the carcinogenic action of continuously increased plasma prolactin levels. More mammary tumours developed earlier in the presence of progesterone. The results in the mice on 1 *mg/l oestrone in their drinking water indicated a "protective" action by higher dose levels of oestrone against the induction of mammary tumours. The induction of pituitary tumours—to a size incompatible with survival by compression of the brain before the induction of mammary tumours—could not satisfactorily explain this for all cases, since in progesterone supplemented mice the development of tumorous enlargement of the pituitary by* 1 *mg/l oestrone in drinking water was completely prevented and yet the mammary tumour incidence declined.*

INTRODUCTION

As far back as 1913 the hormonal changes involved in pregnancy in mice were implicated in the genesis of mammary carcinoma by Lathrop and Loeb [1]. After Lacassagne [2] had described the induction of mammary carcinomas by prolonged administration of oestrone, oestrogens were considered the main

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group of hormonal agents involved. The observation that transplants of pituitary glands could induce mammary gland development and increase the incidence of mammary carcinoma in female mice, whereas such transplants had little effect on the mammary gland of either castrated females or intact or castrated males [3], was considered to be in line with the concept that oestrogens played the major role; the transplants were thought to act by stimulating the ovaries to an increased secretion of oestrogens.

Attention was focussed on prolactin when the hormonal state of isograft-bearing female mice was elucidated by vaginal smear studies [4]. Mammary tumour formation in this experimental model was considered to take place through excessive stimulation by prolactin and progesterone in the presence of more or less normal amounts of other hormones such as oestrogens, growth hormone, corticosteroids, etc.

A semi-quantitative estimation of the prolactin activity of the hypophyseal isograft was found by expressing numerically the sequences of the vaginal oestrous cycle per 100 days interval. On this basis, a complete correlation was found between graft activity and effectiveness in mammary tumour induction in each individual strain or hybrid studied [5]. An additional observation was that increase of graft activity in time completely correlated with the gradual increase in size of the isografts. Later experiments showed that the growth of the grafts was oestrogen dependent. In castrated males without oestrogen treatment the grafts remained small, suggesting that little or no prolactin was produced. Oestrogen treatment of castrated males with a pituitary isograft stimulated the growth of the grafts and concomitantly the production of prolactin [6].

For the present experiments the (C57BL X CBA)F_1 hybrid was chosen on the basis of previous studies: it was known that the pituitary *in situ* is sensitive to high dose oestrogen treatment and that the hypophyseal isografts in the kidney show a rapid growth rate in the presence of oestrogens [6].

The development of a radioimmunoassay based on extraction of the "granular fraction" of transplanted oestrogen-induced pituitary tumours [7] allowed a quantitation of the effects of isografts in female mice [8], that endorsed the conclusion reached by the vaginal smear technique and even indicated that the prolactin levels in the circulation at 18 months reached "pharmacologically" high values (ranging from 30 to 120 μg/ml, compared to physiological peak values, e.g. at pro-oestrus of 0·3 μg/ml).

However, difficulties which were due presumably to proteolytic enzymes, prevented to obtain regularly preparations of mouse prolactin of a quality needed for a satisfactory routine radioimmunoassay. Recently an improvement in the isolation procedure was reported [9], which yielded preparations that showed acceptably low "immuno-damage" after iodination.

MATERIAL AND METHODS

Orchidectomized (C57BL X CBA)F_1 mice were used for the experiments. The mice were operated at the age of 6–8 weeks and received a pituitary isograft under their left kidney capsule. The mice were kept in macrolon cages of 17·5 × 11·5 × 12·5 cm, three animals to a cage. The diet consisted of commercial food pellets (Standard Laboratory Diet, Hope Farms, Woerden, Holland) and oestrogenized tap water *ad libitum*.

Forty-eight mice were divided over 2 groups; one group of mice received 3 pellets of 3 mg progesterone per mouse per week subcutaneously. Each group of mice was divided into 4 sub-groups, which received oestrone in drinking water at four dose levels: respectively 1 mg/l, 250 μg/l, 12·5 μg/l and 1·25 μg/l. Fresh oestrone solutions were made once weekly (Thursday). Blood samples were collected from the retrobulbar venous plexus as described elsewhere [10], on days 7, 21, 190, 270, 360 and 450 of the experiment (always on Monday and/or Tuesday; day of operation and start of treatment = day 0).

Two hundred and ninety mice were divided over 2 groups; one group was treated with progesterone as above, the other not. Each group was divided over 3 sub-groups, which received oestrone in drinking water at three dose levels: respectively 1 mg/l, 250 μg/l and 12·5 μg/l. The number of mice per sub-group ranged from 47 to 50. The percentage of mice in each sub-group developing mammary tumours and the average tumour age as well as the average age of those not having developed mammary tumours were calculated. For each sub-group the average sizes of both the *in situ* hypophyses as well as the isografts at the age of 400 days were estimated by extrapolating the individual autopsy findings on growth curves published earlier [11]. The findings were classified as either + +, +, ±, or −, respectively, for estimated weights at 400 days of ± 100 mg, ± 20 mg, a distinct increase in

weight, but estimated as less than 5 mg, and no increase at all.

Radioimmunoassays were carried out with mouse prolactin and antisera raised in rabbits against this hormone according to the procedures described elsewhere [10].

RESULTS

(a). *Mice not receiving additional progesterone treatment*

The prolactin levels of the blood samples taken on day 7 were below the limit of sensitivity of the assay (0·015 μg/ml plasma) in the mice of the two sub-groups receiving the lower doses of oestrone. The sub-groups on 1 mg/l and on 250 μg/l showed mean plasma prolactin levels of 0·044 (±0·012) μg/ml and 0·035 (±0·020) μg/ml respectively.

As from day 21 onward the prolactin levels in all blood samples were well within the assayable range, the assay results of the four sub-groups are summarized in Fig. 1. They suggested a dose dependence, i.e. at each time when blood samples were collected, the mean plasma prolactin value was found to be correlated with the oestrone dose. The only exception being that on day 450 the mean prolactin value found for the sub-group receiving the highest oestrone dose was slighely lower (statistically not significant) than that of the sub-group on 250 μg/l.

The results also suggested within each sub-group an increase of the mean plasma prolactin levels in the course of time, with one exception that in the sub-group on the lowest oestrone dose a slight (statistically not significant) decline was observed between day 360 and day 450.

(b). *Mice treated with progesterone pellets*

Due to a failure of a freezer unit the plasma samples collected on day 7 and day 21 were lost for the assay. The results of the remaining samples are summarized in Fig. 2.

Compared to the previous group a number of more or less striking differences can be noticed: (a) all mice except one in this group had to be killed before day 450 of the experiment due to the development of mammary tumours; (b) the mice receiving progesterone pellets of the

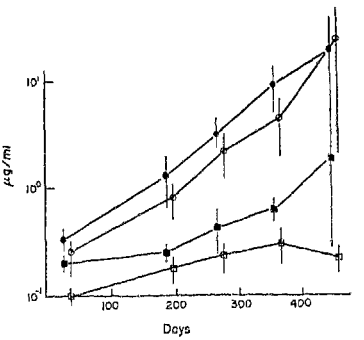
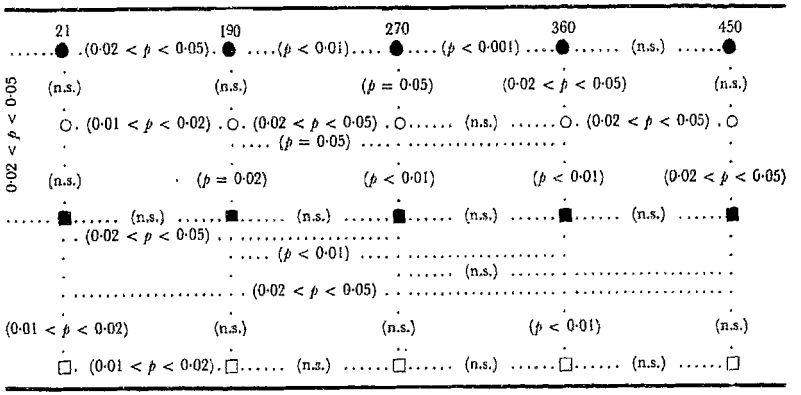


Fig. 1. Prolactin levels in plasma of four groups of isograft-bearing (C57BL X CBA)F_1 hybrid mice on different doses of oestrone dissolved in their drinking water. On the vertical axis the levels are plotted logarithmically in μg prolactin per ml plasma; the horizontal axis indicates the duration of oestrogen treatment at the time of sampling. The mean prolactin levels are represented by:
● *for the mice on 1 mg oestrone per 1 drinking water*
○ *for the mice on 250 μg oestrone per 1 drinking water*
■ *for the mice on 12·5 μg oestrone per 1 drinking water*
□ *for the mice on 1·25 μg oestrone per 1 drinking water*
The vertical bars show the standard deviation. The statistical significance of differences of the assay results between the experimental groups of mice as well as with the lapse of time calculated with the Student's t-test:



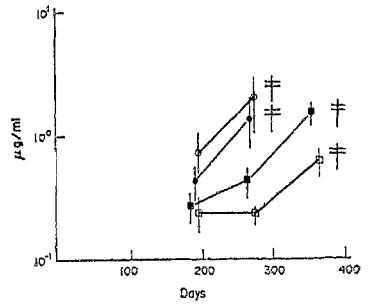
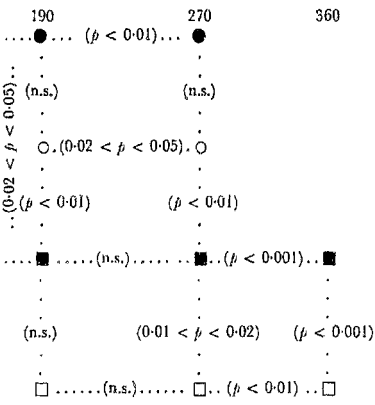


Fig. 2. Prolactin levels in plasma of four groups of isograft-bearing, progesterone pellet implanted (C57BL X CBA)F$_1$ hybrid mice on different doses of oestrone dissolved in their drinking water. For further details see legends to Fig. 1. The statistical significance of differences of the assay results between groups of mice as well as with onset of time according to Student's t-test:



1 mg/l sub-group showed distinctly lower plasma prolactin levels than those in the corresponding one not receiving progesterone (for day 190 as well as for day 270 the differences observed were statistically valid at the $0{\cdot}01 < p < 0{\cdot}02$ level); (c) in contrast to the results in the previous group the mean levels observed in the 250 μg/l sub-group were higher than those found in the 1 mg/l sub-group. The differences on both day 190 and day 270, however, were statistically not significant; (d) in the sub-groups of both groups that were on the three lower dose levels of oestrone in their drinking water the mean prolactin levels observed in the plasma samples of day 190 as well as in those of day 270 were practically the same in each corresponding pair, indicating that progesterone in the dose employed did not affect the prolactin levels induced in these mice by the rather wide range (250 μg to 1·25 μg) of oestrone per litre of drinking water; (e) in the two surviving sub-groups treated with progesterone, however, this steroid hormone apparently caused a distinct rise in the plasma prolactin levels induced by the two lower dose levels of oestrone in drinking water, over the next 3 months period. The increase was in the 12·5 μg/l sub-group from 0·6 μg to 1·5 μg ($p < 0{\cdot}001$) and in the 1·25 μg/l sub-group from 0·3 to 0·6 μg/ml ($0{\cdot}02 < p < 0{\cdot}05$).

(c). *Mammary tumour incidence and growth of pituitary tissue and other data from the group of* 290 *mice.*

The results of the experiment employing 290 mice to establish mammary tumour data and estimates of increases in pituitary tissue of both the *in situ* as well as the isografted glands are summarized in Table 1.

Additional progesterone treatment distinctly resulted in the development of more mammary tumours and in a much shorter time at all three dose levels of oestrone employed in this experiment. Another interesting result was that the highest dose of oestrone (1 mg/l) decreased the incidence of mammary tumours in the sub-group not receiving additional progesterone as well as in the one receiving progesterone. Although in the former sub-group this effect could at least partly be ascribed to the lowering of the average age of the mice in this sub-group attained due to the development of pituitary tumours *in situ*, it was evident that this could not be the case in the progesterone sub-group since here the development of pituitary adenomas was completely inhibited. The third striking observation is that although the reduction of the oestrone dose from 250 μg/l to 12·5 μg/l in their drinking water resulted in a significant decrease in size of the isografted pituitary, in the two sub-groups not receiving additional progesterone the mammary tumour incidence (56 % and 59 % respectively), as well as the average tumour age (484 and 493 days respectively), remained virtually the same; in the two sub-groups with progesterone the mammary tumour incidence remained 100 % and the 20-fold lower dose of oestrone in the drinking water resulted only in a slight retardation of the development of the mammary tumours (from 276 days to 306 days).

DISCUSSION

When comparing the results of the sets of experiments started to follow the changes of prolactin levels in the circulation in sub-

Table 1. *Mammary tumour incidence and growth rate of hypophyseal tissue in orchidectomized (C57BL X CBA)F_1 mice after oestrone treatment and with or without progesterone ("tumour biology" group)*

Treatment	No. animals	With tumour			Without tumour	Growth rate of hypophyseal tissue	
		No.	%	Average age (days)	Average age at death (days)	*in situ*	isograft
1 mg/l oestrone	50	9	18	409	447	++*	++*
1 mg/l oestrone + progesterone†	48	37	77	336	366	±	++
250 μg/l oestrone	48	27	56	484	517	±	++
250 μg/l oestrone + progesterone†	48	48	100	276	—	±	++
12·5 μg/l oestrone	49	29	59	493	537	−	+
12·5 μg/l oestrone + progesterone†	47	47	100	306	—	−	+

*++ = ± 100 mg at 400 days of age.
\+ = ± 20 mg at 400 days of age.
± = variable, but less than 5 mg at 400 days.
− = no increase in weight.
†3 x 3 mg weekly s.c.
N.B. Since treatment was started at the age of 6–8 weeks the age in days minus 50 days gives the duration of treatment approximately; see also under discussion.

groups of mice on four different dose levels of oestrone in their drinking water, with or without progesterone treatment, with those started to establish mammary tumour incidence and other tumour biology data it should be stressed that the latter set of experiments were carried out over a period of several years as part of a much larger complex of experiments designed to establish the contribution of various other biological factors (e.g. MTV, genetical constitution, etc.) to mammary carcinogenesis. Therefore standardized rules, which defined the criteria when an animal that developed a mammary tumour had to be killed for histological verification and classification of its tumour, had to be adhered to. In contrast the experiments with the smaller "assay" sub-groups were set up primarily to follow the effect of the respective treatments on the plasma prolactin levels; therefore any mouse was kept alive when the general condition was considered good enough to meet the next target date set for blood sampling, irrespective whether it had developed a mammary tumour or not. Table 2 represents the tumour incidence and other "tumour biology" data of the 8 assay sub-groups. For the reasons mentioned above the average ages attained were in general higher than in the comparable sub-groups represented in Table 1.

However, it was evident that the mice of two of the "assay" sub-groups survived their respective treatments for a much longer time than did the mice in the corresponding sub-groups represented in Table 1: (a) the sub-group on 1 mg/l oestrone without progesterone supplementation showed an average age of 409 days and 447 days for the 9 mice that developed mammary tumours and the 41 animals that did not do so, respectively. None of the 6 mice of the corresponding "assay" sub-group developed mammary tumours and showed an average age of 513 days. Since in the two sub-groups the life-span limiting factor was mainly the development of tumours of the *in situ* pituitary to a size incompatible with life due to compression of the brain, it was evident that the enlargement of the pituitary gland in the mice of the "assay" sub-group was slower; (b) the sub-group on 12.5 μg/l oestrone in drinking water supplemented with progesterone showed an average tumour age of 306 days in the 47 mice represented in Table 1, whereas the corresponding mice in Table 2 reached an average age of 384 days. Since the 3 large sub-groups supplemented with progesterone showed average ages in their tumour-bearing animals clustering around 300 days and only the two highest doses of oestrone administered to the corresponding "assay" sub-groups showed average ages of around 300 days, whereas the 20-fold reduction from 250 μg/l to 12·5 μg/l increased the mean age to 384 days and a further reduction to 1·25 μg/l drinking water increased it further to 430 days, it was tentatively assumed that the "effective"

Table 2. Mammary tumour incidence and growth rate of hypophyseal tissue in orchidectomized (C57BL X CBA)F_1 mice after oestrone treatment and with or without progesterone ("assay" group)

Treatment	No. animals	With tumour: No.	With tumour: %	With tumour: Average age (days)	Without tumour: Average age at death (days)	Growth rate of hypophyseal tissue: *in situ*	Growth rate of hypophyseal tissue: isograft
1 mg/l oestrone	6	0	0	—	513	++*	++*
1 mg/l oestrone + progesterone†	6	5	83	305	349	±	++
250 µg/l oestrone	6	4	67	502	542	±	++
250 µg/l oestrone + progesterone†	6	6	100	307	—	±	++
12·5 µg/l oestrone	6	3	50	538	536	–	+
12·5 µg/l oestrone + progesterone†	6	6	100	384	—	–	+
1·25 µg/l oestrone	6	2	33	387	531	–	±
1·25 µg/l oestrone + progesterone†	6	6	100	430	—	–	±

*++ = ± 100 mg at 400 days of age
+ = ± 20 mg at 400 days of age
± = variable but less than 5 mg at 400 days
– = no increase in weight
†3 x 3 mg weekly s.c.
N.B. Since treatment was started at the age of 6–8 weeks the age in days minus 50 days gives the duration of treatment approximately; for comparison with the results represented in Table 1 see also under discussion.

oestrone dose taken up by the mouse in the smaller "assay" groups was lower than that in the mice of the larger "tumour biology" sub-groups. However, since all mice in the 1 mg/l "assay" sub-group developed pituitary tumours of their *in situ* pituitary gland albeit approximately 2 months later than in the corresponding "tumour biology" sub-group, the conclusion could be drawn that the "effective" oestrone dose in the 1 mg/l "assay" sub-group was reduced by a factor distinctly less than 4, because none of the mice in the 250 µg/l "tumour biology" sub-group developed tumourous enlargement of their *in situ* pituitary gland.

During the same period that the set of "assay" experiments were carried out, a slowing down in the supply of prolactin-producing pituitary tumour transplants for the isolation of prolactin was noticed. Pseudomonas pyoceanea was found to grow in the water bottles on the cages, causing opalescence within a few days of changing the bottles. After addition of HCl to bring the drinking water to pH 4 the drinking water remained clear and the supply of tumour transplants was restored.

In experimental conditions leading to a continuously sustained stimulation of the prolactin-producing cells, proliferation of these cells is triggered when the stimulus is strong enough. In all such cases, both in rats as well as in mice, the prolactin levels in the circulation were always found to become high within a short time to increase further after a shorter or longer period. The start of this second increase always appeared to coincide with the start of the proliferative outgrowth of pituitary tissue [8, 10, 12]. In rats, as well as in mice, the *in situ* gland did so only after continuous administration of rather high doses of oestrogens (compared to the respective substitution dose, i.e. the oestrogen dose needed to restore vaginal cornification). The *isografted* pituitary gland, however, exhibited an extreme difference in sensitivity to oestrogen stimulation between rats and mice. In *rats* the isograft could only be stimulated by rather massive doses of oestrone. The effect of high doses of oestrone on isograft-bearing rats having their *in situ* gland could only just be demonstrated, since the isografts were found to have started to enlarge by the time the tumourously enlarged *in situ* gland about killed the rat by its size. The weights of the isografts were lagging behind those attained by the *in situ* gland by almost a factor 10 [10, 13]. One had either to resort to isografting a number of pituitary glands [14] or to isografting pituitary glands under the kidney capsule of hypophysec-

tomized animals to unequivocally ascribe increases in prolactin levels in the circulation to prolactin release by the isografts [15]. In contrast, the extreme sensitivity of isografted pituitary tissue to oestrogen stimulation in mice was unequivocally demonstrated in the present experiment: even as low a dose as 1·25 μg/l of oestrone dissolved in drinking water brought the prolactin levels within 3 weeks to levels which were distinctly higher than those found either in anoestrous female mice having normal oestrous cycles or in intact or castrated male mice not treated with oestrogens. It raised these levels further to reach values well within the range found during the pro-oestrus/oestrus surge of prolactin observed in female mice with normal 5-day cycles by day 190 and sustained these increased levels in the circulation up to day 450 of oestrone administration.

A close correlation between increases of pituitary tissue and prolactin levels in the circulation was again found in the present experiments; apparently the isografts were already maximally stimulated by 250 μg oestrone per litre drinking water and this stimulation did not appear to be affected by progesterone. The isografts of the mice on 1 mg/l and those on 250 μg/l were judged to have reached the same degree of enlargement, irrespective of having received additional progesterone pellets or not. The *in situ* gland, however, was stimulated sufficiently to grow out tumourously by the 1 mg/l dose only. Moreover the stimulatory action of (this high dose of) oestrone on the *in situ* gland was completely blocked by the administration of progesterone This indicates that the inhibitory effect of progesterone was mediated via the hypothalamus. The observations on the growth stimulatory action of oestrone on hypophyseal tissue were fully endorsed by the results of the assay experiments; e.g. in the three groups of mice, in which only the isografts grew out maximally the prolactin levels at each of the sampling times were virtually identical (the 250 μg/l sub-groups with and without progesterone), or at least did not differ in a statistically significant way (the 1 mg/l sub-group with progesterone in comparison to the two above mentioned ones).

Although the assay results of the four subgroups not receiving progesterone pellets in general showed, at each time when blood samples were collected, mean plasma prolactin values which were highest in the mice on 1 mg/l, somewhat lower in the 250 μg/l sub-group, again lower in the 12·5 μg/l subgroup and lowest in the mice on 1·25 μg/l, suggestive of an oestrone dose dependence of the prolactin response over the whole range of oestrone doses employed, the higher mean plasma prolactin levels observed in the mice on 1 mg/l had to be ascribed to the contribution of the (maximally) stimulated *in situ* pituitary gland on top of the amounts of prolactin secreted by the isografts. A real oestrone dose dependent prolactin response of the prolactin-producing hypophyseal cells (probably restricted mainly to those in the isografts) was demonstrated in the present experiments starting in the region of somewhat less than 250 μg of oestrone per litre drinking water going down markedly when the oestrone dose is decreased to levels as low as 1·25 μg/l. This dose dependence was observed in the sub-groups on the three lower oestrone doses employed irrespective whether the mice received progesterone or not. Here again there appeared to be a good correlation between the graded response indicated by the prolactin assays and the graded growth response of the pituitary tissue by the estimates of the enlargements of the isografts. This indicates that a stimulus acting on the prolactin cells had to be so strong as to induce a maximal secretory response as reflected in the prolactin levels assayed in plasma to be effective to trigger cellular multiplication of the pituitary cell type involved and that a less than maximal stimulation, which was reflected by lower levels of prolactin in the circulation, within a certain range still could trigger cellular multiplication. The subsequent pituitary enlargement in such cases took longer to become noticeable; either because less cells were involved in the process or because either the multiplication started later or the multiplication rate was slower.

In contrast to this demonstration of a dose response relationship between oestrone on the one hand and plasma prolactin levels and outgrowth of the pituitary tissue involved on the other hand, only suggestive evidence was obtained for a dose response relationship between either the oestrone dose or the height of the prolactin levels resulting from it on the one hand and mammary tumour induction on the other hand. The tumour incidence data compiled in Table 2 suggested such a relationship: (a) an increase in mammary tumour incidence with increasing oestrone dose was found in the group of mice not supplemented with progesterone for the oestrone dose range between 1·25 μg/l to 250 μg/l in drinking water; (b) the group of mice receiving pro-

gesterone pellets in addition showed a 100% tumour incidence in this oestrone dose range. Nevertheless the mice in these 3 sub-groups also provided suggestive evidence for a dose response relationship, since the average ages attained by the mice as a consequence of their mammary tumours decreased with increasing oestrone dose.

When the data were compared to those obtained in the much larger groups of mice represented in Table 1, however, no supporting evidence was found for such a dose response relationship. Mammary tumour incidence in the sub-groups on 250 μg/l and on 12·5 μg/l in the mice not supplemented with progesterone was 56 and 59 per cent respectively (the 1·25 μg/l dose was not included in this earlier experiment). The mice in the group supplemented with progesterone again showed a mammary tumour incidence of 100 per cent. The average ages attained by the mice in the 12·5 μg/l sub-group and in the 250 μg/l sub-group were 306 and 276 days respectively. It should be noted that the average ages of the mice in the corresponding sub-groups represented in Table 2 were 384 and 307 days respectively.

The failure to find supportive evidence for a dose response relationship between the oestrone dose (or presumably the prolactin levels in the circulation induced by it) and either mammary tumour incidence or average age of the tumour-bearing mice from the mice represented in Table 1 either indicated that the observations suggestive of such a dose response relationship reflected in the data compiled in Table 2 were due to random variation on too small a number of mice in the assay experiments, or could be related to the earlier mentioned tentative conclusion that the "effective" oestrone dose reaching the mice was distinctly higher in the experiments represented in Table 1 compared to that in the experiments represented in Table 2.

The most important conclusions that unequivocally could be drawn from all the experiments presented were: (a) For the induction of mammary tumours it was not necessary that prolactin in the circulation increased to the "pharmacologically" high levels that were reported earlier [8] in isograft-bearing intact female mice and which were observed in the present experiments too in orchidectomized mice treated with oestrone doses that were in the range needed to restore vaginal cornification in spayed mice; oestrone dose levels reduced to 10- to 100-fold less than that necessary to restore vaginal cornification in spayed mice when given to orchidectomized male mice bearing a pituitary isograft over a long enough period were found to increase prolactin levels to values encountered in intact untreated female mice during the pro-oestrus/oestrus prolactin surge and were found to be able to keep the plasma prolactin levels at these levels over long periods as long as the oestrone dose was administered; such sustainedly increased levels of prolactin *remaining within the "physiological" range* proved to be effective to induce mammary tumours in a number of mice. (b) Progesterone administration supplementary to the oestrone treatment raised the mammary tumour incidence dramatically to 100 per cent in all sub-groups on oestrone in dose levels of 1·25 μg/l, 12·5 μg/l and 250 μg/l of drinking water and in most cases shortened the average age attained by the mice bearing mammary tumours; since in general the progesterone administered did not affect prolactin levels in the circulation (with the exception of the mice on 1 mg/l oestrone when it suppressed the contribution of the *in situ* gland to plasma prolactin levels and in the plasma samples taken on day 360 from the isograft bearing mice on 1·25 μg/l and 12·5 μg/l oestrone when the progesterone supplemented mice showed distinctly higher prolactin values than the mice not supplemented with this hormone) in sharp contrast to oestrone, it is highly improbable that progesterone affected mammary tumour induction via raising the prolactin levels. (c) The highest dose of oestrone employed in the experiments presented provided evidence for an adverse effect on mammary tumour induction, or a beneficially "protective" effect on the mice. In the mice developing a tumourous enlargement of their *in situ* pituitary gland at least this "protective" effect could be ascribed to early development of these tumours to a size incompatible with survival before mammary tumours could develop. However, in the mice on the high dose of oestrone supplemented with progesterone (progesterone showing in these mice a two-fold action: (1) completely blocking the tumorous enlargement of the *in situ* pituitary and (2) speeding the induction and development of the mammary tumours) the mammary tumour incidence was consistently lower than in the mice on the 3 lower dose levels of oestrone. The mechanism of the "protective" action of administration of oestrone at dose levels of 4 to 8 times the dose needed to restore vaginal cornification in spayed mice is not clear; an inhibitory action of large amounts of oestrogen on mammary gland growth has been described already in 1941 [16].

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Hexamethylmelamine—

A New Drug with Activity in Solid Tumors

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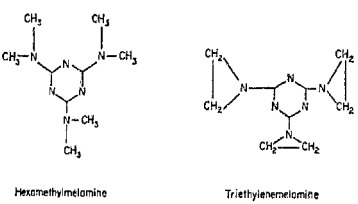
Abstract—*Hexamethylmelamine* (NSC 13875) *is a triazine that has completed phase* I–II *trials under the sponsorship of the Division of Cancer Treatment, National Cancer Institute. It was selected for clinical trial based on superior activity against the Walker* 256 *carcinosarcoma. In the clinical trials reported, the dose schedules used were* 4–15 *mg/kd/d for* 21–90 *days. Dose limiting toxicity was gastrointestinal. Also seen were leukopenia, thrombocytopenia, and central nervous system toxicity. The overall response rate in* 784 *evaluable patients was* 17%. *Greater than* 20% *response rates were seen in the following tumor types: small cell (oat) carcinoma of the lung, ovarian adenocarcinoma, lymphoma, and breast cancer. Further controlled clinical trials in certain tumor types are warranted.*

INTRODUCTION

HEXAMETHYLMELAMINE, an antitumor triazine, has been evaluated in extensive phase I and II clinical trials. The National Cancer Institute, sponsor of the Investigational New Drug Application with the U.S. Food and Drug Administration, is the supplier of the clinical material used in these studies. In this review, we have summarized the data from the literature, as well as data on file at the Cancer Therapy Evaluation Branch, National Cancer Institute. This information, collated by disease, would be useful as a data base for the development of further clinical evaluation in phase III controlled comparative trials.

BIOCHEMISTRY

Hexamethylmelamine closely resembles triethylenemelamine (NSC 9706; TEM), a known alkylating agent, in structure. A comparison of the two structures is shown in Fig. 1. However, it has been suggested that hexamethylmelamine may not function as an alkylating agent, but perhaps as a pyrimidine antimetabolite [1], as certain *s*-triazines are known to act. Data on pharmacologic effects in humans suggest that the metabolism of this compound differs significantly from that of TEM (CF: Pharmacologic Studies in Man).



Hexamethylmelamine Triethylenemelamine

Fig. 1. *Structures of hexamethylmelamine and triethylenemelamine.*

Heere and Donnelly [2] demonstrated that hexamethylmelamine selectively inhibited the incorporation of labeled precursors into RNA and DNA to a greater extent than was observed with the uptake of appropriate protein precursors. If the cells were preincubated with hexamethylmelamine, the inhibition of DNA and RNA incorporation of labeled precursors was lost. The authors suggested that this *in vitro* effect might parallel the *in vivo* tumor inhibitory effect. Borkovec [1] proposed that hexamethylmelamine may demethylate to form the weak alkylator, formaldehyde, but this mechanism was presented as a possible alternative. The exact mechanism of action was unknown.

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ANTITUMOR ACTIVITY IN ANIMALS [3]

At the maximum tolerated dose (MTD) in the Walker 256 carcinosarcoma system (intramuscular) of 80 mg/kg/day, given IP × 10 to 12 days starting the day after transplantation, the drug inhibited tumor growth by 79%, but it also caused considerable weight loss in the hosts.

The growth of Sarcoma 180 was decreased by 25% when hexamethylmelamine was given IP at 125 mg/kg/day × 7. This was the MTD.

Hexamethylmelamine was inactive against 3-methyl-cholanthrene induced rat breast cancer, B16 melanoma and mouse leukemia L1210.

PHARMACOLOGIC STUDIES IN MAN

Bryan *et al.* [4] used an ultraviolet spectrophotometric analytic method to study the plasma levels and urinary metabolites of hexamethylmelamine in cancer patients after oral administration. Maxima plasma levels after a single dose of 4 mg/kg were obtained two to three hours later and averaged about 2·75 μ/ml. No intact drug was present in the plasma, but two metabolites of the drug were detected. Hemolyzed erythrocytes contained no hexamethylmelamine or drug metabolites. Nearly 19% of a single oral dose was excreted in the urine during the first 24 hr after administration, with an additional 12% appearing between 24 and 48 hr. After prolonged, continual daily administration of the drug, 50 to 65 per cent of a daily dose was recovered from the urine as metabolites. At least three major metabolites of hexamethylmelamine were found in the urine, but no unmetabolized compound was recovered.

In mice [5], cyanuric acid was the sole urinary metabolite found after the IP injection of ^{14}C-ring-labeled TEM. Cyanuric acid could not be identified in the study of Bryan *et al.* as a urinary metabolite of hexamethylmelamine. They [4] concluded that "the ultraviolet absorption and colorimetric behavior of these metabolites suggest that they may retain an *s*-triazine ring structure that does not correspond to cyanuric acid."

Worzalla [6] studied the metabolism of $^{14}C_6$-methyl-hexamethylmelamine in rodents and man. When the drug was given IP to rats, 30% of the labeled carbon was recovered as $^{14}CO_2$. But when the drug was given PO via the stomach tube, only 13% was recovered in the first 24 hr. Two patients received the labeled material with about 10% of the ^{14}C recovered as $^{14}CO_2$ in the first six hours.

When the urine was collected in patients who received hexamethylmelamine, three distinct metabolites were identified chromatographically: N^2, N^4-dimethylmelamine, N^2, N^4, N^6 trimethylmelamine, and melamine. The data suggested that hexamethylmelamine is N-demethylated to the lower homologues and that a significant amount of the methyl groups are removed from the parent compound and oxidized to CO_2.

CLINICAL EXPERIENCE

The Midwest Cooperative Chemotherapy Group carried out a phase I study of hexamethylmelamine in 1964–1965. Louis *et al.* [7] reported the results of this trial in which the primary objectives were to determine the dose which 80% of patients would tolerate continuously for 42 days, the quantitative and qualitative toxicity of the drug and the feasibility of testing the agent in man for oncolytic activity. They found that the dose tolerated as defined was 10 mg/kg/day. The principal adverse effects requiring discontinuation of therapy were gastroenteric and hematologic. Marrow aplasia developed in one patient who received 64 mg/kg/day × 22 days. Despite discontinuation of therapy, a latent effect associated with prolonged treatment was peripheral neuropathy which in two patients was not reversible. This neuropathy manifested as paresthesias, hypesthesias, hyporeflexia, motor weakness, decreased position and vibratory sense, and decreased tactile and proprioceptive sense. This complication developed in seven of 124 patients entered on the study. None of the seven received less than 12 mg/kg/day. An electromyogram indicated muscle rather than neuronal failure in one patient.

In 1965, Wilson and de la Garza [8] reported results of a Central Oncology Group phase I study of hexamethylmelamine. The drug was given orally in doses from 6 to 15 mg/kg/day × 14–21 days. Twelve mg/kg/day × 21 days, given in four divided daily doses, was found to be the maximal tolerated dose regimen and was given to 22 patients. Thirteen of the twenty-two had nausea and vomiting associated with drug administration, which in general was well controlled with antiemetics. However, nausea and vomiting were severe in four patients, and in two of them, therapy was stopped after a week. This gastrointestinal toxicity was the primary limiting factor in drug administration. A white blood cell (WBC) count < 4000 was

Table 1. *Toxic effects of hexamethylmelamine* [9]* (12 *mg/kg/d* × 21 *days*)

Degree	Blood†	GI‡	GU	Liver	CNS	Oral
			All Patients			
None	281 (73)	178 (46)	375 (97·4)	362 (94)	359 (93·2)	375 (97·4)
Slight–Moderate	74 (19)	118 (31)	9 (2·4)	18 (5)	10 (2·6)	7 (1·8)
Severe	30 (8)	89 (23)	1 (2·0)	5 (1)	16 (4·2)	3 (0·8)

*Figures in parentheses are percentage of total. GI = gastrointestinal. GU = genitourinary. CNS = central nervous system.

†Blood: Slight–Moderate: WBC 3000–4500/mm^3 and/or platelet count 75,000–100,000/mm^3. Severe: WBC less than 3000 and/or platelets less than 74,000.

‡Gastrointestinal: Slight–Moderate: frequent nausea and occasional vomiting that may require the use of antiemetics. Severe: continuous nausea and frequent vomiting requiring almost continuous administration of antiemetics.

seen in six of the twenty-two; one patient had a fall to between 1000 and 2000. Two patients had thrombocytopenia, defined as a drop in platelet count to < 100,000, associated with therapy. There was no weight loss attributable to the drug at this dose level, but significant weight loss was seen at 15 mg/kg/day × 21. No evidence of renal or hepatotoxicity was observed.

Significant tumor regressions were seen in a number of patients, including three with carcinoma of the ovary, two with carcinoma of the lung and one each with carcinoma of the breast, prostate and unknown primary. In all of them, the benefit extended through the observation period (treatment plus four weeks of follow-up). There was no apparent correlation of response with hematologic toxicity of the drug.

Wilson *et al.* [9] then reported the results of a phase II evaluation of this drug by the Central Oncology Group. Patients selected for this protocol had to have histologically proved, inoperable malignant disease, an estimated life expectancy of at least three months and WBC > 4500 and platelet count > 150,000 at the beginning of hexamethylmelamine treatment. The dose regimen was 12 mg/kg/day orally × 21 days with the drug divided into four doses each day. Complete response in this study was defined as disappearance of all measurable disease and partial response as > 50% regression of measurable disease.

Tables 1 and 2 summarize the toxic effects and the response seen in Wilson's study. A total of 385 patients were entered on the protocol, and 257 cases were evaluable ("acceptable study patients"). Of the 128 unsatisfactory trials, 92 were disqualified because of discrepancies in dosage. Twelve of these had upward deviations of dosage, and most of the others had insufficient dosage because of gastrointestinal toxicity. Other causes for classifying a case as unsatisfactory were death during the study or not enough measurements. No deaths were attributed to the drug. (Both leukopenia and thrombocytopenia were reversible.)

As can be seen from the tables, a response rate greater than 20% was seen in cancer of the lung and ovary. The overall response rate in the 257 patients was 16%, with a demonstrable correlation between response and hematological toxicity.

Wilson and his co-workers [10] in the Central Oncology Group then reported on an evaluation of an additional 320 patients at a dose of 8 mg/kg/day over a prolonged period of time,

Table 2. *Responses to hexamethylmelamine* [9]* (12 *mg/kg/d*)

Site	Responses Complete	Partial	Total
Head and neck	0	6(19)	32
Colon–rectum	0	3(11)	27
Lung	0	10(26)	38
Breast	0	3(18)	17
Melanoma	0	1(5)	21
Sarcoma	0	0	26
Ovary	0	5	12

*Figure in parentheses indicates percentage.

in most cases 90 days. The therapeutic effect and toxicity were compared to their earlier study of 12 mg/kg for 21 days.

Four hundred and thirty-seven patients were evaluated for toxicity. The incidence of hematopoietic toxicity was 48% for the 8 mg/kg dose compared to the 28% incidence in the

earlier study. The incidence of gastrointestinal toxicity was also higher for the lower prolonged dose administration regimen with 62% incidence compared to 54%.

CNS toxicity was seen in two patients as ataxia and one patient as a Parkinsonian-like syndrome. The CNS toxicity seemed dose related and disappeared with discontinuance of the drug.

Of the 437 patients entered, 117 were not evaluable for response. Of the 117 patients, 50 patients died secondary to progression of their primary disease, and 30 patients were removed because of drug intolerance. The response rates are tabulated in Table 3.

One hundred and twenty-eight additional patients were evaluable, but the number of patients per tumor type was insufficient to determine a response rate. These cases are tabulated by tumor type in Table 7. The overall response rate for the 320 patients evaluated in this study was 23%.

De la Garza, Carr and Bisel [11] evaluated the efficacy of hexamethylmelamine in patients with metastatic lung cancer. The patients received 8–12 mg/kg daily in divided oral doses for about 21 days. The dose was adjusted in the face of toxicity. The 19% overall response was analyzed by cell type on Table 4. Survival correlated with degree of response.

Table 3. Responses to hexamethylmelamine [10] (*8 mg/kg/d up to 90 days*)

Tumor	Patients evaluable	Responses CR	PR	%
Lung	94	2	17	20
Breast	31	5	5	32
Ovary	10	1	2	30
Colon–rectum	44	0	8	18
Lymphoma	13	1	10	85
Totals	192	9	12	27

Table 4. Hexamethylmelamine in lung cancer [11] (*8–12 mg/kg/d* × ≃ 21)

Cell type	Number evaluable	Responses CR	PR	%
Squamous	27	0	4	15
Small cell	19	1	4	26
Adenocarcinoma	9	0	1	11
Large cell	12	0	3	25
Alveolar	3	0	0	—
Totals	70	1	12	19

Takita [12] treated 38 patients who had inoperable bronchogenic carcinoma with hexamethylmelamine. Most patients were treated with 12 mg/kg/day as divided daily doses for 21 days, but four patients were treated with 6 mg/kg/day for 42 days. Twenty-five of the 42 patients entered developed gastrointestinal toxicity of varying degree. It was tolerable in 17, severe in 8, necessitated dose reduction in 2 and led to the discontinuance of the drug in 3 patients.

The hematopoietic toxicity was transient and dose related. Twelve of the 42 patients had moderate reduction in WBC to the range of 3000–5000, but only one patient developed a count below 3000. Thrombocytopenia was seen in only a mild degree in 2 patients. No other toxicities were reported.

Of 39 evaluable patients, there were two complete responses, four partial responses and a 15% overall response rate. Table 5 summarizes the results by cell type. Although the overall response was similar to de la Garza's data, there was a difference by cell type.

Table 5. Hexamethylmelamine in lung cancer [12] (*12 mg/kg/d* × 21)

Tumor	Patients evaluable	Responses CR	PR	%
Squamous	9	0	0	—
Adenocarcinoma	7	0	0	—
Anaplastic	10	0	1	10
Oat cell	12	2	2	33
Totals	38	2	3	15

Bergevin *et al.* [13] have entered 38 patients on a protocol randomizing patients between Regimen A (4 mg/kg/day × 42 days) vs. Regimen B (8 mg/kg/day × 42 days). There was a provision for crossing over from the lower dose to the higher dose. From the mixture of previously untreated and treated patients, 28 patients were evaluable with a total of 5 objective regressions. These responses included 2 of 11 patients with bronchogenic carcinoma (both responders had squamous cell carcinoma), of four responses in gastrointestinal carcinoma, one of three responses in lymphoma and a response in hepatic carcinoma.

There was no difference in the response rate in previously treated patients or in the two doses evaluated. All responses became evident in the first treatment course of 42 days. The range of the day of greatest response was

between 22 and 38 days. They concluded that there was no rationale for crossing over to higher doses, nor for treatment for longer than 42 days in those patients showing no response.

Bergevin observed significant drug intolerance to the 8 mg/kg dose. Ninety-two per cent of the patients who were crossed over to the higher dose exhibited dose limiting GI or CNS toxicity. This was in contrast to 62% of the patients in Regimen B and 37% in Regimen A alone. As another reflection of dose limiting toxicity, 60% of the total dose was given in Regimen B, but 86% was given in Regimen A. All drug toxicities were dose related, clinically obvious and reversible.

The Western Chemotherapy Group [14] was using hexamethylmelamine at 12 mg/kg/day for 21 days in divided doses, repeating the course after a four week rest. One hundred and thirteen patients have been entered with 72 patients evaluable for response and 97 patients evaluable for toxicity. Sixty-eight patients (70%) had gastrointestinal toxicity, including nine patients with severe effects. Thirty-two patients (38%) had hematopoietic depression, including 2 patients who developed life threatening leukopenia. Seventeen patients (18%) had CNS toxicity, including 3 patients with severe effects. Descriptive detail of the toxicity has not been compiled, as this was an ongoing study. The overall response rate in 72 evaluable patients is one complete and five partial response, or 8%. The responses for particular tumors are included in Table 7. The Western Group was also combining hexamethylmelamine with imidazole carboxamide against bronchogenic carcinoma, malignant melanoma and other tumors. There have been no responses in the 17 patients evaluated.

Wampler *et al.* [15] evaluated hexamethylmelamine in a phase-II study. Ninety-seven patients were entered into study, but only 54 were evaluated for tumor response. Four additional patients were dropped from study after 30 days because of drug toxicity.

The dose schedule was 8 mg/kg/day, but 79% of the patients required dose reductions secondary to drug toxicity. The incidence and severity of drug induced toxicity was similar to that already discussed by other investigators.

Hexamethylmelamine induced objective regressions in the 54 evaluable patients are noted in Table 6.

Three patients responded to hexamethylmelamine after becoming unresponsive to the alkylating agent, chlorambucil. This included the Hodgkin's disease patient who had a complete remission and the 2 patients with the partial remissions seen in ovarian carcinoma.

The Central Oncology Group combined hexamethylmelamine and methotrexate for the treatment of carcinoma of the lung [16]. Hexamethylmelamine was given in daily divided doses of 6 mg/kg/day. Methotrexate was given weekly or biweekly at a dose range of 12·5–50 mg/week. Fifty-three patients were entered, but 8 were disqualified because of protocol violations.

Of 38 evaluable patients, there were 2 complete responses with 11 partial responses recorded, giving a response rate of 35% (13/38). There was no report of an analysis of cell type and the response rate.

In this study, the hematologic toxicity was profound. Eighty-six per cent had significant leukopenia with 16 patients having counts less than 3000. Sixty-five per cent of the patients had significant thrombocytopenia with four patients having counts of less than 75,000. Seventy eight per cent had significant anemia with 22 patients having hemoglobin drops of greater than 4 g. Also, 78% of the patients had significant gastrointestinal toxicity with 16 patients having severe effects.

Table 6. Hexamethylmelamine response by disease [15] (≃8 *mg/kg/d* × 21)

Lung:	Bronchogenic carcinoma: 6 PR* of 30 patients evaluated
	Adenocarcinoma: 5 PR of 6 patients
	Undifferentiated: 1 PR of 1 patient
Ovary:	1 CR† + 2 PR of 7 patients
Breast:	1 PR of 8 patients
Hodgkin's:	1 CR of 2 patients (duration—790+ days)

*Partial Remission (PR): decrease 50% or more of the product of 2 diameters.

†Complete Remission (CR): complete disappearance of all evidence of disease

DISCUSSION

Hexamethylmelamine has been in clinical trials since 1964. In this paper, we have collated and evaluated the available clinical data by investigator in terms of drug induced toxicity and response by tumor type.

The lack of absolute comparability of the various patient populations limited our conclusions. Differences in the clinical status of the patients entered into study, and the varying methods of evaluation and reporting of the data, led in part to this limitation. Legitimate

investigator bias, because of the nonrandomized nature of the trials, further hindered our conclusions.

All of the investigators used hexamethylmelamine in dose ranges of 4–15 mg/kg PO as a daily divided administration for between 21–90 days. None of the investigators demonstrated the superiority of a particular dose schedule. However, Bergevin *et al.* [13] randomized their patients to either a 4 mg/kg or 8 mg/kg as a daily divided dose for 42 consecutive days. Limiting toxicity resulted in significant dose reductions in the 40 evaluable patients, but the median dose for the two groups was statistically different (3·5 mg/kg versus 5·0 mg/kg–$p < 0.006$). Expressed another way, 62% of the patients on the higher dose schedule encountered limiting toxicity compared to 37% of the patients on the lower dose.

Bergevin *et al.* [13] observed that the lower the dose being administered, the later the onset of toxicity. They concluded that toxicity was a function of the total dose administered, rather than a direct function of the dose level being administered. Wilson *et al.* [9, 10] compared two dose levels in a retrospective nonrandomized study, and therefore their conclusions that the chronic low dose schedule resulted in higher overall toxicity were questionable.

The most common toxicity encountered was gastrointestinal which was often dose limiting. Forty to seventy per cent of the patients vomited to varying degrees, but less than ten per cent of the patients required dose modifications because of the severity of vomiting.

Hematological toxicity was seen in about 20–40% of the patients evaluated. The onset was usually in the first week of therapy and reached predictable levels. In all cases, the counts returned to their pretreatment levels after drug discontinuance. The frequency of leukopenia and thrombocytopenia were similar, but leukopenia was more pronounced. There was a low incidence of severe hematological depression, but no drug related deaths were reported.

Central nervous system toxicity, as manifested by confusion, agitation and depression, appeared in approximately 10–20% of the evaluable patients. In one report, CNS toxicity necessitated the downward dose modification in 6 of the 32 patients being treated. All of the toxic manifestations were reversible with recovery usually complete within four weeks after the drug had been discontinued.

Weight loss, dermatological reactions and cystitis appeared infrequently. These toxicities were not serious, nor were their relationship to hexamethylmelamine fully documented.

Table 7 summarizes the therapeutic effect of hexamethylmelamine. For carcinoma of the lung by cell type, response rates for the major cell types ranged from 11% for epidermoid carcinoma to 35% for small cell carcinoma. Adenocarcinoma was intermediate with a 19% response rate. The undifferentiated group, with a 10% response rate, represented patients in whom there was no distinction made between small and large cell undifferentiated tumors. Other chemotherapeutic studies showed similar response rates, although few studies reported response by cell type [19, 18, 19].

Hexamethylmelamine was evaluated in 32 patients with ovarian carcinoma with 2 complete (7%) and 10 partial responses (31%). This response rate was comparable to that of the alkylating agents and 5-FU [17].

Table 7. Summary of hexamethylmelamine single agent activity

Tumor	Number patients evaluated	Responses CR	PR	Overall per cent
Lung cancer,				
Adenocarcinoma	59		11	19
Alveolar	11		0	
Large cell	21		5	24
Small cell	67	7	17	36
Squamous	99		11	11
Undifferentiated	78	1	7	10
Cervix	17		5	29
Ovary	32	2	10	38
Uterus	6		2	33
Bladder	10	1	2	30
Breast	54	4	7	20
Colon	95		11	12
Esophagus	3		1	33
Liver (primary)	8		3	38
Lymphoma	23	2	11	57
Stomach	12		2	17
Head and neck	75		9	12
Kidney	17		1	6
Melanoma	42		2	5
Pancreas	6		0	—
Prostate	6		2	33
Sarcoma	43		1	2
Totals	784	17	120	17

Uterine carcinoma was evaluated in 6 patients, and 2 of these patients showed partial responses. Although most antineoplastic agents have not been adequately

evaluated in uterine cancer, the potential level of activity of hexamethylmelamine, compared to that of cyclophosphamide and 5-FU, warranted further investigation [17].

Seventeen patients with cervical carcinoma were evaluated with hexamethylmelamine therapy with five patients responding for a response rate of 29%. This response rate was comparable to that seen with systemic methotrexate (20%), intra-arterial methotrexate (47%) and systemic alkylating agents (20%) [17]. In four patients for whom data was available, the duration of hexamethylmelamine response was 3+, 3+, 4 and 6 months [14]. Of these latter patients, none had previous chemotherapy.

Lymphoma patients had good responses in about 50% of the 23 patients evaluated, but the investigators did not specify the duration and degree of response nor the patients' prior chemotherapy. Also patients with bladder, breast, colon and stomach cancer responded at significant levels with responses of 30%, 20%, 12% and 17% respectively.

From the 700 patients reviewed in the phase II trials, we have adequate numbers of patients in the following tumor types: adenocarcinoma of the breast and colon, bronchogenic carcinoma, malignant melanoma and lymphoma. The level of antitumor activity warranted controlled comparative trials in all of these tumor types except malignant melanoma.

In addition, analysis of the data from this review suggested types of respones like those seen for the alkylating agents. This observation, coupled with the biochemical evidence that hexamethylmelamine could perhaps function as an alkylator, points up the need for a clinical evaluation of cross-resistance between hexamethylmelamine and the classical alkylating agents [1]. If, in fact, there is clinically demonstrable lack of cross-resistance, then hexamethylmelamine may well have a useful role in cancer chemotherapy.

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Acceptor Control Ratio of Mitochondria

Factors Affecting it in Morris Hepatoma 5123 and Yoshida Hepatoma AH-130

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Abstract—*Mitochondria from AH-130 Yoshida ascites hepatoma and 5123 Morris hepatoma show high acceptor control and ADP:O ratios approaching the theoretical values when suspended in a reaction mixture containing EDTA. Bovine serum albumin or NaF do not completely substitute for EDTA in achieving high acceptor control. The ATPase activity of these mitochondria is stimulated by Mg^{2+}; this metal ion also has harmful effect on the acceptor control and the ADP:O ratios. EDTA induces a strong inhibition of the ATPase basal activity (unstimulated by dinitrophenol or added Mg^{2+}). Hepatoma mitochondria, incubated in the presence of EDTA, respond well to different regulatory mechanism such as: changes in mitochondrial concentration, phosphate potential, and endogenous substrates content. The phosphorylative apparatus of hepatoma mitochondria exhibits, even in the presence of protective agents, a higher lability than that of mitochondria from neonatal and adult rat livers. It is suggested that, although a slight degree of functional and perhaps structural damage occurs in hepatoma mitochondria* in vitro, *it is possible to obtain a high degree of coupling if protective agents are added to the suspensions and appropriate experimental conditions are selected.*

INTRODUCTION

PREVIOUS investigations [1] on oxidative phosphorylation of mitochondria from tumour, have shown the tumour–phosphorylative system to be very instable. Nevertheless, mitochondria from certain tumours show acceptable P:O ratios in reaction mixtures containing Mg^{2+}, the glucose–hexokinase "trap" system for ATP, and/or the adenosinetriphosphatase (ATPase) inhibitor, sodium fluoride [1–3]. The beneficial effect of fluoride on both P:O and acceptor control ratios (ACR) of tumour mitochondria, incubated in Mg^{2+}-containing media [2, 4], probably indicates a lack of full "latency" of the Mg^{2+}–ATPase. Good ADP:O and ACR were found in tumour mitochondria by including bovine serum albumin in the isolation medium [5, 6]. This suggests that long-chain-fatty acids present in tumour homogenates [7] could be involved in the ATPase activation. According to recent evidence [8, 9], however, the acceptor control and ADP:O ratios of mitochondria from rapidly-growing tumours are impaired, even if bovine serum albumin is included in the isolation and incubation media.

These contrasting results demonstrate our rather sketchy information on the regulatory role exerted on the acceptor control and the oxidative phosphorylation of tumour mitochondria by factors such as: dilution of mitochondrial suspensions, level of endogenous substrates, Mg^{2+} added to the reaction medium in the absence of ATPase inhibitors, and changes in phosphate potential.

In this communication we present the results of studies on several conditions affecting the ACR of tumour mitochondria *in vitro*. The data indicate that it is possible to isolate tightly-coupled mitochondria from rapidly-growing tumours. However, the phosphorylative apparatus of tumour mitochondria is very labile,

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and shows a great sensitivity to factors such as: Mg^{2+} in the medium, age of mitochondria, and their content of endogenous substrates.

MATERIAL AND METHODS

Materials

Barium salts of the nucleotides (Sigma Chemical Co. Ltd., St. Louis, Mo.) were dissolved in the presence of IR-120 Amberlite (H^+ form). The resin was allowed to settle and then washed with water; the collected supernatants were neutralized with KOH. Tris-(hydroxymethyl) aminomethane ("purissimum") was purchased from Fluka (Buchs, Switzerland), triethanolamine from Boehringer (Mannheim), crystalline bovine serum albumin and albumin fraction V from Sigma Chemical Co. Ltd.; the albumin was defatted according to Goodman [10]. The albumin solutions were dialyzed overnight against water prior to use. Rotenone (K. & K. Laboratories, Plainview, N.Y.) was recrystallized three times from ethanol. Other chemicals were A. R. grade whenever possible. All solutions were made with glass-doubly-distilled water.

Animals and tumours

Long–Evans rats, weighing 200 to 250 g, were used either as a source of normal liver or to transplant the AH-130 Yoshida ascites hepatoma; female Buffalo rats, weighing 200–250 g, were used to bilaterally transplant 5123 Morris hepatoma. Neonatal rat liver was obtained from 5- to 7-day old Long–Evans rats.

Preparation of mitochondria

AH-130 and 5123 hepatomas were used, after 5–6 days and 14–17 days of growth, respectively. At this time tumour-bearing animals were decapitated and bled. Heavy mitochondria from AH-130 hepatoma were prepared as described previously [11] in an isolation medium containing 70 mM sucrose, 220 mM mannitol, 20 mM Tris-Cl (pH 7·4), 2 mM Tris-EDTA, and 0·2% (w:v) bovine serum albumin (fraction V). 5123 hepatoma was rapidly dissected free from non-tumorous tissue, immersed in ice-cold isolation medium. The minced tissue was suspended in a volume of isolation medium equivalent to 2 times the weight of the tissue. It was homogenized in a Potter–Elvehjem homogenizer, with one stroke of a loosely-fitting pestle, running at 1500 rev./min. From the homogenate, diluted with the medium to obtain a 20% (w:v) suspension, heavy mitochondria were isolated with the same procedure used for the AH130 hepatoma. The homogenization procedure used for 5123 hepatoma was also adopted for both adult and neonatal rat liver. Heavy mitochondria were isolated from these tissues according to De Duve *et al.* [12]. All mitochondrial preparations were washed twice; bovine serum albumin was present in all fractionation steps and EDTA was omitted in the two final washes. The maximal isolation period was 90 min.

Determination of the respiratory rate and acceptor control

Mitochondrial respiration was measured with a vibrating platinum electrode (Oxygraph, G.M.E., Middleton, Wis.) coated with collodion. Mitochondria (2 to 2·5 mg protein, unless otherwise stated) were added to 2 ml of assay medium containing 145 mM KCl, 20 mM Tris-Cl (pH 7·4), 10 mM substrate (Tris-salt), 1 mM Tris-phosphate, and 5 mM Tris-EDTA. In some experiments this medium was submitted to various modifications (see Results). The ADP:O ratio and ACR were determined according to Chance and Williams [13]. The exact concentration of the ADP solutions was estimated by an enzymatic method [14].

To determine the maximum inhibitory effect of EDTA on tumour mitochondria adenylate kinase (E.C. 2.7.4.3.), 1·23 mM AMP was added to mitochondria (about 3 mg of protein) suspended in 2 ml of assay medium containing different amounts of EDTA. The ability of AMP to activate the respiratory rate was taken as an indirect measure of the adenylate kinase activity. In the "depletion" experiments, mitochondria (about 3 mg of protein) were suspended in a medium containing 145 mM KCl, 20 mM Tris-Cl (pH 7·4), 5 mM EDTA, 1 mM phosphate, 2 mM ADP. When a respiratory rate of about 2 mμ atoms of oxygen per min, per mg of protein, was reached, various amounts of substrates were added as shown in the individual experiments. In the experiments performed with "aged" mitochondria, the particles were submitted to different incubation times, at 0°, in a medium containing 290 mM sucrose, 20 mM Tris-Cl (pH 7·4), 4 mM EDTA, 1% (w:v) bovine serum albumin, and, where indicated, 1 mM pyruvate plus 0·5 mM L-malate.

Adenosinetriphosphatase assays

The ATPase (E.C. 3.6.1.3.) activity was determined by incubating the mitochondria (0·2–0·4 mg of protein) in 1 ml of reaction medium containing 145 mM KCl, 20 mM triethanolamine-Cl (pH 7·4), 5 mM ATP, and, where indicated, various amounts of EDTA,

Mg^{2+}, or 2:4-dinitrophenol. To obtain deoxycholate-treated mitochondria, a mitochondrial suspension in the isolation medium (4–8 mg of protein per ml) was mixed with equal volumes of a solution containing 290 mM sucrose, 20 mM Tris-Cl (pH 7·4), and 0·2% (w:v) deoxycholate. After 5 min at 0°, 50 μl aliquots of this mitochondrial suspension were used as a source of enzyme. The reaction was started by the addition of ATP, and, after 15 min incubation at 23°, was blocked by the addition of 0·2 ml of 1600 mM $HClO_4$, followed by cooling in ice and centrifugation. Data are expressed as mμ moles of phosphate split per min, per mg of protein.

Analytical methods

Inorganic phosphorus was determined according to Lowry and Lopez [15], using NaH_2PO_4 as standard. The proteins were determined by a biuret procedure [16], using crystalline bovine serum albumin as standard.

RESULTS

Acceptor control of mitochondria in media of different composition

It appears from data in Table 1 that the ACR and ADP:O ratios of freshly-isolated mitochondria from AH-130 hepatoma approached those of normal liver mitochondria with all substrates tested, excepting succinate plus rotenone. The ACR, with succinate as substrate, and rotenone was about 81% lower in hepatoma than in liver mitochondria. This clearly resulted from the failure of hepatoma mitochondria to perform low State 4 rates at the end of the ADP-induced respiratory jump. In general, the State 3 rates of hepatoma mitochondria were slightly lower than in normal-liver mitochondria, however, they were similar to those found by others in mitochondria from different tumours [4,5,9,17,18]. Mitochondria from 5123 hepatoma exhibited normal ADP:O ratios, but the State 3 rates and ACR were depressed compared to either AH-130 or normal-liver mitochondria. Rotenone caused a 35% decrease of the ACR in the presence of succinate as substrate.

Table 2 shows the effect of changes in the composition of the reaction mixture on the acceptor control and ADP:O ratios of hepatoma and liver mitochondria. Mg^{2+} ions largely affected the ACR of hepatoma, but not of liver mitochondria. In the presence of Mg^{2+} the ACR of AH-130 hepatoma mitochondria was about 73% lower than that measured in the EDTA-containing medium, and about 50% lower than that measured in the EDTA-free medium. In mitochondria from 5123 hepatoma these percentages were 67% and 35%, respectively. The highest values of ACR of hepatoma mitochondria were obtained in the presence of EDTA. Neither 10 mM NaF nor 1% (w:v) bovine serum albumin could completely substitute for EDTA in obtaining high acceptor control. The ACR of normal-liver mitochondria was not influenced by Mg^{2+} or EDTA in the medium.

Table 1. *Acceptor control and ADP:O ratios of hepatoma and rat liver mitochondria oxidizing different substrates*

Substrate	Source of mitochondria	No. of exp.	Respiratory rate		ACR	ADP:O
			State 3	State 4		
Glutamate	AH-130	6	57·00 ± 12·04	7·10 ± 2·24	8·0 ± 2·00	2·90 ± 0·50
	5123	7	28·97 ± 4·60	7·14 ± 1·22	4·6 ± 0·51	2·80 ± 0·16
	Liver	4	60·21 ± 8·31	6·69 ± 0·76	9·0 ± 1·31	2·74 ± 0·76
Succinate	AH-130	4	77·10 ± 12·86	11·21 ± 3·06	6·9 ± 0·97	2·28 ± 0·50
	5123	4	27·24 ± 3·62	10·61 ± 1·03	2·6 ± 0·26	2·32 ± 0·28
	Liver	4	89·12 ± 14·61	10·75 ± 0·84	8·2 ± 0·36	1·92 ± 0·12
Succinate plus rotenone	AH-130	3	64·80 ± 14·00	48·00 ± 6·21	1·3 ± 0·28	1·97 ± 0·12
	5123	3	28·06 ± 5·00	16·40 ± 2·21	1·7 ± 0·00	1·90 ± 0·34
	Liver	4	80·10 ± 11·06	9·41 ± 1·06	8·5 ± 0·92	1·98 ± 0·06
α-Ketoglutarate	AH-130	6	56·20 ± 14·02	8·10 ± 2·60	6·9 ± 1·20	2·64 ± 0·12
	Liver	4	66·31 ± 10·08	8·74 ± 1·04	7·6 ± 0·40	2·68 ± 0·11
β-Hydroxybutyrate	AH-130	6	64·20 ± 12·06	8·10 ± 1·60	7·9 ± 1·00	2·97 ± 0·63
	Liver	4	59·00 ± 1·00	8·55 ± 2·12	6·9 ± 0·72	2·66 ± 1·03

The reaction mixture contained 145 mM KCl, 20 mM Tris-Cl (pH 7·4), 5 mM EDTA, 1 mM phosphate, in a final volume of 2 ml. The additions were: 4 mM L-glutamate, 10 mM succinate, 10 mM α-ketoglutarate, 10 mM β-hydroxybutyrate, 4×10^{-4} mM rotenone. The reaction was started by addition of 0·2 ml of mitochondria (2·0–2·5 mg of protein). The State 3 was induced by 0·25 mM ADP. Temperature, 23°C. The respiratory rate is expressed as mμ atoms of oxygen taken up per min, per mg of protein. The results are mean values ± standard deviation.

Table 2. Acceptor control and ADP:O ratios of hepatoma and rat liver mitochondria in reaction mixtures of different composition

Additions	Source of mitochondria	No. of exp.	Respiratory rate State 3	Respiratory rate State 4	ACR	ADP:O
None	AH-130	7	50·13 ± 13·00	1·85 ± 3·02	4·4 ± 1·30	2·90 ± 0·31
	5123	7	18·44 ± 3·40	8·00 ± 3·00	2·3 ± 0·70	2·53 ± 0·16
	Liver	3	64·10 ± 5·61	7·81 ± 1·04	8·2 ± 0·64	2·79 ± 0·07
EDTA*	AH-130	6	57·00 ± 12·00	7·10 ± 2·24	8·0 ± 2·00†	2·90 ± 0·50
	5123	7	28·97 ± 4·60	7·14 ± 1·22	4·6 ± 0·51†	2·80 ± 0·16
	Liver	3	60·21 ± 8·31	6·69 ± 0·76	9·0 ± 1·31	2·74 ± 0·76
Mg^{2+}	AH-130	4	45·49 ± 14·32	0·50 ± 5·27	2·2 ± 0·67	2·30 ± 0·16
	5123	4	21·11 ± 5·70	4·30 ± 2·20	1·5 ± 0·20	2·15 ± 0·13
	Liver	3	64·26 ± 3·12	9·19 ± 1·03	7·0 ± 1·12	2·60 ± 0·21
NaF	AH-130	3	40·41 ± 12·60	7·60 ± 2·31	5·3 ± 1·12	2·94 ± 0·18
	5123	3	15.22 ± 3·00	6·40 ± 1·36	2·4 ± 0·74	2·78 ± 0·23
Albumin	AH-130	3	46·20 ± 14·00	8·85 ± 3·79	5·2 ± 1·22	2·80 ± 0·30
	5123	3	18·80 ± 2·87	6·10 ± 1·28	3·1 ± 0·74	2·84 ± 0·20
Albumin plus EDTA	AH-130	1	60·20	6·64	9·0	2·83
	5123	1	31·23	6·86	4·5	2·92

*Data reported from Table 1.

†Significantly different from data in lines 1 and 2 (no additions) and 7 and 8 (Mg^{2+} added), (at least $p < 0.025$).

The reaction mixture contained 145 mM KCl, 20 mM Tris-Cl (pH 7·4), 1 mM phosphate, 4 mM L-glutamate, and mitochondria (2·5–2·8 mg of protein). When indicated, 5 mM EDTA, 1%(w:v) crystalline bovine serum albumin, 10 mM NaF, or 4 mM $MgCl_2$ plus 0·5 mM EDTA were included in the medium. Other conditions as in Table 1. The respiratory rate is expressed as mμ atoms of oxygen taken up per min, per mg of protein. The results are mean values ± standard deviation.

From data in Table 2, it seems that the composition of the reaction mixture modified less markedly the ADP:O ratios than the ACR. ADP:O ratios near to the theoretical values could be measured in all reaction mixtures, excepting that containing Mg^{2+} ions. In this medium the ADP:O ratios of AH-130 and 5123 hepatoma mitochondria were 23% and 28% lower than the theoretical values; this phenomenon was not observed with liver mitochondria.

The role of Mg^{2+}-stimulated ATPase on the impairment of the acceptor control

As can be seen from data in Fig. 1 (tracings a and b), Mg^{2+} added to AH-130 hepatoma mitochondria induced a low increment in the State 4 respiration of mitochondria having undergone a State 3. The length of this respiratory increment was constantly correlated to the total amount of added ADP (not shown). Oligomycin inhibited the effect of Mg^{2+}, but the respiratory response to the permeant cation Ca^{2+} was left unchanged by the inhibitor (Fig. 1, tracings c and d). Similar results were obtained with mitochondria isolated

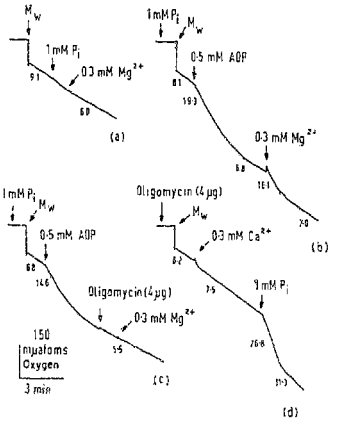

Fig. 1. Effect of Mg^{2+} on the respiratory rate of AH-130 hepatoma mitochondria. The reaction mixture contained 100 mM KCl, 90 mM sucrose, 20 mM Tris-Cl (pH 7·4), 4 mM L-glutamate, and mitochondria (4 mg of protein) in a final volume of 2·2 ml. Other additions as indicated in the figure. Temperature, 23°C. The numbers on the left of the tracings indicated mμ atoms of oxygen taken up per min, per mg of protein.

from 5123 hepatoma (data not included in the figure).

One possible interpretation of the experiment cited in Fig. 1. was that the respiratory increment by Mg^{2+} resulted from stimulation of ATPase activity. This was verified by studying the ability of Mg^{2+}, in concentrations corresponding to those used in the polarographic assays, to elicit an ATPase activity in hepatoma mitochondria. For comparison, experiments were performed with rat liver mitochondria. It appears from data in Table 3 that the ATPase of intact liver mitochondria was activated by dinitrophenol, but very little by Mg^{2+}. When the mitochondria were preincubated with deoxycholate, the ATPase did not respond to dinitrophenol but could be elicited by Mg^{2+}. The ATPase of freshly-isolated hepatoma mitochondria was stimulated by either Mg^{2+} or dinitrophenol, but responded only to Mg^{2+} and not to dinitrophenol after the deoxycholate treatment. Thus no differences between liver and hepatoma mitochondria could be observed after the disruptive action of deoxycholate.

It can be seen from Table 2 that the absence of EDTA from the reaction mixture was sufficient to induce an appreciable decrease of the ACR in hepatoma mitochondria. In order to test the possibility that the favourable influence of EDTA depended upon inhibition of adenylate kinase and/or ATPase activities, the effect of different concentrations of EDTA on the ACR, as well as, on the activities of the two enzymes was studied in AH-130 hepatoma mitochondria. Data in Table 4 show that EDTA, at concentrations as low as 0·5 mM, was without effect on the ACR, but almost completely inhibited the respiratory increment by AMP. In the presence of 3–5 mM EDTA, maximal increments of ACR, together with maximal inhibition of ATPase were obtained.

Table 3. Activation by Mg^{2+} and 2:4-dinitrophenol of the ATPase of hepatoma and rat liver mitochondria

Additions	Preincubation with DOC	Source of mitochondria: AH-130 hepatoma	5123 hepatoma	Rat-liver
		(Specific activities)		
None	—	17·20 ± 3·58 (6)	26·00 ± 10·90 (3)	5·06 ± 1·10 (3)
Mg^{2+} (0·3 mM)	—	28·21 ± 5·24 (6)		
Mg^{2+} (4 mM)	—	131·00 ± 22·10 (6)	70·00 ± 19·59 (3)	10·00 ± 2·31 (3)
DNP (0·05 mM)	±	88·50 ± 16·41 (6)	70·00 ± 12·43 (3)	89·06 ± 6·10 (3)
None	+	2·03 ± 0·12 (5)	1·06 ± 0·12 (3)	0·00 (3)
Mg^{2+} (4 mM)	+	186·04 ± 20·12 (5)	84·12 ± 10·02 (3)	124·04 ± 9·81 (3)
DNP (0·05 mM)	+	4·03 ± 0·32 (5)	7·62 ± 2·26 (3)	1·03 ± 0·08 (3)

The reaction mixture contained 145 mM KCl, 20 mM triethanolamine-Cl (pH 7·4), Mg^{2+} or 2:4-dinitrophenol (DNP) as indicated, and mitochondria (0·2–0·4 mg of protein), in a total volume of 1 ml. To perform the treatment with deoxycholate (DOC), mitochondrial suspensions in isolation medium (4–8 mg of protein per ml) were mixed with equal volumes of a solution containing 290 mM sucrose, 20 mM Tris-Cl (pH 7·4), and 0·2% DOC. After 5 min at 0°, 50 μl aliquots of these suspensions were used as a source of enzyme. The reaction was started by addition of 5 mM ATP. After 15 min incubation at 23°C, the reaction was stopped by addition of $HClO_4$. Specific activity is expressed as mμmoles of phosphate split per min, per mg of protein. The results are mean values ± standard deviation. Numbers of experiments are given in parentheses.

Effect of the mitochondrial concentration on the acceptor control

As shown in Table 5, there was an increase of ACR in both AH-130 and 5123 hepatoma mitochondria oxidizing glutamate, by raising the mitochondrial protein. In the same Table experiments 1–3 gave the highest ACR values obtained in this study with AH-130 hepatoma mitochondria. In general, increasing the mitochondrial concentration decreased the State 4 rate with no changes, or a slight increase, in the State 3 rate. These experiments were performed with protein amounts ranging from 0·25 to 1·4 mg per ml, so as to avoid a drop in sensitivity of the oxygen electrode caused by protein [19]. Similar effects of the mitochondrial concentration on the ACR were observed by using higher amounts of protein (1·8 to 3·0 mg/ml), nevertheless a lack of reactivity of the electrode in proportion to the amount of

Table 4. Effect of increasing concentrations of EDTA on the acceptor control, the respiratory release induced by AMP and the ATPase activity of AH-hepatoma mitochondria

EDTA (mM)	ACR	Respiratory increment by AMP (mμ atoms oxygen per min, per mg)	ATPase (Specific activity)
0·00	3·26	17·30	22·00
0·02		12·00	18·31
0·05	3·26	2·22	16·72
0·1	4·32	0·00	
1·0	4·35		
3·0	5·60		3·12
5·0	6·79		0·00

The acceptor control of mitochondria oxidizing L-glutamate was determined in the same conditions as in Table 1, except that different concentrations of EDTA were used as indicated. The respiratory release induced by AMP was measured in the same reaction mixture used for the determination of the acceptor control, but 1·23 mM AMP was added, in place of ADP, to State 4 respiring mitochondria. The ATPase activity was determined in the same conditions as in Table 3, but the indicated amounts of EDTA were included in the medium in place of Mg^{2+} or dinitrophenol. The specific activity is expressed as mμ moles of phosphate split per min, per mg of protein. The data are mean values of 4 assays from two different mitochondrial preparations.

Table 5. Influence of the mitochondrial concentration on the acceptor control of hepatoma mitochondria.

Exp. No.	Source of mitochondria	Protein (mg/ml)	Respiratory rate State 3	Respiratory rate State 4	ACR
1	AH-130	0·33	60·00	7·90	7·6
		0·66	64·00	6·16	10·4
		1·00	76·61	4·48	19·5
2	AH-130	0·67	51·23	8·02	6·4
		1·34	56·80	4·56	12·4
3	AH-130	0·38	54·51	10·30	5·2
		1·32	69·00	4.60	15·0
4	AH-130	0·25	59·20	14·20	4·2
		1·01	59·02	8·80	6·7
5	5123	0·58	22·20	9·25	2·4
		1·16	30·80	6·17	5·0
6	5123	0·35	26·52	10·20	2·5
		0·70	26·80	9·22	2·9
		1·40	32·21	7·06	4·6

The test system contained 145 mM KCl, 20 mM Tris-Cl (pH 7·4), 4 mM L-glutamate, 1 mM phosphate and 5 mM EDTA in a final volume of 2 ml. The reaction was started by the addition of 0·2 ml aliquots of mitochondria corresponding to the indicated amounts of protein. Temperature, 23°C. The State 3 was induced by 0·25 mM ADP. The respiratory rate is expressed as mμ atoms of oxygen taken up per min, per mg of protein.

protein could not be excluded as a possible mechanism involved in the decrease of the State 4 rate. In fact, low decrease of the electrode sensitivity could be only evidenced when the oxygen consumption is slight (i.e. the State 4 rate).

Influence of ATP on the respiratory rate of hepatoma mitochondria

One criterion by which to ascertain the integrity of the function and structure of mitochondria is their ability to respond to changes in the phosphate potential in the incubation medium [20]. To investigate if this regulatory mechanism operates in tumour mitochondria, we measured the respiratory response to ATP added during both controlled and activated states. As shown in Fig. 2, high amounts of

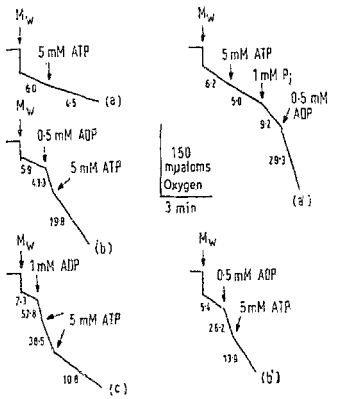

Fig. 2. Effect of ATP on the respiratory rate of hepatoma mitochondria. All conditions as in Table 1, excepting that 3 mg of protein per 2 ml of reaction mixture were used; phosphate was omitted in the assay (a), it was added as indicated in the assay (a'). Substrate, 4mM L-glutamate. Tracings (a), (b) and (c) refer to AH-130 hepatoma mitochondria, tracings (a') and (b') to 5123 hepatoma mitochondria. The numbers on the left of the tracings indicate mμ atoms of oxygen taken up per min, per mg of protein.

ATP extensively inhibited the State 3 rate; they also induced a slight inhibition of the State 4 rate in both types of mitochondria. By comparing tracings (b) and (c) in Fig. 2, it appears that the inhibitory effect was quantitatively linked to the relative amounts of ATP and ADP in the reaction mixture.

Control by endogenous substrates of the respiration of hepatoma mitochondria

The addition of ADP to hepatoma mitochondria oxidizing endogenous substrates induced high respiratory release, indicating an active phosphorylation of the phosphate acceptor by endogenous substrates (Fig. 3, tracing a). α-ketoglutarate and glutamate, even at high concentrations, did not stimulate further the oxygen consumption supported by the endogenous supply (tracings b and c). A low respiratory increment was obtained with succinate (tracing d). This behaviour was also observed when low substrate concentration was used (tracing e), however the accelerated rate continued only a few minutes. Thereafter the respiration progressively decreased until further substrate was added. α-ketoglutarate, glutamate, and succinate, at concentration as low as 0·05 mM in the presence of ADP, stimulated the endogenous respiration of liver mitochondria (Fig. 4, tracings a–c). Phosphorylation of ADP also took place during the oxidation of endogenous substrates, but the respiratory activation by ADP was lower than in hepatoma mitochondria (tracing d). The possibility to induce an additional respiratory jump by exogenous substrate, indicated that the mitochondrial preparation did not contain enough endogenous substrate to support the phosphorylation of all added ADP (about 48 mμ moles/mg of protein).

The respiratory rates were measured in liver and AH-130 hepatoma mitochondria with a series of concentrations of α-ketolutarate, glutamate, and succinate in the presence of ADP. The ADP was added after or before substrates, in the latter case time intervals sufficient to obtain maximal depletion of endogenous substrates (i.e. maximal respiratory inhibition) elapsed between the ADP and the substrate additions. As can be seen from Fig. 5(A), low

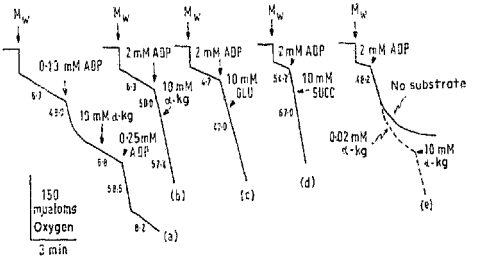

Fig. 3. Control by substrate of the respiratory rate of AH-130 hepatoma mitochondria. The reaction mixture contained 145 mM KCl, 20 mM Tris-Cl (pH 7·4), 1 mM phosphate, 5 mM EDTA, in a final volume of 2 ml. Where indicated, 0·02 mM α-ketoglutarate was included in the medium used for the assay (d). The reaction was started by addition of 0·2 ml of mitochondria (3·0 mg of protein). Other additions are indicated in the figure. Temperature, 23°C. The numbers on the left of the tracings indicate mμ atoms of oxygen taken up per min, per mg of protein.

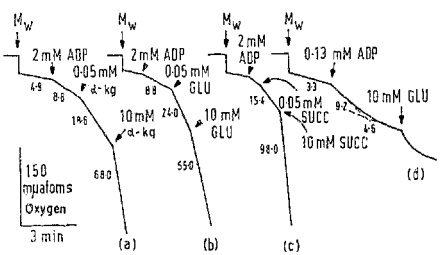

Fig. 4. Control by substrate of the respiratory rate of rat liver mitochondria. All conditions as in Fig 3, excepting that 2·6 mg of mitochondrial protein were used. The numbers on the left of the tracings indicate mμ atoms of oxygen taken up per min, per mg of protein.

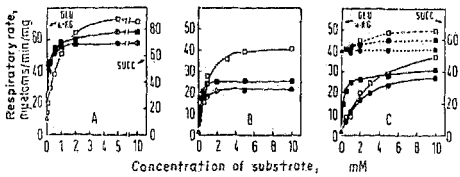

Fig. 5. Effect of the concentration of substrates on the respiratory rate of AH-130 hepatoma and rat liver mitochondria. The test system contained 145 mM KCl, 20 mM Tris-Cl (pH 7·4), 5 mM EDTA, 1 mM phosphate, the indicated amounts of substrates and mitochondria (2·0–2·7 mg of protein). The final volume was 2·2 ml. Temperature, 23°C. The reaction was started by 2 mM ADP. The substrates were added before ADP only in graphs A and C (dotted lines). In the experiments reported in graphs B and C (straight lines), the mitochondria were allowed to respire in the presence of ADP until the oxygen uptake reached values of about 2 mμ atoms of oxygen per min, per mg of protein. At this point the substrates were added and the respiratory increment was recorded. The substrates were: L-glutamate (●), α-ketoglutarate (■) and succinate (□). The symbols on the ordinates indicate the endogenous respiration, in the presence of ADP, before (△) and after (▲) the depletion of endogenous substrates. Each point represents the mean value of four experiments.

substrate concentrations showed high stimulatory effect on the endogenous respiration of liver mitochondria in the presence of ADP. Therefore, maximal acceleration of the State 3 rate was observed with high concentrations of substrates; saturating concentrations were between 5 and 10 mM for succinate and α-ketoglutarate, and between 2 and 5 mM for glutamate. After depletion of the endogenous reserves, saturating concentrations of the substrates were no longer able to elicit "maximal" State 3 rates near to those measured in "fresh" mitochondria (Fig. 5b). Only after depletion of the endogenous reserves did exogenous substrates induce a high respiratory stimulation in hepatoma mitochondria (Fig. 5c, straight lines). In the "fresh" mitochondria high values of endogenous respiration in the presence of ADP, poorly increased by added substrates, were observed (Fig. 5c, dotted lines). Experiments performed with 5123 hepatoma mitochondria gave essentially identical results as regards the effect of endogenous substrates on the oxidative rate of both "fresh" and "depleted" particles.

When the results in Fig. 5 were expressed in terms of K_m (Table 6) a comparison of "fresh" and "depleted" mitochondria was obviously possible only in liver mitochondria. The depletion of the endogenous substrates caused a 4-to 10-folds increase in the apparent K_m for the substrate oxidation. The comparison of the K_m values found in "depleted" mitochondria from liver and hepatoma revealed a higher K_m for the oxidation of glutamate in hepatoma mitochondria.

Stability of the phosphorylative apparatus of hepatoma mitochondria

According to Weinbach [21] the elevated stability of the liver mitochondria phosphorylative apparatus relates to the high content of endogenous substrates of these particles. Since our hepatoma mitochondria preparations show

Table 6. Parameters describing the rates of respiration in the presence of ADP as a function of the substrate concentration

Substrate		Liver mitochondria "Fresh"	Liver mitochondria "Depleted"	AH-130 hepatoma mitochondria "Depleted"
Glutamate	K_m	0·05	0·22	1·20
	V_{max}	59	22	37
α-Ketoglutarate	K_m	0·04	0·40	0·50
	V_{max}	66	25	41
Succinate	K_m	0·33	1·12	1·80
	V_{max}	88	42	59

The conditions were the same as in the Fig. 4. The K_m is expressed as μ moles per liter, the V_{max} as mμ atoms oxygen taken up per min, per mg of protein

good control by substrate and seem to have a large endogenous supply, we studied the stability of their phosphorylative system. The results were compared with those obtained with mitochondria from adult and neonatal rat livers. The data in Table 7 clearly show that the ACR fell much more rapidly in tumour than in normal-liver mitochondria during incubation at 0° in the presence of EDTA and albumin. Attempts to prevent the fall of ACR of tumour mitochondria, by adding 1 mM pyruvate plus 0·5 mM L-malate to the storage medium, were partially successful. Under these conditions the ACR decreased about 35% after 4–5 hr and 69% after 24 hr. In tumour mitochondria stored without substrates, these percentages were about 50 to 60% and 80 to 100%, respectively.

genous substrates, presence of Mg^{2+} and EDTA, and mitochondrial age greatly influence the ACR of normal mitochondria [23–28]. Our results indicate that some of these regulatory mechanisms operate in hepatoma as in liver mitochondria. The influence of the mitochondrial concentration on the ACR of rabbit-heart mitochondria was first shown by Tarjan and Von Korff [25]. We observed a similar effect in hepatoma mitochondria. With the low protein concentration used in our experiments it seems unlikely that the decrease in State 4 rate as the protein increased, was related to a loss in reactivity of the oxygen electrode. In fact, we used a collodion-coated electrode whose sensitivity does not seem to be appreciably reduced by relatively high amounts of mitochondria protein [19]. The mechanism

Table 7. Effect on ageing on the acceptor control of mitochondria isolated from AH-130 and 5123 hepatomas, neonatal and adult rat livers

Source of mitochondria	No. of exp.	Time of storage at 0° (hr)				
		0	1·5–2	4–5	8–9	17–24
AH-130	3	7·2 ± 0·79	5·8 ± 0·55	3·0 ± 0·47	2·9 ± 0·00	1·3 ± 0·01
5123	3	4·3 ± 0·67	2·7 ± 0·67	2·2 ± 0·45	1·0 ± 0·00	
Neonatal liver	3	6·8 ± 0·07	6·6 ± 0·04	6·6 ± 0·60	4·0 ± 0·53	3·3 ± 1·20
Adult liver	3	8·9 ± 3·31	7·8 ± 3·30	8·0 ± 2·00	5·7 ± 1·32	2·0 ± 1·20

*Values of 1·0 indicate the absence of acceptor control.

The mitochondria (20 mg of protein) were stored at 0° in a medium containing 290 mM sucrose, 20 mM Tris-Cl (pH 7·4), 4 mM EDTA, 1% bovine serum albumin. After different times the ACR was determined as in Table 1, with 4 mM L-glutamate plus 2 mM L-malate as substrates. The results are mean values ± standard deviation.

DISCUSSION

The present study shows that normal phosphorylating activity and good ACR may be measured in mitochondria from AH-130 and 5123 hepatomas oxidizing different substrates, with the only exception being succinate with rotenone present. An ACR with succinate lower than that with NAD-linked substrates was already observed in both normal and tumour mitochondria [5, 22] In the preparations of hepatoma mitochondria used in this study, the ACR in the presence of succinate and rotenone was lower than in liver mitochondria. The significance of this low regulation by the respiratory control of the succinate oxidase system in hepatoma mitochondria is not clear. Perhaps it reflects a marked lability of the second site of phosphorylation in these mitochondria.

It is known that factors such as: nature of the exogenous substrate, mitochondrial concentration, phosphate potential, content of endo- by which the mitochondrial amount regulates the ACR is unknown at this time. As pointed out by Klingenberg [29], in concentrated mitochondrial suspensions, the release of endogenous ATP leads to an increment of the ATP:ADP ratio in the extramitochondrial space. Consequently, an apparent increase of K_m for ADP and of phosphate potential occurs. One would expect that since the exchange between ADP and ATP is fast, due to the presence of high amounts of mitochondria, a rapid fall of respiration in State 4 would occur. In this respect it is noteworthy that ATP, added to tumour mitochondria, may induce a decrease in respiration, i.e. a more controlled state, analogous to that first described by Klingenberg and Schollmeyer [24] in mitochondria from normal skeletal muscle.

The importance of endogenous substrates in maintaining a high respiratory rate and ACR was emphasized by several investigators [23, 25, 26, 29–31]. We found that the endo-

genous substrates of hepatoma mitochondria support a high oxygen uptake and efficient phosphorylation. Clearly, the exogenous substrates were not oxidized, in the presence of ADP, at a higher rate than the endogenous. This indicates that the respiratory enzymes of hepatoma mitochondria are functioning at their maximal rate on endogenous substrates. The addition of exogenous substrates to "fresh" hepatoma mitochondria, however, was necessary to permit the accelerated rate of respiration in the presence of ADP to be continued for several minutes. This behaviour of hepatoma mitochondria contrasts with the low endogenous respiration in the presence of ADP of freshly-isolated mitochondria from normal tissues, observed in this study as well as by others [32, 33]. It seems probable that the low respiratory stimulation in "fresh" hepatoma mitochondria is a consequence of the interference of the oxidation of the endogenous substrates with the utilization of the exogenous, rather than of an altered penetration and accumulation of substrates into the mitochondria. In fact, the exogenous substrates highly stimulated the respiration in the presence of ADP of "depleted" hepatoma mitochondria. Respiratory stimulation was also observed by adding substrates to "depleted" mitochondria from liver, so that there was not a great difference between the two types of "depleted" mitochondria. This could indicate that the differences between freshly-isolated hepatoma and liver mitochondria, as it concerns the response to added substrates, primarily regard the content of endogenous substrates. Although at present such differences are not defined quantitatively and qualitatively, this phenomenon appears interesting. In fact, it is in line with the proposal that mitochondria in intact tumour cells may maintain high respiratory rate on endogenous substrate alone [24]. The endogenous substrates were shown to control, through interaction with the exogenous, the nature of the over-all metabolism of isolated mitochondria [25, 32]. They could also regulate the degree of accumulation of exogenous substrates into the mitochondria [35], and, consequently, the rate of oxygen uptake. The existence of a regulatory mechanism of the mitochondrial oxidations by the endogenous reserves was shown, in our rat liver mitochondrial preparations, by the decrease in affinity for the substrate oxidation after "depletion". Although it was not possible to determine the K_m values for the oxidation of substrates by "fresh" hepatoma mitochondria, our data clearly indicate that such a regulatory mechanism does exist in these mitochondria. This was shown by the ability of low amounts of substrates to maintain, for several minutes, the accelerated respiratory rate during the oxidation of the endogenous reserves, while in "depleted" mitochondria high concentrations of substrates were needed to obtain "maximal" oxidative rates.

There are some other mechanisms regulating the degree of coupling which seem to behave differently in hepatoma and liver mitochondria. In fact, the low resistance to ageing and the harmful effect of Mg^{2+} were only observed with tumour mitochondria. The so-called "latency" of the ATPase is a well-known feature of intact liver mitochondria [36–38]. While dinitrophenol activates the enzyme of freshly-isolated mitochondria, Mg^{2+} induces either very little or no activation. If mitochondria are submitted to various disruptive treatments, the ATPase activity can be elicited by Mg^{2+} but not dinitrophenol. The ability of ATPase to respond to both dinitrophenol and divalent cation was taken as a sign of slight damage of mitochondria [39]. The latter phenomenon was shown in our preparations of hepatoma mitochondria in spite of the presence of protective agents in the isolation medium. This could indicate that a slight functional and, perhaps, structural damage exists in freshly-isolated tumour mitochondria. This damage could result in the high instability of the phosphorylative apparatus as well as in the decrease of ACR when EDTA is not present. The beneficial effect of EDTA could depend upon inhibition of the ATPase activity. The basal activity of ATPase (unstimulated by dinitrophenol or added Mg^{2+}) was found to be higher in hepatoma than in liver mitochondria. This increased activity could depend upon the existence of a spontaneous swelling in freshly-isolated hepatoma mitochondria [6, 9, 11] coupled to an increment and unmasking of mitochondrial ATPase [40]. Furthermore, a slight increase in the endogenous Mg^{2+} of hepatoma mitochondria [41] could also be involved in the increase of the ATPase activity. Under these circumstances EDTA could operate either as a membrane stabilizer [42] or as a chelating agent for endogenous Mg^{2+}. The latter mechanism seems unlikely, however, since Settlemire *et al.* [43] demonstrated that EDTA cannot penetrate the mitochondrial matrix and consequently remove Mg^{2+} from the inside of the inner membrane where the ATPase is located [44]. This accords with recent evidence that EDTA does not remove endogenous Mg^{2+} from any of the different

mitochondrial compartments [45]. The other possibility is that EDTA could ensure a better conformational organization of the mitochondrial membrane resulting in a decrease of the State 4 rate and of the ATPase activity. This is consistent with the observation that EDTA can prevent the swelling of hepatoma mitochondria [6]. An analogous mechanism was invoked to explain the ability of Mg^{2+} to make high acceptor control possible beyond a few minutes in rat liver mitochondria [39, 46]. It is obvious that this Mg^{2+} effect cannot operate in hepatoma mitochondria due to the lack of latency of their Mg^{2+}-ATPase.

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Activity of a New Glycosidic Lignan Derivative (VP 16-213) Related to Podophyllotoxin in Experimental Tumors

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Abstract—*VP* 16-213 (*4'-demethyl-epipodophyllotoxin ethylidene-β-D-glucoside*) *is closely related to VM* 26 *in chemical structure and biological activity: it inhibits entry of cells into mitosis as does VM* 26. *It produces higher increases in life span than VM* 26 *in the mouse leukemias P-*815, *P-*1534, *and L-*1210. *Depending on number of tumor cells inoculated, site of tumor implantation, treatment schedule, and route of drug injection, VP* 16-213 *increases life span in L-*1210 *by more than* 300% *or cures more than* 90% *of the mice. A schedule of twice weekly drug treatment was usually superior to other schedules in L-*1210. *The percentage of L*1210 *cells killed with a single i.p. application of VP* 16-213 *was found to be* 99·9997%, *calculated from the proportion of survivors. The drug is active by the parenteral and the oral route.*

INTRODUCTION

In earlier reports cytostatic activities *in vitro* and *in vivo* of a podophyllotoxin glucoside derivative (VM 26) with a new mechanism of action were described [1, 2]. VM 26 differs from previously known podophyllotoxin derivatives in that, in tissue cultures, it does not produce accumulation of arrested metaphases; instead, it inhibits entry of cells into mitosis. Clinically, VM 26 has so far been shown to be effective in malignancies of the lymphatic system [3, 4, 5] and in some solid tumors [5, 6, 7].

In this report results obtained with another glycosidic lignan derivative, VP 16-213, shall be described. It is closely related to VM 26 as far as chemical structure is concerned and probably also in mechanism of action, but its antitumor effect in experimental systems has a larger therapeutic index. A brief summary of some effects of VP 16-213 has been reported [8].

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MATERIAL AND METHODS

1. *Drug, solvents*

VP 16-213, 4'-demethyl-epipodophyllotoxin ethylidene glucoside, (or: 4'-demethyl-1-0-[4,6-0-ethylidene-β-D-glucopyranosyl] epipodophyllotoxin) was synthesized in our chemical research laboratories [9]. To obtain aqueous solutions, VP 16-213 was dissolved either in DMSO and Tween 80 or in a mixture consisting of polyethylene glycol 300, ethanol and tween 80; such solutions could then be diluted with water or balanced salt solutions for use in cell cultures or animals. In animal experiments, the groups of mice and rats receiving different doses of drug were always treated with the same amount of solvent as the highest dosage group. Animals of the control groups also received that amount of solvent.

2. *Animals and tumors*

These were the same as described in an earlier publication [2], except that for some of

the experiments (MF2 × DBA/2)F_1 mice were used instead of (SIM × DBA/2)F_1 mice (MF2 = (BALB/c × MIW)F_2). The hybrid mice are always designated MDF in the Tables. There was no difference between these two mouse strains as to the effect of VP 16-213 in leukemia L-1210 inoculated into those animals.

RESULTS

1. *Activity* in vitro

The effects of VP 16-213 on vertebrate cells *in vitro* will be reported elsewhere (Stähelin, in preparation). It should only be mentioned here that the concentration necessary for 50% inhibition of multiplication of mouse P-815 mastocytoma cells [10] *in vitro* (ED-50) is 0·046 mg/l (mean value of 11 experiments, S.E.M. ± 0·006). Human tumor cells (HeLa) are somewhat less sensitive, DE-50 being 0·14 mg/l (method see [10]). Thus, VP 16-213 is about 10 times less active *in vitro* than VM 26. Qualitatively, both lignan derivatives seem to act similarly on chick embryo fibroblasts in interphase and in mitosis, as well as on other tissue culture cells.

2. *Toxicity*

(a) *Single application.* The LD-50 of VP 16-213 in mice, rats and rabbits are given in Table 1. With high doses as they are used in toxicity experiments, the toxicity of the solvents posed some problems. Slow injection reduced toxicity of the solvent. The values given in Table 1

Table 1. Acute toxicity

	LD-50, mg/kg		
	i.v.	i.p.	p.o.
Mouse	118	105	>800
Rat	68	57	750
Rabbit	>80	—	—

for the 50% lethal dose are considered to be not or only slightly influenced by the solvent, since the solvent alone produced little or no lethality in the controls. While there is no big difference between the i.v. and i.p. route, the rat seems more susceptible to the toxic effects of the drug than the mouse and rabbit. The rabbit has not been used so far in therapeutic experiments with this drug.

Compared to VM 26, 16-213 is about 3–4 times less toxic for the mouse by single i.p. administration.

(b) *Repeated applications.* The LD-50 of VP 16-213, when given to mice daily or twice weekly, are tabulated in Table 2. It becomes

Table 2. Subacute toxicity, mouse

Schedule (number of injections)		LD-50 mg/kg injection	
Once daily	(12×)	13	i.v.
		13	i.p.
Twice weekly	(4×)	53	i.v.
		56	i.p.
Twice weekly	(6×)	58	i.v.

evident that equitoxic total doses for a two-week period are smaller when the drug is given daily (12 × 13 = 156 mg/kg) than when it is given twice weekly (4 × 53 = 212 mg/kg).

3. *Effects on transplantable rodent tumors*

(a) *Solid tumors.* Exploratory experiments were done with two mouse sarcomas. Weight of the subcutaneously growing tumors was reduced by maximal, non-toxic doses of VP 16-213 by 61–72% compared to solvent treated controls, 9 days after tumor inoculation (Table 3). A dose is considered non-toxic if not more than 1 out of 6 animals dies during the experiment and if the average loss of body weight due to the drug during the experiment (9 days) is less than 10%.

The Walker tumor is inhibited by more than 2/3 either by giving the drug twice weekly by i.v. injection or orally; intravenous infusion seems to produce superior results compared to injection (Table 3). No difference in effect was observed between s.c. and i.m. inoculated tumors; therefore, they were not differentiated in the Table.

(b) *Leukemias P-815 and P-1534.* Survival time increase over controls produced by VP 16-213 treatment in these two leukemias is 83–110%, as seen in Table 4. There were no cures. Survival time of controls was 15 days (average) for P-815 and 20 days for P-1534. The latter tumor was transplanted by s.c. trocar injection of 2 mm pieces of solid tumor.

(c) *Leukemia L-1210.* Table 5 gives results obtained with 10^6 L-1210 cells inoculated subcutaneously (0·1 ml of cell suspension into the right hind leg, into or near the m. gastrocnemius). ILS decreases with different routes of drug application in the order i.v. > i.p. > p.o.; it should, however, be noted in Table 5 that p.o. application of VP 16-213 resulted in a few cures while no survivors were seen with i.p. treatment (this applies to s.c. tumor inoculation only). L-1210 cells inoculated into

Table 3. Effect of VP 16-213 on growth of 3 solid transplantable tumors

Tumor	Host	Treatment (days)	Dose (mg/kg)	Reduction of tumor weight (%)	No. of exp.
Sarcoma 37	MDF	2–8	17 i.v.	62	1
s.c.		1, 3, 7	37 i.v.	72	1
Sarcoma 180	MDF	2–8	11 i.v.	61	1
s.c.		1, 3, 7	25 i.v.	64	1
Walker carcinosarcoma	Wistar	2–8	8·0 i.v.	51	3
s.c. or i.m.	rat	4–8	10* i.v.	89	3
		2–8	40 p.o.	35	2
		1, 3, 7	9.0 i.v.	70	5
		1, 3, 7	180 p.o.	71	4

*In these 3 experiments, the drug was given as a daily i.v. infusion of 30 min. duration, simulating mode of application in man.

Treatment days are those days at which the animals received injections of the drug (day of tumor inoculation is day 0). Animals were not injected on Sundays, thus daily treatment on days 2–8 means that animals received only 6, not 7 injections, and with treatment on days 1–14, animals received 12 injections.

ILS = increase in life span over controls.

If not indicated otherwise, 30 animals were used in each experiment; a group of six served as controls, 4 groups of six animals received different drug doses. Only the optimal dose (the dose producing maximal ILS) and the ILS obtained with that dose are recorded. Where more than one experiment of the same type was performed, the arithmetic mean of dose and anti-tumor effect are given in the Tables (for Table 3, ILS has to be replaced by reduction of tumor weight). As to cures (definition see foot note to Table 6), their number is taken from the entire experiment, not only from the group treated with the optimal dose. For calculation of ILS, survivors are excluded; but no ILS is given when more than half of the animals were cured.

Table 4. Effect of VP 16-213 on survival time in mouse leukemias

Tumor	Host	Treatment (days)	Dose (mg/kg)	ILS (%)	Cures	No. of exp.
Mastocytoma	MDF	1–14	11 i.v.	86	0	2
P-815		1, 4, 8, 11, 15, 18	27 i.v.	83	0	2
10^7 cells s.c.						
Leukemia	DBA/2	1–14	7,5 i.v.	89	0	2
P-1534, s.c.		1, 4, 8, 11, 15, 18	22 i.v.	110	0	2

Legend: see Table 3.

Table 5. ILS in early leukemia L-1210. Inoculum: 10^6 cells s.c. (hind leg)

Host	Treatment (days)	Dose (mg/kg)	ILS (%)	Cures	No. of exp.
MDF	1–14	8·9 i.v.	218	0	8
	1–14	11 i.p.	183	0	3
	1–14	34 p.o.	144	3	7
	1, 4, 8	29 i.v.	217	2	2
	1, 4, 8, 11	32 i.v.	318	2	3
	1, 4, 8, 11, 15, 18	34 i.v.	352	16	5
	4, 7, 11, 14, 18, 21	40 i.v.	294	4	1
	1, 4, 8, 11, 15, 18	40 i.p.	188	0	2
	1, 4, 8, 11, 15, 18	106 p.o.	131	4	7
	1, 8, 15	63 i.v.	69	0	1
	2, 9. 16	43 i.v.	122	0	1
	2, 9, 16	50 i.p.	71	0	1
DBA/2	1, 4, 8, 11, 15, 18	24 i.v.	251	3	2

Legend: see Table 3.

Table 6. ILS in early leukemia L-1210. Inoculum; 10^5 or 10^6 cells i.p.

Inoculum	Host	Treatment (days)	Dose (mg/kg)	ILS (%)	Cures	No. of exp.
10^5	MDF	3–16	6·0 i.v.	201	3	1
		1–14	6·0 i.p.	169	9	1
		1, 4, 7	30 i.p.	—	42/45*	4
10^6	MDF	1, 4, 7	29 i.v.	295	3	1†
		1, 4, 7	10–50 i.p.	—	45/84‡	2
	DBA/2	1, 4, 8, 11, 15, 18	24 i.p.	226	7	1

*In these 4 experiments, a total of 45 mice were treated with 30 mg/kg i.p. on days 1, 4 and 7 after i.p. tumor implantation. Forty-two of them were cured (survived for more than 30 days after cessation of treatment, with no macroscopically detectable tumor), while all 45 control mice died of leukemia.
†Twelve mice per dose were used in this experiment.
‡In these 2 experiments, a total of 84 mice were treated with different doses (from 10 to 50 mg/kg) of which 45 were cured.

Legend: see Table 3.

Table 7. ILS in leukemia L-1210 with single application of VP 16-213. Host: MDF mice

Inoculum	Time of drug applic.*	Dose (mg/kg)	ILS %	Cures	No. of exp.
10^5 cells	−1 hr	15–100 i.v.	0	0	2
i.v.	+1 hr	45 i.v.	104	2	2
10^5 cells	−3 hr	64 i.v.	3	0	1
i.p.	−1 hr	45 i.v.	20	0	1
	−3 hr	135 i.p.	35	0	1
	−1 hr	76 i.p.	151	3	4
	+1 hr	40 i.p.	82	16	3
	+1 d	30 i.p.	300	7	1
	+3 d	42 i.p.	70	10	2
	+3 d	160 p.o.	96	0	1
10^6 cells	−1 hr	40 i.v.	19	0	1
s.c.	+1 hr	58 i.v.	99	0	1
	+1 d	45 i.v.	71	0	3
	+3 d	83 i.v.	102	0	3
	+5 d	58 i.v.	92	0	1
	+6 d	40 i.v.	51	0	1
	+7 d	40 i.v.	40	0	1
	+1 d	120 i.p.	98	1	1
	+3 d	60 i.p.	73	0	2
	+1 d	160 p.o.	74	0	1
	+3 d	240 p.o.	80	0	2

*Relative to tumor inoculation; −1 hr = drug given 1 hr before tumor implantation, etc.

Legend: see Table 3.

DBA/2 mice show that ILS and cures are, in the host isologous for these tumor cells, of the same order as in the hybrid host (Table 5). The sex was always the same for all animals of an experiment (in the majority of cases female); but since the sex of the host in comparative experiments did not influence the results, this factor is not mentioned in the tables.

As a comparison of Table 6 with Table 5 shows, site of tumor inoculation is an important factor in therapeutic trials with L-1210. The number of cures is much larger when L-1210 cells are implanted i.p. compared to s.c. inoculation, particularly when the drug is also given intraperitoneally. Daily application cures some of the i.p. inoculated animals, also when VP 16-213 is injected i.v. More than 50% of hybrid mice survive an i.p. inoculation of 10^6 L-1210 cells when VP 16-213 is injected i.p. on days 1, 3 and 7; more than 90% are cured under these conditions when the inoculum is reduced to 10^5 cells. In the isologous host (DBA/2) the effect is similar to that in the hybrid host (Table 6), but an extensive comparison between the two hosts was not made.

When VP 16-213 is injected only once into L-1210 mice, the route of tumor inoculation, of drug application and the time relationship between the two are of importance (Table 7). No ILS is observed when the drug is given i.v. and 1 hour later the tumor cells by the same route; reversal of the time sequence in this system results in a marked ILS (104%) and a few cures. There is almost no ILS when the drug is given i.v. 3 or 1 hr before i.p. tumor implantation (the value of 20% ILS with drug application 1 hr before tumor inoculation is, however, statistically significant ($p < 0.001$), due to the small variation of the survival time among the animals of a treatment group). When cells and drugs are both given i.p., VP 16-213 injected 3 hr before the tumor cells still kills a significant portion of the latter, resulting in ILS of 35% ($p < 0.0025$); a time interval of 1 hr in this system results in a high ILS (151%) with a few cures. Drug given once i.p. one hour after i.p. tumor implantation produces a considerable number of cures, as is also the case with drug injected 3 days p.i. (post inoculation). A better effect is obtained when VP 16-213 is given 1 day p.i. Single oral treatment 3 days after i.p. tumor inoculation produces roughly a doubling of survival time. The ILS do not vary very much when a single i.v. treatment is given from one hour to 5 days after s.c. L-1210 implantation (Table 7), but it is small when VP 16-213 is given one hour before the tumor cells and it is only 40–51% with very delayed treatment (6–7 days after tumor inoculation). With single i.p. and oral treatments 1 or 3 days p.i., ILS between 70 and 100% are obtained in animals inoculated s.c.; one cure was observed with i.p. treatment. One single survivor is assumed to be significant since there were no survivors among more than 600 L-1210 inoculated control mice in the experiments with VP 16-213.

As mentioned in the previous paper [2], the percentage of tumor cells killed [11] by a single drug application can be calculated from ILS when doubling time of L-1210 cells in the respective host is known, when treatment does not change doubling time of surviving tumor cells, and when animals die at the moment when the number of leukemic cells per mouse reaches a certain level. Although there are indications that the second of the above mentioned conditions is not always fulfilled (see also [12]), the per cent kill based on Skipper's concept [11] was calculated for VP 16-213. This calculation shows that L-1210 cell kill is about the same as with VM 26 [2], namely 99·998% (or 1 surviving cell out of 46,000) for a 100% increase in survival time which is obtained with a single non-toxic i.v. dose (see Table 7) in s.c. inoculated mice (mean survival time of controls was 9·49 days, doubling time was found to be 0·61 days). For the kill percentage in i.p. inoculated mice see below (discussion).

In 17 of the L-1210 experiments reported above, a number of non-inoculated mice equal to the tumor-bearing mice were treated with the drug. From the mortality of these "normal" mice a LD-50 and LD-17 was calculated according to Reed and Muench [13]. In one of these experiments, the dose producing maximum ILS was higher than LD-50; and in 3 out of the 17 experiments it was higher than LD-17. Thus, in the majority of cases the doses listed in Tables 5–7 can be considered non-toxic or of low toxicity. From such toxicity data (LD-17) and the minimal effective dose (producing 40% ILS), a therapeutic index can be computed [14]. For the treatment schedule of twice weekly i.v. drug injection, starting one day p.i. and continued for 3 weeks, the therapeutic index of VP 16-213 is 42/3 or 14 (42 mg/kg = LD-17; 3 mg/kg = dose resulting in 40% ILS).

A number of mice cured from L-1210 by treatment with VP 16-213 were reinoculated with L-1210. Of 55 cured mice reinoculated with 10^5 or 10^6 L-1210 cells, only 3 survived this second tumor challenge (observation period: more than 30 days), while 8 out of 55 cured mice survived a second s.c. inoculation of 10^4 L-1210 cells. The 3 "cured" animals which survived a second tumor dose of 10^5 cells came from an L-1210 experiment in which treatment was started only 4 days after tumor implantation. These mice had thus more time to develop an immune response to the tumor cells before the drug began to eliminate the antigen and to depress the immune system. None of the 8 DBA/2 mice which had been cured and then reinoculated with L-1210 survived the second tumor challenge.

In a number of experiments, ILS in L-1210 by treatment with VP 16-213 was compared to that obtained with amethopterin (methotrexate, Mtx, NSC-740), mice for both drugs being inoculated from the same cell suspension, treated with the same schedule and by the same route. ILS produced by Mtx ranged from 58 to 116%, depending on treatment schedule and route of application (tumor inoculum: 10^6 cells s.c.). There were no cures with Mtx in the four comparative experiments, while 18 mice survived after VP 16-213 treat-

ment. Taking ILS produced by Mtx as 100, then ILS obtained with VP 16-213 was, in these comparative experiments, between 200 and 390.

DISCUSSION

Podophyllotoxin and its glucoside, as well as aldehyde condensation products of podophyllotoxin glucoside, inhibit mitosis in metaphase [15]. Later studies have shown that demethylation in position 4′ and epimerisation in position 1 of the molecule together with aldehyde condensation of podophyllotoxin glucoside [9] leads to products which prevent cells from entering mitosis and which, at the same time, show enhanced activity in experimental tumor systems [2]. Condensation of 4′-demethylepipodophyllotoxin glucoside with a short chain aliphatic aldehyde has produced a drug (VP 16-213) which is less active *in vitro* and *in vivo*, mg for mg, than other aldehyde condensation products (see ref. [9]); but the new compound seems to have, particularly in mouse leukemia L-1210, a higher therapeutic index, indicating the possibility of useful activity in certain human malignancies.

As to mechanism of action, VP 16-213 seems to be very similar to VM 26 [2, 8, 16]; but since no resistant cell or tumor lines have been developed so far with these drugs, evidence for the similarity is only circumstantial (besides being, of course, suggested by chemical similarity). Whether the higher therapeutic index of VP 16-213 is due to differences in pharmacokinetics or to different affinities for different tissues (compared to VM 26) or to other causes is not known.

The spectrum of tumor inhibition of VP 16-213 is also similar to VM 26. The more recent drug is somewhat more active in sarcomas 37 and 180, while in the Walker carcinosarcoma VM 26 shows better tumor inhibition. Growth of Ehrlich's ascites tumor is macroscopically completely suppressed by both drugs, but no cures are obtained (results for VP 16-213 not shown in the tables). Survival time of mice inoculated with leukemias P-815 (mastocytoma), P-1534, and L 1210 is increased to a higher degree by VP 16-213 than by VM 26, with the exception of intracerebrally inoculated L-1210 where VM 26 produces higher ILS (results for VP 16-213 not shown in the tables). Perhaps this is related to the more lipophilic nature of VM 26 as compared to VP 16-213

Activity of VP 16-213 in leukemia L-1210 has been studied in about 80 experiments, most of them involving 30 mice. An interval of 3–4 days between doses seems to be optimal. No experiments were done with the so called "priming dose" schedule [17]. Given orally, VP 16-213 produces ILS of up to 200% (in single experiments), in contrast to VM 26 which showed moderate and variable oral activity in mice and rats. Intraperitoneal drug injection resulted in a somewhat lower ILS compared to i.v. injection when tumor cells were implanted s.c. Intraperitoneally inoculated L-1210, however, was best influenced by i.p. drug treatment. This becomes evident when the percentage of cures is considered; it does not show up in the ILS values because mean ILS was calculated excluding cured mice. The higher susceptibility to i.p. drug treatment of i.p. inoculated tumors, compared to s.c. inoculated ones, has been observed in our laboratories repeatedly with a number of drugs. Probably this observation results from the fact that $C \times T$ (concentration × time) [12] to which tumor cells are exposed is highest with drug application close to the site of tumor inoculation.

The kill percentage was highest with a single dose given i.p. one day after i.p. implantation of 10^5 L-1210 cells: 4 out of 6 animals were cured with 30 mg/kg. For a host survival of 4 out of 6 animals, 0·264 tumor cells per animal are expected to have survived treatment (according to Skipper's formula [11]), thus there is one surviving cell out of $10^5/0{\cdot}264$ or out of about $3{\cdot}8 \times 10^5$ cells (per cent kill = 99·9997). In this experiment the reservations mentioned under "results" with respect to calculation of cell kill percentage do not apply since in this case the percentage of cells killed was calculated from the number of cures and not from ILS, and since animals were observed up to day 60. ILS and most likely cell kill are lower when the drug is given later than 5 days after tumor implantation. This could be due to the fact that probably a smaller proportion of cells is in a sensitive phase of the cell cycle at this advanced stage of tumor growth [12] and furthermore to intracranial metastases and growth of the tumor cells where the drug apparently (see above, intracerebral inoculation) is less effective [11]. The observation that drug application before tumor inoculation is ineffective when VP 16-213 is given i.v., but that there is a considerable ILS with some cures when drug and tumor cells are given i.p., suggests again that, with this arrangement, tumor cells are exposed to a high $C \times T$. This latter value is very likely to be a decisive one for the proportion of proliferating cells which are killed by treatment with a cytostatic or cytotoxic agent.

Clinical trials with VP 16-213 are under way.

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The Relationship of Embolic Homogeneity, Number, Size and Viability to the Incidence of Experimental Metastasis*

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Abstract—*B_{16} melanoma cells grown* in vitro *were used to study factors influencing experimental metastasis. Groups of C57 BL/6, mice were given intravenous injections of varying numbers of viable B_{16} melanoma cells, with or without gamma-irradiated B_{16} cells, viable B_{16} cells with or without C_{57} embryo cells, and equal number of viable B_{16} cells suspended singly or in clumps.*

The data permitted the following conclusions: The number of lung metastases was proportional to the number of viable tumor cells injected i.v. Mice injected with clumps of B_{16} tumor cells had a higher number of lung metastases when compared to mice injected with an equal number of single B_{16} cells. Mice injected with viable B_{16} tumor cells together with dead B_{16} cells or viable C_{57} embryo cells had a statistically significant higher number of pulmonary tumors as compared to mice injected with viable B_{16} cells alone. The mechanism responsible for these phenomena might be the induction of pulmonary vasospasm by arrested emboli bringing about an increased trapping of tumor cells in the lung capillaries. These results point out the absolute necessity for a standardized and homogenous systems for studies of metastasis. In addition, they call attention to the possible role of circulating dead tumor cells in the formation of secondary tumors.

INTRODUCTION

METASTASIS involves the invasion of blood vessels by primary cancer cells, the release of tumor emboli into the blood stream, arrest of the emboli in distant organs, and their final development into secondary tumors. Information on many aspects of this process is lacking. The presence of circulating tumor cells does not correspond to their ability to form metastasis [1]. Our previous quantitative study has demonstrated conclusively that most tumor emboli are arrested in the lung immediately after arrival, but only a few survive to form metastases [2]. Some emboli can pass unarrested through the lung and appear in the systemic circulation [3]. These cells may then be trapped in other organs where their development is impeded [4] or they may be rearrested in the lung capillaries.

Most past studies of experimental metastasis were carried out with transplantable animal tumors propagated *in vivo*. Obviously, tumor cell suspensions prepared from such solid tumors usually contain some normal stromal cells and vary greatly in viability from one preparation to another. It seemed likely that the above disadvantages could be eliminated by the use of a homogeneous population of tumor cells grown

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in vitro, as this would allow for precise, standardized tumor cell suspensions of equal viability and metastasability. Past studies demonstrated that the number of metastases is proportional to the number of viable emboli released into the circulation [5]. The utilization of *in vitro* cultured tumors could determine whether tumor embolic size and viability have similar effects on the formation of metastases. Specifically, do dead tumor emboli or other emboli play a role in enhancing pulmonary metastases? Does the number of resultant metastases depend on total number of emboli or only on the number of viable tumor emboli? It is the purpose of this report to present evidence which suggests that the outcome of experimental metastases could be readily influenced by all the above factors.

MATERIAL AND METHODS

Animals

C_{57} BL/6$_J$ mice, 8–10 weeks old were obtained from Jackson Laboratories, Bar Harbor, Maine, and utilized throughout these studies.

Tumor cell culture

The transplantable B_{16} melanoma was adapted to growth in tissue culture as described previously [2]. Stock cultures were maintained on glass in Eagle's minimum essential medium, supplemented with 10% fetal calf serum, vitamin solution, sodium pyruvate, non-essential amino acids, penicillin–streptomycin, and L-glutamine (CMEM) (Grand Island Biologicals, Grand Island, New York). The cells were cultured in a humidified 37°C incubator containing 5% CO_2 atmosphere.

C_{57} embryo cultures

Primary embryo monolayers were derived from 15–18 days old feti. Pregnant mice were killed and submerged in consecutive washes of iodine, 70% ethanol and saline. Feti were removed, decapitated and minced in cold Hank's basic salt solution (HBSS). The tissues were then trypsinized for 15 min in 0·1% trypsin, at 37°C. After 2 washes, cellular pellets were resuspended in CMEM and plated. Cultures were maintained at 37°C, humidified incubator, 5% CO_2 atmosphere.

General procedure for study of experimental metastasis

B_{16} melanoma cells grown *in vitro* were harvested by 1 min trypsinization (0·25% trypsin-EDTA solution), washed, centrifuged and resuspended in HBSS. The number of viable tumor cells was determined with the aid of a counting chamber utilizing the trypan blue exclusion test. Inoculum volume for all experiments was 0·2 ml suspension per mouse, injected intravenously into the tail vein. Mice were sacrificed 14 days after tumor cell injection and the number of resultant pulmonary metastases was determined with the aid of a dissecting microscope.

Preparation of single and clumped tumor cell suspensions

B_{16} melanoma monolayers were harvested from culture flasks with a rubber policeman. Large resulting cell clumps were broken up by gentle pipetting. The cell suspension was then placed in a test tube and allowed to stand at room temperature for 15 min. The supernatant suspension was removed and filtered through 4 layers of sterile gauze. The precipitated portion was diluted with HBSS. Microscopic examination demonstrated that the supernatant contained practically only single tumor cells, while the suspension derived from the precipitate consisted of clumps averaging 4–5 cells each. Both tumor cell suspensions were diluted to yield an inoculum dose of 50,000 cells per 0·2 ml.

Preparation of dead tumor cell suspension

B_{16} melanoma cells were harvested from culture flasks with trypsin solution as described above. The cell suspension in HBSS was placed in a glass tube and exposed to 12,000 *R* gamma-irradiation from a Cesium 137 source emitting at a constant rate of 118 rad/min (Gamma Cell 40, Atomic Energy of Canada, Ltd.). This irradiating dose was previously demonstrated by us to effectively prevent the tumor cells from further dividing, or indeed surviving, in the recipient animal [2].

Preparation of C_{57} embryo cell suspension

C_{57} mouse embryo monolayers were trypsinized, washed, centrifuged and resuspended in HBSS. Total cell number and viability was determined as described above.

Statistical analysis

Statistical analysis was carried out by the Student's-*t*-test.

RESULTS

The viability of both tumor and embryo cells cultured *in vitro* was always above 95%. On the

other hand, 80% of the gamma-irradiated tumor cells were heavily stained (trypan blue) 30 min post irradiation. Samples of these irradiated cells placed in 37°C incubator and tested 24 hr later were practically 100% stained.

Relationship of number of injected cells to pulmonary metastases

The results of this experiment are shown in Table 1. The data demonstrated that the resultant number of pulmonary metastases is directly related to the number of tumor cells injected i.v. This finding agrees with earlier work of Zeidman *et al.* [5].

Table 1. *Relationship of the number of viable B_{16} melanoma cells injected i.v. in C_{57} mice to the number of resultant pulmonary metastases*

Number of viable B_{16} cells injected i.v.	Average number of pulmonary metastases* ± S.D.	
100	0·3	(0–1)
1,000	12·1 ± 3·4	(9–18)
10,000	71·6 ± 16·2	(52–96)
50,000	205·5 ± 34·3	(166–260)
100,000	394·8 ± 51·6	(338–502)

* Nine mice per each group. Pulmonary metastases were counted on day 14 post i.v. injection with the aid of a dissecting microscope.

Embolic size and resultant pulmonary metastases

The effect of embolic size and resultant pulmonary metastases is clearly demonstrated in Table 2. Mice injected with 10,000–12,000 clumps of 4–5 cells each (total of 50,000 tumor cells) had a higher number of pulmonary metastases as compared to those injected with 50,000 single tumor cells. Specifically, inocula of B_{16} clumps produced an average of 33·3 ± 8 (range 21–41) lung tumors while inocula of single B_{16} cells produced an average of 11·5 ± 3·4 (range 5–14). This difference was statistically significant ($p < 0{\cdot}05$).

Table 2. *The effect of B_{16} melanoma embolic size on resultant pulmonary metastases in C_{57} mice*

Total number of embolic B_{16} cells injected i.v.	Number of cells per embolic clump	Average number of resultant pulmonary metastases* ± S.D.
50,000	1	11·5 ± 3·4 (5–14)
10,000–12,000	4–5	33·3 ± 8 (21–41)

* Eight mice per each group. Pulmonary metastases were counted on day 14 post i.v. injection with the aid of a dissecting microscope.

Injection of live and/or dead tumor cells and pulmonary metastases

Previous studies from our laboratory demonstrated that most tumor emboli are trapped by the lungs immediately following injection, and a small fraction may pass through and recirculate. Furthermore, most of the injected cells failed to survive to form metastases [2]. Since the number of resultant metastases is proportional to the number of viable tumor emboli and their size, it is of interest to clarify whether the number of metastases is dependent only on the number of viable circulating tumor cells or on the total number of injected tumor cells. The data in Table 3 clearly indicates that the number of resultant pulmonary metastases is dependent on the total number of cells injected and not solely on the number of viable tumor cells injected. Specifically, an injection of 50,000 viable B_{16} cells yielded an average of 38 ± 9·2 (range 28–46) pulmonary tumors while 100,000 viable B_{16} cells as expected, yielded a proportionately higher number of tumors, averaging 205 ± 35 (range 182–269) On the other hand 50,000 live and 50,000 gamma irradiated B_{16} melanoma cells (a total of 100,000 injected cells) produced an average of 159 ± 29 (range 130–187) pulmonary tumors. The increase in the number of lung tumors between the animals receiving 50,000 viable B_{16} cells and those

Table 3. *Number of lung metastases in C_{57} mice following the i.v. injection of live and/or dead B_{16} melanoma cells*

Number and type of B_{16} cells injected i.v.	Average number of pulmonary metastases† ± S.D.
a. 50,000 live B_{16} cells	38 ± 9.2 (28–46)†
b. 100,000 live B_{16} cells	205 ± 35 (182–269)‡
c. 50,000 live and 50,000 dead* B_{16} cells	159 ± 29 (130–187)‡
d. 50,000 dead B_{16} cells	0

* B_{16} melanoma cells exposed to 12,000 R *x*-irradiation prior to i.v. injection.

† 8 mice per each group. Pulmonary metastases were counted 14 days post injection with the aid of a dissecting microscope.

‡ The increase in pulmonary metastases in groups b and c was highly significant ($p < 0{\cdot}001$) as compared to group a.

receiving 100,000 viable B_{16} or 50,000 viable and 50,000 dead B_{16} cells was statistically significant ($p < 0.001$). A final control included animals injected with 50,000 gamma irradiated B_{16} cells which obviously produced no pulmonary metastases.

Injection of B_{16} tumor cells with or without C_{57} embryo cells and resultant metastases

In similar experiments, groups of mice were injected with either 50,000 viable B_{16} cells; 100,000 viable B_{16} cells; 50,000 viable B_{16} and 50,000 viable C_{57} embryo cells; and 50,000 viable C_{57} embryo cells alone. The results of this experiment are shown in Table 4. Again, the data established that the number of resultant lung metastases corresponds to the total number of emboli and not necessarily only to the number of viable tumor cells. Specifically, 50,000 viable B_{16} cells produced an average of 75 ± 19 (range 56–107) lung tumors; 100,000 viable B_{16} cells produced proportionately higher number of lung tumors averaging 395 ± 66 (range 287–485); 50,000 C_{57} embryo cells produced no tumors but 50,000 viable B_{16} cells and 50,000 C_{57} embryo cells injected i.v. increased the number of resultant tumors to an average of 186 ± 31 (range 147–228). The increase in lung metastases was statistically significant $p < 0.001$ for 100,000 viable B_{16} cells and $p < 0.05$ for 50,000 B_{16} and 50,000 C_{57} embryo cells as compared to the control group receiving only 50,000 viable B_{16} cells.

Table 4. Number of lung metastases in C_{57} mice following the i.v. injection of B_{16} melanoma cells and/or C_{57} embryo cells

Number and type of cells injected i.v.*	Average number of pulmonary metastases† ± S.D.
a. 50,000 B_{16} cells	75 ± 19 (56–107)‡
b. 100,000 B_{16} cells	395 ± 66 (287–485)‡
c 50,000 B_{16} + 50,000 C_{57} embryo cells	186 ± 31 (147–228)‡
d. 50,000 C_{57} embryo cells	0

* B_{16} and C_{57} embryo cultured *in vitro*.

† 8 mice per each group. Pulmonary metastases were counted 14 days post injection with the aid of a dissecting microscope.

‡ The increase in pulmonary metastases in group b was highly significant as compared to group a ($p < 0.001$) group c differed from a to a lesser degree ($p < 0.05$).

DISCUSSION

Cancer metastasis is probably influenced by many factors relative to the host and intrinsic qualities unique to the tumor cell itself. The latter include the invasive nature of the primary cancer, the ability of the tumor emboli to circulate or to be arrested in distant organs, and establishment of a new microenvironment favorable to its growth [6, 7]. Finally, the tumor cell has to escape or circumvent the immune surveillance of the host [8, 9]. Cells from the same tumor traverse different organs at different rates [10] moreover, cells from different tumors exhibit different patterns of arrest and recirculation from the same capillary bed, i.e. lungs [3]. We have demonstrated that most B_{16} melanoma (labelled with ^{125}I-iodo-deoxyuridine) injected intravenously were trapped by the first major capillary bed encountered. However, most trapped tumor cells rapidly died while some cells continued to recirculate [2]. It is well known that the majority of injected tumor cells fail to survive to form tumors [2, 5, 6, 7, 10, 11].

Our present studies demonstrated that the outcome of experimental metastasis is influenced by factors unique to the tumor cell preparation. The number of resultant pulmonary tumors was proportional to the number of injected viable tumor cells, which agrees closely with earlier reports [5, 6]. However, the number of lung metastases was not determined by the number of viable tumor emboli but rather by the total number of and/or size of emboli. It was of great interest to note that when non-viable tumor cells or indeed, viable C_{57} embryo (syngeneic cells) were injected simultaneously with viable B_{16} tumor cells, the resultant lung metastases increased significantly in number.

Recirculation of tumor cells depends on intrinsic qualities of the tumor cell itself and is not regulated by mechanical host mechanisms [2]. When tumor cells are introduced into the blood stream by injection or by natural processes, pulmonary embolization followed by pulmonary vasoconstriction occur [12, 13]. The physiological response to pulmonary embolization has been divided into three successive components: (1) primary mechanical obstruction of vessels; (2) secondary local vasoconstriction; (3) spread of vasoconstriction to other lobes of the lung. The vasoconstriction brings about a rise in pulmonary pressure which in turn triggers the opening of pre-existing arteriovenous communications. Other studies employing a microscopic examination of the rabbit lung demonstrated pulmonary vasospasm following the i.v. injec-

tion of a suspension of the V_2 carcinoma. Circulation was arrested even in areas that were not obstructed mechanically [14]. It is possible that the opening of the A–V shunts brings about a decline in the number of arrested tumor cells by their release into the systemic circulation. Once the crisis is over and relief of anoxemia occurs, the A–V shunts close. This closing could lead to re-trapping of tumor cells in the lungs.

It is highly probable that the degree of pulmonary vasospasm is directly related to the degree of embolization. Higher numbers of emboli or larger emboli could bring about a more pronounced pulmonary vasospasm resulting in increased trapping of tumor cells in the pulmonary vascular bed. The subsequent appearance of increased lung metastases is probably related to this increased tumor cell arrest. Similarly, we proposed recently that the mechanism responsible for enhancement of pulmonary metastases following total body *x*-irradiation is the increased arrest of tumor cells in damaged capillaries [15]. Our current findings point out the absolute requirements for uniform suspensions of tumor cells utilized in studies of metastasis. Embolic homogeneity, viability, number and size must be considered to insure accurate and reproducible results. In addition, the data call attention to the possible role dead tumor cells may play in the formation of clinical metastases.

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Combination Chemotherapy: the Antagonism of Methotrexate and Cytosine Arabinoside

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Abstract—*Combination treatment with methotrexate and cytosine arabinoside resulted in antagonistic effects against murine lymphoma cells growing in culture. If cytosine arabinoside preceded methotrexate treatment by six hours, the antagonism was reduced, and it is concluded that clinically these drugs should be scheduled in this sequence. A biochemical basis for the interaction is presented.*

THE SUPERIORITY of combinations of drugs over single drugs in the treatment of a number of human malignancies has been established during the last few years [1]. A variety of drug interactions ranging from antagonism to synergism can result from the combination of two drugs that are separately cytotoxic, and to use two drugs together optimally it is necessary to understand their mechanisms of action and interaction.

Methotrexate (MTX) and cytosine arabinoside (Ara C) are two widely used anticancer drugs whose mechanisms of action have received detailed study. Methotrexate has been shown to be a stoichiometric inhibitor of dihydrofolate reductase, the enzyme responsible for the synthesis of tetrahydrofolate [2]. 5–10 Methylene tetrahydrofolate is a substrate in the synthesis of thymidylate (dTMP) from uridylate (dUMP), catalysed by thymidylate synthetase; other reduced folates are sources of one carbon fragment in the purine biosynthetic pathway, and for the biosynthesis of methionine [3].

Cytosine arabinoside (Ara C), following its metabolism to the 5′ triphosphate (Ara CTP), is a potent competitive inhibitor of DNA polymerase, and the inhibition is reversed by one of the natural substrates for the enzyme, deoxycytidine triphosphate (dCTP) [4].

MTX and Ara C therefore have separate sites of action in the DNA synthetic pathway, and on the basis of sequential blockade, it might be anticipated that in combination these drugs might exert an additive cytostatic effect. We have studied the effects of combinations of MTX and Ara C on the growth of suspension cultures of L5178Y lymphoma cells. Our results have shown strong antagonism when the drugs are administered at the same time. A possible biochemical basis for this antagonism emerged when we measured the deoxyribonucleoside triphosphate pool changes in cells exposed to MTX: a three-fold elevation in the dCTP content occurred following MTX treatment. MTX-treated cells also had reduced levels of deoxycytidine kinase, and their uptake of ^{3}H Ara C into DNA was substantially reduced.

MATERIAL AND METHODS

Ara C was obtained from the Upjohn Co. and MTX from Lederle Laboratories. ^{3}H Ara C was purchased from New England Nuclear, Polygram Cel 300 PE I from Camlab and nucleosides from Boehringer.

Cell culture

The line of L5178Y cells used in the experiments and the culture conditions have been

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described previously [5]. The sensitivity of the cell line to MTX and Ara C alone and in combination was determined from dose-response curves of cells growing in suspension culture. The concentrations of each drug alone and in combination required to achieve 50% inhibition of growth at 48 hr were then compared and the result plotted in the form of isobols, to determine the type of interaction produced [6].

The nucleoside triphosphate pools were measured using a modification of the assay

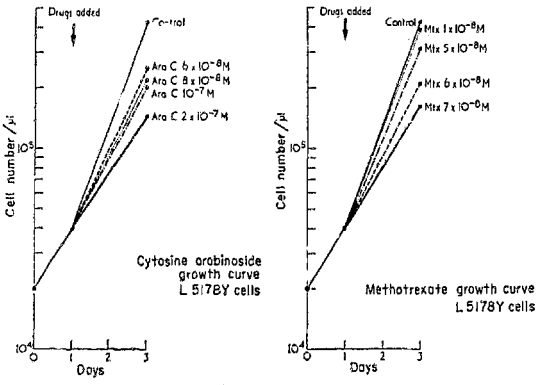

Fig. 1. Dose-response curves for Ara C and MTX against L5178Y cells in vitro.

described by Solter and Handschumacher [7]. *Deoxycytidine kinase* was assayed in crude cell homogenates using the method of Momparler and Fischer [8]. The uptake of ^{3}H Ara C was measured using the method of Chu and Fischer [9]. Protein concentration was determined by the method of Lowry *et al.* [10].

RNA and DNA were extracted by the method of Schmidt and Tannhauser [11], and assayed colorimetrically [12]

RESULTS

Dose-response curves for different concentrations of MTX and Ara C are shown in Fig. 1 Figure 2 shows the interaction resulting from a combination of MTX and Ara C when the drugs are added simultaneously and sequentially. The striking antagonism seen when methotrexate precedes Ara C treatment is also observed when the drugs are added together. When Ara C is added first and MTX 6 hr later there is only slight antagonism.

The deoxycytidine triphosphate (dCTP) pool changes in cells exposed for varying times to 10^{-6}M MTX are shown in Table 1. The elevation in dCTP concentration which develops 1–2 hr after MTX treatment is also produced by growing cells in a medium enriched with 4×10^{-5}M deoxycytidine. This concentration of deoxycytidine partially overcomes the inhibition of growth which cytosine arabinoside produces, as shown in Fig 3.

The results of studies on the transport and incorporation of labelled cytosine arabinoside into nucleic acids are shown in Table 2. The striking fall in ^{3}H Ara C transport into cells grown in a deoxycytidine-enriched medium

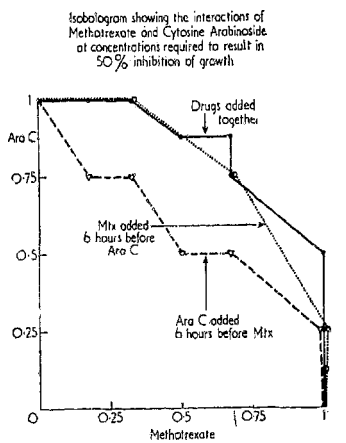

Fig. 2. The concentration of each drug alone required for 50% inhibition of growth is determined from dose response curves (Fig. 1). The fractions of these concentrations which in combination produce 50% inhibition are plotted to determine the type of interaction. If the plotted data results in a straight line connecting the two points 1 and 1, the drug combination has resulted in an additive interaction. Curves to the left of this line indicate a synergistic interaction, and curves to the right, an antagonistic interaction.

Table 1. dCTP pools of L5178Y cells in logarithmic growth.

		dCTP μmoles/μg DNA
Control		0·81
10^{-6}M MTX	30 min	1·6
	1 hr	2·3
	2 hr	3·0
	6 hr	3·2
	12 hr	2·4
deoxycytidine 4×10^{-5}M	2 hr	3·2

Table 2. The effect of deoxycytidine and methotrexate treatment on the incorporation of ^3H cytosine arabinoside by L5178Y cells into different cell fractions.

Compounds	Acid soluble extract (cpm)	(%)	RNA (cpm)	(%)	DNA (cpm)	(%)
None	5287	100	392	100	528	100
Deoxycytidine (4×10^{-5}M) (2 hr)	794	1.5	369	94	380	71
Methotrexate (10^{-6}M) (2 hr)	6990	130	400	102	400	77

Table 3. Deoxycytidine kinase activity of L5178Y cells.

Cells	Activity mμmole dCMP produced/ min/mg protein
Control	0·99
Methotrexate 10^{-6}M 2 hr	0·39
Deoxycytidine 4×10^{-5}M 2 hr	0·45
Cytosine arabinoside 10^{-6}M 2 hr	0·66

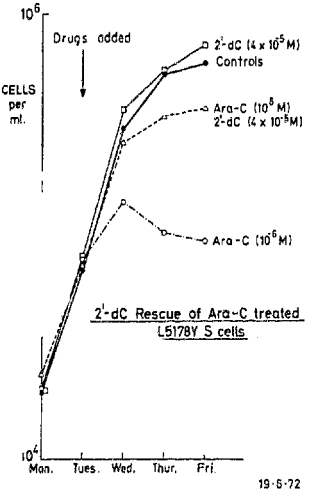

Fig. 3. Growth curve of L5178Y cells treated with Ara C ± deoxycytidine.

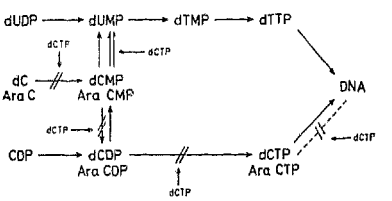

Fig. 4.

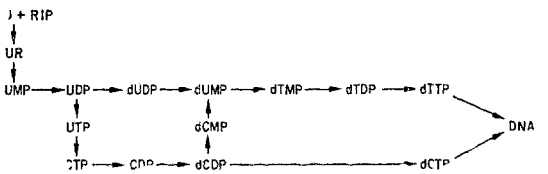

Fig. 5.

Figs. 4 and 5 Symbols used.

U = uracil
RIP = ribose 1 phosphate
(d)UM(D,T)P = (deoxy)uridine mono (di, tri) phosphate
(d)CM(D,T)P = (deoxy)cytidine mono (di, tri) phosphate
dTM(D,T)P = deoxythymidine mono (di, tri) phosphate
Ara CM(D,T)P = arabinofuranosyl cytidine mono (di, tri) phosphate
dC = deoxycytidine

may be due to competition by deoxycytidine for the transport mechanism involved in Ara C uptake. MTX-treated cells and cells grown in a deoxycytidine enriched medium show reduced incorporation of labelled Ara C into DNA.

Deoxycytidine kinase activity is reduced in cells exposed to MTX, Ara C and deoxycytidine as shown in Table 3.

DISCUSSION

The sequence of administration of two drugs may have a marked effect on the outcome of their interaction. Methotrexate and cytosine arabinoside when administered simultaneously antagonise each other, confirming the result of Grindey and Nichol [13]. When cytosine arabinoside treatment precedes methotrexate treatment by several hours, the effects are almost additive, but when MTX precedes Ara C by a similar period there is marked antagonism. Deoxycytidine has been shown to protect cells from cytosine arabinoside toxicity. Moreover, dCTP has been shown to antagonize deoxycytidine kinase activity, to activate deoxycytidine deaminase, and to reduce the binding of Ara CTP to DNA polymerase (Fig. 4). The finding that methotrexate treatment of cells leads to an elevation in the intracellular deoxycytidine triphosphate pool suggested to us that this might be the mechanism by which methotrexate and cytosine arabinoside antagonise each other. Data presented here supports this hypothesis, and the sequence studies *in vitro* clearly indicate how these drugs should be scheduled clinically for their optimal effectiveness.

The mechanism by which methotrexate treatment produces an elevation in dCTP is not known. It is tempting to speculate that a hold-up in the utilization of dUMP would lead to an accumulation of precursors in the *de novo* pyrimidine synthetic pathway and to their conversion to cytosine nucleotides (Fig. 5), thus accounting for the observed accumulation of dCTP.

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Letter to the Editor

Serum Dopamine-β-Hydroxylase Activity in Patients with Leukemia and in Patients with Hepatoma*

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DOPAMINE-β-hydroxylase (DβH) is the enzyme which catalyzes the terminal step in norepinephrine synthesis. Evidence was presented that this enzyme is present in human serum [1, 2, 3]. Recently we have shown elevated serum DβH activity in patients with neuroblastoma [4], a disease known to be associated with the overproduction of catecholamines. The question arises whether serum DβH is a useful index of sympathetic activity in both normal and disease states. We therefore assayed serum DβH activity in disorders which are not characterized by abnormal catecholamine metabolism.

Serum DβH activity was determined by a previously described sensitive enzymatic assay [1]. One unit defined as the enzyme activity that forms 1 mμmole of product per milliliter of serum per 20 min incubation time. Since serum DβH activity is influenced by age [5] the results obtained from the leukemic patients were compared with control subjects of the same age group.

Table 1 shows levels of serum DβH activity in nine leukemic patients; one untreated, and eight of whom were treated with a combination of anti-cancer agents for varying periods of time. Six of nine leukemic patients had DβH values at least two standard deviations above the mean values for age matched controls.

In a separate study we found that two out of three patients with hepatomas had extremely high serum DβH activities (3–5 standard deviations above the mean values for age matched controls, Table 2). It is noteworthy that a patient with azathioprine (Imuran) induced liver hepatitis had extremely high DβH activity (3 standard deviations above the mean value for age matched controls), while two other patients with hepatitis secondary to alphamethyldopa therapy had DβH activities within the range of normals.

Since abnormalities in sympathetic functions have not been associated with leukemic or hepatoma patients, it is possible that the elevated DβH values may be due to an impairment of the degradation process of the enzyme. It remains to be determined whether these elevations can be related to the pathology of the disease or the chemotherapy.

The steady state of levels of circulatory DβH activity can be considered the result of a balance between rates of release of DβH into the circulation and of its removal from the circulation (rates of the degradation of the enzyme). Although the mechanisms and sites involved in this degradation are unknown, it is conceivable that DβH is removed from the circulation by the reticulo-endothelial cells of the liver and spleen which phagocytize serum proteins [6]. Thus, variations in the rate of DβH degradation may be in part the cause for the large differences in circulatory DβH activity found in the normal population [5].

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Table 1. Serum DβH activity in patients with acute lymphoblastic leukemia

Patient-age (years)	Serum DβH (units)*	*P* (values)†	SGOT (units)‡	Concurrent clinical status		
				Bone marrow	Duration of disease (years)	Previous chemotherapy
DP–8	100	< 0·001	48	Remission	4	§
EL–8 1/2	90	< 0·001	72	Remission	6	§
JB–5	52	< 0·001	53	Relapse	at diagnosis	None
SC–2 1/2	44	< 0·001	31	Relapse	1·5	§
HT–5	32	< 0·01	80	Remission	2	§
LM–4	25	< 0·05	49	Remission	0·2	vincristine methotrexate daunomycin
RM–4 1/2	16	Not significant	299	Remission	2	§
EG–5	19	Not significant	70	Remission	3	§
HF–7	19	Not significant	33	Remission	1·5	§

*DβH activity for controls: (units ± S.D.) age groups 1–5 years, 7·9 ± 6·6 u; 6–10 years, 11·5 ± 5·8 u.

†Indicates statistically significant difference from control subjects.

‡SGOT: Upper limits of normal are 53 units/ml.

§These patients all received the following drugs in various sequences and combinations during the duration of their disease courses: vincristine, daunomycin, prednisone, methotrexate, cytosine, arabinoside, thioguanine, hydroxyuria, cyclophosphamide, L-asparaginase and 1,3-*bis*(2-chloroethyl)-1-nitrosouria.[7]

Table 2. Serum DβH activity in patients with hepatoma

Patient-age (years)	Serum DβH (units)*	*P* (values)†	SGOT (units)‡	Concurrent clinical status		
				Bone marrow	Duration of diseases (years)	Previous chemotherapy
TB–8	24	Not significant	51	—	1/2	5-tri fluoromethyl-2′-deoxyuridine
MM–15	166	< 0·001	79	—	2	5-tri fluoromethyl-2′-deoxyuridine, cyclophosphamide, 5-fluorouracil
BB–12	150	< 0·001	219		at diagnosis	None

*DβH activity for controls was the same as in Table 1.

†Indicates statistically significant difference from control subjects.

‡SGOT: Upper limits of normal are 53 units/ml.

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Dose–Response and Ultrastructural Alterations in Dioxane Carcinogenesis.

Influence of Methylcholanthrene on Acute Toxicity

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Abstract—*A dose–response study with dioxane revealed the hepatocarcinogenicity of this compound in male rats to be a function of the total oral dose administered. The progressive development of these tumors was studied histopathologically, and detailed electronmicroscopy was carried out on precancerous liver tissue and on hepatocellular carcinoma produced by dioxane.*

The LD_{50} dose of dioxane in male rats (5·60 g/kg body weight) is significantly decreased (5·18 g/kg) in animals pretreated with 3-methylcholanthrene. This suggests that a metabolite is involved in the toxicity and possibly carcinogenicity of dioxane, and that both may be potentiated by enzyme inducers. Because certain hydroperoxides are known to be carcinogenic, it was shown by micro-iodimetric titrations that the dioxane used does not contain a dioxane hydroperoxide and that no peroxide is formed under the conditions of administration.

INTRODUCTION

THE HEPATOCARCINOGENICITY of dioxane was first described in rats by Argus *et al.* [1] in 1965, and later in guinea pigs by Hoch-Ligeti and Argus [2]. The testing of this compound was undertaken at that time because physicochemical studies [3, 4, 5, 6] had revealed dioxane to closely resemble the potent carcinogens dimethyl- and diethylnitrosamine in their role as agents of protein denaturation. Moreover, in 1934 Barber [7] had reported the death of five workers employed in the manufacture of artificial silk, all of whom had central necrosis of the liver and symmetric necrosis of the renal cortex that were attributed to dioxane vapor exposure. More recently, in 1947, Hohl [8] reported that at identical concentrations the carcinogen, urethan, and dioxane produced the same chromosomal abnormalities in plant root cells. It is of interest to note that the dioxane molecule represents the central segment of 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin (also known as "dioxin"),‖ a contaminant of the herbicide 2, 4, 5-T and one of the most toxic synthetic compounds known [9, 10].

In the present study the dose–response relationship of the incidence in rats of hepatic tumors as a function of the total oral dose of dioxane administered at four concentration

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‖ Not to be confused with 2, 6-dimethyl-*m*-dioxan-4-ol acetate, which according to The Merck Index (8th edition, Merck, Rahway, N.J., p. 373) has the trivial brand names "Dioxin" and "Dimethoxane".

levels for the same time period has been established. Electron microscopic investigations of precancerous liver tissue and of hepatocellular carcinoma produced by dioxane have been carried out. Because dioxane is used as a solvent in many industries as well as in laboratory tissue processing and scintillation counting, the question of human exposure to dioxane arises. As a beginning toward investigating a degree of safety for dioxane use, LD_{50} studies were carried out and the effect of the microsomal enzyme-inducing agent, 3-methylcholanthrene (MC), on the acute toxicity of dioxane was studied.

MATERIAL AND METHODS

Dioxane carcinogenicity

Five groups (28–32 each) of male rats (Charles River CD strain, random bred, Sprague–Dawley descendent 1950), 2–3 months old and weighing 110–230 g at the beginning of the experiment were used. All animals were housed 2 to a cage and received Purina laboratory chow *ad libitum*. Four groups of rats received in the drinking water 0·75, 1, 1·4 or 1·8% dioxane (Eastman Organic Chemicals #2144) for 13 months. These solutions were prepared fresh daily. One additional group of 30 rats served as controls. At the beginning and on two occasions during the study the fluid consumption per rat was determined for each group over a 3-day period and was found to average 30 ml daily. The weight of the rats, which was recorded weekly, increased normally (initially about 25 g per week). All rats surviving 16 months were killed with ether. Complete autopsies were performed on every rat that died or was killed. For histological investigation, all tissues were fixed in Mossman's fixative. The slides were stained routinely with hematoxylin and eosin. Periodic acid-Schiff or trichrome stains were used on the kidneys in addition.

Electron microscopy

Ten male, Sprague-Dawley rats (Holtzman Co.) were administered 1 per cent dioxane in the drinking water. The animals were sacrificed by neck stroke, 5 rats after 8 months and another 5 rats after 13 months of dioxane administration. In the latter group, 2 rats had well-defined hepatocellular carcinomas. Small pieces of tissue from these tumors and from the livers of the rats sacrificed at 8 months were fixed in phosphate-buffered 4% glutaraldehyde at 4°C for 2 hr. Following several washings in phosphate buffer, the tissues were post-fixed in osmium tetroxide for 1 hr, dehydrated in an ascending series of ethanol, and embedded in Maraglas. In the processing, tissue of each experimental animal was simultaneously paralleled by tissue of a control animal. Thin sections were stained with uranyl acetate and lead citrate, and were examined with an Elmiskop I electron microscope.

Determination of acute toxicity

For determination of the LD_{50} values fourteen groups of 10 male, Sprague-Dawley rats (Holtzman Co.) each, weighing 130–170 g and fed Purina Laboratory chow, were used. Eight groups received i.p. in 0·9% saline, dioxane alone at graded levels from 5·30 to 5·80 g/kg body weight. In the other 6 groups, each rat received i.p. 10 mg MC (Eastman Organic Chemicals #4383) in corn oil, 23 hr prior to the i.p. injection of dioxane at levels from 4·90 to 5·30 g/kg body weight. The LD_{50} values with fiducial limits at a 95% probability, based on deaths occurring 48 hr following dioxane administration, were calculated from probit regression lines [11]. Based on the null hypothesis for a true difference between the LD_{50} values, the probability was calculated using the pooled variances.

For histopathological investigations 15 additional male, Sprague–Dawley rats were injected i.p. as follows: 5 rats received 10 mg MC alone; 5 were administered 5·18 g dioxane/kg body weight; and 5 were injected with 5·18 g dioxane/kg 23 hr after receiving 10 mg of MC. All animals were sacrificed 71 hr after administration of MC or 48 hr after administration of dioxane. Histopathological investigation was carried out on the following organs: liver, spleen, kidney, thymus and stomach.

Micro-iodimetric titrations

Tap water solutions of dioxane at concentrations of 0·75, 1, 1·4 and 1·8% were allowed to stand for 48 hr in inverted animal watering bottles, capped with rubber stoppers which were fitted with steel syphons (i.e. under the same conditions as existed for the actual animal experiments). These solutions, as well as tap water alone as control, and a 10% solution of dioxane, were titrated iodimetrically [5] in an attempt to detect the presence of dioxane hydroperoxide. Using a series of dilutions of H_2O_2 (3–0·0001%), it was determined that the method was sensitive to the presence of peroxide at a concentration as low as 15·0 μM.

RESULTS

Dioxane carcinogenicity

As was reported previously [1] in a study on the continuous administration of 1% dioxane for 63 weeks, the gross and microscopic appearance of liver tumors produced in rats by dioxane in the present study resembled those induced by dimethyl- and diethylnitrosamine [12, 13]. An opportunity to study the progressive development of these tumors was now at hand since the changes in the liver resulting from administration of various dose levels of dioxane showed definite gradation of severity.

The changes in the liver were generally diffuse. The earliest effect observed was an increase in the nuclear size of hepatocytes, mostly in periportal areas. Groups of large cells with reduced cytoplasmic basophilia gave the liver a slightly nodular appearance under the microscope. In addition, two other types of circumscribed nodules occurred. One consisted of large cells, apparently filled and distended with fat. The small compact nuclei were located peripherally, suggestive of dislocation by the fatty changes in the cytoplasm (Fig. 1). The third type of nodules consisted of finger-like strands, 2–3 cells thick, of rather smaller hepatocytes with large, hyperchromic nuclei and dense cytoplasm. These nodules were designated as incipient hepatomas, since they showed all the histological characteristics of the fully developed hepatomas (Fig. 2). These lesions, which generally were not larger than a low-power microscopic field, were multiple, and it seemed that the larger hepatomas developed often by their confluence. All three types of nodules were generally present in the same liver. Grossly, these lesions were noted on the liver as small whitish spots. Cirrhosis of the liver was not observed.

Hepatomas were manifest grossly as white lesions of 0·5 to 2 cm in diameter. Microscopically these lesions consisted of long strands of large cells, 2–4 cells thick. In some tumors conglomeration of small nodules was discernible. In two rats the malignant character of the hepatocellular lesion was manifest by invasion of veins (Fig. 3). In one rat a tumor composed of glandular structures was present (Fig. 4). Metastases were not observed.

Both incipient liver tumors and hepatomas were included in the dose–response curve (Chart 1). There were 4 incipient tumors in the group receiving 0·75% dioxane, 9 incipient tumors in the group receiving 1% dioxane, 13 incipient tumors and 3 hepatomas in the group receiving 1·4% dioxane, and 11 incipient tumors and 12 hepatomas in the group administered 1·8% dioxane. The minimum effective tumor dose (TD_5), the 50% tumor dose (TD_{50}), and the maximum effective dose (TD_{95}) of dioxane in rats under the present experimental conditions are 72 g, 149 g and 260 g respectively, as evaluated from the probit plot of the dose–response.

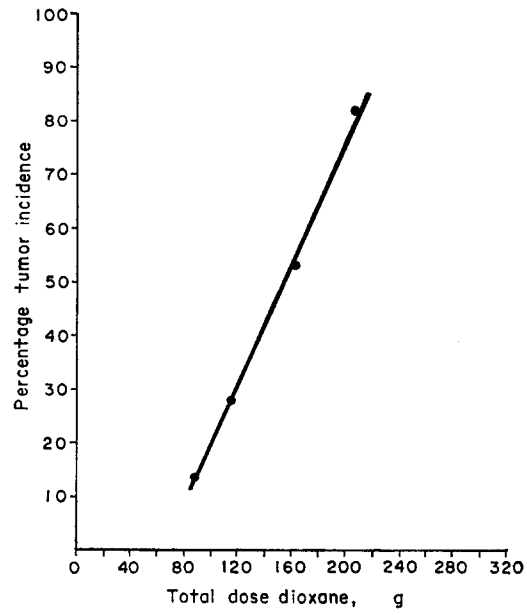

Chart 1. *Hepatic tumor incidence in* 4 *groups of rats* (28–32 *animals each*) *as a function of the total oral dose of dioxane.*

On all dose levels of dioxane the kidneys of rats showed marked alteration. In many kidneys the changes were those of glomerulo- and pyelonephritis, with characteristic epithelial proliferation of Bowman's capsule, periglomerular fibrosis and distension of tubules. Many tubules were filled with proteinaceous eosinophilic material. Another alteration consisted of striking focal enlargement of epithelial cells of distal tubules. The enlarged cells have large atypical nuclei (Fig. 5). Numerous multinucleated giant cells were present.

Tumors were not found in the lung. In one rat receiving 1·4% dioxane, the lung showed early peripheral adenomatous change of alveolar epithelium (Fig. 6). Papillary hyperplasia of bronchial epithelium was present in another rat receiving the same level of dioxane.

The induction of carcinoma of the nasal cavity in one rat receiving 0·75% dioxane, one rat receiving 1% dioxane, two rats receiving 1·4% dioxane, and two rats receiving 1·8% dioxane has been previously described [14].

Electron-microscopic observations

The structure of the normal parenchymal cells of the rat liver was similar to that described previously by Bruni and Porter [15]. Rough endoplasmic reticulum consisted of parallel layers of flattened cisternae studded with ribosomes (Fig. 7). Smooth endoplasmic reticulum was sparsely distributed and consisted of vesicular and tubular structures which were seen mainly in the apical region of the cells or in relation to the glycogen-rich areas. The Golgi components, mitochondria, microbodies, lysosomes and nuclei were essentially normal in distribution and appearance.

In none of the rats sacrificed after 8 months administration of 1% dioxane were the small white spots on the liver (which were indicative of the incipient tumors found in the histopathology study after administration for 13 months) grossly evident. Figures 8 and 9 illustrate the precancerous changes found in these livers by electron microscopy. The parallel arrays of the cisternae of rough endoplasmic reticulum were very rarely observed. The isolated cisternae were interspersed between the mitochondria which they frequently surrounded. Some of the cisternae were partly dilated. As compared to the controls there was a reduction in the per unit length distribution of the ribosomes associated with the membranes of endoplasmic reticulum. The population of free ribosomes in the cytoplasmic matrix was increased. There was a corresponding increase in the smooth endoplasmic reticulum which occupied extensive areas of the cytoplasm; a great decrease in the glycogen content of the hepatocytes was noted (Fig. 8). The lumen of the smooth endoplasmic reticulum showed a moderate electron density. A significant increase was noted in the lipid droplets within the hepatocytes (Fig. 9). Many lipid droplets were bound by a limiting membrane. The size and shape of the mitochondria remained essentially unchanged; however, intracristal space was enlarged in many mitochondria. The nuclear membrane in some cells was irregular in contour. The nucleoli showed a diffuse hypertrophy in most of the cells.

In hepatocellular carcinoma, resulting from feeding dioxane for 13 months, the cisternae of the rough endoplasmic reticulum were no longer organized in groups but were distributed randomly throughout the cytoplasm (Fig. 10). The mitochondria were reduced in size and their matrix was extremely dense. Vacuolated cristae were seen within some mitochondria (Fig. 10). The cisternae were dilated in certain areas along their length; their lumen was quite electron-dense. The membranes of the rough endoplasmic reticulum were relatively depleted of ribosomes. The free ribosomes occurring in polysome form greatly increased in number, sometimes occupying extensive areas of the cytoplasm (Fig. 11). In some cells concentrically-layered membranes enclosing lipid droplets were seen (Fig. 11). Golgi components were weakly developed in most cells (Fig. 11). Glycogen was absent in most of the cells; however, in some cells the glycogen particles were seen in the peripheral region of the cells and were interspersed between the tubules of the smooth endoplasmic reticulum (Fig. 12). The smooth endoplasmic reticulum was prominent in a few cells (Fig. 12); however, in most cells it was greatly reduced. Discontinuities were present in the plasma membrane of many cells (Fig. 12). Many of the nuclei were extremely irregular in contour.

LD_{50} and systemic manifestations of acute toxicity. Noninvolvement of dioxane hydroperoxide.

The LD_{50} dose of dioxane to male, Sprague–Dawley rats was found to be 5·60 ±0·06 g/kg body weight, while in animals receiving 10 mg MC, i.p. 23 hr prior to dioxane treatment, the LD_{50} was 5·18 ±0·06 g/kg body weight. There was a statistically significant decrease ($p < 0{\cdot}001$) in the tolerance to dioxane as a result of MC pretreatment.

Histopathological investigation from rats receiving the latter LD_{50} dose of dioxane and pretreated with 10 mg MC showed mild plastic peritonitis (thickening of the peritoneum and infiltration with mononuclear cells). These changes were observed both in sections of the liver and of the stomach; they were also present in the animals receiving MC alone, but not in those receiving dioxane alone. The kidneys in the MC-injected animals did not show any significant changes, showing that at the level used MC has no histopathologically detectable toxic effect on this organ. In two rats administered dioxane the tubular epithelium of the kidney showed vacuolation. However, in all rats receiving both MC and dioxane there was extensive vacuolation of the tubular epithelium so diffuse and severe that it probably contributed significantly to the death of the rats receiving both compounds in the LD_{50} study.

The iodimetric titrations showed that dioxane, freshly prepared as a 10% solution, as well as at the series of concentrations used in the tumor dose-response study and allowed to stand for 48 hr (under conditions approximating the actual feeding experiments), was devoid of any oxidizing power. This indicates

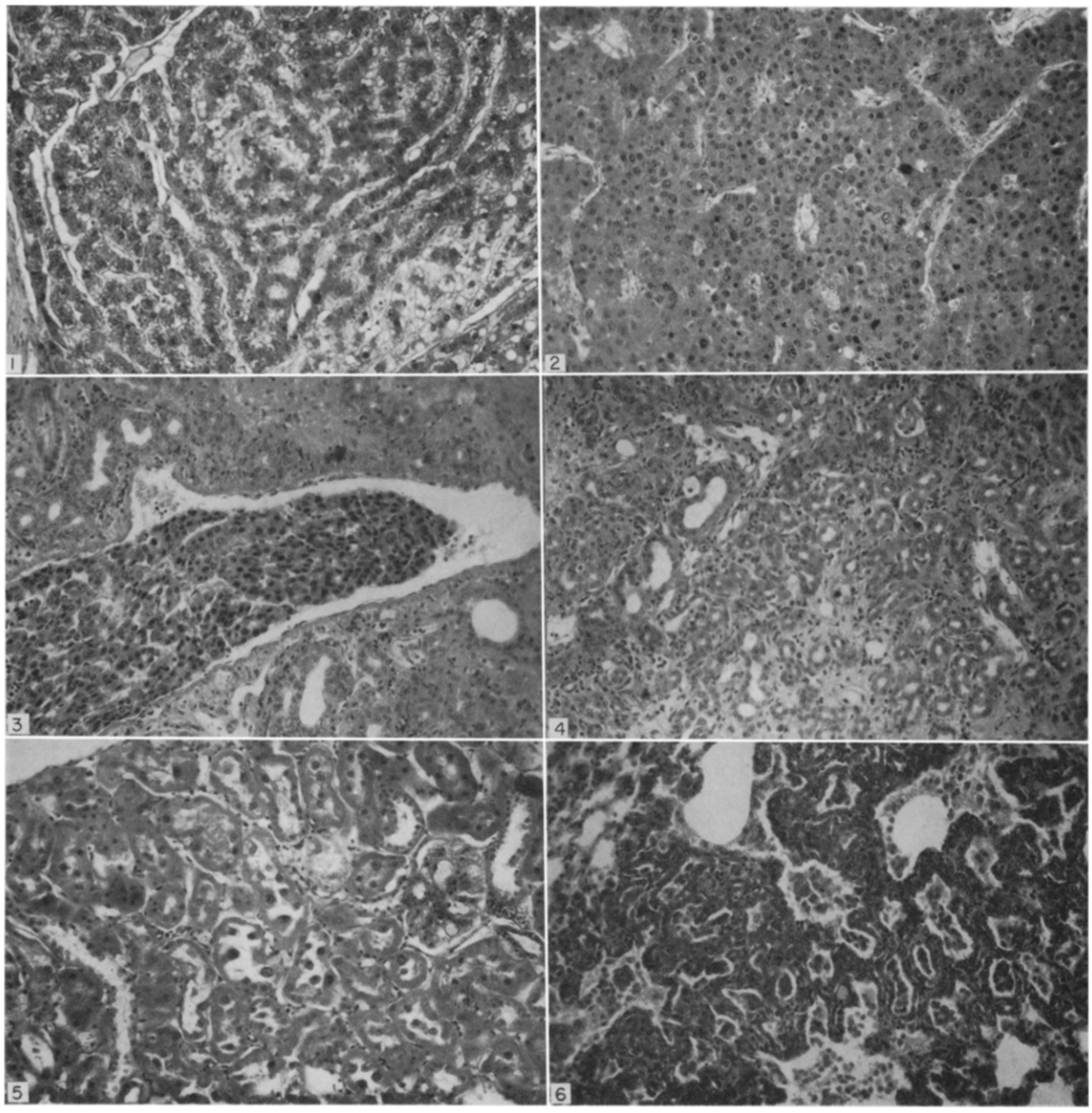

Fig. 1. *Liver nodule composed of large, fat-filled hepatocytes and of small, compact cells at the periphery, from rat receiving* 1% *dioxane in the drinking water. H and E,* × 200.

Fig. 2. *Liver nodule consisting of* 2–3 *cell-thick strands of relatively small hepatocytes with large nuclei, from rat administered* 1·4% *dioxane. H and E,* × 100.

Fig. 3. *Hepatocellular carcinoma in a small hepatic vein, from rat receiving* 1·8% *dioxane. H and E,* × 100.

Fig. 4. *Cholangiocarcinoma from rat administered* 1·8% *dioxane. H and E,* × 100.

Fig. 5. *Section through kidney showing increased nuclear size in renal tubules, from rat receiving* 0·75% *dioxane. H and E,* × 200.

Fig. 6. *Adenomatous change in alveolar epithelium of rat administered* 1·4% *dioxane. H and E,* × 100.

(to face p. 240)

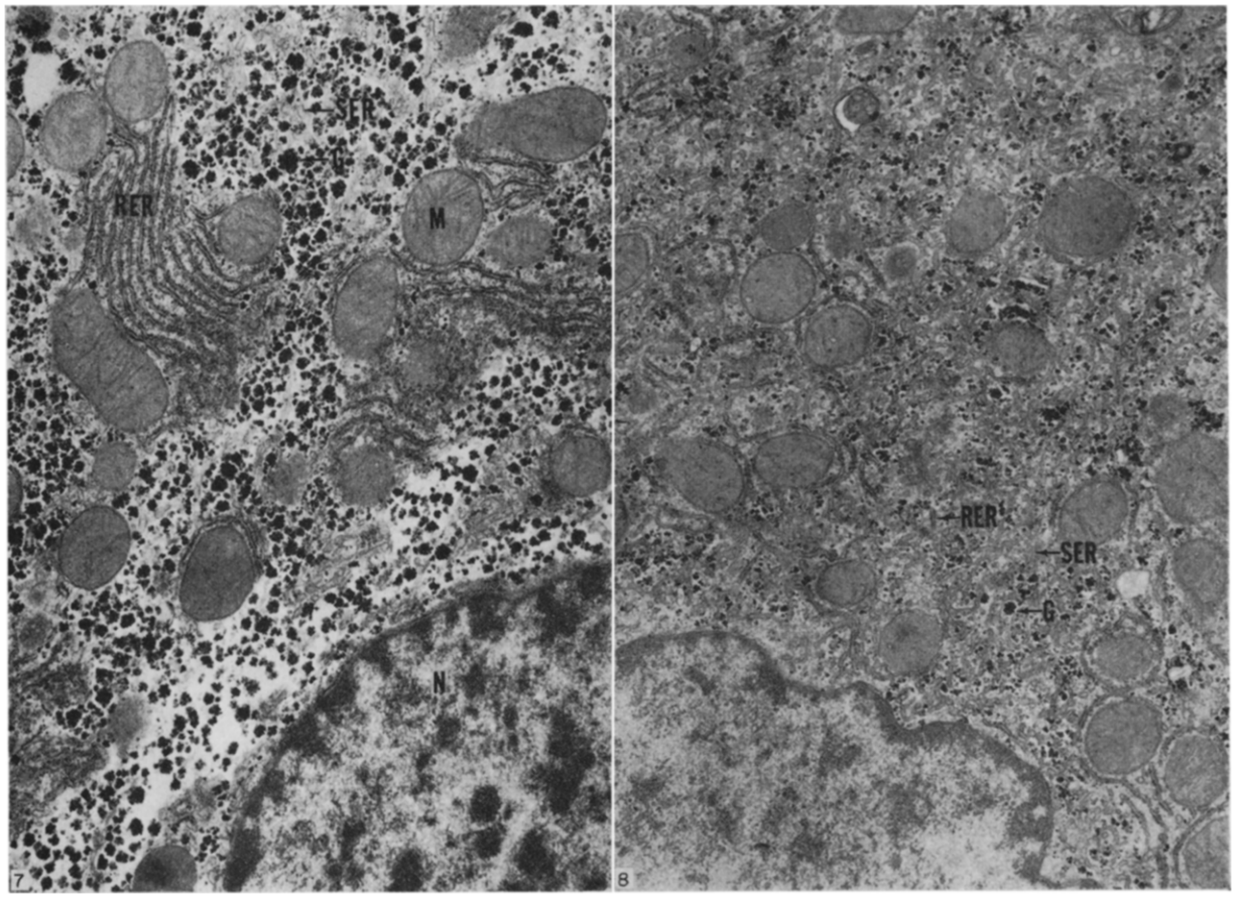

Fig. 7. Electron micrograph of a hepatic parenchymal cell from a control rat showing parallel arrays of rough endoplasmic reticulum (RER) cisternae. The smooth endoplasmic reticulum (SER) is distributed in association with glycogen (G) rosettes. Mitochondria (M) and nucleus (N) are normal in appearance. × 24,000.

Fig. 8. Electron micrograph of a hepatocyte from a rat administered 1% *dioxane for* 8 *months. The cisternae of the rough endoplasmic reticulum (RER) are mostly seen in isolated form, frequently surrounding the mitochondria. Smooth endoplasmic reticulum (SER) shows hypertrophy with a corresponding decrease in the glycogen (G) content. The nuclear surface shows minor indentations.* × 24,000.

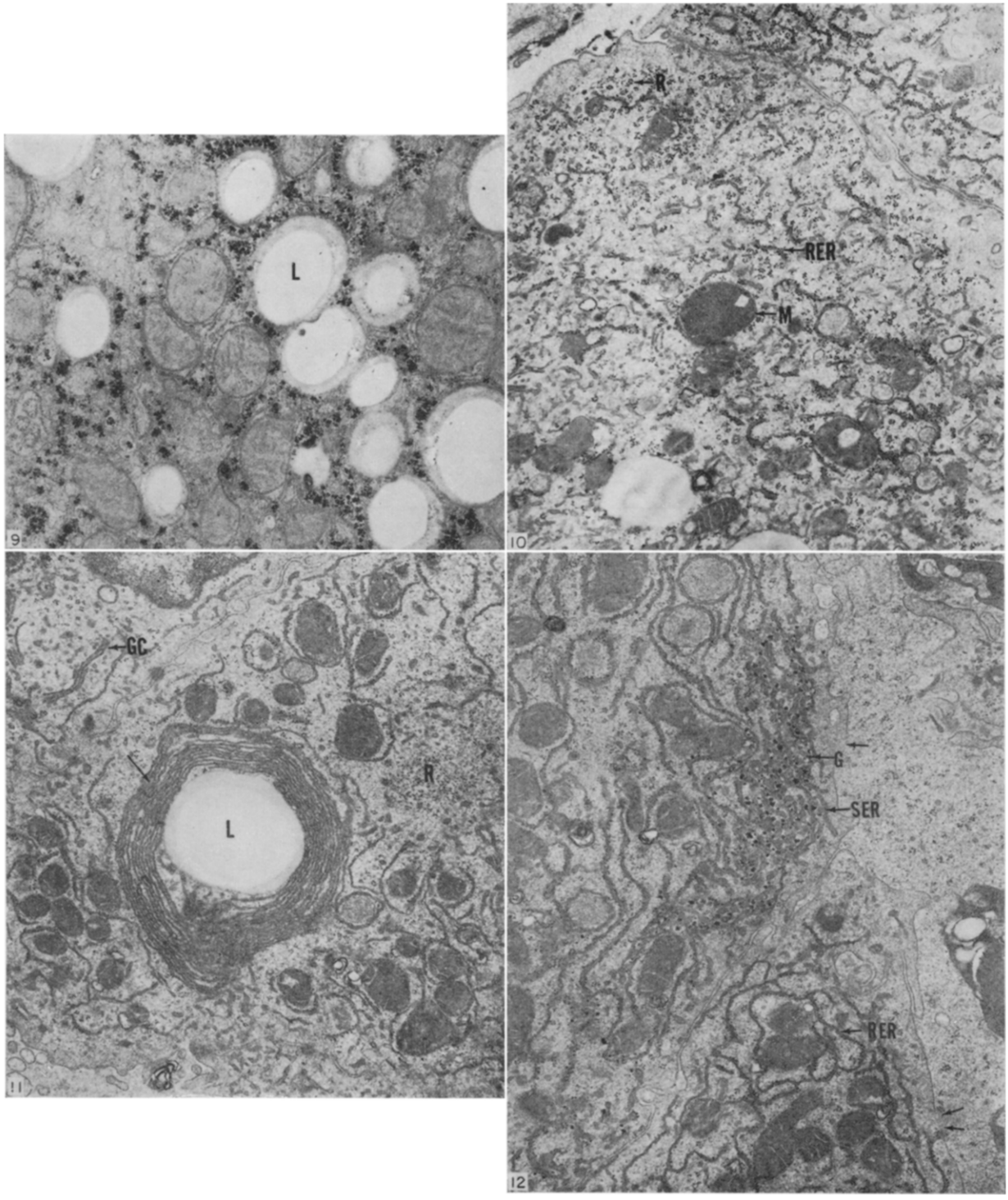

Fig. 9. *Electron micrograph of a hepatic parenchymal cell from a rat receiving* 1% *dioxane for* 8 *months, showing numerous lipid* (*L*) *droplets within the cytoplasm.* × 24,000.

Fig. 10. *Electron micrograph of hepatocellular carcinoma from a rat administered* 1% *dioxane for* 13 *months. The cisternae of the rough endoplasmic reticulum* (*RER*) *are seen only in the isolated form. The substance within the lumen of the cisternae has a high electron density. An increased number of free ribosomal particles* (*R*) *within the cytoplasm is seen. The mitochondria* (*M*) *show a very dense matrix. Glycogen particles are not seen in this section.* × 24,000.

Fig. 11. *Electron micrograph of hepatocellular carcinoma from a rat receiving* 1% *dioxane for* 13 *months showing an array* (*arrow*) *of concentric paired membranes enclosing a lipid* (*L*) *droplet. The Golgi components* (*GC*) *are poorly developed. A free ribosomal* (*R*) *rich area of the cytoplasm is noted.* × 26,000.

Fig. 12. *Electron micrograph of hepatocellular carcinoma from a rat receiving* 1% *dioxane for* 13 *months showing breaks in the plasma membrane* (*arrows*). *An area of smooth endoplasmic reticulum* (*SER*) *associated with glycogen* (*G*) *particles is seen. The substance within the lumen of SER tubules is quite electron-dense. The cisternae of rough endoplasmic reticulum* (*RER*) *show considerable branching.* × 31,200.

that the toxicity and carcinogenicity of dioxane cannot be imputed to a dioxane hydroperoxide which may have been present in the original material or may have been formed in prolonged contact with possible oxidizing additives in the tap water or by oxidation by air present in the water.

DISCUSSION

The hepatocarcinogenicity of dioxane to rats does not appear to be strain-specific since Wistar [1] as well as Sprague–Dawley rats, in the present study, have been shown to be susceptible. One Wistar rat out of 26 receiving 1% dioxane developed a transitional cell carcinoma of the kidney pelvis [1]. Of the 120 Sprague–Dawley rats now administered dioxane no kidney tumor was found. However, striking changes in the epithelial cells of the distal tubules in the kidney, similar to those now described after continuous administration of dioxane, have been observed under different experimental conditions: by treating rats [16, 17] or Syrian hamsters [18] with certain carcinogenic compounds such as nitrosamines, by feeding young rats a diet deficient in choline or methionine [19], or by injection of certain high-molecular gelatin preparations to rats [20]. These changes were interpreted in the experiments in which carcinogens were used as precancerous. In the experiments in which choline or methionine deficient diets were fed, a relationship between metabolism of the methyl group and development of the pathological changes of the kidney was suggested. The numerous giant cells seen in the kidneys of rats receiving dioxane at all dose levels are reminiscent of the findings of Levis and Nadai [21] that giant cells are induced by treating guinea pig kidney cell cultures with the carcinogen, nitrogen mustard.

The precancerous ultrastructural changes seen in the liver after administration of dioxane for 8 months were roughly intermediate in the progression toward the hepatocellular carcinoma obtained after administration for 13 months. The changes brought about by dioxane in the ultrastructure of the liver cell are in most respects comparable to those seen with a number of other hepatic carcinogens. Flaks [22, 23] has shown with 2-acetylaminofluorene, and Svoboda and Higginson [24] with aflatoxin B_1, dialkylnitrosamines, ethionine, lasiocarpine, 3′-methyl-4-dimethylaminoazobenzene, tannic acid and thioacetamide that the ultrastructural changes fall into a common pattern: fragmentation of the rough endoplasmic reticulum cisternae and gradual disappearance of their typical parallel arrays; detachment of the ribosomes and their dispersion in the cytoplasm; increase in the smooth endoplasmic reticulum; poorly developed, often empty vesicles of the Golgi complex; initial swelling of mitochondria or increase of intracristal space, abnormal disposition of the cristae, and these are often followed by deposition of electron-dense material (probably calcium) in the matrix; partial or almost total loss of glycogen; increase in lipid droplets, many vacuoles in close association with membranes; irregular contour of the nuclear membrane; breaks in the plasma membranes and irregularities in the relationship between membranes of adjacent tumor cells. Essentially, all these facets are seen in dioxane-induced hepatic carcinogenesis. A great many of the detached ribosomes in the cytoplasm were seen as polysomal aggregates, which was also noted in hepatic carcinogenesis by diethylnitrosamine [25]. Nucleolar changes were unremarkable in dioxane carcinogenesis.

Since MC is a well-known inducer of enzyme synthesis [26, 27, 28], and since MC pretreatment accentuates the toxicity of dioxane, it is reasonable to assume that a metabolite of dioxane (possibly of radical or carbonium ion nature) is responsible for the acute toxicity. While these experiments do not as yet provide a clue that the same metabolites are also responsible for the carcinogenic effects of dioxane, it is possible that this might be the case as it has been observed, e.g., with CCl_4 [29–33]. In the present study dioxane has been found to be a weak to moderate hepatocarcinogen; however, if an individual were repeatedly exposed to dioxane vapor and at the same time exposed to enzyme inducers in the environment, and/or in the diet [29, 34], these latter factors could increase the toxicity and possibly the carcinogenicity of dioxane.

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On the Functional State of Rat Liver Ribosomes Following Administration of the Carcinogen Dimethylnitrosamine

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Abstract—*The depolymerization of the polyribosomes, which takes place in the rat liver after a single dose of* 100 *mg/kg b.w. of the carcinogen dimethylnitrosamine, gives rise to a large amount of monoribosomes of the monomer type, as indicated by their susceptibility to be dissociated into* 40 *S and* 60 *S subunits by high ionic strength salt solutions. Tests on the capacity of these subunits to recombine into* 80 *S couples and to support the polyuridylic-primed polyphenylalanine synthesis have indicated that a fraction of these subunits is inactive. The ribosomes constituting the residual polysomes are found to be unaffected by the drug. The correlation between these effects and the alkylating properties of the dimethylnitrosamine metabolites is briefly discussed.*

INTRODUCTION

THE HEPATOTOXIC effects observed after a single dose of the carcinogen dimethylnitrosamine (DMN) concern mainly the protein synthesis machinery. A strong inhibition of protein synthesis appears within 30 minutes after administration of the drug [1, 2] and is associated with a large depolymerization of the polysomes [3, 4] and a parallel increase in the amount of the lighter components (mainly, 80 S units). These effects have been ascribed to several causes: breakdown or loss of messenger RNA, alteration of the interactions between ribosomes and template, or functional impairment of the ribosome itself. As for the last point, evidence has been produced regarding some functional and structural changes induced on the ribosomes by the nitrosamines [5, 6], as well as by other toxic compounds, which are able to produce quite similar effects on rat liver [7]. On the other hand, since the polyribosomes from DMN-poisoned rat livers support the polyuridylic-acid-primed synthesis to the same extent of the controls [3, 8, 9] it has been assumed that the ribosomes released from the polysomes in the course of the toxic action are functionally intact.

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In the present investigation, we have studied the functional state of both the monoribosomes observed *in vivo* after DMN administration and the ribosomes constituting the survival polysomes. We have observed that the monoribosomal population is mainly composed of monomers, a number of which are not able to support the polyU-directed polyphenylalanine synthesis, and that no functional defects seem to occur in the ribosomes enclosed in polysomal aggregates.

MATERIAL AND METHODS

Animals and treatments

Wistar male rats, weighing 200–250 g and fed a synthetic diet, were fasted 16 hr before being sacrificed by decapitation. The drug, diluted with sterile saline solution, was administered intraperitoneally at a dose of 100 mg/kg body weight and at appropriate times before sacrifice of the animals.

Chemicals

N-nitrosodimethylamine (DMN) was obtained from Eastman Kodak Co., U.S.A., and was redistilled before use.

ATP, creatinephosphate, creatinephosphokinase and polyuridylic (polyU) acid were purchased from Boehringer, F.R. Germany.

GTP was obtained from Fluka, Switzerland, and dithiothreitol (DTT) from Calbiochem Inc., U.S.A.

L-phenylalanine-^{14}C (specific activity 513 mCi/mM) was purchased from the Radiochemical Center, England.

Isolation of polyribosomes from rat liver

The livers were promptly removed and homogenized in 3 volumes of cold 0·25 M sucrose in TKM (Tris-HCl 50 mM, pH 7·6; KCl 25 mM; Mg acetate 5 mM) in a Potter–Elvejhem homogenizer by ten strokes of a loose-fitting teflon pestle. All subsequent operations were performed at 4°C. The homogenate was centrifuged for 15 min at 12,000 **g** and the post-mitochondrial supernatant was carefully removed after discarding the upper layer of lipid material. The post-mitochondrial fraction was made with 4% Triton-X-100 and 0·2% deoxycholate and layered on a discontinuous sucrose gradient, made with 10 ml of 0·5 M sucrose and 8 ml of 2 M sucrose, both containing rat liver cytosol antiRNAse factor [10].

Centrifugations were carried out in a Spinco rotor 60 Ti for 14 hr at 45,000 **g**; these long runs lead to an almost quantitative recovery of the lighter ribosomal components.

The pellets (that is the total polyribosomes) were stored at −80°C and dissolved in TKM buffer just before use.

Isolation of ribosomal subunits

Large and small ribosomal subunits were prepared by the procedure of Falvey and Staehelin [11] with minor modifications. The control subunits were obtained from normal polysomes after a preliminary incubation of 30 min at 35°C in a complete protein synthesis system in order to release a sufficient amount of ribosomes disassociated by a high ionic strength salt solution. In the case of DMN administration, the subunits were obtained from polyribosomes prepared from treated animals having omitted the pre-incubation step. Aliquots of normal and treated samples, prepared in a final volume of 5 ml of 0·5 M KCl, were layered on a 15–40% sucrose linear gradient containing Tris-HCl 20 mM, pH 7·6; KCl 300 mM; Mg acetate 3 mM; and DTT 5 mM. The runs were carried out in a Spinco rotor SW 27 for 14 hr at 20,000 **g**. The fractions containing the subunits were carefully separated and the pooled samples, suspended in 8 mM Mg acetate, were centrifuged at 55,000 **g** for 6 hr. The pellets were rinsed with deionized water and stored at −80°C. Just before use, the subunits were resuspended in Tris-HCl 20 mM, pH 7·6; NH_4Cl 100 mM; Mg acetate 5 mM; DTT 10 mM; at a concentration giving about 100 $A_{260\,m\mu}$/ml.

Sucrose density-gradient analysis of polyribosomes and isolated subunits

The ultracentrifugations were performed in 15–40% linear sucrose gradients prepared in two different saline buffers. The buffer indicated as "low salt" was Tris-HCl 50 mM, pH 7·6; KCl 25 mM; Mg acetate 5 mM; and buffer indicated as "high salt" was Tris-HCl 20 mM, pH 7·6; KCl 300 mM; Mg acetate 3 mM. The samples were prepared in 0·5 M KCl before layering them on the "high salt" gradient. All the runs were performed in a Spinco rotor SW 27, at 4°C, for the indicated length of time. The profiles were obtained by monitoring the effluents at 254 mμ in a continuous flow-analyzing apparatus (Uvicord LKB, Sweden).

In vitro *polyphenylalanine synthesis*

The cell-free incorporating system content per 0·2 ml was: Tris-HCl (pH 7·6) 4 μMoles; NH_4Cl 20 μMoles; Mg acetate 1 μMole; (2,4 μMoles when polyU was present); DTT 2 μMoles; ATP and GTP 0,2 μMoles each; creatine phosphate 2 μMoles; creatinephosphokinase 10 μg; post-microsomal supernatant (S_3) from rat liver 50 μl (equivalent to 1·5 mg of protein); pH 5 enzyme 50 μl (equivalent to 1 mg of protein); polyU 200 μg (when present); and L-phenylalanine-^{14}C 0·5 μCi.

The amounts of ribosomes or subunits, specified in the figure legends, were expressed as ribosomal RNA, determined according to Fleck and Munro [12]. The incubations were carried out at 37°C and, at various intervals of time, 60 μl aliquots were withdrawn, adsorbed on a Whatmann 3 MM paper disk, and processed according to Mans and Novelli [13].

The disks were dried and counted in 5 ml of diluted Permafluor (Packard Instrument Co., U.S.A.) solution with a Nuclear Chicago Mark 1 scintillation spectrometer with a counting efficiency of 62%.

Analysis of rRNA extracted from subunits

The extraction was performed according to Noll and Stutz [14]. The sedimentation analysis was carried out in a 5–40% linear sucrose gradient prepared in acetate buffer 10 mM

pH 5·1; NaCl 100 mM; and EDTA 1 mM in a Spinco rotor SW 27 at 26,000 **g** for 16 hr at 4°C.

Cesium chloride buoyant densities of the isolated subunits

The preparations of the particles and the determinations of their densities were carried out according to Hamilton [15].

RESULTS

The exposure of mammalian ribosomes to 0·3 M KCl and 3 mM Mg^{2+} is known to dissociate the ribosome into its constitutive large and small subunits, provided that the particle is not carrying peptydil-tRNA and mRNA. This behaviour, therefore, may be adopted as a test for ascertaining the functional state of the ribosome itself. In fact, both polysomal ribosomes and monosomes derived from nucleolytic polysomal degradation are stable in high ionic strength salt solutions.

The panels of Fig. 1 show the sedimentation profiles in "low salt" and "high salt" sucrose gradients of liver polyribosomes isolated from DMN-treated and control rats. In "low salt" gradients, the 120, 105, and 80 S peaks increase progressively in size at various intervals of time after treatment with the drug. In "high salt" gradients, however, the corresponding profiles are markedly modified by the conversion, for the most part, of the lighter fractions into 40 S and 60 S peaks. Evaluation of the size distribution patterns in "high salt" gradients with the aid of a curve resolver (Du Pont mod 310, U.S.A.) shows that the size of the 80 S peak does not change appreciably at various time intervals after treatment, while a 90 S peak, scarcely detectable in "low salt" gradients, becomes progressively more visible. The finding in "high salt" gradients of an 80 S peak is to be related to the presence of monoribosomes of monosomal type, including those formed by nucleolytic degradation of the polysomes. The 90 S peak, however, represents a tightly adhering "high salt"-stable ribonucleoprotein particle, probably composed of a monomer with a 40 S subunit [16].

On the basis of their behaviour in salt solution of high ionic strength, the ribosomes from DMN-treated rat livers can be divided into two different populations: (a) "high salt"-dissociable ribosomes, devoid of mRNA and peptydil-tRNA's, which progressively accumulate *in vivo* during the action of the drug; and (b) "high salt"-stable ribosomes constituting the polysomal aggregates and which are still engaged in translating the endogenous mRNA. As the dissociation of mammalian ribosomes by 0·3 M KCl and 3 mM Mg^{2+} gives rise to structurally and functionally intact subunits [11], the subunits obtained from the monomers formed *in vivo* after DMN treatment were tested for their capacity to reassociate into 80 S couples and to support the polyU-programmed polyphenylalanine synthesis. These subunits have been isolated from polyribosomes without any pre-incubation step. On the contrary, the control subunits, because of the scarcity of dissociable monomers in normal animals, were prepared from polysomes incubated in a complete protein synthesis system; in such a way, the polysomes were able to terminate and release their polypeptide chains. Therefore, the control subunits were derived from *in vitro* produced ribosomes (run-off ribosomes), while the DMN-subunits were isolated from *in vivo* formed monomers.

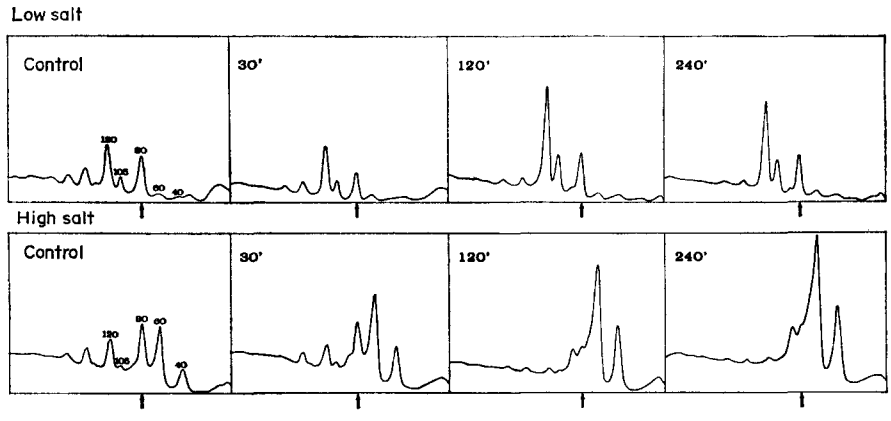

Fig. 1. Sedimentation profiles in low (a) and high (b) salt concentration sucrose gradients of total polyribosomal samples prepared at different time intervals (from 0 to 240 min) after a single dose of 100 mg/kg b.w. of DMN. Centrifugations were carried out in 15-40 per cent linear sucrose gradients, at 26,000 rpm, for 4 hr, at 4°C. Arrows mark the 80 S peak.

When mixed together in a suitable ionic condition, the large and the small subunits are able to reassociate into 80 S couples, in the absence of any artificial template, transfer RNA's and cofactors [11]. As shown in the sedimentation profiles shown in Fig. 2, a spontaneous association occurs in a large number of DMN and control subunits, but the 80 S peak generated by the recombined DMN subunits is less pronounced when compared to the controls. These findings indicate that a certain amount of subunits derived from DMN-induced monomers are not able to undergo a physical recombination with their partners subunits. The sedimentation profiles obtained by mixing the large DMN subunits with the small ones isolated from normal livers and *vice versa* (Fig. 2) show that, in both cases, the extent of the reassociation is intermediate between the values observed for either all normal or all DMN particles. The capacity of these subunits to support the polyU-directed polyphenylalanine synthesis is consistent with their recombination patterns. In fact, the incorporation values reported in Table 1 show that the capacity of DMN subunits to synthetize in the presence of an artificial messenger is about 30% less than that observed for control subunits and that a 20% reduction occurs in samples containing the mixed subunits.

These results indicate that a number of subunits isolated from the *in vivo*-formed DMN-induced monomers are no longer able to reassociate and to support the polyU-primed synthesis. Furthermore, the incorporation

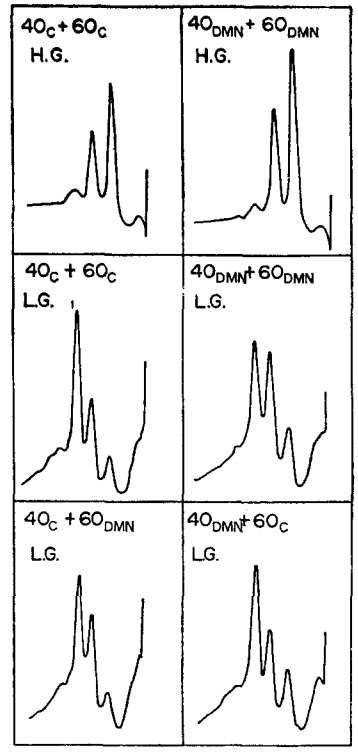

Fig. 2. Spontaneous reassociation into 80 S couples of isolated subunits from control and DMN-treated animals. Isolated 40 S and 60 S subunits suspended in Tris-HCl 20 mM, pH 7·6; NH_4Cl 100 mM; Mg acetate 5 mM; DTT 5 mM were mixed together and directly analyzed in "high salt" concentration sucrose gradients (H.G.) or incubated 20 minutes at 30°C and then analyzed in "low salt" concentration sucrose gradients (L.G.). The $A_{260\,m\mu}$ unit ratio for 40 S and 60 S subunits was 1:2·7 in "low salt" and 1:1 in "high salt" concentration. Centrifugations were carried out in 15–40% linear sucrose gradients at 26,000 g for 4 hr at 4°C.

Table 1. Polyuridylic acid-directed polyphenylalanine synthesis of the subunits isolated from DMN and control† polyribosomes*

Subunit species tested	rRNA input μg	Radioactivity cpm/assay	% activity‡
$40_{C\ (control)}$	10	486 ± 21	—
40_{DMN}	10	452 ± 36	—
60_C	10	6021 ± 979	—
60_{DMN}	10	4921 ± 701	—
$40_C + 60_C$	10 + 10	15218 ± 894	100
$40_{DMN} + 60_{DMN}$	10 + 10	11599 ± 1689	72
$40_C + 60_{DMN}$	10 + 10	12540 ± 1460	82
$40_{DMN} + 60_C$	10 + 10	12799 ± 1830	83

*The subunits have been isolated without any preincubation of the polyribosomes.
†The subunits have been isolated from polysomes incubated at 35°C for 30 min in a complete protein synthesis system.
‡The relative activities have been calculated by subtracting the endogenous activities of both subunit species.

Incubations and preparations of samples for radioactivity analysis were carried out as described in the Methods.
The molar ratio between small and large subunits was 2:1.
The results are expressed as the means of two separate experiments.

experiments performed by mixing normal and DMN subunits (Table 1) show that the functional defect following the drug action does not affect selectively either the large or the small subunit.

The ribosomes constituting the residual polysomes after DMN treatment, having been separated from the lighter components by two additional ultracentrifugations in "high salt" buffer, were tested for their incorporating capacity in the absence or the presence of artificial template (Table 2). In the absence of polyU, the DMN polysomes appear to be slightly less active than the control polysomes; this fact may be ascribed to the higher content of shorter polysomes in the treated samples. When polyU is present, DMN polysomes do not differ significantly from the control ones. On the basis of these results, the ribosomes composing the surviving polysomes appear to be fully competent to translate the natural template and to support the polyU-primed synthesis. In order to test the occurrence of some structural changes in a number of subunits isolated from DMN-induced monomers—particularly in the subunits which fail to recombine into 80 S couples—we carried out a zonal sedimentation analysis in sucrose gradient of their constitutive RNA's and determined the buoyant densities in CsCl of these subunits. The sedimentation profiles of the 28 S and 18 S peak did not show any difference between DMN and control subunits. Furthermore, both the subunits obtained after exposure to the drug sediment as symmetrical peaks, without any anomalous components, and their densities—1·61 for the large and 1·54 g/cm^3 for the small subunit—are similar to those observed for the normal subunits, prepared in the same way.

DISCUSSION

As described elsewhere [3, 4], the depression of liver protein synthesis, following a single large dose of DMN, is associated with the conversion of polysomal aggregates into lighter components. In the present investigation, this lighter fraction is shown to be largely made up of monoribosomes devoid of mRNA and peptidyl-tRNA, as indicated by their susceptibility to dissociate into subunits in salt solutions of high ionic strength. These monomers, therefore, behave like the run-off ribosomes which are released from normal polysomes incubated *in vitro* to terminate their nascent polypeptide chains. The same conclusion has been suggested by Vernie *et al.* [17] after their observation that, following a DMN treatment, a large fraction of 80 S ribosomes—77 S according to these authors—can be reversibly converted into 59 S particles at low Mg^{2+} concentration (0·5 mM), thus displaying the same features which have been found in the run-off ribosomes derived from normal polysomes. The large increase of monomers in the liver of DMN-poisoned rats suggests that the polysomal depolymerization is related to a block of the initiation step in the translation cycle, rather than a consequence of an extensive breakdown of the mRNA. This fact may be ascribed either to a functional impairment of the ribosome *per se* or to a failure of any of the multiple factors—still scarcely understood—which are involved in the initiation step. Ribosomes from DMN-treated livers appear to retain their functional capacities, since their polyU-primed synthesis attains values close to or even greater than those for the controls [3, 8, 9]. This inference, however, cannot be considered as definitive, being based on a generic criterion of

Table 2. Endogenous and polyuridylic acid-directed incorporating activities of the total polyribosomes and of the residual polysomes isolated from normal controls and DMN-treated rats

	No polyU added		PolyU added	
	Control	DMN	Control	DMN
Total polyribosomes	6642 ± 137	4291 ± 629	39669 ± 1399	36700 ± 1137
Residual polysomes	11105 ± 1125	8748 ± 174	45629 ± 743	47501 ± 323

Total polyribosomes were isolated as described in the text. Residual polysomes were isolated in "high salt" concentration buffer by two consecutive runs of 300,000 **g** each.

Incorporations and preparations of samples for radioactivity assay were carried out as described in the Methods.

The ribosomal concentration in the incorporating system was 10 $A_{260\ m\mu}$/ml. All values are expressed as counts/min/100 μg of rRNA and are the means of two separate experiments.

functionality which could be valid only for the artificial conditions of the *in vitro* assay.

However, according to this criterion and on the basis of our results, the ribosomes which constitute the residual polysomes after DMN treatment may be regarded as functionally intact, while a number of the monomers accumulating *in vivo* after exposure to the drug must be considered devoid of activity.

For what concerns the mechanism by which some ribosomes are inactivated by DMN, the property of this drug to methylate several cellular components must be emphasized. Villa Trevino [4] reported that the microsomal RNA of rats treated with ^{14}C-DMN shows a fast labelling rate and that 3 hr after the injection of 30 mg/kg b.w., the rRNA attains the highest specific activity without any difference in the labelling distribution between the 28 and 18 S peaks. According to these findings, it appears that DMN acts directly on the ribosomes, and that a functional deficiency of those ribosomes in which the degree of alkylation of their nucleic and protein constituents reaches a critical level should be expected. Anyhow, the defective ribosomes arising in the course of DMN action represent only a fraction of the total monomer population and, therefore, their contribution to the polysomal depolymerization is not to be considered as relevant. Several different conditions are known to promote polysome breakdown in rat liver, such as treatment with antibiotics, drugs (including non-alkylating compounds), starvation, thermal stress, etc. Therefore, in order to elucidate the mechanism of the polysomal depolymerization induced by DMN, more information is needed on the sequence of events in the reformation of polysomes and on the functional state and fate of the mRNA's released from polysomes.

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The Biological Behaviour of the Transmissible Venereal Tumour in Immunosuppressed Dogs

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Abstract—*The biological behaviour of the Transmissible Venereal Tumour of the dog (TVT) was studied in whole body X-irradiated dogs. The tumour showed a regular malignant biological behaviour in dogs which had been given 200 rads whole-body X-irradiation before tumour transplantation. In most of these dogs the TVT became ulcerated and metastasized. In control dogs the tumour showed a more benign and irregular biological behaviour which ranged from no tumour growth or growth followed by regression in some dogs, to metastases and ulceration in others. Sera, collected at intervals from experimental and control dogs, were tested for the presence of anti-TVT antibodies using the Indirect Membrane Immunofluorescence Test. Only minor differences in anti-TVT antibody activity could be detected between the two groups.*

INTRODUCTION

THE Transmissible Venereal Tumour (TVT) is a naturally occurring-coitally transmitted neoplasm of the dog which affects the external genitalia of both sexes. This neoplasm is also experimentally transplantable to immunocompetent allogeneic hosts [1].

Although several attempts have been made to investigate the reasons for the transmissibility of the TVT, the etiology and the mechanism of transmission of this neoplasm are as yet uncertain.

Some authors claim to have transmitted the disease using cell free extracts of the TVT [2, 3, 4]. On the other hand, it has been repeatedly demonstrated that highly significant and constant karyotypic differences exist between normal dog cells and the cells of this tumour [5, 6, 7]. These constant chromosome abberations are regarded as being suggestive of a cellular mechanism of transmission of this neoplasm.

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The malignancy of the naturally occurring and the experimentally transplanted TVT is very variable. The tumour is usually localized and after initial rapid growth its increase in size is slow. In a proportion of TVT affected dogs the tumour regresses spontaneously [1] whereas in others it can grow progressively, metastasize and kill the host [8, 9]. It has been shown that this tumour is antigenic in the dog. Complete regression of the tumour is followed by transplantation immunity [10] and passive transfer of immune serum prevents the growth of the experimentally transplanted TVT [11]. Furthermore, humoral antibody directed against the tumour cell membrane has been recently demonstrated [12].

If, as seems likely, the TVT is transmitted by the transplantation of cells, the immune reaction mounted against this tumour is primarily directed against its histocompatibility antigens.

The present study was carried out in order to elucidate the role played by the immune response in determining the course of this disease. The biological behaviour of the TVT was studied in 200 rads whole-body X-irradiated

dogs. It could be demonstrated that the tumour showed a regular malignant behaviour in X-irradiated dogs as compared to a more benign and variable behaviour in non-irradiated animals.

MATERIAL AND METHODS

Outline of the experiment

Eight X-irradiated and seven control dogs were observed over a period of eight months after the transplantation of the tumour. One day before TVT transplantation the dogs of the experimental group were irradiated. On the following day all dogs were injected subcutaneously into each inner thigh with $4{\cdot}5 \times 10^7$ trypsinized TVT cells. The dogs were carefully inspected at weekly intervals and eight months after tumour transplantation euthanasia was carried out on all dogs and post-mortem examinations performed.

Origin of tumour

The transmissible venereal tumour was obtained from a 3-year-old male Labrador dog which contracted the disease in Malaya. The tumour was located on the base of the penis and appeared as a nodular lump. Histological examination, chromosome analysis and the successful transplantation of the neoplasm confirmed the clinical diagnosis.

Preparation of cell suspensions

Single cell suspensions were prepared by the trypsinization of solid tissues at 37°C. Trypsin was used as a 0·25% solution in Hank's buffered salt solution (BSS) ph 7·2–7·4, to which a few drops of a 0·02% solution of deoxyribonuclease in Hank's BSS (DN-C Deoxyribonuclease I, Sigma Chemical Company, London, England) were added. After trypsinization the cells were washed and resuspended in Hank's BSS. Cell counts were performed in an improved Neubauer counting chamber. In all experiments 90–100% of the cells were viable as assessed by trypan blue exclusion.

Experimental animals

Adult male and female dogs of mixed breeds were used and fed on a proprietary diet with water *ad libitum*.

Transplantation of the tumour

Serial transplantation of the TVT was carried out by subcutaneous injection of single-cell suspensions in Hank's BSS. For routine transplantation dogs were usually injected with $1\text{–}2 \times 10^8$ cells into each of two different sites.

Sera

Samples of blood were collected from the cephalic vein; serum was removed and stored at −20°C.

The indirect membrane immunofluorescence test

The indirect membrane immunofluorescence (IMIF) test was carried out essentially as described by Möller [13].

In all tests dog sera were used undiluted and the treatment of the cells with fluorescein isothiocyanate conjugated to rabbit anti-dog Ig (RAD/FITC) (Nordic Pharmaceuticals and Diagnostics, Tilburg, The Netherlands) was carried out with fluorescein conjugates at a dilution of 1 : 5.

The controls which were included in each test were: (a) cells incubated in Hank's BSS; (b) cells incubated in the pretransplantation serum.

One hundred cells were counted in every sample and the results expressed as the fluorescence index (FI).

$$\text{Fluorescence index} = \frac{\left(\begin{array}{c}\%\text{ negative cells}\\ \text{incubated in normal dog serum}\end{array}\right) - \left(\begin{array}{c}\%\text{ negative cells}\\ \text{incubated}\\ \text{in test serum}\end{array}\right)}{\begin{array}{c}\%\text{ negative cells incubated}\\ \text{in normal dog serum}\end{array}}$$

Background fluorescence (i.e., percentage of fluorescing cells after treatment with Hank's BSS or normal dog serum) of up to 20% was regarded as acceptable. Cell suspensions with fluorescence indices of 0·30 or more were regarded as showing a positive reaction.

X-irradiation

Anaesthetized dogs were given 200 rads whole-body X-irradiation under the following conditions: a Tomaximar therapeutic X-ray machine with an output of 220 kV and 15 mA was used with the beam filtered through 1 mm aluminium at a focal distance of 100 cm. The mean output of the machine was 10 r/min, giving a total irradiation time of 20 min. Whenever large dogs were used, first the anterior and then the posterior half of the body was irradiated. The unexposed half was shielded with 2 mm lead.

Criteria used for the assessment of the biological behaviour of the TVT

(1) *Metastasis.* Growth of the tumour in a site other than the transplantation site was regarded as a metastasis. During the experiment the submaxillary, pre-scapular, axillary, inguinal and popliteal lymph nodes of all dogs were palpated at weekly intervals; their increase in size and the appearance of a nodule distant from the injection site was recorded. The exact nature of these nodules was established by post-mortem examination.

(2) *Regression.* A regression was recorded when the following conditions were satisfied:

(a) the disappearance of the two existing tumour nodules;

(b) the decrease in size of both nodules not followed by growth which exceeded the previous maximal volume of the tumour;

(c) one of the two tumour nodules following the pattern described in (a) and the other that described in (b).

(3) "*Take*". A tumour nodule which could be palpated in at least one of the two transplantation sites was recorded as a "take".

(4) *Ulceration.* A non-healing wound of the skin overlying one or both primary tumours was recorded as ulceration.

Statistical analysis of the differences in the incidence of these criteria between the two groups was performed using the 2×2 contingency table [14].

(5) *Tumour growth.* Three perpendicular diameters of every tumour nodule were measured at weekly intervals for 95 days using calipers. From the three diameters, after allowing for a double 1·5 mm skin thickness, the volume of the tumour was calculated as

$$= \frac{\pi}{6} (\text{mean diameter})^3$$

The volume of each nodule was either interpolated or extrapolated wherever necessary in order to obtain the volumes for 10-day intervals. The means of the logarithms of the tumour volume were obtained for each group at 10-day intervals between 25 and 95 days post-transplantation. Because of the heavy ulceration of the tumour nodules it was not possible to measure tumour volume beyond 95 days post-transplantation.

The homogeneity of the variance within the control and the experimental groups was examined using the Bartlett test [15]. No significant differences were found within the groups and consequently pooled variance was used for each group. The standard error of the means was calculated for $p = 0{\cdot}05$. The significance of the difference between the mean variance of the two groups was calculated using the Fisher test [15].

RESULTS

The biological behaviour of the TVT in immunosuppressed dogs

The biological behaviour of the TVT in immunosuppressed dogs was assessed in the two groups as follows:

(a) The incidence of metastases, regressions, "takes", and ulcerations of the skin overlying the tumour was recorded.

(b) The growth of the tumours was compared in the immunosuppressed and control dogs.

The results of (a) are summarized in Table 1,

Table 1. *The biological behaviour of the TVT in X-irradiated and control dogs*

	X-irradiated dogs	Control dogs	Probability
Metastases	6/8	1/7	$0{\cdot}050 > p > 0{\cdot}025$
Regressions	0/8	3/7	*NSD
"Takes"	8/8	5/7	NSD
Ulcerations	7/8	1/7	$0{\cdot}050 > p > 0{\cdot}025$

*NSD: No significant difference.

and demonstrate that the incidence of metastases in the X-irradiated group was significantly higher than that of the control group ($0{\cdot}050 > p > 0{\cdot}025$). Ulcerations of the skin overlying the tumour were also significantly more frequent in the experimental group ($0{\cdot}050 > p > 0{\cdot}025$). On the other hand, the incidence of regressions and "no takes" was higher in the control group. These latter differences, however, were not statistically significant.

The growth of the TVT in the two groups was used as a further criterion for the assessment of the biological behaviour of the tumour. In Fig. 1 the means of the logarithms of the tumour volume with probable error ($p = 0{\cdot}050$) were plotted against time (days after transplantation). The means of the logarithms of the tumour volume in the X-irradiated group were signi-

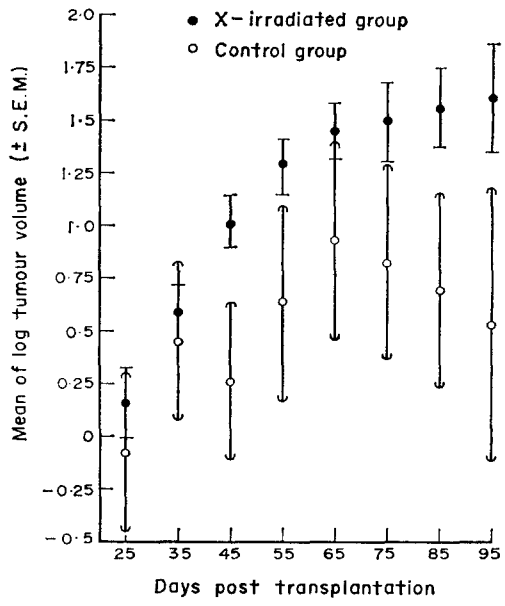

Fig. 1. *The growth of the Transmissible Venereal Tumour in X-irradiated and control dogs.*

ficantly greater than those of the control group at 45, 55, 75, 85 and 95 days after transplantation.

The mean variance of the control group was significantly greater than that of the X-irradiated group ($p < 0{\cdot}005$).

The detection of anti-TVT antibody

Sera from the X-irradiated and the control dogs were collected at intervals after transplantation and their anti-TVT activity was assessed using the Indirect Membrane Immunofluorescence (IMIF) test.

The results summarized in Table 2 demonstrate that no positive fluorescence indices (indices above 0·30) were obtained in either group in sera collected between 17 and 24 days post-transplantation. At 35 to 47 days post-transplantation sera from three out of eight X-irradiated dogs and five out of seven control dogs contained anti-TVT activity. All the sera collected at 52 days or more after transplantation contained, with one exception, anti-TVT antibody. The serum which did not contain anti-TVT activity was collected from a dog in which the tumour did not "take".

The state of the tumour at serum collection is also recorded in Table 2. At 52 days or more after transplantation anti-TVT activity could be detected in the serum of dogs irrespective of whether the tumour was growing progressively or regressing.

The course of the experiment and pathological findings

During the course of the experiment most

Table 2. *The detection of anti-TVT antibody at intervals in X-irradiated and control dogs*

Dog No.	17–24 days P.t. F.I. of sera		State of tumour	35–47 days P.t. F.I. of sera		State of tumour	52–61 days P.t. F.I. of sera		State of tumour	100–240 P.t. F.I. of sera		State of tumour
X-irradiated Group												
62	0·01	0·02	P	0·43	0·21	P	0·63	0·61	P	0·93	0·82	M P
64	−0·02	−0·02	P	0·03	−0·01	P	0·81	0·59	P	—	—	M U
73	0·00	−0·05	P	0·72	0·40	P U	—	—	P U	0·78	0·87	M U
75	−0·06	0·06	P	0·64	0·46	P	—	—	U	0·76	0·97	U
77	0·03	−0·11	P	0·21	−0·07	P	—	—	P	0·69	0·88	M U
82	0·02	0·03	P	0·13	0·02	P	0·53	0·82	P	0·93	0·73	M U
83	−0·01	−0·02	P	0·75	0·46	P	0·76	0·89	P U	0·90	0·61	M U
117	−0·03	−0·01	P	0·03	0·08	P	0·21	0·14	P	0·65	—	U
Control group												
65	0·05	0·13	P	0·81	0·83	R	0·80	0·72	R	0·80	0·84	R
74	−0·01	−0·01	P	0·92	0·78	P	—	—	M P	0·72	0·93	M
76	0·21	0·22	P	0·88	0·80	P	—	—	P	0·92	0·93	U
84	0·09	0·04	P	0·12	0·01	R	0·70	0·81	R	0·95	0·81	R
116	0·03	0·03	P	0·46	0·50	R	0·40	0·37	R	0·57	0·53	R
118	0·03	0·05	N.T.	0·02	0·03	N.T.	0·11	0·07	N.T.	0·13	0·08	N.T.
119	0·05	0·13	N.T.	0·52	0·44	N.T.	0·32	0·41	N.T.	0·34	0·32	N.T.

P.t. = Post transplantation R = Tumour regressing or regressed P = Tumour growing progressively
N.T. = No take M = Tumour metastasized U = Tumour ulcerated
F.I. = Fluorescence Indices
(indices between 0·30 and 1·00 are regarded as a positive reaction)

of the dogs in both groups were clinically in good health and were not severely affected by the presence of the tumour. Some animals from the X-irradiated as well as the control group lost weight during the period of observation.

The appearance of metastases, as detected by palpation, occurred between the third and fifth month post-transplantation. The size of the metastases at the end of the experiment varied from 1·5 cm to 7 cm in diameter. Ulceration of the skin overlying the tumour appeared in the X-irradiated animals between one-and-a-half and five months after transplantation. Following ulceration the tumour nodules became partially necrotic. The ulcerations appeared mainly in the larger tumours and seemed to be a result of the expansion of the tumour nodules. In all dogs in which metastases could be detected they appeared in the inguinal lymph nodes and replaced most of the lymphoid tissue. In three different cases, the iliac lymph node, the axillary lymph node and the spleen were also affected.

DISCUSSION

In the present study it was demonstrated that 200 r whole body X-irradiation before the transplantation of the TVT caused a marked increase in the malignancy of the tumour. The immunosuppressed dogs showed a significantly higher incidence of metastases ($0{\cdot}050 > p > 0{\cdot}025$) and a significantly higher growth rate ($p = 0{\cdot}050$) than the control group. Other differences between the two groups in the biological behaviour of the TVT included a significantly increased incidence of ulcerations of the skin overlying the tumour ($0{\cdot}050 > p > 0{\cdot}025$) and a slightly decreased incidence of regressions and no "takes" in the X-irradiated group as compared to the control group. The fact that temporary immunosuppression before the transplantation of the TVT results in increased malignancy of the tumour and a higher growth rate demonstrates that the immune response inhibits the growth and the spread of this neoplasm in immunocompetent animals.

The biological behaviour of the TVT among the dogs of the control group was very irregular and ranged from "no takes" and regressions to metastases. Previous studies of the transplanted and the naturally occurring TVT also showed the variable behaviour of this neoplasm [1, 8, 9]. Furthermore, the variable biological behaviour of the tumour in the control group could be demonstrated by the fact that the mean variance of the logarithms of the tumour volume was significantly greater in this group [$p < 0{\cdot}005$] than that of the experimental group. On the other hand, the biological behaviour of the TVT in the X-irradiated group was fairly regular. The tumour grew progressively and metastasized in a high proportion of the dogs. The different pattern of the disease in the two groups can be attributed to the immunosuppression. It seems, therefore, that the variable biological behaviour of the TVT in the control group is the result of differences in the immune response of individual dogs to the TVT.

Should the TVT be indeed transmitted in nature as well as in the laboratory by the transplantation of cells [5, 6, 7], it would be a naturally occurring allogeneic tumour. In such a case the degree of disparity between the histocompatibility antigens of the tumour and those of the host might determine the intensity of the immune response and, consequently, the biological behaviour of the tumour in a given host.

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Transplantable Immunoglobulin-Secreting Tumors in Rats—III

Establishment of Immunoglobulin-Secreting Cell Lines from LOU/Wsl Strain Rats*

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Abstract—*Continuous cell lines were derived from a number of transplantable immunoglobulin-producing tumors of rat origin.*

The cultured cells retained the morphological and biosynthetic properties of the original tumor cells and are more capable of inducing tumors comparable to the original growths when injected intraperitoneally or subcutaneously into histocompatible animals.

INTRODUCTION

IT HAS been mentioned in the preceding paper that the LOU/Wsl strain of rats displays an unusually high incidence of spontaneous tumors originating in the ileocecal lymph node [1].

By histopathologic criteria, these tumors might be classified as reticulum cell sarcomas or undifferentiated malignant lymphomas of Type A according to Butler (The Anderson Hospital classification [2]). They might equally well be described as immunocytomas, in the sense that, at least in the majority of the cases, their cells are actively engaged in the biosynthesis and secretion of monoclonal immunoglobulins or fragments thereof. Sera of animals carrying such tumors usually show the presence of a homogeneous immunoglobulin component in the β or γ area, and frequently a Bence-Jones protein is detected in the urines. Further details concerning the biochemical aspects of this immunoglobulin production, together with a discussion on problems of nomenclature, will be found in the other papers of this series [1, 3].

As already described [1], these immunoglobulin-producing tumors readily lend themselves to serial transfer in histocompatible rats. The present article describes the establishment of long-term cultures, which were originally planned with two major aims in mind. Firstly, it was thought that such cultures would provide a valuable tool for the unmasking of a putative oncogenic virus. In this respect immunocytoma cells offer the unusual advantage of leaving no doubt concerning their true origin and identification, as they are readily recognizable by the

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unique monoclonal immunoglobulin which they secrete. Secondly, such cultures should furnish large amounts of pure, molecularly homogeneous, rat immunoglobulins, which should be valuable material for the study of the biosynthesis and structure of these proteins. Work along these lines is currently in progress.

MATERIAL AND METHODS

The procedure for establishing cell-line cultures from LOU/Wsl immunocytomas was essentially the same as that previously described for the long-term culture of hematopoietic cells of human origin [4, 5].

Preliminary attempts involved cells from solid tumors as the starting material. It was found that the problem of dealing with necrotic material could be minimized by removing the transplanted neoplasm before it had reached a volume of about 1 cm^3. Fat and fibrous tissue were removed with care, the tumor was cut into small pieces, and the cells were teased apart by stirring in 150 ml saline containing 0·25% trypsin.

In subsequent experiments it was found that ascites fluid provided a more suitable starting material. Ascites forms of the rat tumors were produced by intraperitoneal injection of cancer cells, as described elsewhere [1]. All tumors lent themselves well to this form of passage, except Tumor S 207, for which a solid graft had to be used for the establishment of the corresponding cell line.

In general, the ascites fluid was harvested one week after intraperitoneal grafting. The fluid was aspirated using a heparinized syringe. Usually from 20 to 30 ml were obtained, yielding about 5×10^8 viable tumor cells. After 10 minutes of low-speed centrifugation at room temperature, the sedimented cells were washed once with RPMI 1640 Culture Medium. They were then suspended in the same medium supplemented with 20% of fetal calf serum. Each culture bottle received 5×10^7 cells, for a total volume of 50 ml. If cells were available in excess of this amount, they were stored for further use by suspending them in a 10% solution of dimethylsulfoxide in complete culture medium, followed by freezing in liquid nitrogen.

The best results were obtained when the cell concentrations were maintained between 5×10^5 and 10^6 cells per ml. For this purpose cells were counted after centrifugation every 10 days, and suitable adjustments were made when resuspending them in freshly prepared medium. In between, the cultures were fed every third day by adding 25% (vol/vol) of fresh medium. Additional fresh medium was provided if the glucose level of the culture was found to fall below 1·5 mg/ml.

RESULTS

Until now, ten different rat immunocytomas have been established as long-term suspension cultures of lymphoblastoid cell lines (Table 1).

A two-phase pattern could be distinguished during these cultures.

During a first period, which usually exceeded six weeks, the total number of viable cells per flask would fluctuate about its original value, undergoing a succession of wide and erratic changes (Fig. 1). At that time the smears

Table 1

Cell line	Original tumor	Secretion product
1	S203	*Ig*(*g*−1)*
2	S204	Light chains, type κ
3	S205	*IgA*
4	S207	*Ig*(*g*−2)
5	S208	*Ig*(*g*−1)
6	S208	Light chains, type κ
7	S209	none
8	S210	Light chains, type κ
9	S211	Light chains, type κ
10	IR3	none

*$Ig(g-1)$ and $Ig(g-2)$ are used as provisional designations for two different (sub)classes of rat immunoglobulins whose relationship to known mammalian *IgG* subclasses and to each other is the subject of current investigation at this laboratory.

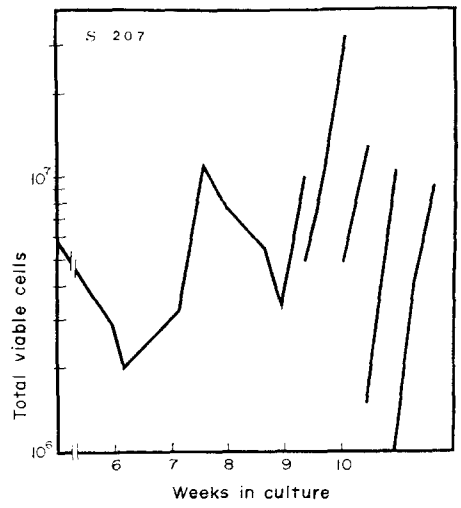

Fig. 1. Pattern of kinetic studies of S204 and S207 cell culture for population growth.

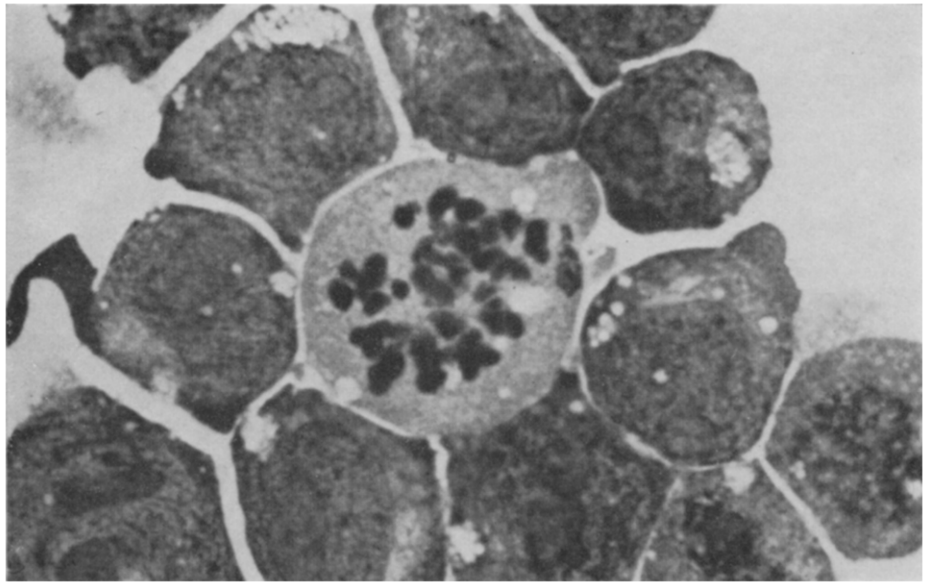

Fig. 2. *Smear preparation of No.* 4 *cells (May-Grümvald-Giemsa stain).*

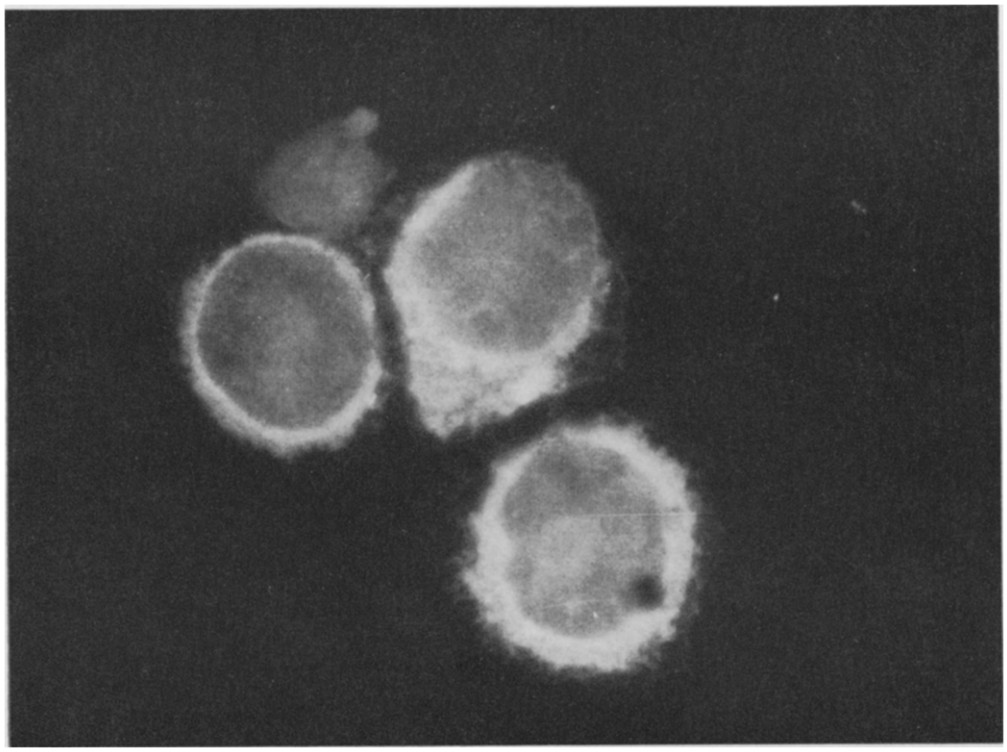

Fig. 3. *Immunofluorescence photomicrograph of No.* 5 *cells, showing specific cytoplasmic staining for rat immunoglobulin.*

(to face p. 260)

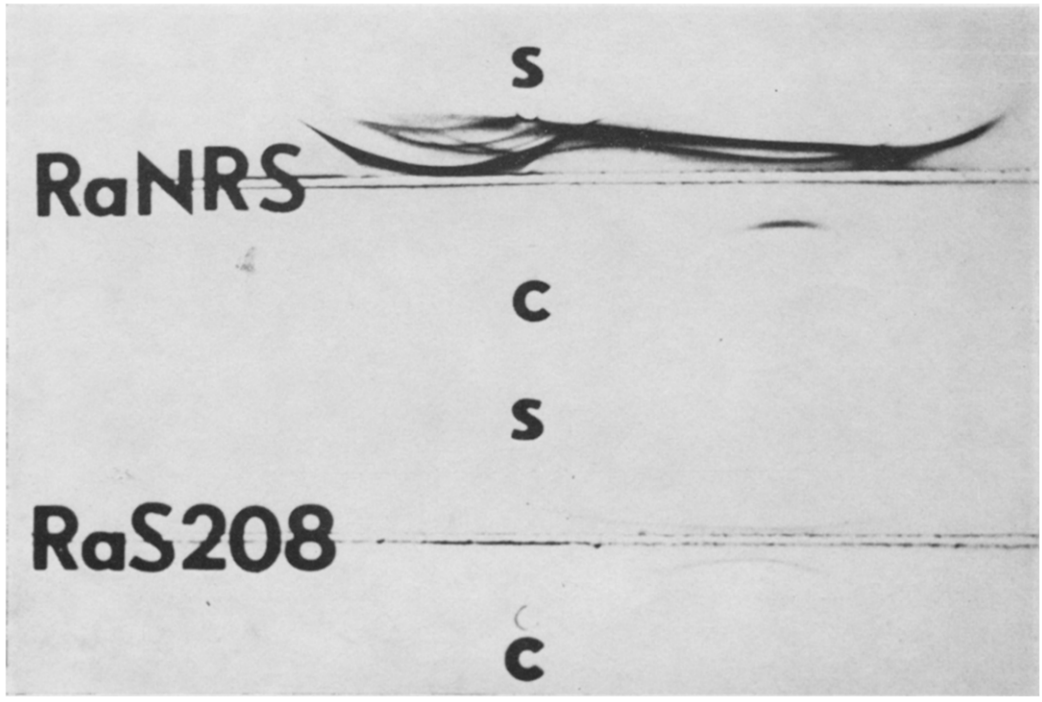

Fig. 4. Immunoelectrophoresis showing, by comparison with a normal rat serum (S), the S208 type immunoglobulin in the culture medium (C). RaNRS: Rabbit anti-normal rat serum. RaS208: Rabbit anti-S208 immunoglobulin.

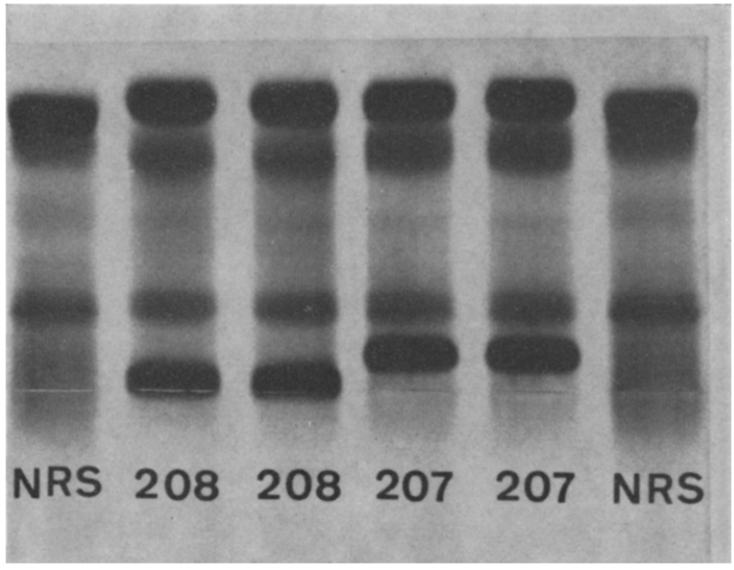

Fig. 5. Agarose electrophoresis showing, by comparison with normal rat serum (NRS), the serum of tumor-bearing animals grafted with S208 or S207 immunocytoma cells from solid growths (at left) or cell cultures (at right).

showed many dead cells and debris, whereas only a few mitoses were found. Most of the living cells were very similar in appearance to the original tumor cells, but the cultures also contained many macrophages, small lymphocytes, and even some polymorphonuclear-like cells. The glass surfaces were covered by a discontinuous layer of fibroblastic cells. Most of the cells, however, were found to float freely in the medium or to form isolated clumps.

During a subsequent stage the population was seen to expand exponentially with a constant doubling time, at least if optimal environmental conditions were provided. At this stage the cells tended to form large clumps and their metabolism resulted in rapid acidification of the medium, so that they had to be supplied with fresh medium every day. Smears stained with May-Grünwald-Giesma showed cells resembling primitive large lymphoblasts, with a high nucleocytoplasmic ratio, finely dispersed chromatin, a prominent nucleolus, and a strongly basophilic cytoplasm (Fig. 2). Some of the cells had several nuclei, and mitoses were relatively frequent. In general these cells resembled the lymphoblastoid elements from human hematopoietic cell lines described by other workers [4, 6].

At the exponential stage, the biosynthesis and secretion of immunoglobulins could be demonstrated by a variety of techniques. Washed and centrifuged cells were found to stain with fluorescein-labeled antisera to rat immunoglobulins. The specific apple-green fluorescence was seen to have a granular distribution in the cytoplasm (Fig. 3). In another experiment, an exponential culture of line No. 5 (Table 1) was centrifuged two days after being transferred to fresh medium, and the supernatant was concentrated 25 times by precipitation with half-saturated ammonium sulfate. This material was analyzed by immunoelectrophoresis and was shown to consist of proteins precipitable by rabbit antiserum to whole rat serum proteins, as well as by rabbit antiserum specific for the heavy chains of the subclass of rat Ig to which the original S208 protein belonged (Fig. 4).

Quantitative data on the rate of secretion of rat Ig by the cell line from Tumor S208 were obtained by measuring the concentration of this protein in the culture medium. For this purpose the single radial immunodiffusion method of Mancini [7] was used, employing a specific antiserum. It was found that a culture with an initial count of 5×10^5 per ml produced 43·9 μg of Ig per ml over a period of three days.

Samples containing 25×10^6 cells from cultures that had been exponentially expanding for over 6 months were inoculated intraperitoneally into adult female (August/Cen female $\times$ LOU/Wsl male)F_1 hybrid rats, using two recipients for each experiment. Cell lines No. 1 (S203), 4 (S207), 5 (S208), 6 (S208 BJ) and 8 (S210) were tested in this way. In all animals inoculated, a tumor producing a monoclonal immunoglobulin indistinguishable from that of the original tumor appeared after one to two weeks (Fig. 5). These ascites tumors have now been transferred serially over more than 10 passages and have exhibited all the features typical of the original tumors. All the cultured cell lines were found to give rise to tumor nodules when inoculated subcutaneously into histocompatible hybrids in the same dose as employed for the intraperitoneal tests.

Histological examination showed no difference between nodules produced with the cell lines and those obtained by serial transfer of the original tumor.

The experiments described in the preceding section seem to leave little room for doubt that the cell lines studied in the present work truly represented the original malignant immunoglobulin-producing cells of the tumors from which they were initially derived.

With these cultures it would seem that we now dispose of a valuable starting material for the studies on immunoglobulin-producing tumors enunciated in the introduction to the present paper. Similar investigations on material of mouse origin have been in progress at other laboratories for a number of years. The rat material will probably prove just as rewarding with the additional advantage that the tumors studied here have all appeared spontaneously, at a high incidence, in a special strain of inbred rats.

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The Organization of the Plasma Membrane in Mammalian Cells*

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Abstract—*The plasma membrane in animal cells consists of a lipid bilayer and of particulate protein-glycoprotein entities which may span the membrane width. The fluidity of the lipid phase and other factors may favor the motion and the rearrangement of the particulate entities. The association within the liquid-like lipid phase of identical or quasi-equivalently related proteins might result either in oligomeric complexes or in bidimensional lattices of repeating particles.*

THE ORGANIZATION of cells into tissue and the regulation of the "social behavior" of cells are mediated by two main functions, that of transport and that of cell contact. These functions are essentially dominated by the plasma membrane which is the main site for processing signals for cell recognition and homeostatic control.

The mechanism of such regulation, which is altered in the neoplastic transformation, is still obscure, primarily because the precise molecular arrangement of the membrane components in the plane and within the thickness of the membrane is not fully clarified.

In comparison with the limited variety of efficient designs available for the assembly of biological containers such as helical tubes of icosahedral shells, consisting of a well-defined number of identical or equivalently related protein molecules, the principle of structuration of biological membranes, in particular of the plasma membrane in animal cells, is not readily apparent.

The plasma membrane is an infinite boundary separating two asymmetrical environments or distinct phases and consisting of a great variety of chemical components including different molecular species of lipids, proteins and carbohydrates, held together by several types of interactions, primarily non-covalent.

Yet, certain progress in the field of membrane structure has been made, and in recent times most of the established generalizations on the membrane model have been reconsidered. A more direct experimental approach suggests new ways of thinking about the principles of plasma membrane design in animal cells.

Transverse heterogeneity of the plasma membrane

An essential feature of the plasma membrane architecture concerns the existence of a transverse polarity based on stereospecific asymmetrical localization of constituents. Asymmetrical membrane models had already been deduced from the earliest studies of Robertson [1] and of Sjöstrand [2].

A number of cytochemical and ultrastructural studies on intact cell surfaces and on isolated plasma membranes has further shown that the outer and inner membrane surfaces are non-identical in composition and structure.

In particular one aspect of this asymmetry is the distribution of oligosaccharides on both surfaces of the membrane. The asymmetric distribution of oligosaccharides has been studied using "positively" charged colloidal iron staining, which has revealed that the carboxyl groups

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of *N*-acetyl-neuraminic acid are exposed at the outer surface of the plasma membrane [3, 4].

Furthermore, using saccharide-binding concanavalin A and ricin labelled with ferritin, the non-reducing sugar residues reacting with the lectins are found to be exposed exclusively at the outer surface of the plasma membrane of various cell types [5]. In contradistinction, the protein "spectrin" has been localized on the inner surface of the erythrocytes cell membrane by specific ferritin-conjugated antibodies [6].

The structural polarity of membrane constituents and the vectorial flow of matter are also demonstrated by the localization of some enzymic end-products as revealed by electron microscopic cytochemistry on isolated plasma membrane. For the nucleoside phosphatases activities, according to the type and region of the cell membranes, the reaction products were found either at the outer [7] or at the inner surfaces of the membrane element [8, 9].

Cytochemical methods and electron staining techniques almost exclusively could visualize chemical or/and structural features exposed either on the outer or inner membrane surface. However, by the same methods no direct nor consistent demonstration of a discontinuity or asymmetrical heterogeneity within the plasma membrane core could be provided. This limitation was particularly disappointing since the existence of a complex molecular arrangement of lipid and protein components within the membrane element could be deduced from the results of other biochemical and physical methods. For example, evidence compatible with asymmetrical localization of cholesterol at the external side of the membrane bilayer has been obtained from X-ray diffraction analyses of myelin [10].

Furthermore, by selective chemical labels in combination with specific phospholipase action, evidence of the asymmetrical concentration of sphingomyelin and phosphatidyl choline in the external side of the membrane, and of phosphatidyl serine and phosphatidyl ethanolamine in the inner cytoplasmic half of the bilayer, has been provided [11].

Advances in the field of molecular arrangement of membrane proteins have been realized by the technique of membrane inversion (inside-out membrane vesicles, or right-side-out membrane vesicles) allowing the application of proteolytic selective digestions respectively to the inner or to the outer surface of the plasma membrane vesicles [12, 13, 14].

The accessibility of the membrane proteins either from the exterior or from the interior of the plasma membrane has also been tested by the use of a selective label, the radioactive acylating agent formyl-methionyl sulfone methyl-phosphate, combined with the protease approach [15, 16] or by iodination catalyzed by lactoperoxidase of the exposed tyrosine and histidine of membrane proteins [17].

At least in the type of plasma membranes studied so far, a variety of membrane proteins seems to be asymmetrically oriented since they are accessible either from the interior of isolated membrane or from the exterior of intact cells [14]. Furthermore, in contrast with earlier membrane models, a considerable amount of protein, in particular glycoprotein, exposed at both outer and inner surfaces, probably spans the entire thickness of the membrane [13, 15, 16, 18].

The results of these studies, which almost exclusively have been carried out using intact red blood cells and vesicles originated from erythrocytes ghosts, are however controversial and require further elucidation.

The demonstration that intact red blood cells are not modified by phospholipases A_2 and C treatment, whereas the same action hydrolyses phosphoglycerides present in the erythrocyte ghost membranes, indicates that the conformation and the exposure of the membrane constituents might be perturbated during the isolation and inversion of the vesicles [19].

A more direct contribution to the study of the transverse heterogeneity of the plasma membrane core has been realized by the application of the freeze-etching technique. This method provides useful information on the structure of the membrane by fracturing and replicating these structures in the frozen state without the utilization of chemical fixation or drastic dehydration and staining of the specimens.

Studies on the path of cleavage through biological as well as artificial membranes and thermodynamic considerations of the weakened hydrophobic bonding at low temperature are consistent with the original hypothesis of Branton that the membrane is fractured along its internal hydrophobic matrix [20, 21, 22] (Fig. 1). In particular the development of a technique for obtaining replicas of both sides of the fractured object has clearly shown that the membrane splits in two asymmetrical complementary halves [23, 24]. Therefore, the true external or inner cytoplasmic surfaces of the plasma membrane are visualized only by deep-etching, which, lowering the ice level by sublimation, exposes the non-fractured faces [21, 22] (Fig. 1). It is, however, still questionable

whether the cleaving occurs precisely along a plane where the methyl terminals of the phospholipid fatty acids of individual monolayers are located, rather than along a not yet definite level comprised within the membrane hydrophobic core [25].

In several types of plasma membranes the hydrophobic matrix exposed by the fracture is heterogeneous and shows smooth areas corresponding to lipid layer and particulate components. In animal cell plasma membranes the particles average 80–90 Å in diameter [7, 21,26].

The fracture of the membrane element produces two distinct types of faces, characterized by a marked asymmetry in the distribution of particles. The inner aspect of the leaflet adjacent to the cytoplasm [Face A or (+) or outer fracture face (OFF)] is covered by a number of particulate entities, whereas the inwardly directed fracture face of the outer leaflet [Face B or (−) or inner fracture face (IFF)] is studded with fewer particles [21, 23, 24, 27] (Fig. 2). The particle density may vary according to the type of cell [21, 26, 28].

The intramembranous particulate entities

The nature of the intramembranous particles is not yet fully clarified. The identity of the subunits observed on freeze-etched membrane with the globular projections revealed in negatively stained preparations of plasma membranes isolated from different types of cells is not readily apparent. The similar dimension and shape of the particles support the view that they may be related structures revealed by two different techniques [7]. However, the particles visualized by negative staining, which are associated with enzyme activities [29], appear to be localized only in certain differentiated areas of plasma membrane and to be exposed to the hydrophilic environment rather than ubiquitory comprised within the internal hydrophobic membrane matrix [7].

In a few appropriate instances [30] it has been shown that protuberances or bumps are exposed by deep-etching on the true surface of the plasma membrane and that they are topographically correlated with the underlying particles. Very likely the surface bulges as well as intramembranous particles may exist either as associated entities or as independent structural units according to the complexity of the plasma membrane differentiation. It might also be that the intramembranous particles in some instances, such as during the negative staining, are displaced and exposed at the outer surface. Consistent with the latter possibility are the observations of Wigglesworth *et al.* [31], showing that, in the inner mitochondrial membranes, the repeating subunits associated with ATPase activity [32] are probably intercalated into the apolar matrix of the membrane, rather than exposed to the hydrophilic environment, since in the frozen material they are visualized by the fracture but not by the etching.

The chemical identity of the intramembranous particles in plasma membranes has not yet been sufficiently elucidated. It seems very unlikely that the presence of the intramembranous particles in freeze-etched membranes reflects the occurrence of localized lipid phase changes including extrusion of cholesterol, since these events are most probably prevented by the rapid cooling of the samples [33, 34]. The results of attempts to remove the particles by low-ionic-strength solution [7, 18], by lithium diiodosalicylate [35], by proteases [7, 21, 36], by neuraminidase [7] and by phospholipase C [37] have so far been ambiguous. Most of the aforementioned treatments cause only a marked aggregation and clustering of the membrane subunits. In the case of red blood cell membranes the particles were lost after prolonged digestion with pronase [21, 26]. Papain digestion, using a concentration compatible with the maintenance of the isolated rat liver plasma membrane structure, did not remove the intramembranous particles visualized by freeze-etching [7], although it dissociates from the membrane the particles revealed by negative staining [29, 38].

The association between the intramembranous particles and membrane proteins or glycoproteins has been investigated by mapping the distribution of the particles and that of the surface antigens and specific receptors. For this purpose ferritin-labelled lectins or antibodies and virus particles were used as labelling agents on freeze-etched preparations. The results of these experiments tend to prove that the membrane particulate component revealed by freeze-etching on fracture face A are topographically correlated with surface antigens and receptors sites visualized by the labelling agents on the etched outer plasma membrane surface [36, 39, 40].

Noteworthy in erythrocytes, the number of particles ($5–6 \times 10^5$/cell) is consistent with the number of phytohemagglutinin receptors ($4–5 \times 10^5$/cell) and of A_1, A_2 and B blood groups sites ($5–8 \times 10^5$/cell) [36, 39]. The number of particles on face A is also close to the estimated number of copies per cell (5×10^5) of the two distinct protein and glycoprotein components identified by Bretscher [15, 16] which extend from the outer to the inner surface of the cell membrane.

If these experiments at all demonstrate the identity of the intramembranous particles with the surface antigens and receptor sites, rather than a merely topographic coincidence of unrelated membrane entities, then a consistent fragment of the particles should emerge on the outer hydrophilic surface of the leaflet, whereas the other part should deeply penetrate the membrane hydrophobic matrix, being more strongly attached to the inner half of the bilayer. As we have mentioned before (see p. 264), the existence of this type of amphipatic proteins in the membrane has been asserted by several authors [13, 16, 18]. Marchesi *et al.* [40] have further reported that in the major glycoprotein of the erythrocyte membrane, the carbohydrate-containing peptides, concentrated at the *N*-terminal of the molecules, are exposed at the outer surface, whereas the C-terminal peptide, which does not contain carbohydrates and is rich in hydrophobic amino-acids, residues buried within the membrane matrix.

Similar sialoglycoprotein, for which, however, no C- or *N*-terminal residues could be detected, has been recently identified, together with two other glycoproteins in red blood cell membranes. These proteins, which represent half of the total erythrocyte ghost protein, probably extend through the membrane [18].

All these observations and the results derived from X-ray diffraction analysis of biological membranes [41, 42], indicate that a considerable amount of the protein penetrates the membrane core rather than coats the bilayer. Possibly the difference in distribution of the intramembranous particles, revealed by freeze-etching on the two complementary A and B fracture faces, suggests that some proteins almost entirely span the lipid bilayer, whereas others penetrate only half way through [42].

Tangential heterogeneity of the plasma membrane

Several experiments have demonstrated the existence within the plane of the plasma membrane of a tangential heterogeneous distribution of various chemical constituents.

These studies on the mutual position of individual classes of membrane associated antigens in a single cell type, using ferritin- or virus-labelled antibody, have shown that most of the antigens investigated so far are distributed over the plasma membrane in a two-dimensional heterogeneous arrangement. Some antigens may either reside, as do the Rh_0 (D) antigens [43, 44] and H-2 mouse alloantigens [45, 46], in circumscribed patches spread in a rather periodic pattern, or cover, as do the A_1 and B blood group antigens, large areas uniformly dispersed in an aperiodic arrangement [36, 43, 47]. The distribution of ferritin-labelled concanavalin A or phytohemagglutinin reveals again that the lectin receptors may be uniformly spread as single entities over the surface of non-malignant cells [36, 48], whereas in virus-transformed [48] or trypsin-treated cells [36, 48] the lectin binding sites are randomly distributed in large clusters.

The heterogeneous distribution and the density of cell surface antigens or lectins receptor sites may vary according to the cell differentiation [49] and to the cell cycle [50, 51, 52].

It is noteworthy that by freeze-etching experiments it has been shown that the number of plasma membrane associated particles in mechanically or chemically synchronized mammalian cells *in vitro*, decreases during telophase and early G1 and shows a gradual increase on both fracture faces during mid and late G1 and S periods [28].

However, the correlation between the changes in particle density and the variation of the cell surface constituents in conjunction with the cell cycle is not easily apparent.

Besides the increase of the intramembranous particle density observed during late G1 and S periods [28], a preferential accumulation on the cell surface of glycoproteins has been detected during the M phase [50, 52, 53]. Yet, the rate of synthesis and the assembly program for a large number of antigenic constituents and for different cell surface glycoproteins need not be the same, even within a single cell type [52, 53, 54].

Dynamic aspects of the heterogeneity in the plane of the plasma membrane

Substantial evidence has been accumulated showing that membrane constituents are free to reorient and likely to intermix and cluster [55].

Direct evidence of the motion of membrane protein has been provided by the studies carried out on visual receptor membrane. The rhodopsin, probably submerged in the hydrocarbon part of the lipid bilayer, and with only the sugar groups extruding into the aqueous environment, can rotate free, probably around an axis perpendicular to the plane of the membrane [56, 57].

The motion of the membrane intercalated protein, which very likely depends on the degree of fluidity of the lipid environment [58], is probably reflected by the rearrangement of membrane antigenic sites shortly after virally-induced cell fusion [59] and by the redistribution of lymphocyte-surface immunoglobulin

1 I

FRACTURE MECHANISM IN PLASMA MEMBRANE

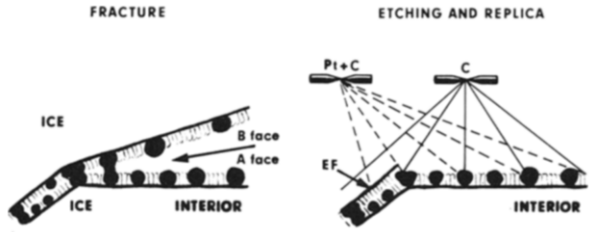


Fig. 1 *I. Diagrammatic representation of the probable fracture mechanism in plasma membrane. After the fracture and the etching* (1′–2′ *at* −100°C) *the replica is made by carbon-platinum shadowing and reinforced by carbon evaporation. The arrow* (*EF*) *points to the face exposed by deep-etching.*

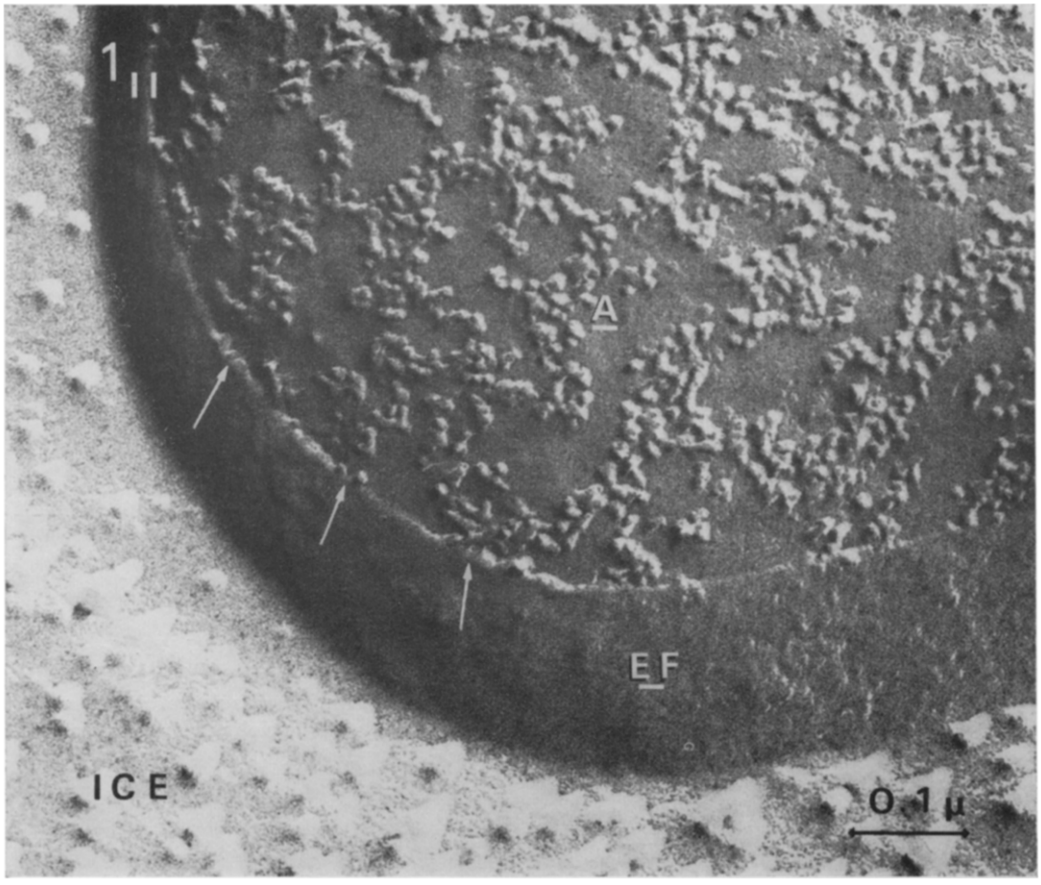


Fig. 1 *II. Replica of isolated calf lens fibre plasma membrane* (*cf. ref.* 73). *The face associated with the particles is produced by the fracture* (*face* A) *and the rather smooth face* (EF) *is exposed by the deep-etching. The arrows point to the particles spanning the edge of the fracture.*

(to face p. 266)

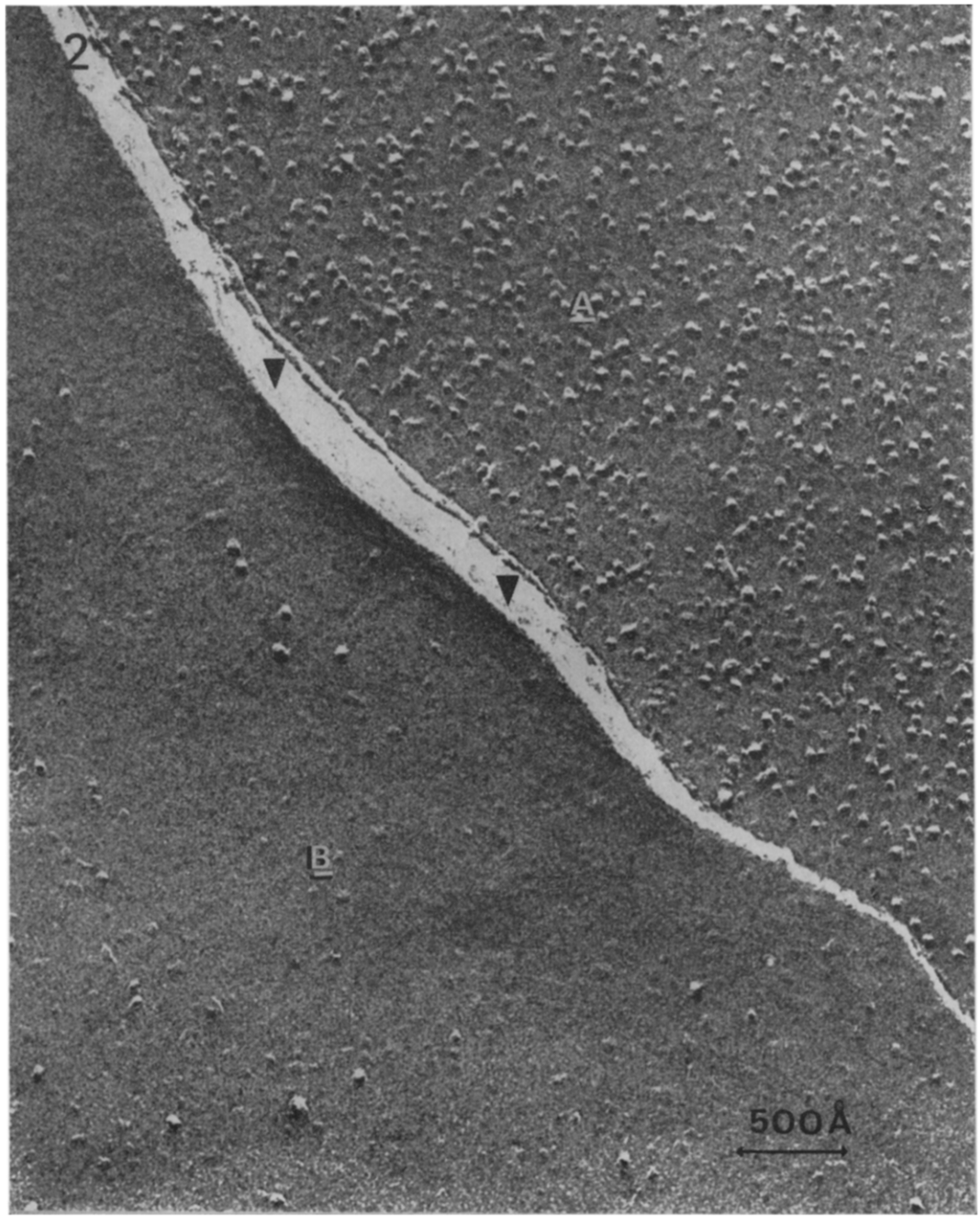


Fig. 2. Replica of fibroblast-like variant line from BALB/C mouse plasmocytoma MOPC 173. Large fracture surfaces of the plasma membranes of two adjoining cells are exposed. The arrow-heads are pointing to the intercellular space. The outwardly directed fracture face A *of the inner leaflet of one plasma membrane is characterized by numerous globular particles. The inwardly directed fracture face* B *of the outer leaflet of the adjoining plasma membrane shows very few particles.*

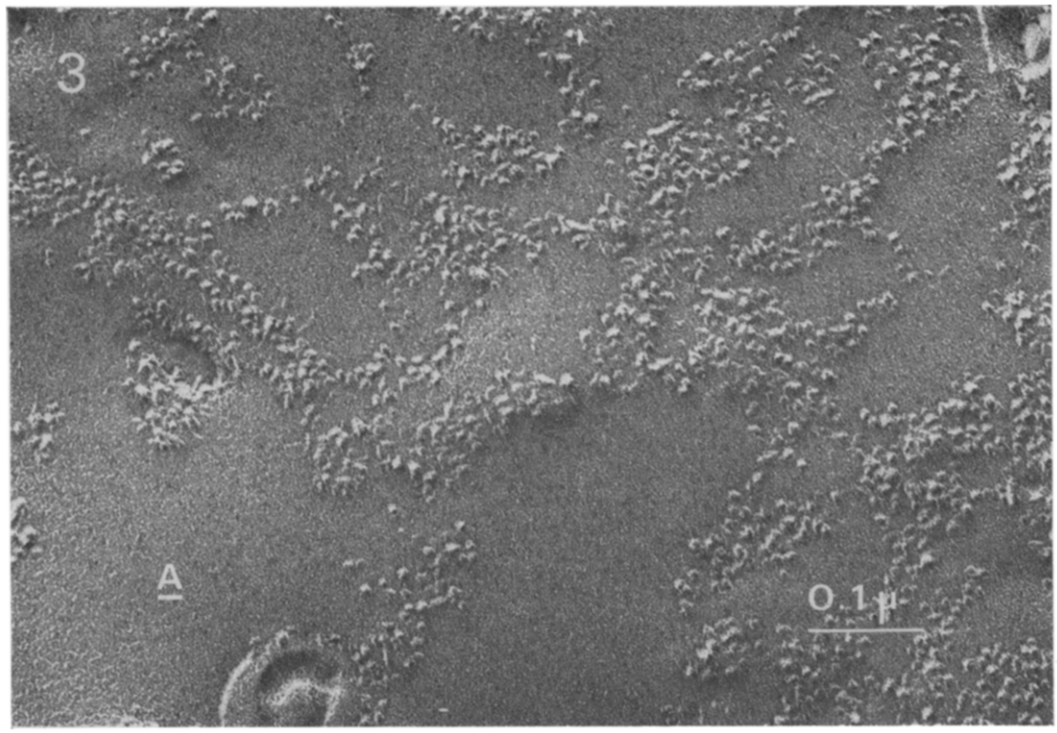


Fig. 3. *Replica of fibroblast-like variant cell from BALB/C mouse plasmocytoma MOPC* 173. *Incubation with concanavalin A* 250 γ/*ml*/5×10^6 *cells*, 45′ *at* 20°*C. The fracture face* A *shows aggregates of globular particles* (*cf. the uniform distribution of particles on fracture face* A *in fig.* 2).

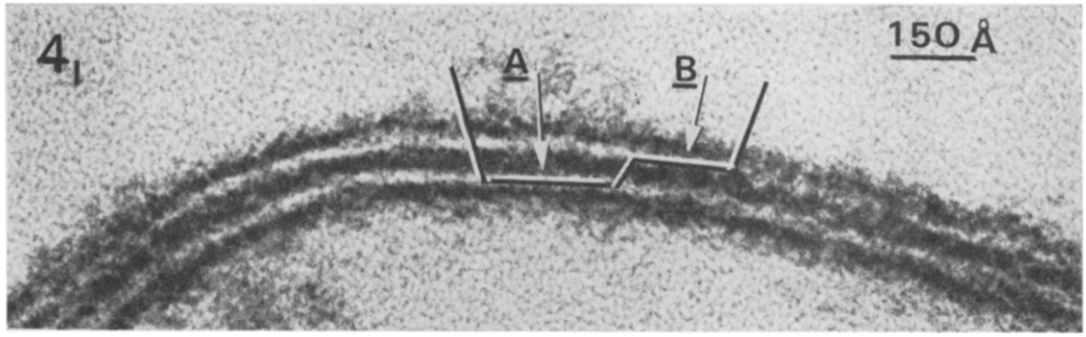


Fig. 4 *I. Isolated gap junction* (*cf. ref.* 72). *Stained with uranyl acetate and lead citrate. The section is perpendicular to the plane of the junction. The line depicts the path of cleavage exposing respectively the outwardly directed fracture face* A *and the inwardly directed face* B.

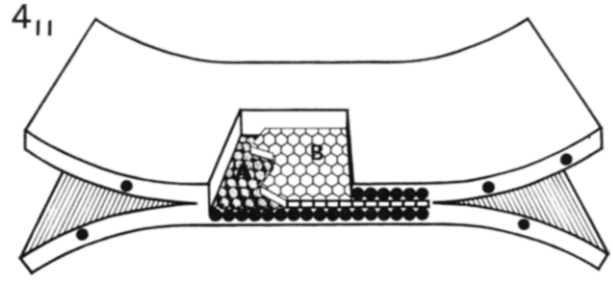


Fig. 4 *II. Tentative diagrammatic representation of the fracture faces* A *and* B *produced by the cleavage.*

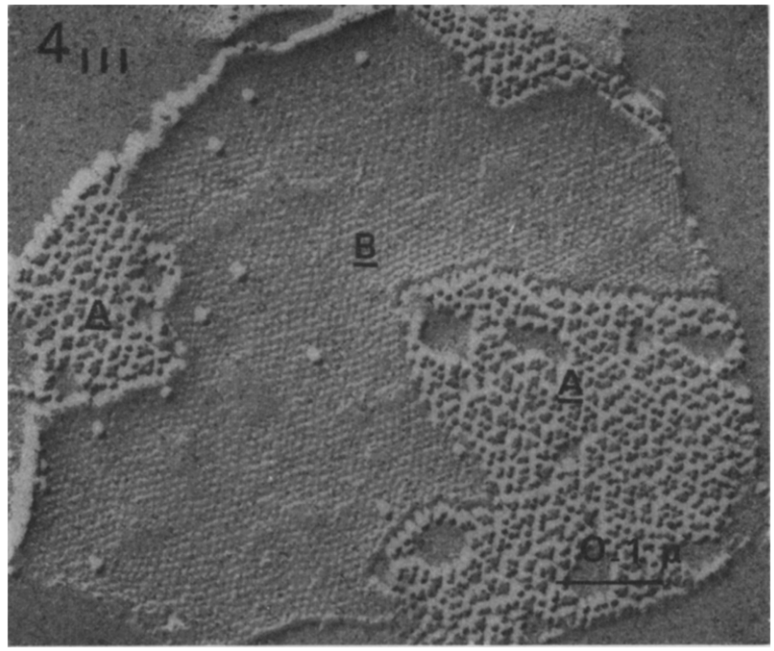


Fig 4 *III. Freeze-etched replica of isolated gap junction showing that the fracture face* B *consists of a hexagonal lattice of pitted facets and the fracture face* A *of regular array of packed globular particles.*

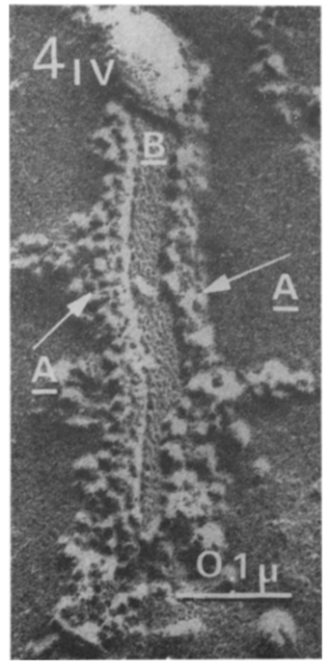


Fig. 4 *IV. Oblique view through an isolated gap junction by freeze-etching. The fracture and the deep-etching expose two layers consisting of array of globular particles (face* A*) and one interlocking hexagonal lattice of pitted facets (face* B*).*

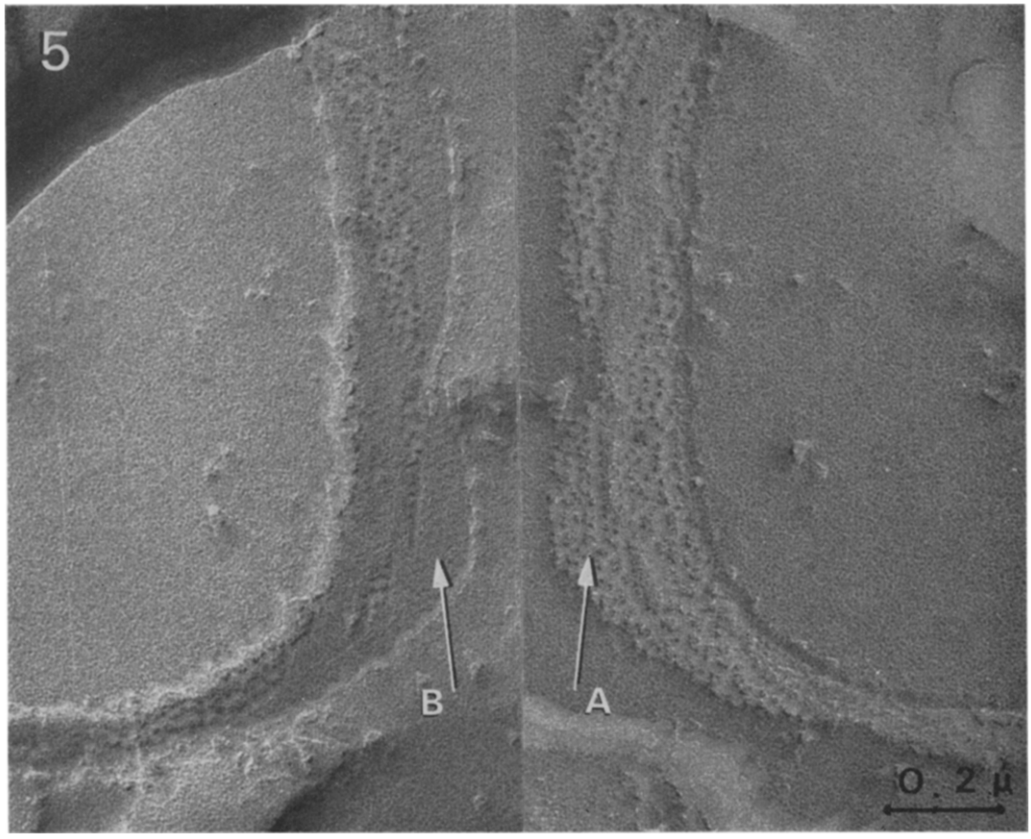


Fig. 5. *Isolated gap junction (cf. ref.* 72). *Double replica showing two matching fracture faces. The fracture face* A *associated with the array of particles is complementary to the fracture face* B *consisting of a hexagonal lattice of pitted facets.*

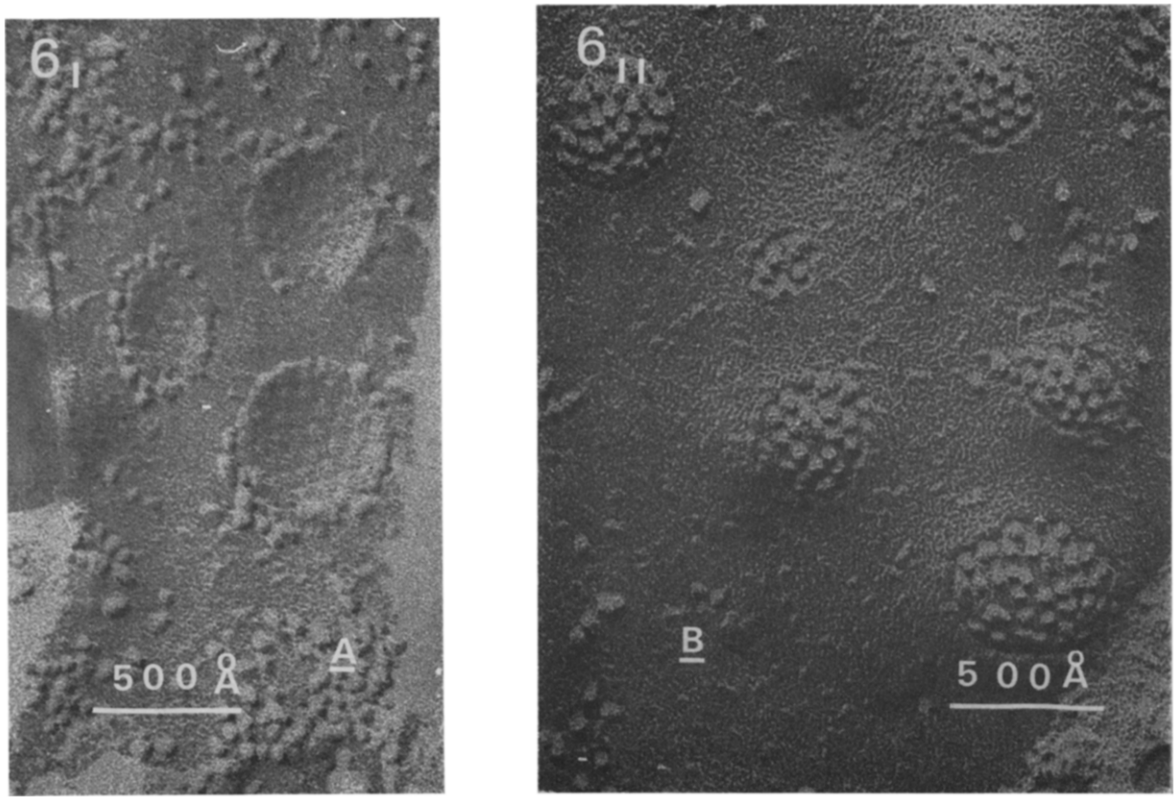


Fig. 6 *I and II. Replica of junctional areas present in isolated lens fibre plasma membranes (cf. ref.* 73). *A fracture plane through the junction illustrated in Fig.* 6 *I shows round shaped plaques characterized by a repeating substructure probably correspondent to the arrays of geometrically packed subunits visualized in Fig.* 6 *II.*

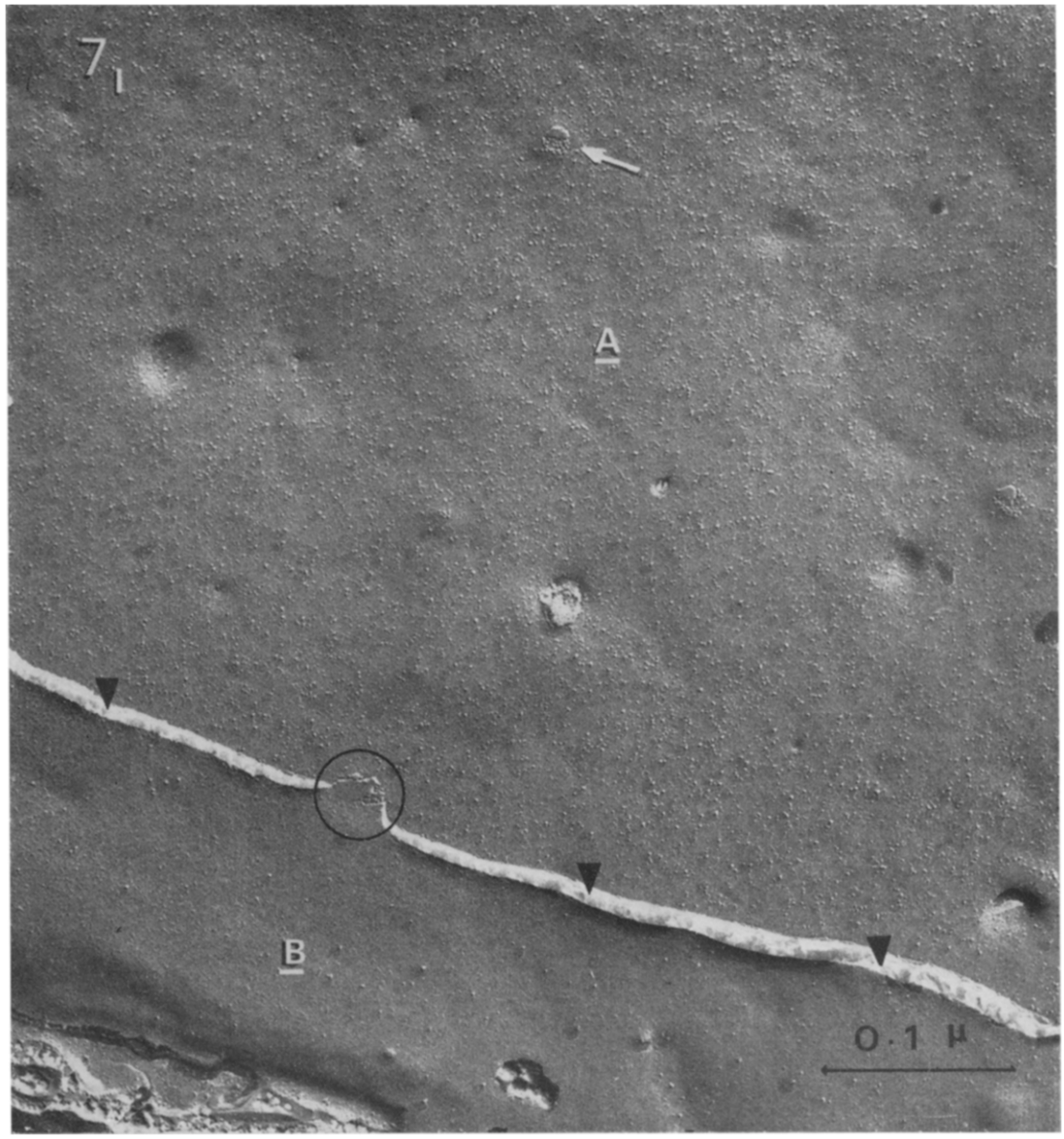
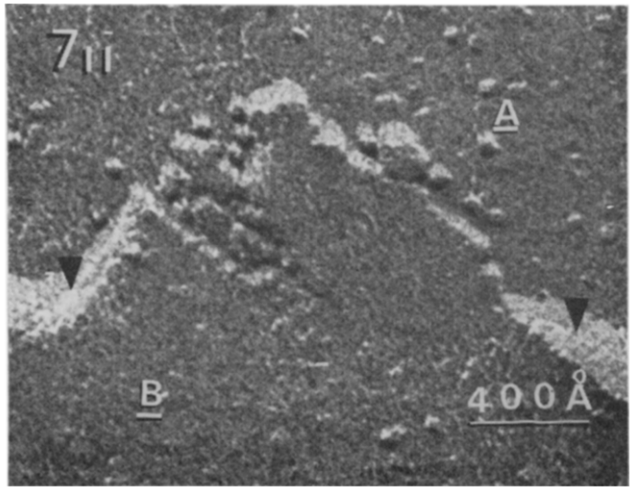


Fig. 7 I. Replica of BHK 21 cell transformed by Schmidt Ruppin Rous Sarcoma virus. Large area of the plasma membrane fracture face A *of one cell and area of the plasma membrane fracture face* B *of the adjoining cell are exposed. The arrow-heads are pointing to the intercellular space. A triangular shaped area suggestive of a gap junction between the two plasma membranes is visible (circle). At the upper region of the fracture face* A *another small junction is present.*



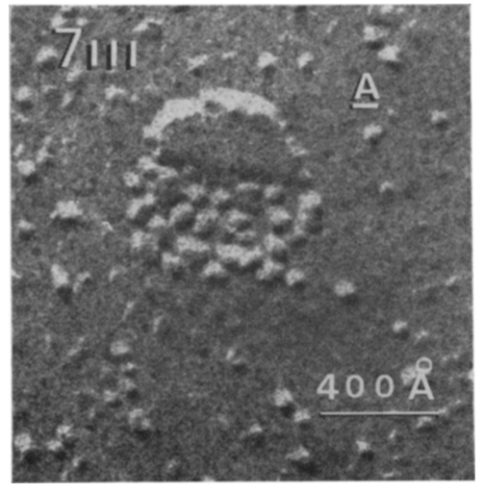


Fig. 7 II and Fig. 7 III illustrate the aspect of the junctional areas of higher magnification. Both areas are characterized by a packing of particles associated with the fracture face A *and covered by a rather smooth layer continuous with fracture face* B.

Fig. 8 I. Replica of fibroblast-like variant cell from BALB/C mouse plasmocytoma MOPC 173. Several areas of particle aggregates suggestive for gap junction are associated with the fracture face A.

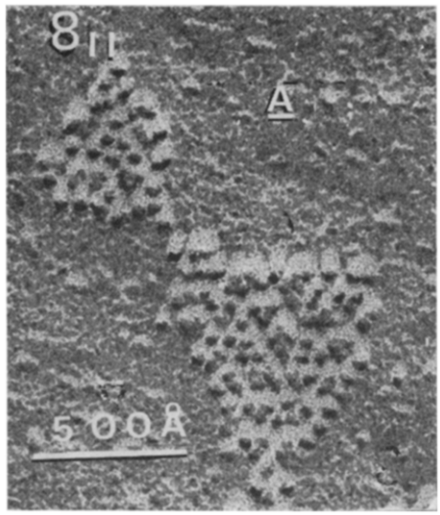


Fig. 8 II. High magnification of the fig. 8 I. The junctional areas consist of regular array of globular particles.

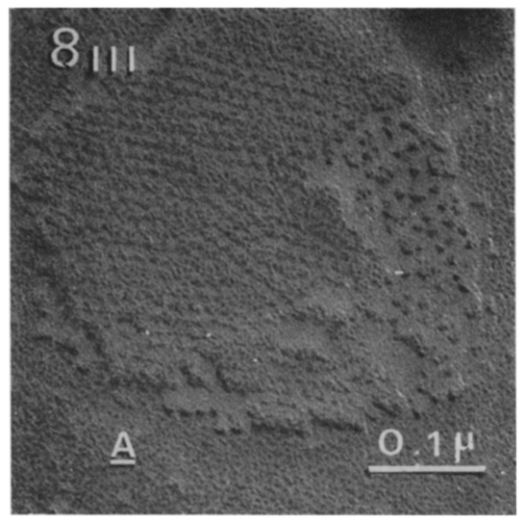


Fig. 8 III. Gap junction in epithelial-like variant cell from BALB/C mouse plasmocytoma MOPC 173. The regular packing of globular particles on fracture face A is covered by a hexagonal lattice of pitted facets.

molecules induced by anti-immunoglobulin antibody [60].

These observations have been tentatively explained by the consideration that the redistribution and clustering of cell surface antigens are due to the displacement of membrane protein within a fluid lipid environment, rather than to the synthesis and reinsertion of new components. Freeze-etching studies have not been done, so far, in order to correlate the redistribution of the intramembranous particles with that of the cell surface antigens*. This correlation has been established in the case of the displacement of lectin and virus receptors and intramembranous particles induced by mild tryptic digestion [36].

In fact, also due to the action of several other proteases [26] and some lipases [37] the intramembranous particles show progressive alteration of their distribution (cf. p. 265). It is unclear whether these results reflect the same phenomenon of free diffusion and intermixing of lipid and protein within the fluid lipid matrix intact plasma membrane, rather than a radical and irreversible rearrangement of membrane constituents as a consequence of the progressive loss of membrane protein and lipid.

Reversible particle aggregation has been induced by changing the pH of the aqueous environment at the cell surface [61]. The clustering of the intramembranous particles visualized by freeze-etching, which occurs at pH 5–4 within 2–3 min, is completely reversible if the membranes are brought back in a neutral fluid environment [61]. The clustering of the particles is also observed to accompany cell agglutination by concanavalin A [62] (Fig. 3).

In a more general sense all these phenomenons might be interpreted by the assumption that the membrane architecture can be readily diversified in multiple features, reflecting the changes of the relative proportion of protein and lipid, and the type and character of the molecular species of the membrane components. The environmental condition of the cell surface seems to play also an important role in this respect [61].

Singer and Nicolson [55] have proposed that, if the lipids form the matrix of the fluid mosaic, the long-range distribution of the protein would be preferentially random.

The presence of ordered plasma membrane arrangement

It should be stressed that the accumulation of identical or equivalently-related protein entities within the membrane core could favor the oligomeric association or even the formation of a highly-ordered bidimensional lattice of repeating subunits. The latter type of membrane organization is not uncommon in specialized areas of the cell surface [63, 64] and in the membranes which are involved in cellular interaction leading to electrotonic and metabolic coupling (synapsis and nexus or gap junction) [65, 66, 67, 68, 69, 70, 71].

In the latter type of cell junctions, negative staining of isolated plasma membranes [72] and the introduction of a low molecular weight electron opaque tracer through the intracellular spaces of intact tissues [66] have shown the existence of a bidimensional hexagonal pattern of repeating subunits spanning the occluding portion of the junctions.

The freeze-etching technique has further revealed that the differentiation of the junction involved the rearrangement of both the hydrophilic domain as well as of the hydrophobic core of the junctional membranes.

In the nexus the cleavage usually passes along two paths (Fig. 4 I–II). First the cleavage splits one of the two plasma membranes. In this circumstance the inwardly directed fracture face of the outer leaflet is exposed (Face B) (Fig. 4 III). Then the cleavage passes in a stepwise fashion to the interior of the adjacent membrane. Now the fracture face (Face A) is characterized by regular array of 90 Å particles (Fig. 4 III–IV) whereas the corresponding face B consists of a hexagonal lattice of regular facets showing in the best shadowing conditions a central pit or a hollow space with a center-to-center distance of 90 Å.

The application of the study of the gap junction of the complementary replica technique, which allows the direct observation of both sides of the fracture, gives conclusive evidence that the globular repeating units visualized on face A are actually complementary to the interlocking structure of the junction consisting of the hexagonal lattice of pitted facets [27] (Fig. 5).

Although the basic structural aspects of the nexus (gap junction) are common to almost all tissues studied so far, variants of the nexus features are observed, which are always consistent with the presence of a bidimensional lattice of repeating subunits [73, 74] (Fig. 6).

The molecular intermembrane mechanism leading to the formation of specialized areas of cell surfaces characterized by the presence of an ordered subunit mosaic is not yet clarified.

The experimental evidence, derived from rapidly growing cell systems, indicates in particular that the formation of a junction allowing a stable cell-to-cell contact and electric or

*Note added to proof.

metabolic coupling, occurs within a short time period of the order of a few minutes [75, cf. ref. 74]. The fact that the junctions are rapidly assembled, and probably also disassembled, might indicate that the local membrane differentiation is related to the accumulation and rearrangement of pre-existing randomly dispersed membrane components, rather than to the synthesis and insertion of newly formed membrane constituents into the junctional areas.

It is noteworthy that freeze-etching technique applied by us [62] to a variety of animal cells in culture, including transformed cells, where probably junctions are rapidly assembled and disassembled, has revealed on the fracture face very small particle-aggregates and arrays of particles representative for gap junction, extending over areas of variable dimensions (Figs. 7, 8). All these features might represent different stages of the process of junctional differentiation. The chemical nature of the junctional membrane repeating subunits is not yet fully clarified. In a previous study it has been shown that the general plasma membrane of rat liver is almost totally solubilized by DOC, while the gap junctions were resistant to the same treatment [72].

Our further yet unpublished results, and those of Emmelot *et al.* [76] on rat liver plasma membrane, and the most recent studies of Evans and Gurd [77] and of Goodenough and Stoeckenius [78], tend to indicate that the nexus-rich fraction, in contrast with the general plasma membrane, is characterized by high protein, very low phospholipid content, and by a preponderance of neutral lipid including free and esterified cholesterol and triglyceride. Furthermore, Goodenough and Stoeckenius [78] have shown that one single major protein component with an apparent molecular weight of 20 000 can be separated from mouse liver gap junctions by gel electrophoresis.

It is thus conceivable that the process of junctional differentiation triggered by a variety of cell surface and environmental events [79, 80, 81] initiates with a convergent accumulation of pre-existing particulate entities consisting of a single or at least very few equivalently related proteins.

The clustering of protein entities will provide the element of a regular and ordered lattice, in which selected molecular species of lipids may be accommodated in a proper configuration very likely other than a bilayer.

The displacement and rearrangement of membrane particulate entities within the fluid lipid domain might also explain various other membrane events and membrane mediated functions. In particular some recent observations of Satir *et al.* [82] on the mechanism of secretion in *Tetrahymena* show that the process of membrane fusion is very often characterized by the rearrangement and accumulation of intercalated membrane particles.

In conclusion, the multiple features of the membrane configuration result from the dynamic association of at least two main components: a lipid phase primarily occurring as a bilayer [41] and protein-glycoprotein intercalated particulate entities. The preferential association of identical or closely related polypeptides might either form specific oligomeric complexes [83] or bidimensional lattices of repeating subunits. However, full understanding the dynamics of the internal plasma membrane constituents, the metabolic state not only of the lipid-protein layer but also of the glycocalix and of the ectoplasmic domain should be taken into account.

NOTE ADDED TO PROOF

In recent and extensive contributions to Unanue *et al.* [84] and of Karnovsky *et al.* [85] on the movement of surface antigenic and lectin receptors in tymocyte and lymphocyte, no correlation between aggregation (capping) of rabbit anti-mouse lg labelled with ferritin or hemocyanin and clustering of intramembranous particles could be detected. These results may indicate that lg molecules are superficial to the liquid-like lipid bilayer, whereas other antigenic or receptor sites are more strongly anchored to the membrane core. However, their special distribution in the plane of the plasma membrane may or may not favour their movement in the fluid lipid domain after the binding with the ligands.

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Progressive Karyotypic Changes *In Vitro* in the Ehrlich Lettré Ascites Subline SZEL

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Abstract—*Cells of the subline SZEL, of Ehrlich Lettré ascites hyperdiploid (ELD) origin, were monitored at intervals throughout their first two years* in vitro *for changes in number and composition of the stemline karyotype. After* 6 *months, the stemline chromosome number had increased to* 56–57 *from the* in vivo *mode of* 43. *Successive changes included increases in the number of biarmed chromosomes and reduction in the stemline number to* 49. *After two years* in vitro *the characteristic "A" chromosome had disappeared from the stemline karyotype and from most cells of the population as a whole. The two minutes were constant.*

The ELD stock, from which the SZEL subline was derived, was maintained by serial transfers in mice. Its karyotype exhibited high stability of stemline number and of all marker chromosomes.

No obvious changes in growth rates in vitro *were apparent, although a reduction in optimal cell density may have occurred. After two years* in vitro, *SZEL cells were still capable of producing tumours in adult mice, but the incidence of tumour formation had clearly declined.*

INTRODUCTION

THE establishment *in vitro* of permanent sublines of Ehrlich Lettré ascites hyperdiploid (ELD) origin has been reported by a number of investigators, but only rarely has information been provided regarding progressive monitoring of stemline chromosome numbers [1–3] or karyotype patterns [2, 3]. No detailed analysis of number and arrangement of chromosomes of any Ehrlich Lettré ascites subline over a prolonged period of time *in vitro* appears to have been reported.

It is now well-known that tumour cell lines cultured *in vitro* for long periods are typically heteroploid and that the stemline cells often have near-tetraploid chromosome numbers [4]. The limited information available indicates that a variety of karyotypic conditions has been found in ELD sublines *in vitro*. These conditions have included: (a) stemline complements with greatly increased numbers of chromosomes after 2–3 months in culture [1, 2]; (b) the retention of a hyperdiploid mode and the concurrent development of a subsidiary polyploid population within 2 years [3]; and (c) high stability of a hyperdiploid mode throughout 16 months *in vitro* [5].

The present report is based on a 2-year study of the chromosomes of a newly established subline, SZEL, of Ehrlich Lettré hyperdiploid origin. The subline was begun in January, 1970, and adaptation to *in vitro* conditions took approximately two months. After two years, the cells had been subcultured 170 times. The karyotype of the parent stock, ELD, carried in C57Bl mice, was analyzed at the time of initiation of the subline, and again at the end of two years. Karyotype analysis of SZEL cells was carried out after 6 months (50 passages), 14 months (100 passages) and 2 years (170 passages) *in vitro*. Tumour-forming capacity in mice and growth rates *in vitro* were also monitored at intervals during the two-year period.

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MATERIAL AND METHODS

1. *Animals and tumour cells*

Our original stock of hybrid mice inoculated with cells of an Ehrlich Lettré hyperdiploid ascites tumour line, ELD, was obtained early in 1969 from the Australian Atomic Energy Research Establishment at Lucas Heights, N.S.W. Although we traced the history of this mouse-passaged tumour within various Australian laboratories, we were unable to discover its exact overseas source. In our laboratory, the tumour was propagated by serial transfer at 7-day intervals in C57B1 adult male mice. ELD cells were grown continuously in this mouse strain for over 6 months before being used to initiate the SZEL subline *in vitro*.

2. *Initiation of* in vitro *subline*

Approximately 2×10^6 washed cells removed from the peritoneal cavity of a mouse were seeded in a 30 cm^2 flat glass prescription bottle, the surface of which had been thinly coated with collagen. After an initial medium change and removal of dead cells, the residue of viable cells was maintained in the same bottle with minimal disturbance and very few changes of medium for approximately two months. After active proliferation occurred, routine subculturing using a 0·25% trypsin–versene solution for dissociation was carried out approximately twice weekly.

3. *Cultivation*

Eagle's Minimum Essential Medium as supplied in powdered form (F 11) by the Grand Island Biological Co., N.Y., was used routinely, supplemented with 10% young calf serum (Commonwealth Serum Laboratories, Melbourne) and 0·7% lactalbumin hydrolysate. Standard antibiotics included sodium penicillin G (40 I.U./ml), streptomycin sulphate (50 μg/ml) and amphotericin B (Fungizone) (2·5 μg/ml), but at intervals these were replaced by kanamycin (100–500 μg/ml) for several passages, followed by a period of growth in antibiotic-free medium. After some months in culture, the cells were regularly monitored for the possible presence of mycoplasma by the Fogh orcein staining technique [6] and by plating the cells on enriched PPLO agar [7].

4. *Growth*

Growth curves *in vitro* were obtained by seeding Falcon plastic culture flasks (25 cm^2) at initial densities of 3×10^5 cells/flask. The medium was changed at 24 hr intervals. Each day for 5 days, cells from 10 randomly selected flasks were trypsinized and counted in a Fuchs–Rosenthal counting chamber. From these figures the mean population doubling times were calculated [8]. Figures from the first 24 hr after seeding were omitted from these calculations.

5. *Tumour-formation* in vivo

Tumour-forming capacity was assessed by injecting known concentrations of SZEL cells intraperitoneally into mice, incubating them for a minimum of 7 days (the standard interval used between routine ELD tumour transplants in this laboratory) and then killing all experimental and control (ELD-inoculated) mice. Ascitic fluid and suspended cells were removed by pipette from the peritoneal cavity of each mouse and transferred to individual tubes containing Hanks' Ca^{2+}-and-Mg^{2+}-free saline. The cavity was then flushed repeatedly with saline to remove further cells. Total counts of viable cells were obtained for each mouse. Serial dilutions were prepared when necessary. In assessing results, only counts in which the number of cells recovered was greater than that of the inoculation dose were scored as successful "takes".

6. *Karyotypes*

Karyotype preparations of SZEL cells grown in flasks were obtained by standard methods of colchicine treatment, trypsinization, hypotonic treatment and acetic-methanol fixation. Washed ELD cells removed from mice injected with colchicine 3–4 hr previously [9] were similarly treated. The fixed, air-dried preparations were stained with propionic orcein. One hundred consecutive photographs of countable metaphase spreads from each cell type and passage required were taken with a Zeiss Photomicroscope II at a magnification of × 1000.

RESULTS

Karyotype

The parent *in vivo* line ELD at the time of *in vitro* adaptation of the subline SZEL was characterized by a stemline or modal number of 43, a single "A" chromosome [10], 2 metacentric markers or "B" chromosomes [10] and 2 minutes or "m" chromosomes (Table 1, Figs. 1e and 2e). The proximal region of the "A" chromosome was shorter than the distal segment. After 2 years and approximately 100 intraperitoneal passages in male C57B1 mice, the same stemline number and pattern of chromosomes was clearly retained (Table 1,

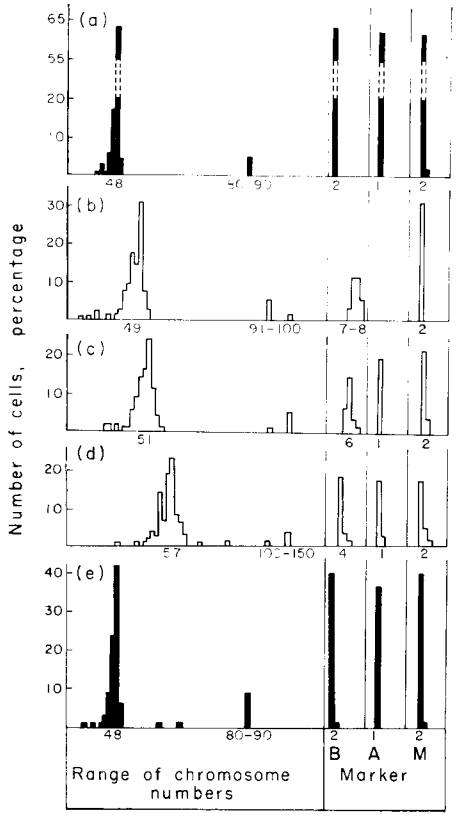

Fig. 1. *Comparison of chromosome numbers of ELD cells* in vivo *and SZEL cells* in vitro *over a* 2*-year period. All histograms are based on counts of* 100 *cells. The marker chromosomes shown illustrate the incidence of markers in cells with stemline karyotypes only.*

(*a*) *ELD* in vivo *at end of* 2*-year period.*
(*b*) *SZEL after* 2 *years* in vitro (*Passage* 150).
(*c*) *SZEL after* 14 *months* in vitro (*Passage* 100).
(*d*) *SZEL after* 6 *months* in vitro (*Passage* 50).
(*e*) *ELD* in vivo *at beginning of* 2*-year period.*

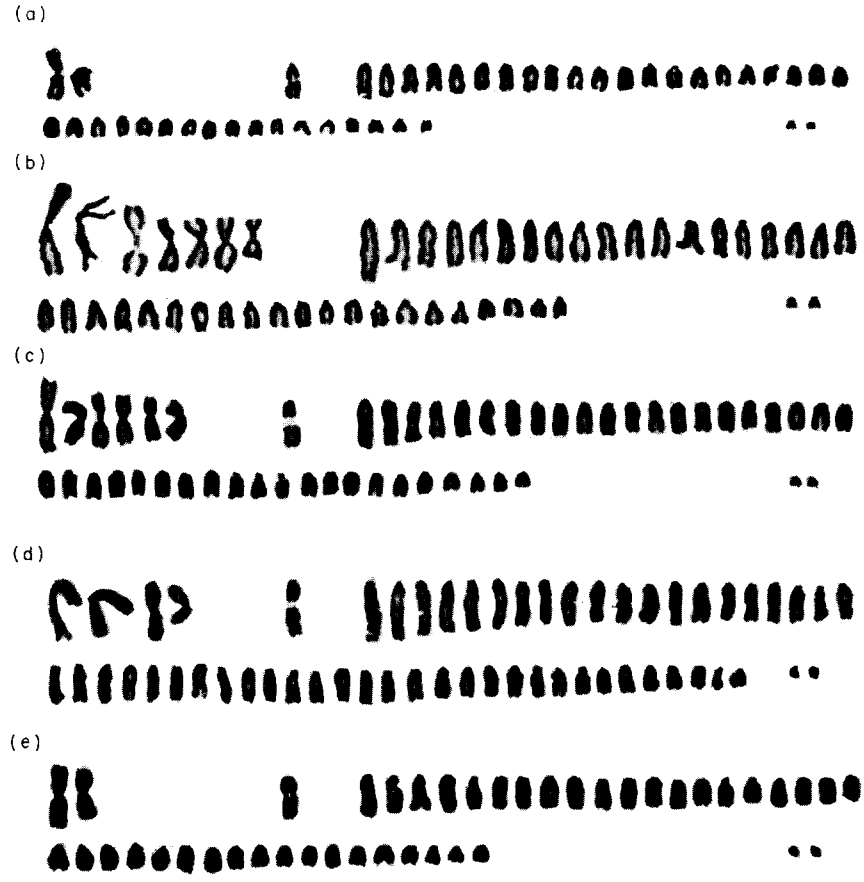

Fig. 2. *Stemline karyotypes of ELD cells* in vivo *and SZEL cells* in vitro *over a* 2*-year period* (× 1100).

(*a*) *ELD* in vivo *at end of* 2*-year period.*
(*b*) *SZEL after* 2 *years* in vitro (*Passage* 150).
(*c*) *SZEL after* 14 *months* in vitro (*Passage* 100).
(*d*) *SZEL after* 6 *months* in vitro (*Passage* 50).
(*e*) *ELD* in vivo *at beginning of* 2*-year period.*

(to face p. 274)

Table 1. Numerical changes in chromosome markers in ELD and SZEL populations throughout a 2-year period

Line or passage or age	Biarmed ("B") chromosomes											"A" chromosomes				Minutes						
	1	2	3	4	5	6	7	8	9	10	11–16	0	1	2	3	0	1	2	3	4	5	6–9
ELD (at same time as SZEL P170)	1*	91	5	3								5	91	4		–	5	88	6	1		
SZEL P170 (2 years *in vitro*)				1	3	5	35	30	15	4	7	97	3	–		–	14	75	4	7		
SZEL P100 (14 months *in vitro*)				3	24	42	22	4	–	2	3	23	70	6	1	–	7	75	11	6	1	
SZEL P50 (6 months *in vitro*)	–	2	7	57	19	7	3	2	2	1		20	74	6		–	5	70	18	5	–	2
ELD—Parent line *in vivo* (at time of initiation of SZEL subline)	2	77	14	4	3							22	71	7		1	10	79	5	5		

*Numbers are percentages, based on counts of 100 metaphase spreads.

Figs. 1a and 2a). In contrast, SZEL populations exhibited considerable change over the same 2-year period. Analyses were not made until the 50th passage (6 months *in vitro*) had been reached, when a wide variation in chromosome number and an increase in the number of metacentric markers were apparent (Table 1, Figs. 1d and 2d). Two stemline populations, the larger with 57 chromosomes and the smaller with 56, were present. Further analyses (Table 1, Figs. 1b and 1c; 2b and 2c) at 14 months (Passage 100) and 2 years (Passage 170) showed the following trends:

(*a*) a progressive increase in the number of biarmed or metacentric markers;

(*b*) a decrease in the number of acrocentric or telocentric chromosomes;

(*c*) the emergence of a single, clear-cut stemline or modal population;

(*d*) a decrease in the total modal number;

(*e*) no change in the number of minutes;

(*f*) eventual elimination of the highly characteristic "A" chromosome from the stemline population and almost entirely from the population as a whole;

(*g*) a decrease in the total number of arms present in the chromosomes of the stemline population from 60 or 61 after 6 months (Fig. 2d) to 57 after 14 months (Fig. 2c) and 56 (Fig. 2b) after 2 years.

Growth rates in vitro

Growth curves of SZEL cells after 10 months (70 passages) and 2 years (170 passages) are shown in Fig. 3. The overall population doubling times for the exponential growth period from Day 1 to Day 4 were $19{\cdot}19 \pm 0{\cdot}57$ hr (mean $\pm 95\%$ confidence limits) at the 70th passage and $19{\cdot}50 \pm 0{\cdot}69$ hr at the 170th passage. Growth curve data related to changes in daily doubling times provided some evidence, supported by regression analyses of population doubling times on cell density obtained from counts of cells grown on coverslips (Stephenson and Stephenson, unpublished), that the optimal population density as indicated by the shortest doubling time had decreased compared with its level after 8–10 months in culture.

Tumour-forming capacity in mice

ELD stock cells maintained *in vivo*, when inoculated in control doses identical with those of SZEL cells shown in Table 2, typically produced in 90–100% of adult mice ascites tumours in which the counts of cells recovered were greatly in excess of the inoculation number. This capacity did not change throughout a 2-year period. Over the same period, SZEL cells exhibited a progressive decline in incidence of tumour formation in adult mice (Table 2). A less pronounced decline in tumour incidence and in the total number of tumour cells recovered was also seen in neonatal mice. In isolated cases, tumorigenicity of relatively high virulence could still be observed (Table 2) after 27 months in culture.

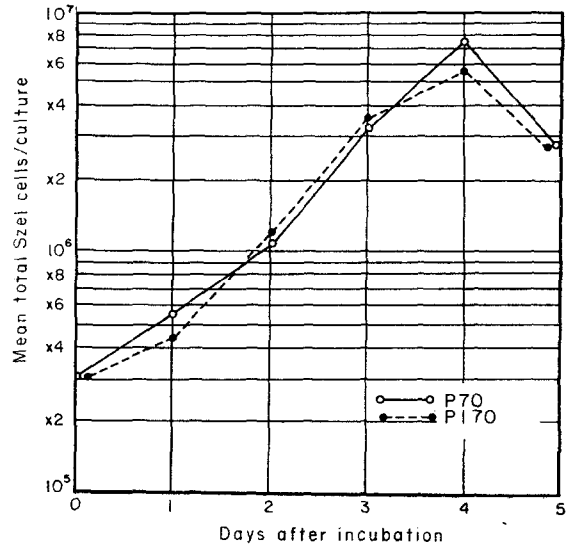

Fig. 3. Growth curves of SZEL cells after 10 months (70 cell passages) and 2 years (170 cell passages) in vitro. Each point represents the mean of 10 cultures.

DISCUSSION

Karyotype

Although our stock *in vivo* line ELD had a total stemline number of only 43 compared with the stemlines of 45 and 46 originally described for Ehrlich Lettré hyperdiploid cells [10], the presence of all three characteristic types of markers, particularly the "A" chromosome, confirmed its derivation. The observed reduction in total chromosome number might be partly explained in theory on the basis of centric fusion of two originally acrocentric or telocentric chromosomes to form a second metacentric and by the loss of a minute, but other types of structural changes cannot be excluded. The "A" chromosome differed from that of the original Ehrlich Lettré ascites tumour [10–12] in having the proximal segment shorter than the distal portion. This characteristic was previously described for the Landschütz subline [11] but has also been

Table 2. Progressive decline in incidence of tumour formation in mice by SZEL cells

Length of time *in vitro*	Inoculation dose/mouse	Days between inoculation and cell recovery	No. of mice inoculated (No. of "takes"*)	Mean total cells recovered/mouse (Range)
11 months	7×10^6	7	5(2)	$2{\cdot}7 \times 10^7$ ($2{\cdot}7 \times 10^6$–$7{\cdot}2 \times 10^7$)
14 months	10^7	7	10(5)	$5{\cdot}3 \times 10^7$ ($2{\cdot}8 \times 10^6$–$16{\cdot}8 \times 10^7$)
19 months	7×10^6	7	2(1)	4×10^7 (0–8×10^7)
24 months	10^7	7	15(0)	6×10^5 (0–$4{\cdot}1 \times 10^6$)
27 months	5×10^6	20–30	5(1)	$1{\cdot}8 \times 10^7$ (0–9×10^7)
11 months	5×10^5	14	13(13) Neonatal (9 dead†)	$2{\cdot}6 \times 10^7$ ($1{\cdot}5 \times 10^6$–5×10^7)
25 months	5×10^5	15	20(7) Neonatal (3 dead†)	$2{\cdot}7 \times 10^6$ (4×10^4–$2{\cdot}2 \times 10^7$)

*Based on criterion of tumour cell multiplication *in vivo* to produce a higher number of cells than in the inoculation dose.

†Dead mice showed massive abdominal distension, ascitic fluid and thick suspensions of tumour cells. Recorded cell counts were obtained only from surviving mice.

illustrated in a number of Ehrlich Lettré ascites strains or sublines from different laboratories [2, 13, 14]. In contrast to Moore *et al.* [3] who observed a progressive reduction in chromosome number in their stock ELD_a cells *in vivo*, we found a high degree of stability in our ELD cells *in vivo* over a 2-year period.

The initial increase in modal chromosome number of SZEL cells was followed by progressive reductions in total stemline number. This numerical decline appeared to be related to progressive increases in numbers of biarmed chromosomes. Reference to the number of arms present in the stemline karyotype in relation to the total number of chromosomes shows that the change in total stemline number from 51 to 49 (57 to 56 arms) between passages 100 and 170 can be theoretically explained by centric fusion of two acrocentric or telocentric chromosomes to form an additional metacentric and by the loss of the "A" chromosome. The transition, from the condition at the 50th passage (56 or 57 chromosomes and 60 or 61 arms) to that at the 100th passage (51 chromosomes and 56 arms) cannot be explained purely on the basis of centric fusion of four acrocentric chromosomes to form two additional biarmed chromosomes. Other types of structural chromosome changes and actual loss of chromosomes have probably occurred.

The stemline number of the SZEL subline *in vitro* may originally have exceeded 57, but no information is available for the first few months in culture. The initial rise in chromosome number seen in SZEL cells and in some other comparable sublines [1, 2] may possibly be analogous to the production of polyploid ELD sublines *in vivo* by exposure to adverse environmental situations [15]. Hsu [16] has suggested that the double stemline cells produced *in vivo* were more resistant than near-diploid cells to the suboptimal conditions they experienced. It seems possible that initial transition of ELD cells to *in vitro* conditions, typically a relatively prolonged process involving degeneration and death of numerous cells, may also have a selective effect. It has been suggested [3] that the retention of a hyperdiploid stemline number in

two *in vitro* sublines [3, 5] may have been influenced by the increased concentration of vitamins and amino acids used in both cases.

Total loss of the "A" chromosome, a specifically characteristic marker [10], from the stemline population and almost entirely from the *in vitro* subline as a whole, does not previously seem to have been recorded except in selected and cloned cells. In these it has been demonstrated [2] that typical ascites tumours can be produced by A-deficient cells.

Growth rates in vitro

Biczowa *et al.* [8] did not observe any significant changes in the shape of ELD_e growth curves *in vitro* or in the population doubling time during the exponential growth phase throughout a 2-year period. This appeared also to be the case with cultured SZEL cells, although a probable lowering of optimal cell density was noted during the second year *in vitro*. No explanation is so far available for this change.

Tumorigenicity

With increased time *in vitro*, SZEL cells exhibited a decreasing capacity for tumour production in both adult and neonatal mice. A similar trend has previously been recorded for other *in vitro* sublines of Ehrlich hyperdiploid origin [1, 3], although some investigators [5, 17] have found that cultured cells have retained a high level of tumorigenicity for considerable periods *in vitro*. Moore *et al.* [3], in relation to a comprehensive series of experiments with ELD_e and ELT_e cells, discussed the decreasing implantability of cultured Ehrlich ascites cells in mice and suggested antigenic changes as a probable cause.

The time interval that we selected for the majority of tests, although useful for making comparisons with the standard response of ELD cells within the same period, may have eliminated some slowly-developing SZEL tumours. This suggestion was supported by the presence of viable SZEL cells, reduced in number from the original inoculation dose and therefore scored as negative within the standard time interval, in at least some of the injected mice. Later experiments, allowing increased incubation time, indicated that SZEL cells after 200 passages and over two years *in vitro* were still to be regarded as potentially lethal, but also supported previous evidence that the incidence of tumour formation had decreased.

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Anti-tumor Effect of the Amino Acid Mimosine*

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Abstract—*Mimosine, an amino acid derived from* Leucaena glauca, *is known to have cytostatic effects against growing hair follicles and reproductive organs. In the present study mimosine in drinking water was shown to have dose-related, specific anti-tumor activity in the Walker* 256 *carcinosarcoma as measured by reduction in tumor size and prolongation in survival. Because mimosine acts as a tyrosine analogue, these results may be of special interest for certain endocrine tumors and melanoma, in which tyrosine has a special metabolic role.*

INTRODUCTION

SHEEP eating the browse tree, *Leucaena glauca*, spontaneously shed their wool. The depilatory agent in *Leucaena glauca* proved to be the amino acid mimosine, an aminopropionic acid [1]. Histologic studies of sheep receiving the diet of *L. glauca* showed a cessation of active cell division in the follicle from which wool fiber grows [1]. Female rats fed mimosine showed reduction in weight of reproductive organs and reversible infertility [2]. This pattern of toxicity, involving tissues with a high mitotic rate and resembling patterns of toxicity observed with several currently used anti-tumor agents led to our interest in possible anti-tumor effects of mimosine and prompted the present study [3].

METHODS

Male Sprague-Dawley rats weighing 60–90 g were injected in the right hind leg muscles with a cell suspension of Walker 256 carcinosarcoma, receiving 5×10^6 cells in 0·1 ml [4]. Purified mimosine was obtained from Calbiochem, Los Angeles, California, and was brought into aqueous solution by alkali titration with desicated sodium hydroxide (final solution pH 8·2–8·7). Mimosine-treated groups received selected concentrations of mimosine in alkaline solution as drinking water offered *ad libitum* while control and weight-matched groups received tap water *ad libitum*.

Control and mimosine-treated groups received Purina laboratory chow *ad libitum*. Rats in weight-matched groups were housed individually and fed chow in amounts planned to result in weight changes equal to those of a matched member of each respective mimosine group. Animals were examined daily for body weight, mean tumor diameter, and survival.

RESULTS

Representative results from this experiment are presented in Table 1 which presents tumor size and body weight observations on day 15 after tumor injection as well as survival results. Rats receiving the lowest level of mimosine showed slight reduction of tumor size and a small increase in survival. Weight-matched controls for this group had no reduction in tumor size or increase in survival. In the group receiving the intermediate level of mimosine there was a more marked reduction in tumor size and survival was markedly increased with 4–6 rats surviving until sacrifice on day 60. Two of these rats were free of tumor on detailed gross and microscopic necropsy examination. Comparison with the

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Table 1. *Tumor size and body weight on day* 15 *and survival response in rats with Walker* 256 *treated with mimosine*

Group designation	Tumor diameter*(mm)	*p* Value	Body weight (g)†	Survival days	Survival significance compared with: *Ad libitum* control	Survival significance compared with: Weight-matcl control
Control	23·7 ± 0·6	< 0·03	146 ± 5	24(13–49)		
0·2 g mimosine*	21·8 ± 0·8	< 0·03	133 ± 4	30(24–42)	N.S.	N.S.
Weight match for 0·2 g	24·5 ± 0·8		128 ± 6	22(20–39)		
0·4 g mimosine	14·7 ± 1·2	< 0·01	108 ± 8	> 60(29– > 60)	< 0·01	< 0·01
Weight match for 0·4 g	21·3 ± 1·3		102 ± 8	24(23–35)		
0·8 g mimosine	10·3 ± 0·5	< 0·01	71 ± 5	33·5(21– > 60)	< 0·10	N.S.
Weight match for 0·8 g	16·3 ± 0·6		70 ± 5	23(18–25)		

*Mimosine dose shown is number of grams per 100 ml drinking water.

†Data shown is mean ± standard error of mean.

weight-matched controls provides evidence that this is a specific tumor inhibitory effect and was not related to reduced caloric intake *per se* in the group receiving mimosine.

The highest level of mimosine tested produced the most marked effect in terms of tumor inhibition with failure of tumor appearance in one rat and complete regression of small developing tumors in three others. Only two rats in this group developed progressively growing tumors. This level of mimosine was toxic to the rats as indicated by weight loss and early death of two rats that were free of tumor. In this group there were two tumor free survivors at sacrifice 60 days after tumor transplant.

DISCUSSION

The mechanism of action of mimosine in the present study is not known. Mimosine is an amino acid containing an alanine side chain bound to the nitrogen atom of a pyridine ring [5]. Crouse and colleagues reported that mimosine acted as a tyrosine analogue, capable of inhibition of tyrosine decarboxylase and competitive inhibition of tyrosinase [6]. The toxic action of mimosine may be due to the inhibition of tyrosine-utilizing enzymes or to the incorporation of mimosine into biologically vital proteins in place of tyrosine. Biochemical study of mimosine content and tyrosine metabolism in tumors from mimosine-treated animals may clarify its mechanism of action.

This study was initiated because the toxicity reported for mimosine suggested an inhibitory effect on rapidly dividing normal cell populations, and it was of interest to determine whether mimosine would also have an inhibitory effect on rapidly dividing tumor cell populations. The results demonstrate a significant anti-tumor effect of this chemical, and suggest that related compounds might have anti-tumor activity. Additional studies with other tumors and host strains have provided evidence that tumor inhibition is a general effect of mimosine and is not unique to the experimental system studied herein [7].

Mimosine may be of special interest in those tumors in which tyrosine plays a special metabolic role. It is known that tyrosine is a precursor of thyroid hormones, catecholamines, and melanin. The adverse effects to the host bearing a tumor producing one of these derivatives of tyrosine may in part be related to the metabolic product of the tumor. Inhibition of formation of the metabolite as well as inhibition of tumor growth might derive from treatment with mimosine. We plan to study this further in animal tumor models, particularly melanomas.

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Lung Carcinogenesis with 1-Acetyl-2-Isonicotinoylhydrazine, the Major Metabolite of Isoniazid

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Abstract—*The continuous administration of* 0·4% *1-acetyl-2-isonicotinoylhydrazine in the drinking water to 4-week-old randomly bred Swiss mice for the remainder of their lifetime gave rise to an increased incidence of lung tumors. It rose from* 12 *to* 77% *in females and from* 10 *to* 58% *in males as compared with the untreated controls. The treatment had no apparent effect on the development of other types of tumors.*

The present study thus proves for the first time the carcinogenicity of 1-acetyl-2-isonicotinoylhydrazine, which in man is the major metabolite of the tuberculostatic drug isoniazid.

INTRODUCTION

ISONICOTINIC acid hydrazide (ioniazid, INH) in man undergoes a major metabolic change in which part of the chemical is acetylated to yield 1-acetyl-2-isonicotinoylhydrazine (Fig. 1). It is estimated that as much as 50–90% of the dose is excreted in acetylated form which is

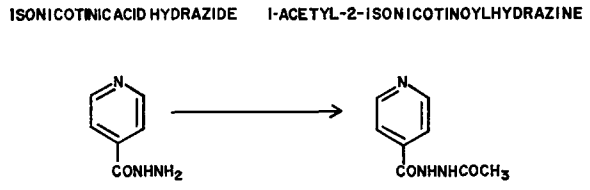

Fig. 1. *Metabolism of INH in man according to Hughes. It is estimated that* 50 *to* 90% *of INH is excreted in acetylated form.*

much less toxic than the parent drug [1]. Man also excretes small amounts of the unchanged drug and isonicotinic acid and its glycine conjugate [2]. In various animal species the metabolism of INH follows a somewhat different pathway, although our knowledge in this area is still fragmentary if not confusing. This seems to be due mainly to the different analytical techniques and tools which have been used in various experiments. For instance, in dogs the main transformation product appears to be isonicotinic acid and nearly 30% of the compound is unchanged [3]. In rabbits a small amount of INH is converted to hydrazine [4], while in rats 1-acetyl-2-isonicotinoylhydrazine, isonicotinuric acid and the isonicotinylhydrazones of pyruvic and α-oxoglutaric acids are formed [5–7].

After the discovery of the carcinogenicity of INH for the mouse lungs [8], studies have been directed towards investigating the actions of the various metabolites of the drug. In mice, in the first study, isonicotinic acid given as sodium salt exerted a very low tumor-inducing capability [9]. In subsequent investigations by the same workers [10] and in another laboratory it was found to be without carcinogenic effect [11]. Hydrazine given to mice and rats by itself and also as sulfate, on the other hand, gave rise to significant incidences of various kinds of tumors [9, 12–15].

The present study records the carcinogenic action of the so far unstudied major metabolite of INH, 1-acetyl-2-isonicotinoylhydrazine, which was administered orally in the drinking water daily for life to randomly bred Swiss albino mice.

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MATERIAL AND METHODS

Swiss albino mice from the colony randomly bred by us since 1951 were used. They were housed in plastic cages with granular cellulose bedding, and separated into groups of ten according to sex. Wayne Lab-Blox regular diet (Allied Mills, Chicago, Illinois) and tap water containing the chemical were given *ad libitum*.

The chemical used was 1-acetyl-2-isonicotinoylhydrazine (AINH), M.W.: 179·18, M.P.: 156°, synthetized for us by Dr. E. W. Alpern, Saber Laboratories, Inc., Morton Grove, Illinois. The treated groups and the controls were as follows:

The toxicity study was carried out prior to the chronic experiment. Five dose levels of AINH such as 0·4, 0·2, 0·1, 0·05, 0·025% were administered in the drinking water for 35 days to 4-week-old Swiss mice. Each group consisted of 8 animals, 4 females and 4 males. By taking into account four parameters, survival rates, body weights, chemical consumption figures and histological changes, the 0·4% was found to be suitable for the life-long treatment. This toxicity technique for chronic carcinogenesis experiment was developed in this laboratory and described recently in detail [16].

Group 1. AINH was dissolved in the drinking water as a 0·4% solution and was given continuously for the life span of 50 female and 50 male mice that were 4 weeks old (31 days) at the beginning of the experiment. The solution was prepared thrice weekly, and the total consumption of water containing AINH was measured at the same intervals during the treatment period. The solution was contained in brown bottles because of the possible light sensitivity of the chemical. The average daily consumption of water containing AINH per animal was 5·4 ml for the females and 6·9 ml for the males. Therefore, the average daily intake of AINH was 21·6 mg for a female and 27·6 mg for a male.

Group 2. As a control, 110 females and 110 males were kept untreated.

All animals were checked and weighed weekly and gross observable changes were recorded at the same time. The mice were allowed to die or were killed with ether when found to be in poor condition. All animals were necropsied. All organs were examined macroscopically and fixed in 10% buffered formalin. Histologic studies were done on the liver, spleen, kidney and at least 4 lobes of the lungs of each mouse as well as those organs that showed gross pathologic changes. Sections from these tissues were stained routinely with hematoxylin and eosin and with additional special methods when necessary.

RESULTS

The survival rate at ten-week intervals is recorded in Table 1. It is clear from this data that AINH treatment shortened the survival when compared with the corresponding controls. The average weekly body weight of treated and control mice was also recorded throughout the experiment and the treatment significantly lowered body weight in both sexes when compared with the controls [14].

Table 2 shows the number, incidences of various types of tumors together with their latent periods. As can be seen, the administration of the compound gave rise to high incidences of lung tumors. In the following, the most important lesions are described in detail.

Lung tumors

In the AINH-treated animals, 37 females developed 157 tumors of this organ with an incidence of 77%. Out of these, 27 mice had 83 adenomas and 10 animals had 58 adenomas and 16 adenocarcinomas. The average latent period for these lung tumors was 79 weeks, the first was found at the 45th week and the last at the 97th week. In the AINH-treated males, 29 mice developed 108 lung tumors with an incidence of 58%. Out of these, 23

Table 1. *Treatment and survival rates in* 1-*acetyl*-2-*isonicotinoylhdrazine* (*AINH*) *treated and control Swiss mice*

Group	Treatment	Initial No. and sex of mice	No. of survivors (Age in weeks)												
			10	20	30	40	50	60	70	80	90	100	110	120	130
1	0·4% AINH in drinking water daily for life	50♀	50	47	47	47	41	33	26	16	7	—	—	—	—
		50♂	49	49	42	39	31	14	9	3	2	—	—	—	—
2	Untreated	110♀	109	109	107	104	96	89	73	57	41	23	11	1	—
	Control	110♂	110	95	91	86	67	55	41	22	6	1	1	—	—

Table 2. Tumor distribution in 1-*acetyl-2-isonicotinoylhydrazine (AINH)—treated and control Swiss mice.*

Group	Treatment	Effective No. and sex of mice	Animals with Lung tumors No.	%	Latent period*	Mal. lymphomes No.	%	Latent period*	Other tumors (Latent period*)
1	0·4% AINH in drinking water daily for life	48♀	37	77	79 (45–97)	3	6	77 (56–97)	1 Hepatoma (51) 1 Angioma of liver (65) 1 Granulosa cell tumor (89)
		50♂	29	58	65 (36–93)	2	4	24 (20–28)	1 Papilloma of forestomach (93) 1 Adenocarcinoma of glandular stomach (96)
2	Untreated	110♀	14	12	90 (64–119)	16	14	77 (39–115)	9 Adenocarcinomas of breast (46, 60, 64, 68, 73, 91, 93, 98, 110) 3 Subcutaneous sarcomas (68, 82, 82) 3 Hemangiomas of liver (69, 77, 84) 1 Sex cord mesenchyma tumor (99) 1 Papilloma of forestomach (112) 1 Luteoma (99) 1 Subcutaneous fibroma (87) 1 Granulosa cell tumor (65) 1 Malignant plasmacytoma (71) 1 Hemangioma of ovary (42)
	Control	110♂	11	10	74 (47–110)	2	1	77 (73–82)	2 Hemangiomas of liver (72, 80)

*Age in weeks

mice had 71 adenomas and 6 animals had 29 adenomas and 8 adenocarcinomas. The average latent period for these lung tumors was 65 weeks, the first was observed at the 36th week and the last at the 93rd week of age. There was no significant difference in the latent periods of adenomas and adenocarcinomas. The adenocarcinomas did not metastasize to other organs.

Macroscopically and histologically these lesions were similar to those described previously in another publication from this laboratory [17].

Malignant lymphomas

In the treated females, only three such tumors were seen, one was classified as lymphocytic type and was found at the 56th week. The other two were diagnosed as histiocytic type and were observed at the 78th and 97th week of age. In the treated males, two lymphoreticular tissue neoplasms were seen. The first one was classified as stem (undifferentiated) cell type and was found at the 20th week, while the other was diagnosed unclassifiable (because of advanced decomposition) and was observed at the 28th week of age.

Grossly and cytologically these tumors were similar to those found and described earlier in this mouse strain [18].

Other tumors

As can be seen in Table 2, a few other types of tumors were also observed in the treated groups. Since they occurred in low incidences, their apprarances cannot be related to the treatment.

The descriptions of some tumors in the control animals are given in a previous publication [14]; nevertheless, their incidences are shown in Table 2 for comparative purposes.

DISCUSSION

The present findings demonstrate that the continuous lifetime administration of 0·4% 1-acetyl-2-isonicotinoylhydrazine in the drinking water daily to 4-week-old randomly bred Swiss mice enhanced the development of lung tumors. The incidence rose from 12 to 77% in females and from 10 to 58% in males as compared with the untreated controls. The treatment had no apparent effect on the occurrence of other types of tumors.

The study thus records for the first time the carcinogenicity of AINH. In fact, this chemical had never been used for carcinogenesis investigation even though it had been known for quite some time that in man it was the major urinary metabolite of the tuberculostatic agent INH [1]. Another metabolite of INH, hydrazine, which was found in rabbit urine but not in man's [4, 7], was also shown to produce different types of neoplasms in mice and rats [9, 12–15]. On the other hand, isonicotinic acid, a third by-product of INH in man and in several animal species, failed to induce significant incidences of tumors in mice [10, 11]. In addition to these three metabolites, INH metabolizes, in various animal species, into other compounds such as isonicotinuric acid, *n*-methyl-isonicotinic hydrazide, di-isonicotinylhydrazine, etc. [5, 7, 19, 20]. This latter group of chemicals, however, have not been studied for tumor-producing capabilities.

The significance of the current AINH investigation lies in the fact that this acetylated metabolite of INH is less toxic than the parent drug and that it exhibits the same carcinogenic spectrum as the original compound. Another metabolite of INH, hydrazine, has been found to be more toxic and carcinogenic than INH

even though the tumor type found with both chemicals was identical. These findings thus clearly show that during its metabolic process a compound may produce metabolites whose toxic and carcinogenic properties vary significantly. In some cases, the toxicity or carcinogenicity or both of metabolites are greater, and in other instances lesser, than the original chemical.

INH has been found to produce tumors in mice by a number of investigators [8, 21–24]. However, when this chemical was administered to hamsters and rats for life, no tumor induction was achieved [25, 26]. From this it was postulated that the compound might break down differently in the various animal species; therefore, the possibility exists that in hamsters and rats no carcinogenic metabolites are formed.

Presently our attention is focused on two main areas. First, we are continuously enlarging our long-term carcinogenesis investigations by adding so far unstudied metabolites of INH. Second, the metabolism study of INH in mice and hamsters is under way, which hopefully may bring an understanding of its carcinogenic mode of action.

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Estrogen and Progestogen Binders in Human and Rat Mammary Carcinoma

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Abstract—*Estrogen and progestogen binders (so-called receptors) were measured in the high speed ultracentrifugal supernatant (cytosol) from* 23 *samples of human mammary carcinoma. Estrogen receptors were also measured in slices in* 15 *of these tumors. With one notable exception, the cytosol and slice techniques gave the same results.*

It was found that estrogen or progestogen cytosol receptors occurred in 11 *tumors. In* 6 *cases there were substantial levels of both receptors, in* 4 *cases only estrogen receptors and in* 1 *case only progestogen receptors. Generally, there was no evidence of a correlation between the amounts of estrogen and progestogen receptors. Thus, the simultaneous measurement of both receptors in human breast carcinoma may give more information on the hormonal sensitivity of a tumor than measurements of only estrogen receptors which has been done previously.*

Breast tumors in rats induced by 7,12-*dimethylbenzanthracene contained cytosol receptors both for estrogens and progestogens.*

INTRODUCTION

DURING recent years considerable work has been devoted to the measurement of specific estrogen binders (so-called receptors) in human mammary carcinoma tissue [1–6]. Since the presence of such receptors is indicative of estrogen responsiveness this method has potential value in selecting patients for endocrine therapy. At present, the prognostic value of the method is not established although some data are available on a favorable clinical course at least initially in the predicted cases [3]. Since the hormonal regulation of the normal mammary gland is complex, involving estrogens, progestogens, insulin and pituitary hormones, all these hormones may also influence mammary tumor growth. Measurements of estrogen receptors only may therefore not give sufficient prognostic information. The present communication describes simultaneous measurements of estrogen and progestogen receptors in samples of human and rat mammary carcinoma.

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MATERIAL AND METHODS

Human tumor material was radical mastectomy specimens from unselected patients treated at the Clinic of Surgery at the University Hospital of Uppsala. The excised tumor material was stored on ice until the start of an experiment, generally 1 to 2 hours. Breast tumors in rats were induced by giving 7,12-dimethylbenzanthracene (DMBA) intravenously to the animals when they were 50, 53 and 56 days of age [2]. The tumors used in the experiments differed widely in size and in growth rate.

Labelled estradiol-17β (6,7-^{3}H, specific activity 40·0 Ci/mole) and progesterone (1,2-^{3}H, specific activity 50·3 Ci/mole) were obtained from the New England Nuclear Corp., Boston, Mass. Nonlabelled norgestrel antipodes were kindly donated by Dr. R. A. Edgren at the Wyeth Laboratories Inc., Philadelphia, Penn.

The method for determining estrogen receptor content in tumor slices has been described elsewhere [7]. Preparation of tumor cytosol was at +4°C. Homogenates were prepared

by mincing tumor slices finely with a pair of scissors, after which the mince was further homogenized in 3 volumes of buffer (0·01 M Tris-HCl, pH 7·4, 0·0015 M EDTA, 0·25 M sucrose) by a Polytron PT10 (Kinematica, Luzern, Switzerland). Three pulses of 5 sec duration at setting 5 and with 2 min interval were given. The homogenate was centrifuged at $129{,}000 \times \mathbf{g}_{av}$ for 1 hr and the supernatant (= cytosol) was saved.

Binding tests were performed essentially according to Wiest and Rao [8] at +4°C in 3 ml centrifuge tubes which contained 0·5 ml of the buffer and either labelled estradiol-17β or progesterone at 6×10^{-10}M. All solutions with labelled progesterone also contained 6×10^{-8}M nonlabelled hydrocortisone (to eliminate eventual binding to corticosteroid binding globulin, CBG).

In order to test the specificity of the binding of labelled steroid, competition experiments with nonlabelled steroids at 6×10^{-8}M were done. With estradiol-^{3}H, estradiol and its antipode, *ent*-estradiol were used, with progesterone, norgestrel (17α-ethynyl-13α-ethyl-17β-hydroxygon-4-en-3-one) and its optical antipode. The antipodes of estradiol and norgestrel are biologically much less active and serve as controls of binding specificity [2,9]. In a typical experiment tumor cytosol is incubated with each of the following 6 mixtures (5 test tubes of each): estradiol-^{3}H, estradiol-^{3}H + estradiol, estradiol -^{3}H + *ent*-estradiol, progesterone-^{3}H, progesterone-^{3}H + norgestrel, and progesterone-^{3}H + *ent*-norgestrel.

To each test tube, 25 μl of the cytosol was added and the tubes were vortex mixed and allowed to stand for 16 hr. Then 0·5 ml of a charcoal suspension (0·05% Dextrane 10 from Pharmacia, Uppsala, Sweden and 0·5% of Norite A from Sigma Co., St. Louis, Mo.) was added to the tubes which were mixed on a vortex mixer, incubated for a further 10 min and finally centrifuged at $3000 \times \mathbf{g}_{av}$ for 10 min. Of the supernatant, 0·5 ml was withdrawn and 5 ml of scintillation solution (3 l dioxan, 300 ml methanol, 375 g naphtalene, 12 g PPO and 0·15 g dimethyl-POPOP) was added. Radioactivity was analysed by liquid scintillation counting. Protein was analysed with the Lowry method [10] with crystalline bovine serum albumin (Sigma Co.) as standard.

Receptor bound steroid was taken as the difference between the means: bound label in tubes estradiol-^{3}H minus (estradiol-^{3}H + estradiol) and progesterone-^{3}H minus (progesterone-^{3}H + norgestrel) respectively. When binding was observed it was stereospecific as predicted for receptor binding.

RESULTS

Estrogen receptors were determined in the tumor cytosol, in some cases also in tumor slices, while progestogen receptors were determined in the cytosol only.

The results of the determinations of receptor content in human carcinoma are shown in Table 1. It can be seen that estrogen and progestogen receptors were detected in 12 of the 23 samples. In 6 of these samples estrogen, and progestogen receptors occurred together. Samples from patients No. 3, 4, 7 and 8 had considerable amounts of estrogen receptors but no significant levels of progestogen receptors, while samples from patients No. 10 and 11 had considerable levels of progestogen receptors but absence or marginal levels of estrogen receptors. Generally speaking, there was no evidence of a correlation between content of estrogen and progestogen receptors in a sample.

Table 1 shows that in the 15 studied cases the cytosol and the slice techniques gave similar information on estrogen receptor content. In one case (No. 12) the slice technique gave a positive result while no receptor binding was detected in the cytosol preparation. In the specimen from patient No. 10 the result with slices was negative but a low level of estrogen receptors was detected in the cytosol.

Cytosol preparations of 6 tumors from DMBA-treated rats showed significant ($p < 0{\cdot}01$) amounts of estrogen and progestogen receptors (Table 2).

DISCUSSION

Very few earlier studies of progesterone-^{3}H binding to human mammary carcinoma are available. After injection, Ellis *et al.* [11] found uptake and retention of estradiol-^{3}H in mammary carcinoma but no evidence for uptake of progesterone-^{3}H (different series of patients were involved with the two steroids). In fact, the tumors showed a more rapid loss of progesterone-^{3}H than the surrounding normal tissue. The reason for this finding may lie in a dominating unspecific accumulation of steroid in fat tissue. A few experiments (Terenius unpubl.) which were carried out with normal mammary tissue from the mastectomy specimens were negative.

Progestogens are required for normal mammary growth and support mammary tumor growth at least in some experimental systems

Table 1. Estradiol binding capacity (EBC) of tumor slices as well as estradiol and progesterone binding capacity of cytosol receptor from human mammary carcinoma

Patient No.	Age (years)	EBC*	Bound to receptor (femtomoles/mg protein) Estradiol	Progesterone
1	73	2·55 ± 0·57	170	69
2	69	1·45 ± 0·21	160	145
3	63	5·33 ± 0·67	145	N.S.†
4	73	1·87 ± 0·56	130	N.S.
5	51	0·77 ± 0·13	92	32
6	70	1·43 ± 0·23	91	151
7	78	—	82	N.S.
8	69	—	56	N.S.
9	80	1·58 ± 0·20	49	42
10	70	N.S.†	34	160
11	61	—	N.S.†	61
12	47	1·72 ± 0·40	N.S.	N.S.
13	61	—	N.S.	N.S.
14	51	—	N.S.	N.S.
15	43	—	N.S.	N.S.
16	80	N.S.	N.S.	N.S.
17	64	N.S.	N.S.	N.S.
18	61	N.S.	N.S.	N.S.
19	69	N.S.	N.S.	N.S.
20	55	N.S.	N.S.	N.S.
21	57	N.S.	N.S.	N.S.
22	62	—	N.S.	N.S.
23	67	—	N.S.	N.S.

*Estrogen binding capacity of slices (cf. ref. 2)

†Not significant ($p > 0{\cdot}01$)

Table 2. Binding of estradiol and progesterone to the cytosol receptors of breast cancer from rats

Tumor	Bound to receptor (femtomoles/mg protein) Estradiol	Progesterone
1	122	42
2	59	13
3	57	11
4	45	33
5	16	34
6	15	12

[12]. The present results suggest a local sensitivity of human mammary carcinoma tissue to progestogens. This assumption is in agreement with the observation that thymidine incorporation in human mammary carcinoma in tissue culture can be influenced by progesterone [13]. These authors described 20 cases of which 5 were sensitive to progesterone, 5 to estradiol but only 3 to both hormones. Thus, in line with the present findings, estrogen and progestogen sensitivity were separate phenomena.

Therapy of mammary carcinoma with progestogens has received comparatively little interest and only a few critical studies are available. The Co-operative Breast Cancer Group has some recent results which indicate that progestogens or testosterone propionate give similar results in advanced breast cancer [14]. Stoll [15] reviewing work by him and by others reports that a positive clinical course to progestogen therapy is seen in 20–40% of treated cases (dependent on agent and dosage). These results deserve attention in view of the relative lack of severe side effects of progestogens [16].

The DMBA-induced mammary tumor in rats is sensitive to progestogens mainly when given together with estrogen. Progesterone given alone slightly increases cancerous processes while the result of a combined treatment with estradiol and progesterone can be a

marked suppression [17], but the effect is highly depending on the dosage. In this connection it can be noted that the combined use of estrogen and progestogen can give remission in patients who fail to respond to estrogen alone [18, 19].

The role of progestogen receptor activation in the growth of breast carcinoma is unknown as is the mechanism by which high doses of progestogen reduce tumor growth. Measurement of both estrogen and progestogen receptor levels, in combination with controlled clinical trials with estrogens, progestogens or both may give important information on the prognostic value of the receptor measurements besides giving information on what hormone sensitivity means.

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Recovery of the Genome of Murine Sarcoma Virus (MSV) After Infection of Cells with Nuclear DNA from MSV Transformed Non-Virus Producing Cells

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Abstract—*The hamster (HT-1 line) and the mouse (D-243 line) cultures are murine sarcoma virus (MSV) transformed cells which do not produce infectious virus. Nuclear DNA was isolated from these cells. This DNA was used to treat normal mouse embryo cells (3T3 line) and Rauscher leukaemia virus (RLV) infected 3T3 cells. Both cell cultures became transformed, but only the RLV infected 3T3 cells released infectious MSV. The nuclear DNA lost its biological activity following treatment with DNase.*

These experiments indicate that vertical transmission of mammalian C-type RNA virus is carried by the nuclear DNA of the viral transformed cells.

INTRODUCTION

THE CLOSE association of the C-type RNA viruses with leukaemia and sarcoma in different animal species is well documented. In avian leukaemia it was observed that both horizontal and vertical transmission may take place in nature, although the experimental evidence indicates that the important and most frequent mode of transmission is vertical. In murine leukaemia and sarcoma the accumulated evidence of the natural transmission of these agents indicates that the principal mode of infection is vertical [1]. Genetic transmission of C-type viruses may take the form of a morphologically complete and biologically active virus with or without the expression of malignancy. On the other hand, the viral genome may be carried in a latent form as part of the cellular genetic information of the malignant, or even the normal cells. Such latent C-type viral information may be expressed spontaneously [2] or following treatment by chemicals [3, 4]. The experimental evidence indicates that this situation exists both *in vivo* and in cultured cells. Already in 1963 Svoboda [5] suggested that the RSV genome may be present in the XC transformed rat cells as an integrated provirus, when he found the RSV genome persisting in the cells for generations in a latent, non-infectious form. However, the explanation of the mechanism enabling an RNA virus to be carried as part of the cellular genetic information was provided by the discovery of reverse transcriptase, the enzyme that can synthesize a DNA copy of the viral RNA [6, 7]. According to Temin [8] the viral coded DNA is integrated at a specific chromosomal site as an integrated DNA provirus. This provirus is replicated together with the rest of the genetic material and thus is passed to daughter cells at mitosis.

Recently it was demonstrated that the DNA of the host XC cells contains the genetic information for coding the RSV genome.

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Infectious RSV was recovered following the inoculation of chick embryo fibroblasts (CEF) with DNA extracted from the non-virus producing XC cells [9–11]. The experiments described in this paper provide evidence that nuclear DNA from MSV transformed, non-viral producing (N–P) hamster and mouse cells, carry the genetic information for the synthesis of this mammalian sarcoma virus.

MATERIAL AND METHODS

Cell culture

HT–1 cell line. These hamster tumour cells carry the defective MSV genome [12]. They do not contain the group specific (GS) antigen of the murine leukaemia and sarcoma viruses and do not release morphologically distinguishable virus particles [13].

D–243 cell line. These are mouse fibroblasts derived from a clone of MSV transformed cells. Occasional morphologically distinguishable viral particles can be detected and the cells carry the GS antigen of the murine leukaemia and sarcoma viruses [14]. These cells were kindly provided by Dr. G. Bassin of the National Cancer Institute.

3T3 cell lines. Normal mouse embryo fibroblasts (MEF) of both NIH and Balb/c strains.

NRK cells. A normal rat kidney cell line kindly provided by Dr. R. Gilden of Flow Laboratories.

All cell cultures were grown in Dulbecoo's modified Eagle's medium (DMM) supplemented with 10% foetal bovine serum (FBS) for growth and with 5% serum when used for focus assay. The media contained 100 units of penicillin, 100 μg of streptomycin and 0·5 μg of Fungizone per ml.

Sendai virus. This virus was kindly propagated by Dr. P. Biggs of the Houghton Poultry Research Station in the chorioallantoic membrane of embryonated eggs of leukaemia-free chickens. The virus was inactivated by 1,3-propiolactone and was used for cell fusion according to a procedure described earlier [15].

Isolation of nuclear DNA. The DNA was isolated from cell nuclei using Marmur's procedure [16] with some modifications. The detailed procedure was as follows. (All operations are done in the cold unless otherwise stated).

About 10^9 cells were suspended in 5 ml 0·075 M sodium chloride, 0·01 M magnesium chloride, 0·025 M sodium phosphate pH 6·8 and 5% v/v Nonidet P40. The suspension was then centrifuged at 1000 **g** for 10 min. The pellets were washed by resuspension with 5 ml of the same solution and repelleted. The final pellet was suspended in 10 ml 1 M sodium chloride, and 2 ml 5% sodium dodecyl sulphate (w/v) was immediately added. This was extracted at room temperature twice with phenol and then with chloroform isoamyl alcohol (24:1 v/v). The water layer was digested with 1 mg pronase (37°, 16 hr). The DNA was precipitated with 2 vol ethanol, dissolved in 5 ml 0·15 M sodium chloride, 0·015 M sodium citrate pH 7·0 and shaken with phenol at room temperature. The water layer was digested with 250 μg ribonuclease for 1 hr at 37° and then precipitated with 2 volumes of ethanol. The precipitated DNA was dissolved in 0·2 ml water, the volume adjusted to 3 ml with 0·15 M sodium chloride 0·15 M sodium citrate and the solution extracted with chloroform-isoamyl alcohol. After the addition of 1/10th vol of 3M sodium acetate and 1 mmol EDTA (pH 7·0), the DNA was carefully precipitated in a Vortex mixer with 0·54 vol isopropanol.

Recovery of MSV genome

Non-infected mouse embryo fibroblasts (3T3 cells) and Rauscher leukaemia virus (RLV) infected 3T3 cells were seeded separately into 50 mm plastic plates (3×10^5 cells per plate). On the following day when approximately 70% of the plate surface was covered with cells, the plates were incubated for 30 min with 5 ml of DMM which contained 20 μg of diethyl-aminoethyl-dextran (DEAE-D) per ml. Following incubation, the cells were washed twice with DMM, and 40 μg of nuclear DNA extracted from HT–1 cells were added to two plates each of non-infected and RLV-infected 3T3 cells. To two further sets of plates of infected and non-infected cells 40 μg of nuclear DNA, extracted from D–243 cells, was added in 0·5 ml volume. Some of the cells (non-infected and RLV-infected) were incubated with nuclear DNA (HT–1 and D–243) that had previously been incubated with DNase. Following one hour of incubation at 37°C inactivated Sendai virus was added to some of the DNA-treated and non-treated plates to increase the efficiency of DNA penetration into the cells. The Sendai virus was left on the cells for 30 min, the fluid was then discarded and DMM which contained 10% FBS was added to the cell culture. Incubation continued at 37°C in a CO_2 incubator (5% CO_2 in air). These cell cultures were trypsinized and passaged at weekly intervals. Prior to cell transfer, culture fluid was collected, filtered through a millipore filter (0·45 μ) and assayed for the development of transformed foci in 3T3 cells of both NIH and Balb/c strains as well as on NRK cells.

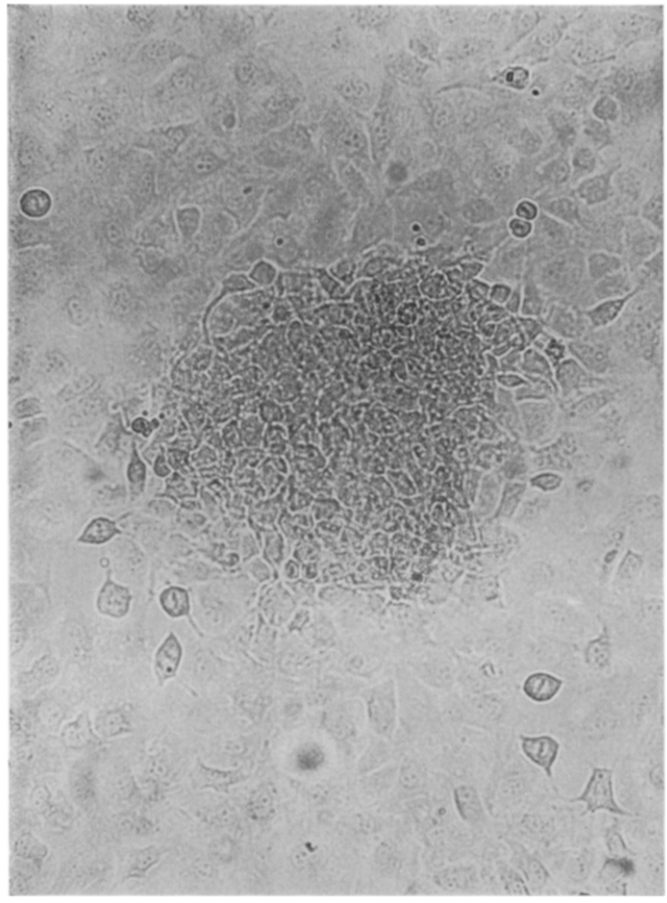

Fig. 1. *Focus of cellular transformation induced in* 3T3 *cells by nuclear DNA derived from D–*243 *cells.*

(to face p. 296)

Focus assay. The assay for the induction of cellular transformation by MSV was carried in 3T3 cells (NIH and Balb/c) as well as on NRK cells. These cells were seeded in 50 mm plates (3×10^5 cells per plate), treated the following day with 100 μg of DEAE-D in 5 ml DMM per plate. After 30 min of incubation the DEAE-D solution was washed off and the cells were exposed to the different filtrate preparation. Incubation continued for one hour, then 5 ml of DMM + 5% FBS was added. The infected plates were observed daily for cellular transformation and their medium was changed three times a week. The plates were kept up to three weeks following the infection.

RESULTS

Normal mouse cells treated with HT1-DNA and D–243–DNA, developed foci of transformed cells (Fig. 1) after five subcultures (six weeks). However, the culture fluid did not yield any infectious MSV when assayed on 3T3 and NRK cells. When these cultures were fused with RLV-infected 3T3 cells an infectious MSV was recovered. Likewise, RLV-infected

Table 1. *Assay for the presence of infectious MSV*

	Release of infectious MSV
3T3 RLV + HT–1–DNA	+
3T3 RLV + D–243 DNA	+
3T3 RLV + HT–1–DNA–DNase	–
3T3 RLV + D–243–DNA–DNase	–
3T3 + HT–1–DNA	–*
3T3 + D–243–DNA	–*
3T3 + HT–1 cells	–
3T3 + D–243 cells	–
(3T3 + HT–1–DNA) + 3T3 RLV	+
(3T3 + D–243–DNA) + 3T3 RLV	+

The cell cultures were treated with both DEAE–D and Sendai virus.

3T3 and NRK cells were used for MSV assay.

+ = infectious MSV. – = no infectious virus.

*These cultures became transformed but did not release infectious MSV.

3T3 cells treated with HT–1–DNA and D–243–DNA synthesized infectious MSV, which transformed both 3T3 cells and NRK cells. Normal and RLV infected 3T3 cells incubated with HT–1–DNA and D–243–DNA pre-treated with DNase, did not become transformed nor did they release infectious MSV. Normal 3T3 cells fused either with HT–1 or D–243 cells did not release infectious MSV. This is in contrast to the situation in XC cells that carry the defective RSV genome that will yield infectious RSV following fusion with any chick cells [17]. The results of the experiments are summarized in Table 1 and indicate that the genetic information for the synthesis of the MSV genome is carried in the nuclear DNA of the N–P hamster and mouse cells. This in turn supports the important mode of vertical transmission of viral information.

DISCUSSION

Direct experimental evidence provided recently indicates that the DNA of the host XC cells contain the avian RSV genome [9–11]. The murine leukaemia and sarcoma C-type viruses have physical, chemical, morphological and biological properties similar to the avian C-type viruses, but are specific mammalian viruses. It was therefore interesting to show that the MSV information is also carried as part of the cellular DNA. The *in vitro* assay for sarcoma viral genetic information is based on the well established biological property that distinguished murine leukaemia viruses from murine sarcoma viruses, namely, the ability of sarcoma virus to transform cells in culture while MLV lacks this property. In the present study the nuclear DNA of two mamalian non-producing cell lines HT–1 and D–243 was used as a source for DNA-integrated MSV genetic information. The hamster cells (HT–1) carry the information for a biologically recoverable MSV, in spite of the absence of any other evidence for viral presence. The mouse D–243 cell line does not produce infectious MSV but contains the GS antigen as well as occasional morphologically distinguishable particles. The procedure used for the isolation of DNA involved the separation of cell nuclei from cytoplasm prior to DNA extraction. This DNA is therefore nuclear DNA as opposed to cellular DNA used previously [9–11]. The method described in this paper is likely to yield purer DNA. The DNA preparation proved to be biologically active when introduced into normal or RLV-infected mouse cells. Our studies show that when MSV-DNA was directly introduced into the mouse cells which were chronically infected with RLV, an infectious and transforming MSV was obtained. Normal mouse cells which were treated with the virus developed focal areas of cellular transformation, but no infectious MSV was recovered from the culture fluid. When these MSV-DNA transformed mouse cells were fused with HLV-infected mouse cells, the RLV was able to function as a helper virus and infectious MSV was released. This is in contrast to the studies with XC-DNA where the infected chick embryo cells yielded an

infectious RSV following the DNA treatment. The recovery of infectious RSV was not surprising since it has been established that even "normal" chick cells carry sufficient genetic information to enable a direct rescue (without the addition of any avian leukaemia virus) following fusion with XC cells. This is not the case of the cells which carry the defective MSV genome. Fusion of either HT–1 or D–243 cells with non-infected mouse cells did not yield an infectious MSV. This indicates that the normal mouse fibroblasts used in the fusion with transformed HT–1 and D–243 cell lines, do not carry sufficient viral genetic information to complement the defective MSV genome.

The biological activity of the extracted nuclear DNA to carry the information for coding for MSV, was lost following treatment with DNase. This further confirms that the nuclear DNA carried the biologically active viral genetic information.

The fact that a biologically active DNA was derived from MSV transformed non-producer cells of two mammalian species carried the genetic information for viral synthesis, may indicate that also a human C-type virus could be isolated *in vitro* from nuclear DNA of malignant cells by similar experimental procedures.

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Different Sensitivity to Actinomycin D of Biopsies of Human Tumors Cultivated *in vitro**

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Abstract—*Primary cultures of* 16 *human carcinomas,* 8 *osteogenic sarcomas,* 12 *sarcomas of different origin have been studied morphologically during the first week of culture. The lethal concentration of actinomycin D after a* 24*-hr treatment was established for both the malignant and the normal population of stromal cells. Results indicate that normal cells have a low sensitivity to actinomycin D in a narrow range of doses, while cancer cells show a higher sensitivity in a significantly wider range of doses. In osteogenic sarcomas the L.E. ranged from* 0·1 *to* 100 *μg/ml in the different patients.*

INTRODUCTION

A NUMBER of previous studies have dealt with determining the sensitivity of human tumors, obtained by biopsy and cultivated *in vitro*, to anti-neoplastic agents [1, 2, 3, 4, 5, 6]. The results have shown a large degree of variability [7, 1, 2, 8], suggesting that tumors considered similar according to morphological criteria, may instead, be quite different in their response to the same drug [11, 12]. In order to obtain more information on this subject, it was decided to investigate the effect of actinomycin D on primary cultures of human biopsies of carcinomas and sarcomas. Whenever possible, the effect of actinomycin D on cancer cells was compared with its effect on normal stromal cells.

MATERIAL AND METHODS

Cancer tissue was obtained from surgical specimens and stored in Medium 199, without bicarbonate, during the few hours needed to send the specimens to the tissue culture laboratory.

Dissection of the specimen was carried out immediately upon arrival in the laboratory, separating the different tissues present in the tumor and identifying the neoplastic parts in the tissue bulk by squashing small fragments of tissue in acetic-orceine. The neoplastic tissue was then cut into sections of about 1 mm each with two sharp blades, and the fragments washed twice in 199 medium before explantation.

Two explants were placed in the center of the lower coverslip of a Rose chamber and covered with a strip of perforated cellophane [13]. The internal diameter of the chamber was 20 mm and the volume, 1·8 ml. A 199 growth medium in Hank's balanced salt solution, supplemented with 20% calf serum and antibiotics, was used. The medium was renewed three times a week and the incubation performed in a CO_2 incubator. Cultures less than 10 days of age were used for all experiments.

Cultures were morphologically scored daily by phase contrast microscopy. When outgrowth of cells started, one microscopic field, containing the cell population to be studied, was marked with India ink on the outside of the coverslip and phase contrast pictures were taken.

Treatment was performed by substituting the growth medium with a medium containing graded concentrations (0·1, 1, 10, 100 μg/ml)

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of actinomycin D. After 24 hr of treatment, cultures were scored again and pictures were taken of the same microscopic field. In a few experiments, a second score was recorded after 48 hr. The study was carried out on 36 human tumors, 16 of which were carcinomas, 8 osteogenic sarcomas, and 12 sarcomas of different origin. It was possible to establish the sensitivity to actinomycin D of normal and cancer cells in some of the 16 carcinoma specimens.

Fifty Rose chambers were set up and the minimal concentration of actinomycin D capable of producing cell lysis [14] was recorded as the L.E. (Lethal End Point).

RESULTS

Carcinoma outgrowth invariably started after 4–5 days of culture and presented a mixed population of endothelial, fibroblastic and epithelial cells that in only a few cases migrated as nests of several cells. In several instances, the number of stromal or epithelial cells was too small to perform the experiment. Table 1 shows that out of 16 carcinomas, only 6 were studied on the epithelial population, only 6 on the fibroblastic, and 4 on both populations.

L.E. of cancer cells ranged from > 10 μg/ml to $< 0{\cdot}1$ μg/ml, while the L.E. of stormal cells was $\geqq 10$ μg/ml. Experiments 13 and 14 were performed on two different metastases of the same pharynx carcinoma.

A $\log_{10}$ scale was used to record the difference between stromal and cancer cells observed during experiments in which both populations were studied. Using this scale, $< 0{\cdot}1$ and > 10 were considered as 0·01 and 100, respectively. By the Student t-test ($t = 4{\cdot}7$ based on 3 df), the hypothesis that no difference existed between L.E. of normal and neoplastic cells was rejected ($p < 0{\cdot}05$). The L.E. of 10 human sarcomas of different origin, and of 7 osteogenic sarcomas are shown, respectively, in Tables 2 and 3. In these experiments, the only features that made it possible

Table 1. In vitro *sensitivity to actinomycin D of human carcinomas and stromal cells*

		Human carcinoma from	Cancer cells L.E. μg/ml	Stromal cells L.E. μg/ml	Difference in log 10
N	1	Lung	—	> 10	
	2	Lung	> 10	—	
	3	Lung	—	10	
	4	Lung	—	10	
	5	Lung	—	> 10	
	6	Lung	> 10	—	
	7	Lung	10	> 10	1
	8	Rectum ..	1	—	
	9	Larynx	> 10	—	
	10	Breast	—	> 10	
	11	Breast	1	> 10	2
	12	Breast	—	10	
	13	Pharynx	< 0, 1	10	3
	14	Pharynx	< 0, 1	10	3
	15	Ovary	> 10	—	
	16	Bone Metastasis	0, 1	—	

Table 2. In vitro *sensitivity to actinomycin D of human sarcomas*

	Human	Sarcomas	L.E. μg/ml
N.	17	Condromyxoma	0·1
	18	Condrosarcoma	100
	19	Condrosarcoma	1
	20	Ewing's Sarcoma	1
	21	Fibrosarcoma	0·1
	22	Fibrosarcoma	< 0·1
	23	Lymphosarcoma	10
	24	Malignant Synovioma	10
	25	Relapse of 24	> 10
	26	Malignant Synovioma	1
	27	Rhabdomyosarcoma	100
	28	Wilm's tumor	1

to define the cell population as malignant was an early outgrowth that became evident within less then 24 hr. In the experiments reported in Tables 2 and 3, the L.E. range was from < 0·1 μg/ml to 100 μg/ml.

Table 3. In vitro *sensitivity to actinomycin D of human osteogenic sarcomas*

	Human osteogenic sarcomas	L.E. μg/ml
N.	29	0·1
	30	0·1
	31	0·1
	32	10
	33	0·1
	34	10
	35	100
	36	10

Table 4 compares the data obtained with the different tissues. Stromal cells are very resistant to actinomycin D, while malignant cells are affected at a lower concentration of the drug.

DISCUSSION

Biopsy material obtained from cancer patients was cultivated *in vitro* to establish the concentration at which actinomycin D would be lethal. The method of morphologically following the first steps of the outgrowth process, and of performing the experiment on extremely young cultures, lead to the recognition of the different cell types before a morphological dedifferentiation and also permitted L.E. determination before cellular growth modified the composition and the metabolic needs of the cell population. This detail explains the different L.E. values shown in our data since cells tend to assume the same morphology [10], and the same sensitivity to chemotherapeutic agents [9] only after a prolonged period of culture. Carcinomas of different origin, sarcomas and osteogenic sarcomas did not show any specific sensitivity to actinomycin D, but there was a large variability of response, extending within more than a 1,000-fold range. It is interesting to note that cancer cells of the same histological type show a different sensitivity to actinomycin D, which is particularly evident with osteogenic sarcomas, where certain tumors proved sensitive to 0·1 μg/ml and others to 100 μg/ml of actinomycin D. This was not related to a lack of reproducibility since, according to previous data [14], two metastases (case 13 and 14) of the same tumor displayed a similar sensitivity to the drug.

The relapse (case 25) of a malignant synovioma (case 24) has also indicated that the same tumor may alter its sensitivity to actinomycin D after a 6-month remission produced by nitrogen mustard treatment.

In 10 carcinoma cases, it was possible to observe that the sensitivity of normal stromal cells to actinomycin D was relatively low. This fact could be related to a stromal cell metabolic peculiarity; however, this type of cell, upon becoming malignant, can become sensitive (cases 21–22). In 2 pharynx carcinoma metastases, the tumor was 1000 times more sensitive to actinomycin D than normal cells.

The causes of the differences in sensitivity observed are not known, although it has been reported that cancer cells either become insensitive [15–16] or show a primary resistance to actinomycin D [15–17]. Sensitive cells accumulate the drug more readily than resistant cells [12], with particular reference to the nuclei [18]. Since actinomycin D does not require any previous metabolization for interaction with DNA [19], the data herein reported may be important in deciding which cancer patient would be more likely to benefit from actinomycin D therapy.

Acknowledgements—We would like to thank Prof. Raymond Roy-Camille, Centre Hospitalier de Poissy, France, for his helpful cooperation on studies of bone tumors.

Table 4. *Lethal effect of actinomycin D on different tissues*

μg/ml	< 0.1	0.1	1	10	> 10
Osteogenic sarcoma	0	4	4	7	8
Other sarcomas	1	3	7	9	12
Carcinomas	2	3	5	6	10
Total malignant	3	10	16*	22	30
Stromal tissues	0	0	0	5	10

The figures indicate the number of individuals having L.E. at the indicated concentration.

*0·05 < p < 0·01 in respect to stromal tissues.

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The Relationship Between Growth Rate, Labelling Index and Histological Type of Human Solid Tumours

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Abstract—*Data on the labelling index of human solid tumours have been collected from the literature and pooled with the authors' own results.*

Mean values for five histological groups have been calculated. For the embryonal tumours and the hematosarcomas, the mean value of the labelling index ($\overline{L.I.}$) is of about 30 per cent and appears to correspond to a growth fraction (G.F.) close to 100%. The $\overline{L.I.}$ for the 3 other groups (squamous cell carcinomas, mesenchymal sarcomas and adenocarcinomas) are significantly lower, the lowest being for adenocarcinomas for which the $\overline{L.I.}$ is of about 2%.

The $\overline{L.I.}$ of these five groups have been compared with the previously evaluated growth rate. The $\overline{L.I.}$ and the doubling time (D.T.) of these five groups are negatively correlated and G.F. appears to be the main parameter of the kinetics of tumour cell proliferation to explain the differences in the growth rates of human cancers.

Cell-loss factors were estimated for the five groups of tumours. Highest cell losses were found in groups with high rates of cell proliferation. In spite of some very high rates of cell loss, this parameter does not seem to explain by itself the differences in growth rates of the various groups.

INTRODUCTION

THE GROWTH rate of human pulmonary metastases varies, as we have previously shown, [1] with histological type. Five histological groups of tumours were distinguished with significantly different doubling times (D.T.). Only a little data is available on the duration of the mitotic cycle and the growth fraction of human primary tumours but the labelling index has been measured either *in vivo* or *in vitro* in a large number of patients.

Nobody, as yet, seems to have surveyed this data in order to see if useful information could be derived from it, which is the aim of this paper. Although these measurements had been carried out by various laboratories with slightly different techniques, an attempt was made to add these to our own results in order to analyse the difference in the kinetics of cell proliferation for the various histological types of tumours.

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MATERIAL AND METHODS

An analysis of the published data has been made for labelling index obtained using small specimens of solid primary tumours, incubated *in vitro* with tritiated thymidine. We have collected data on most of the various histologic types of human tumours. However, we did not find relevant data on nervous tumours and melanomas. On the other hand, we have excluded from the analysis ascitic tumours and leukemias.

The specific activities of the tritiated thymidine that were used varied from 0·5 to 10 μCi per ml, depending on the author, although a value of 1 μCi per ml was that used most frequently. The culture medium was either physiological saline [2–5], medium 199 [6–10] or minimum essential medium (MEM) [11–14] with or without serum, the most common choice being calf serum.

The remaining technical details (i.e. the delay between excision of the tumour and

incubation, length of incubation) varied with the author, but the differences do not appear to be important. Most of the work was performed on small specimens of tumours, not necrotic in appearance and carefully dissected immediately before labelling.

Table 1 shows the labelling index in 156 cases collected from the literature together with the 86 results obtained in our laboratory. For these later results, the following method was employed. The tumour was washed immediately after excision in MEM and pieces 1 mm in thickness were removed and placed in contact with a medium consisting of 5 ml of MEM with 10% calf serum and containing 1 μCi of tritiated thymidine per ml. This preparation was then placed in an air-tight flask in which the atmosphere consisted of 85% air and 15% CO_2. The duration of the incubation was of 30 min at 37°C. The tumour specimens were then washed in medium 199 and fixed in a solution of 3 parts ethyl alcohol and 1 part acetic acid. After embedding in paraffin wax, the fragments were cut into slices 4μ thick. The autoradiographs were made with Ilford K2 emulsion, each slice being exposed for 15 days at 4°C. In general, either a minimum of 100 labelled cells or a total of 5000 labelled + unlabelled cells were counted.

The mean potential doubling time ($D.T._{pot.}$) was calculated using the relationship [15]:

$$D.T._{pot.} = \lambda \frac{T_s}{\overline{L.I.}}$$

A value of 0·75 was used for λ for all tumours. This value has been considered by Steel [15] as the most probable one for human tumours. The median of 22 values obtained for various types of human solid tumours by the method of percent-labelled mitosis curve (P.L.M. curve) was used for T_s [17, 16, 18, 19, 20, 21, 22] (Table 3).

The mean value for cell-loss factor was calculated using as values for D.T. the values obtained in a previous study [1] and using the formula [15]:

$$\phi = 1 - \frac{D.T._{pot.}}{D.T.}$$

The rate of cell loss, L, that is the number of cells which are lost per 10^9 cells per day, is equal to the rate of cell birth multiplied by the cell-loss factor:

$$L = 10^9 \, \phi \, (e^{kt} - 1)$$

where $k = \frac{0{\cdot}6931}{D.T._{pot.}}$ and t = 1 day.

Table 1. *Breakdown by author and pathological type of the different groups of tumours studied*

	Embryonal carcinomas	Hematosarc.	Mesenchymal sarcomas	Squamous cell carc.	Adenocarc.	Total
Published data (other authors) References	—	15 [10, 3, 13]	4 [11, 12, 13]	61 [5, 6, 7, 8, 9, 11, 12, 13]	76 [2, 3, 4, 6, 11, 12, 13, 14]	156
Data from Institut Gustave-Roussy References	6	—	28 (23)	7	45	86
Total	6 5 nephrobl. 1 embr. sarc.	15 13 Burkitt lymph. 1 Hodgkin's disease 1 lymphos.	32 13 fibros. 5 osteos. 4 reticulos. 3 rhabdom. 7 unclass.	68 30 E.N.T. 6 bronchii 19 cervix ut. 4 oesoph. 7 skin 1 bladder 1 kidney	121 75 breast 32 gastro-int. tract. 14 unclass.	242

RESULTS

1. The (geometric) mean values obtained for the labelling index ($\overline{L.I.}$) together with the confidence limits are given in Table 2. The distribution of the individual values is shown in Fig. 1. For values inferior to 0·5%, this measurement is not very accurate. In the group comprising the 121 adenocarcinomas, 25 values

Table 2. Labelling index (geometric mean and 95% confidence interval) for tumours of different pathological type

Pathology	Other authors data			Data for Inst. Gust.-Roussy			Total of the data		
	Number of patients	Geometric means	Confidence interv.	Number of patients	Geometric means	Confidence interv.	Number of patients	Geometric means	Confidence interval
Embryonal tumours	—	—	—	6	30·0	21·9–41·1	6	30·0	21·9–41·1
Hematosarcomas	15	29·2	22·2–38·3	—	—	—	15	29·2	22·2–38·3
Mesenchymal sarcomas	4	0·9	0·4–2·3	28	4·7	3·1–7·2	32	3·8	2·5–5·9
Squamous cell carcinomas	61	8·3	6·2 11·2	7	8·2	6·5 10·3	68	8·3	6·4 10·9
Adenocarcin.*	76	2·3	1·6 3·2	45	1·9	1·3 2·7	121	2·1	1·7 2·7

*Median value (obtained by graphic method) and confidence limits.

were equal or inferior to 0·5%. For this reason, the median $\overline{\text{L.I.}}$ and its confidence interval were estimated graphically. For the determination of the slope of the probit regression line, all the values for the $\overline{\text{L.I.}}$ equal or inferior to 0·5% were pooled together.

The highest $\overline{\text{L.I.}}$ were observed in embryonal tumours and hematosarcomas, the difference between the two not being significant. The $\overline{\text{L.I.}}$ for all the other groups of tumours were significantly different. In decreasing order, then follow squamous cell carcinomas, mesenchymal sarcomas, and adenocarcinomas.

The values obtained for the patients studied at Villejuif for squamous cell carcinomas and adenocarcinomas do not differ significantly from those collected in the literature. On the other hand, for mesenchymal sarcomas, our results on 28 tumours are signicantly higher than the 4 values reported in the literature.

The dispersion of individual values within different histological groups is greater as the $\overline{\text{L.I.}}$ decreases.

The ratio

$$\frac{\overline{\text{L.I.}}97{\cdot}5\%^{+}}{\overline{\text{L.I.}}2{\cdot}5\%}$$

is equal to 8 for embryonal tumours, 11 for hematosarcomas, 23 for squamous cell carcinomas, 142 for mesenchymal sarcomas and 275 for adenocarcinomas (see Fig. 1).

2. T_s was measured using the P.L.M. curve after injection of tritiated thymidine *in vivo* in 22 human tumours (Fig. 2 and Table 3)

$\overline{\text{L.I.}}$ 97·5 and 2·5 are obtained by the probit regression line.

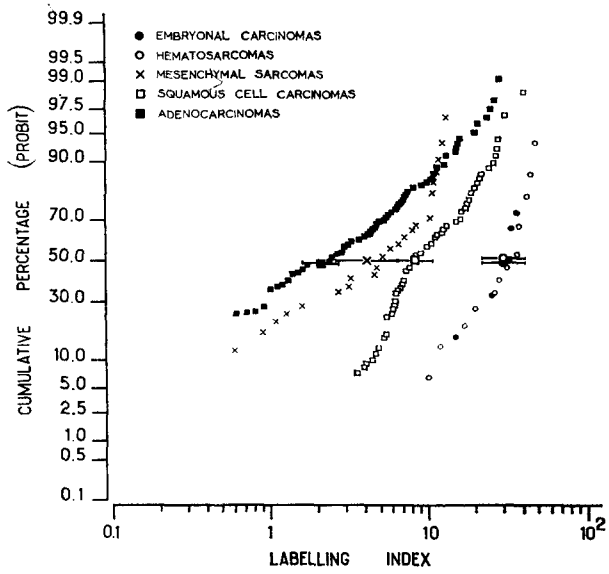

Fig. 1. Distribution of the individual values of the $\overline{\text{L.I.}}$ within different histological groups.

[16, 17, 18, 19, 20, 21, 22]. T_s varies between 11 and 24 hr with a median value of 16 hr. Given the small number of values, it is not possible to determine if T_s varies with the histological type of tumours. Assuming a value for T_s of 16·0 hr for all histological types, the mean $D.T._{pot.}$ of the 5 histological types was calculated and was found to vary considerably between different tumour types (Table 5).

A small variation of T_s (within the range of observed values) would not significantly alter the conclusion.

3. The comparison of $D.T._{pot.}$ obtained from primary tumours and of the mean actual D.T. obtained from a previous study on

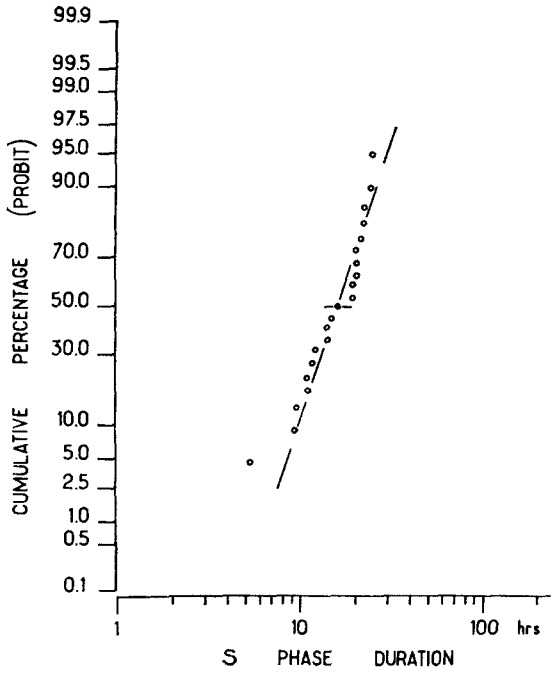

Fig. 2. Cumulative percentage of the different values of T_s measured in human solid tumours by the method of percent-labelled mitosis curve.

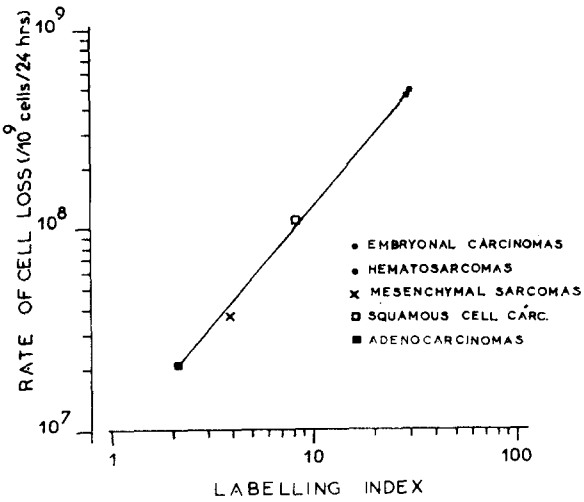

Fig. 3. Rate of cells which are lost in 24 hrs for a population of 10^9 cells as a function of the labelling index.

pulmonary metastases [1] enables the calculation of the cell-loss factor (Table 5). This increases as the cell proliferation becomes more important.

By taking into account both cell-loss factor (ϕ) and the rate of cell birth, it is possible to calculate the rate of cell loss, that is, the number of cells lost in 24 hr for a population of 10^9 cells. The rate of cell loss changes from one histological group to another. There is a difference of a factor of 23 between the 2 extreme groups (Table 5 and Fig. 3).

4. The mean values of D.T. for various histological types are negatively correlated with the $\overline{\text{L.I.}}$ (Fig. 4), the higher the value of D.T., the lower the value of $\overline{\text{L.I.}}$. However, it should be noted that the squamous cell carcinomas which have a higher D.T. than the mesenchymal sarcomas, have also a higher $\overline{\text{L.I.}}$.

DISCUSSION

1. The use of an *in vitro* labelling method raises the problem of validity of the method when applied to tissue separated from its blood supply.

However, the labelling indices obtained after *in vivo* labelling of 23 cutaneous tumours [18, 22] gave a value of 8·3% (6·4% –10·9%) which may be compared to a value of 8·2% (6·5%–10·3%) obtained *in vitro* from skin tumours (Table 2). Similarly, the ratio of $\overline{\text{L.I.}}$ obtained *in vitro* to mitotic index for 28 mesenchymal sarcomas gave a mean value of 12·7 [23] which is in close agreement to the value of 13·0 obtained *in vivo* for 20 superficial tumours [18, 22]. Moreover, for an experi-

Table 3. T_s (in hours) for tumours of different pathological types (method of P.L.M. curve)

Authors	Squamous cell carcin	Adenocarcinomas	Embryonal tumours	Melanomas	Schwannoma
Frindel *et al.*	19–19–11–12	—	—	—	—
Bennington	11·8–9·3	—	—	—	—
Young and De Vita	—	24–19–22	—	24–22	—
Shirakawa *et al.*	—	—	—	20–24	—
Weinstein *et al.*	20	—	—	—	—
Wagner *et al.*	—	—	27	—	—
Terz *et al.*	5·3	14–9·5–14–11	—	21	14·5

Table 4.

Authors	T_c (hr)	T_s (hr)
Frindel *et al.*	97	19
	51·5 (36–67)	19
	27·5 (25–30)	11
	48	12
Bennington	14·5	11·8
	14·3	9·3
Young and De Vita	41·7	24
	82·3	24
	73·6	22
Shirakawa	120 (3–7 days)	20
	144 (3–9 days)	24
Weinstein	217	20
Terz	44·5	14·5
	25·5	11
	26	14
	42	21

Mean value of $\frac{T_c}{T_s} \simeq 3$

mental fibrosarcoma (E. Frindel, unpublished results), the $\overline{\text{L.I.}}$ obtained *in vivo* and *in vitro* are identical. Recently, J. Denekamp observed that for experimental tumours, in spite of a wide dispersion of individual results, the mean values of the $\overline{\text{L.I.}}$ are similar after an *in vivo* or an *in vitro* labelling [24].

Labelling *in vitro* could still introduce serious errors if it happened that for one type of tumour, the *in vitro* labelling index was systematically underestimated. Previous discussion shows that this is probably not the case for squamous cell carcinomas and mesenchymal sarcomas. However there is no direct proof that this type of error is not occurring for adenocarcinomas. For the hematosarcomas and the embryonal tumours the *in vitro* $\overline{\text{L.I.}}$ is so high that it is difficult to imagine that they could be underestimated.

Another problem is due to the differences in the *in vitro* labelling technique used by various authors. However, comparable results have been obtained by various laboratories for tumours of the same type (Table 2). The important differences between the $\overline{\text{L.I.}}$ obtained from different histological types are probably much larger than the dispersion of values introduced by the variations in the labelling technique for a same tumour. It therefore seems valid to calculate averages and confidence limits using results from several authors.

2. The class of tumours for which the $\overline{\text{L.I.}}$ has been studied *in vitro* is not representative of all human tumours [1, 14, 23]. However, the taking into consideration of histological type limits the importance of this bias [1]. The cancers considered consist largely of tumours which are suitable for surgical treatment (tumours of breast, tumours of the gastro-intestinal tract, mesenchymal sarcomas) or those which are easily accessible (skin tumours, E.N.T., gynecological tumours). On the other hand, the tumours considered from the

Table 5.

Pathological type	Actual D.T.* (days)	Labelling index* (in %)	D.T. pot. (days)	Estimated G.F.† (in %)	Cell loss factor (in %)	Rate of cell loss (per 10^9 cells per day)
Embryonal tumours	27 (22–33)	30 (22–41)	1·66	90	94	$487 \cdot 10^6$
Hematosarc.	29 (23–37)	29 (22–38)	1·70	90	94	$473 \cdot 10^6$
Mesenchymal sarcomas	41 (35–50)	3·8 (2·5–5·9)	13·2	11	68	$37 \cdot 10^6$
Squamous cell carcin.	58 (48–70)	8·3 (6·4–10·9)	6·0	25	90	$111 \cdot 10^6$
Adenocarcin.	83 (72–96)	2·1 (1·7–2·7)	23·8	6	71	$21 \cdot 10^6$

*For each pathological type, the mean value and, in brackets, the 95 per cent confidence intervals are indicated.

†The growth fraction is estimated using the equation: G.F. $= \overline{\text{L.I.}} \times \frac{T_c}{T_s}$ (see Table 4).

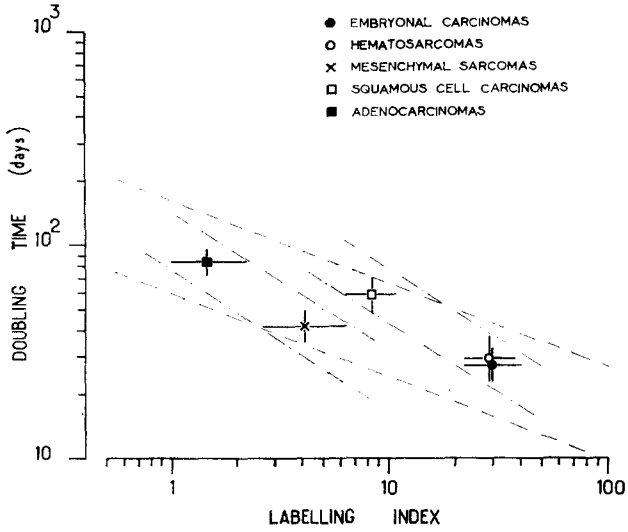

Fig. 4. Relationship between the doubling time and the labelling index for 5 histological groups of human solid tumours.

two types of hematosarcomas and embryonal tumours, which consisted largely of Burkitt's tumours and nephroblastomas, do not comprise more than a small fraction of the tumours normally observed in these two histological groups. It is to be hoped that, in the future, the $\overline{L.I.}$ of other embryonal tumours and hematosarcomas will be reported.

The dispersion of values varies considerably between histological groups. It is negatively correlated with the $\overline{L.I.}$ and is therefore maximal for mesenchymal sarcomas and adenocarcinomas.

Although the use of 2 different methods to appreciate the $\overline{L.I.}$ (geometric mean for 4 groups and median value obtained by graphic method for adenocarcinomas) could introduce a bias, the differences between the $\overline{L.I.}$ of the various histological groups considered are so important that slight variations due to technical factors would not change them.

3. The comparison of the D.T.s of pulmonary metastases to the $\overline{L.I.}$s of primary tumours is open to criticism. It is probable that the D.T. is, on the average, shorter for the metastases than for primary tumours [1]. However, this source of error would be serious only if the relationship between D.T. of the metastases and of the primary tumours varies with histological type. This does not seem to be true [1].

If the D.T.s of the primary tumours are in fact longer, the importance of the cell losses will be slightly underestimated. This discussion shows that, in the future, it would be desirable to measure the D.T. and the $\overline{L.I.}$ simultaneously for the same tumours.

The G.F. was estimated under the assumption that the duration of T_s is about equal to one third of the duration of T_c (Table 4) and that this value is the same for all the pathological groups. In fact, the distributions of T_s and, even more, of T_c are wide even with respect to one tumour. It is therefore difficult to define the ratio T_c/T_s by a single value. Furthermore, so little data exists that it is difficult to justify the assumption that T_c/T_s has the same value in all pathological groups. Nevertheless, in spite of all the sources of inaccuracy, it appears that in hematosarcomas and embryonal tumours, practically all the cells are cycling, whereas in carcinomas and mesenchymal sarcomas, a large proportion of cells are quiescent. Of all the parameters of the kinetics of tumour cell proliferation, the G.F. varies most between various histological types and is the most highly correlated with growth rate. The cell-loss factor varies less from one histological group to another and is the highest in the group with the highest G.F., suggesting that the importance of the cell-loss factor is conditioned by the rate of cell proliferation. The reasons for which the tumours with a high rate of cell proliferation also have a high rate of cell loss are not obvious; it may be that the supply of nutrients does not increase fast enough.

In general, the differences in losses do not explain the differences observed in the D.T.s for various types of tumours. However, there is one exception. Squamous cell carcinomas grow more slowly than mesenchymal sarcomas although they have a higher $\overline{L.I.}$ The increased cell losses from the squamous cell carcinomas

would explain why their D.T. is greater than for the mesenchymal sarcomas. This is similar to observations in experimental tumours obtained by Denekamp [26].

4. In conclusion, the variations in growth fraction (estimated with the previously discussed assumptions) explain most of the differences in growth rate for different types of human tumours. The values estimated for the G.F.s are in contrast to the values measured for tumours labelled *in vivo* and studied by the method of the P.L.M. curve. The values found were of about 50% (24%–82%) [21] and did not seem to vary significantly with histological type. This may be a result of bias since the method of P.L.M. curve has been used only for tumours with a high rate of cell proliferation and a high mitotic index.

5. During the growth of experimental solid tumours, the lengthening of the doubling time which has been observed in all tumours is due mostly to an increase in the cell loss, while the G.F. decreases [27]. This may appear to contrast with the present findings. However, in the present paper, no comparison is made of the kinetics of cell proliferation of a specific histological type of tumour at various stages of its growth. Instead, a comparison is made of the kinetics of various tumours at time of diagnosis, that is at approximately the same phase of their course. Even if later, during the growth of the human tumours, the $\overline{L.I.}$s would decrease, in the fast growing tumours, the cell losses which are already very high could not increase. Even if these changes occurred, they would not alter the conclusions that the G.F. is the main parameter determining the growth rate and that the cell proliferation and the cell loss are correlated.

6. The question remains: why do tumours of various histological types have different kinetics of proliferation? It seems as if the rate of cell proliferation as well as the absolute cell losses reflect in some way what happens in corresponding healthy tissue. The squamous cell carcinomas, the hematosarcomas and embryonal tumours originate from tissues in which the cell turnover is high and, as a result, have a high rate of cell loss. On the other hand, adenocarcinomas (with the exception of the tumours of the gastro-intestinal tract) and the mesenchymal sarcomas originate from tissues which rarely divide except under traumatic shock or hormonal stimulus.

7. It may be noted that it is for the 2 groups, hematosarcomas and embryonal tumours, with highest values for $\overline{L.I.}$ that chemotherapy seems to be most useful.

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Lysosomal Enzymes in 7, 12-Dimethylbenz[a]anthracene-induced Mammary Carcinomas after Ovariectomy

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Abstract—*The activities of the five acid hydrolases together with protein, DNA, and RNA were established in growing tumours and in tumours five days after ovariectomy. Although variations were recorded between the individual enzymes, the general trend was an increase in the specific activity of the five acid hydrolases. The most marked increase in activity was recorded with acid proteinase, an important enzyme involved in the breakdown of tissue components. The results indicate that the pattern observed for the regressing tumour is similar to that observed for normal hormone-dependent tissue undergoing physiological changes.*

INTRODUCTION

MANY investigations have been carried out with rat mammary tumours induced with 7,12-dimethylbenz[a]anthracene and 3-methylcholanthracene to demonstrate that they are hormone-dependent [1, 2]. In this respect, the tumour resembles the growth and regression observed in the remodelling of hormone-dependent tissues undergoing normal physiological changes. Lysosomes are known to be involved in the regression of the uterus [3], the involution of the mammary gland [4, 5, 6], and the atrophy of the prostate [7]. The aim of the present study was to investigate the response to ovariectomy of several acid hydrolases in rat mammary tumours. It was thought that since acid hydrolases are specific marker enzymes for lysosomes, the results would yield information on the role of lysosomes in the regression of hormone-dependent tumours.

MATERIAL AND METHODS

Virgin female rats of Sprague–Dawley strain were fed by intubation with a single dose of a solution containing 20 mg dimethylbenz[a]anthracene (DMBA) in 1 ml sesame oil when the rats were 50 (± 2) days old. The animals were housed in groups of 4 and fed diet and water *ad libitum*. After four weeks the animals were palpated for tumours at weekly intervals.

Ovariectomy was performed, under ether anaesthesia, by incision through the lumbar region. The ovaries were removed from animals in which the tumours had reached a size of 2 cm × 2 cm. Control animals underwent a sham operation.

Removal of tumour and preparation of tissue

Animals were sacrificed five days after ovariectomy. The tumours were then removed, dissected free of fat and cut into pieces of approximately 300 mg wet weight and kept at 0°. From each piece of tumour a small sample was then taken for histology.

Each piece of tumour was homogenized in ice-cold 0·05 M-*tris*-HCl buffer pH 7·4 in a Sorval-Omni Mixer (5 min at full speed). The final concentration of the homogenate was between 2 and 3% (w/v).

Enzyme assays and analytical procedures

The enzyme tests were carried out at 37°C and within 6 hr of the removal of the tumour. All enzyme measurements were linear with time and protein concentration. Initial experiments were carried out to ensure that the homogenization procedure released all the available lysosomal enzymes. This was achieved

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by carrying out the enzyme assays in the presence and absence of 0·1 per cent (w/v) Triton-X100. The inclusion of Triton–X100 did not increase the enzyme activity of the acid hydrolase under investigation.

Acid phosphatase. The determination of acid phosphatase activity was assayed by two different methods.

In one, the amount of phosphate liberated from β-glycerophosphate (0·05 M) at pH 5·0 was measured in a final volume of 1 ml. The reaction was stopped by the addition of 8% (w/v) trichloroacetic acid and inorganic phosphate determined in the filtrate by the method of Fiske and Subbarow [8].

In the second method, acid phosphatase was assayed at pH 5·0 with *p*-nitrophenylphosphate (10 mM) as substrate [9] and preincubating the fractions for 10 min to inactivate microsomal phosphatases as described by Neil and Horner [10].

Acid proteinase (Cathepsin D). Acid proteinase was assayed at pH 3·3 by using haemoglobin (2% w/v) as substrate as described by Barrett [11]. The reaction was stopped by the addition of trichloroacetic acid (3% w/v) and the released tyrosine determined in the filtrate by the method of Anson [12].

β-N-acetylglucosaminadase. Determinations for this enzyme were carried out at pH 5·0 with 4-0-nitrophenyl *N*-acetyl β-D-glucosaminide (4 mM) as substrate. The reaction was stopped by the addition of 3·0% (w/v) trichloroacetic acid and the liberated nitrophenol measured in the filtrate using a glycine-carbonate buffer to develop the colour.

β-Glucuronidase. β-Glucuronidase was measured at pH 5·0 with phenolphthalein glucuronide (1·5 mM) as substrate as described by Giannetto and DeDuve [13].

Nucleic acids were extracted from the total homogenate by the method of Schneider [14]. DNA was determined by the method of Burton [15] and RNA measured by the reaction with orcinol [16].

Protein was measured by the method of Lowry, Rosebrough, Farr, and Randall [17] with bovine serum albumin as a standard.

RESULTS

The effect of ovariectomy on tumour size

The size of the mammary tumours were measured each day after ovariectomy. No significant change in tumour size could be detected up to five days after the removal of the ovaries. This observation is in agreement with the results of Hilf [18].

The effect of ovariectomy on the activity of acid hydrolases

The results of the enzyme assays carried out on tumours taken from ovariectomised and control animals are shown in Tables 1 and 2. Significant increases in enzyme activity were observed for β-glycerophosphatase, *p*-nitrophenylphosphatase, acid proteinase (Cathepsin D), *N*-acetylglucosaminadase, and β-glucuronidase in the tumours taken from ovariectomized animals. These increases in activity are apparent for the total enzyme activity (units/g tissue) (Table 1) and for the specific activity (units/g protein) (Table 2). The ratio of enzyme activity in tumours obtained from ovariectomized animals to those obtained from control animals were: β-glycerophosphatase 7·7, acid proteinase (Cathepsin D) 8·5, *N*-acetylglucosaminadase 2·6, β-glucuronidase 1·9, and *p*-nitrophenylphosphatase 1·8. Thus, the increase in acid phosphatase activity was dependent on the substrate used for the assay. It is interesting to note that although all the enzymes assayed are located within the same sub-cellular particles, the extent of the increase is different for individual enzymes.

Table 1 also shows the DNA and RNA content of the tumour expressed per gram of tissue. Compared with control animals, the DNA content of tumours was significantly increased in ovariectomized animals while the opposite result was observed for RNA. The RNA/DNA ratio was reduced. These results are similar to those reported by Hilf [18]. In view of the slight increase in DNA content of the mammary tumour at five days after ovariectomy, it was thought necessary to express enzyme activity in terms of DNA (Table 3). This reflects the change in activity per cell. When calculated on a DNA basis, the five enzymes showed a significant increase in activity. The ratios between test and control were similar to those calculated using the data in Table 1. Taken together, these results suggest that the increase in enzyme level is due only in part to an increase in cell numbers.

DISCUSSION

Studies relating to the early biochemical changes in hormone-dependent tumours have been mainly concerned with the activities of enzymes involved in carbohydrate metabolism. Hilf and his co-workers [18] have demonstrated significant decreases at five days after ovariectomy of pyruvate kinase, glucose-6-phosphate dehydrogenase and phosphoglucomutase, while the activity of α-glycerophosphate dehydrogenase and glucokinase were not

Table 1. Effect of ovariectomy on biochemical characteristics of rat mammary tumour induced by DMBA

Measurement	Control	Day 5 after ovariectomy
RNA (mg/g)	7·60 ± 0·40*	6·44 ± 0·47*‡
DNA (mg/g)	8·31 ± 0·49*	10·54 ± 0·38*†
RNA/DNA ratio	0·91	0·61
	Enzyme activity.* (Units /g tissue) §	
Cathepsin D	0·45 ± 0·04*	3·82 ± 0·21*†
N-Acetylglucosaminadase	1·00 ± 0·02*	2·60 ± 0·08*†
β-Glucuronidase	0·48 ± 0·05*	0·93 ± 0·04*†
p-Nitrophenylphosphatase	3·07 ± 0·24*	5·57 ± 0·21*†
β-Glycerophosphatase	0·37 ± 0·05*	2·84 ± 0·18*†

*Mean values ± S.E.M.

†Significantly different ($p < 0·005$) from control animals.

‡Significantly different ($p < 0·05$) from control animals.

§Units of activity were: Cathepsin D, μmoles tyrosine equivalents released /min at 37°C; *N*-acetylglucosaminidase, μmoles *p*-nitrophenol released /min at 37°C; β-glucuronidase, μmoles of phenolphthalein released /min at 37°C; *p*-nitrophenylphosphatase, μmoles *p*-nitrophenol released /min at 37°C; β-glycerophosphatase, mmoles of phosphate released /min at 37°C.

Table 2. Effect of ovariectomy on biochemical characteristics of rat mammary tumour induced by DMBA

Measurement	Control	Day 5 after ovariectomy
RNA (mg/g protein)	83.42 ± 5·12*	69·05 ± 4·10*‡
DNA (mg/g protein)	95·52 ± 7·82*	100·92 ± 3·94*†
	Enzyme Activity (Units /g protein)	
Cathepsin D	4·66 ± 0·66*	40·16 ± 2·09*†
N-Acetylglucosaminadase	12·70 ± 2·56*	28·64 ± 1·85*†
β-Glucuronidase	4·63 ± 0·43*	9·50 ± 0·45*†
p-Nitrophenylphosphatase	31·58 ± 2·95*	58·27 ± 3·01*†
β-Glycerophosphatase	3·64 ± 0·56*	30·44 ± 2·24*†

*Mean values ± S.E.M.

†Significantly different ($p < 0·005$) from control animals.

‡Significantly different ($p < 0·05$) from control animals.

Table 3. Effect of ovariectomy on biochemical characteristics of rat mammary tumour induced by DMBA

Measurement	Control	Day 5 after ovariectomy
	Enzyme activity (Units /g DNA)	
Cathepsin D	51·60 ± 6·23*	408·00 ± 21·68*†
N-Acetylglucosaminadase	131·97 ± 31·20*	291·53 ± 13·89*†
β-Glucuronidase	53·92 ± 9·01*	98·41 ± 6·36*†
p-Nitrophenylphosphatase	362·40 ± 49·92*	586·30 ± 37·52*†
β-Glycerophosphatase	54·43 ± 10·67*	291·42 ± 24·63*†

*Mean values ± S.E.M.

†Significantly different ($p < 0·005$) from control animals.

altered. The activity of hexokinase was significantly increased. Thus, no general trend in response to ovariectomy was observed with these important enzymes concerned with carbohydrate metabolism. In the present investigation all the acid hydrolases studied showed a significant increase in specific activity and activity per g DNA. This suggests a selective response of the enzymes associated with the lysosome to ovariectomy in the hormone-dependent tumour.

Lysosomal enzymes in general and acid cathepsin (Cathepsin D) in particular are known to be elevated in a number of types of tissue regression or remodelling including the involution of the rat uterus [3], the rat mammary gland [19], and the prostate gland after castration [7]. Elevation of acid cathepsin together with other acid hydrolases has also been reported in the regression of the Flexner-Jobling carcinoma [20] and the Jensen sarcoma [21]. Cathepsin D, the major acid proteinase of animal tissues, is known to be associated with the subcellular particle termed the lysosome [22]. The enzyme appears to be important in the breakdown of structural and non-structural proteins. In the uterus, which is also an oestrogen target tissue, Cathepsin D is of lysosomal origin, and it increases markedly in concentration during post-partum involution when there is a rapid breakdown of muscle protein, especially collagen, and of extra cellular proteins [23]. The elevated levels of Cathepsin D in the present study suggest that the involvement of the lysosome and Cathepsin D in the process of regression in the DMBA-induced tumour is essentially the same as in other tissues. This is supported by the concommitant increase in the activity of acid phosphatase, β-glucoronidase, and *N*-acetylglucosaminadase, all of which are well-characterized as lysosomal enzymes. Further, several acid hydrolases, including Cathepsin D, were increased in the DMBA-induced tumour undergoing regression after pregnancy [24].

At the present it is not possible to decide on the reason for the changes in enzyme levels reported in this study. A number of explanations are possible. The increase may result from the preferential degradation of non-enzymic and non-lysosomal protein. DeDuve [25] has advanced the view that the lysosomes and their content are conserved in the tissue being degraded. However, such a view would not account completely for the differences in increases of individual enzymes, especially with respect to Cathepsin D and β-glycerophosphatase.

An alternative explanation for the increase is the synthesis of lysosomal enzymes by macrophages after oestrogen withdrawal. No detailed information is available on the morphology of the DMBA-induced mammary tumour. In constrast, the involution of the mammary gland at the cellular level has been extensively investigated [4, 5, 6]. Here there is a marked increase in autophagocytosis during the first three days. Macrophages increase from the third day and appear to be involved in heterophagic processes which remove the debris of dead cells. Quantitative studies by the same authors [19] with acid phosphatase and Cathepsin D indicated that the enzymes increased in units per gram tissue to a peak about five days after involution had commenced. A similar situation in the mammary carcinoma would account for the increase in lysosomal enzymes. Certain metabolic inhibitors used for the treatment of malignant neoplasms cause considerable increase in autophagocytosis in tumours of experimental animals. Such an effect has been reported in mice mammary tumours treated with cyclophosphamide [26].

Weber [27] has demonstrated that tail involution of *Xenopus larvae* is accompanied by increases in the total activity of several acid hydrolases (including Cathepsin D, acid phosphatase, and β-glucuronidase). It appears that this increase in total activity reflects a *de novo* synthesis of such enzymes by activated macrophages since the increase in activity is blocked by the administration of inhibitors of protein synthesis. Helminen and co-workers [7] arrived at a similar conclusion in their studies on castration-induced prostate involution, that the increase in Cathepsin, D but not acid phosphatase, was due to an increase in *de novo* synthesis of the enzyme.

The *de novo* synthesis of enzyme protein is reflected in general by an increase in the activity calculated on a per cell basis (i.e., activity per μg DNA). Such an increase in enzyme activity is reported for the present study, and it remains to be demonstrated whether this apparent synthesis of lysosomal enzymes can be inhibited by actinomycin D or cycloheximide. It is possible that the DMBA-tumour after ovariectomy responds in a manner similar to the prostate after castration and to the normal mammary gland during involution.

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Biochemical Properties of Normal and Neoplastic Cell Surfaces; a Review*

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Abstract—*Changes in the surface of transformed and neoplastic cells are reviewed in relation to data obtained in the author's laboratory. The particular phenotypic pattern of transformed cell growth may stem from a disorder of social interactions between the cells as a result of chemical alteration of the cell periphery. This alteration hampers the establishment between cells of functional contact, the latter being defined as that form of intercell contact that normally triggers off a series of reactions leading to the inhibition of initiation of DNA synthesis. The resemblance with embryonic surface expressions and preponderance of the growth-movement over the resting-maturated state of tumor as in early embryonic cell activity is pointed out.*

More in particular, three types of changes in the transformed cell surface affecting the carbohydrate components of glycosphingolipids and glycoprotein, which seem to be of more than random character, are discussed. The conclusion is reached that in contrast to the dynamic surface expression of the normal cell, which modulates during the cell cycle, (part of) the surface expression of transformed or neoplastic cells is frozen in a conformation adverse to functional contact. The crucial aberration is thought to be that the tumor-cell surface is incomplete (lack of protein "cover"), or unable to modulate, or that the proper surface layer allowing functional contact is constantly being formed but digested by proteolytic action of the tumor cells. The absence of a proper configuration is considered to be of greater importance than the actual surface expression. The absence of a proper configuration, thus allowing surface heterogeneity among different tumor phenotypes, is considered to be of greater importance than the actual surface expression.

The role of cyclic-AMP formed by cell-surface adenylate cyclase in functional contact is discussed, and the tentative conclusion is reached that tumor cells may differ from normal cells in the net formation or assimilation of this signal.

INTRODUCTION

Cell sociology as a function of cell-surface interactions

THE PROBLEM to be considered here is whether a change in cell-surface properties is involved in, or causes cancerous growth. More in particular, this paper will consider whether cancer is a disorder of social interactions between cells which may evolve from chemical alteration of the cell periphery due to structural and/or topological alterations. Any such change should result from a prior event, effected by the carcinogenic stimulus, that alters the genetic information and/or the genetic expression of the cells. Is the cell surface the key site at which the (epi-)genetic changes associated with oncogenesis are expressed?

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Many properties of mammalian cells are expressed at, or mediated by, the cell surface, and among these properties are indeed those which distinguish cancer from normal cells.

Since the malignant cell proliferates more or less unrestrained and is capable of migration leading to invasive and metastatic growth, malignancy may be defined as an escape from both growth and positional controls. Obviously, in view of the manifold neoplastic phenotypes, these controls may be lessened to various extents.

It is evident that for migration to occur, living cancer cells should be shed from the primary tumor and, consequently, released from cell contacts.

However, it is only more recently that the cell surface has been found to participate, if not to play a leading role, in growth control and that changes in transformed (tumor) cell surfaces have been related to the phenotypically altered growth patterns of these cells. These

concepts stem from studies *in vitro* of the so-called contact inhibitions of movement [1] (locomotion; contact paralysis), motility [2], and growth [1, 3, 4] (or replication; also termed topoinhibition [5], or density-dependent inhibition [6] of cell replication), and many other studies which emerged in their wake.

Growth and motility—or membrane movement—appear to be demonstrated mainly by the free cells of a sub-confluent culture, or at least by cells possessing an extended free surface. By contrast, the resting state of cell metabolism, in which both cell movement and replication is suppressed, is exhibited by cells in intimate contact [1, 3] with each other, *viz.*, by cells showing a much reduced free surface area. Now, in tumor cells, as in early embryonic cells, the growth-movement state outranks the resting-matured state of cell activity (cf. "oncogeny is blocked ontogeny" [7]). This also applies to transformed cells growing *in vitro*. It may appear that the, or a main reason for this is the lack on the part of the tumor cells to make proper *functional contact* with each other, as shown by their loss of contact inhibitions. By contrast, such contact between normal cells triggers off a series of reactions leading to cessation of DNA synthesis and proliferation, which in most cases is preliminary to cytodifferentiation. Functional contact, then, is being defined as that form of contact among cells that leads to cessation of ability to initiate DNA synthesis.

It is also worth mentioning that cell-surface expressions may in some way or another be involved in the induction or establishment of cancerous growth. Studies on leukemia [8] and mammary tumor [9] induction in congenic lines of inbred mouse strains have shown that among the H-2 histocompatibility genes which code for cell-surface transplantation antigens (glycoproteins)—*the chemistry of the cell surface is thus genetically determined*—some confer susceptibility, whereas others bestow resistance. This coupling between certain surface expressions and cancer induction (at least by RNA-viruses) may also be one of mechanism: the cell surface may contain specific viral receptors, or antigenic modulation of the tumor-cell surface (or that of cells productively infected with leukemia virus [8]) by which antigen is lost may remove antigenic barriers and allow the cancerous process to proceed. Finally, host immune competence may also be involved [9a].

The capacity of the body to detect foreign expressions on its cells, including those on tumor cells, and to destroy such cells by immune reactions (or even protect them), has in recent years been found to constitute an important factor in cancerous growth (and in the prospect of therapeutic success). This may also be valid for metastases, since immune reactions not only affect primary growth but may also determine secondary growth [10]. Shedding of antigen from rat mammary tumor cells yielded non-immunogenic cells which metastasized, whereas immunogenic cells did not [11]. The number of tumor cells released into the circulation from metastasizing and non-metastasizing tumors being not markedly different (in a number of cases measured), metastatic outgrowth may be controlled by antigenic expressions and immune defence mechanisms [12]. For these reasons it appears imperative to enlarge the concept of malignancy*—especially in regard to the mechanisms involved—beyond that of uncontrolled cell growth and cell migration *per se*, to include also the antigenic alteration of the tumor-cell surface and the immunological response of the host.

Immunological reactions against tumor-cell surfaces may also help therapy, whether in combination with surgery, chemo- and radiotherapy, or alone (immunotherapy proper).

Finally, the occurrence of particular, especially carbohydrate expressions on tumor cells may afford a selective target for cancer chemotherapy (phytohaemagglutinins [13], concanavalin A [14], polycations [15]).

Conclusion

It is now believed that the cell surface is intimately involved in *growth regulation*, *oncogenic viral infection* and *expression* (viral genome-derived or -directed components being inserted in the plasma membrane of virally-induced tumor cells), and, possibly, *oncogenesis at large*.

Since neoplastic changes are being expressed at the cell surface, it follows that altered surface characteristics may not only cause, or at least contribute to uncontrolled growth, but also determine the degree of malignancy of cancer phenotypes and thus affect the course of the disease. Also, in the latter respect, immune mechanisms, may be of importance, if not sometimes decisive for the outcome of the process. Since immuno- and chemotherapy are or may be directed towards particular tumor cell-surface expressions, it thus appears at the present state of knowledge that surface changes may be closely related to cancer in that *many aspects of the origin, behavior,* and *therapy* of neoplastic disease may find their common denominator in the cell surface.

*Malignancy is in essence a clinical concept—it is descriptive and may have prognostic value for the outcome of the disease and therapy, but it does not cover the mechanisms involved.

Since much of our knowledge is still descriptive, a thorough analysis of the cell surface of normal and tumor cells in molecular terms and mechanisms of action would be most promising. This is now being pursued in many laboratories, using either whole cells, surface fragments released from cells by enzymic action, or isolated plasma membranes. The isolation of pure plasma membranes is a problem in itself with which I shall not deal here, nor will the problem be considered as to whether isolated plasma membranes are still in their native conformation. Rather I prefer to present a short review of mostly biochemical data in order to seek the molecular correlate of the biological changes in neoplastic-membrane behavior, and derive some *ad hoc* conclusions. Very likely, these conclusions will have to be changed or refined as investigation proceeds. The present argument makes use of descriptive analogies, or rather some effort has been made to formulate these analogies. However, analogy in behavior does not (necessarily) mean identity of the mechanisms involved. These should be put to experimental test. In doing so, one is faced with the intrinsic difficulty of demonstrating whether the particular process or activity being measured is the cause or the result of the biological behavior under consideration.

Finally, in this paper the terms "transformed" and "tumor" cells are frequently used interchangeable, although the author is well aware that the two concepts (morphological transformation and tumorigenicity, i.e. transplantability) do not always coincide.

CELL-SURFACE CHEMISTRY

The cell surface as studied on isolated plasma membranes contains mainly proteins and lipids, in amounts and composition varying according to tissue. Liver plasma membranes consist for some 65% of protein and 30% of lipid, the rest being mainly carbohydrates. In a comparative analysis of the plasma membranes isolated from mouse and rat livers, a rather anaplastic rat hepatoma and its subline (# 484A), and three well differentiated mouse hepatomas, a marked change in the protein and phospholipid content of the membranes of rat hepatoma—484A was found [16, 18]. All hepatoma membranes showed an increase in their cholesterol content, from 25 to 40%, on a phospholipid-P basis. This increase might be related to the loss of feedback inhibition of cholesterol synthesis by dietary cholesterol demonstrated in hepatomas by Siperstein [17].

Chemically, isolated plasma membranes are characterized—that is, distinct from intracellular membranes, except those of secondary lysosomes—by high cholesterol, sphingomyelin, phosphatidyl serine and sialic acid contents [16, 18], and relative saturation of their phospholipid fatty acids [19]. High cholesterol content and fatty acid saturation may be related to ensure a rigid but flexible membrane structure [19]. Properly isolated plasma membranes do not contain cardiolipin which is a mitochondrial constituent. Differences in the phospholipid profiles of hepatoma plasma membranes are highly strain-specific, and no common differences from normal have been observed either in our material or in comparison with that studied by others [16, 20]; also, no correlations were apparent between membrane phospholipid changes and growth rate or differentiation of the hepatomas (Table 1) [16]. A similar heterogeneity has previously been

Table 1. *Comparison of differences in phospholipid class percentages of hepatoma plasma membranes relative to liver plasma membranes*

	Rapidly growing			Slowly growing		
Phospholipid class	Hepatoma 484A (rat)*	Morris hepatoma 3924A (rat)†	Zajdela ascites hepatoma (rat)‡	Hepatoma 147042 (mouse)*	Hepatoma 143066 (mouse)*	Reuber hepatoma H35 (rat)†
Phosphatidyl choline	−	+	− −	+	0	+ +
Sphingomyelin	0	+ +	0	− −	−	0
Phosphatidyl ethanolamine	0	− −	0	− −	0	− −
Phosphatidyl serine	−	0	+	0	+	0
Phosphatidyl inositol	−	0	0	+	0	0
Lyso compounds	+	−	+	0	0	−

*Van Hoeven and Emmelot [16].

†Selkirk *et al.* [20].

‡Bergelson *et al.* [20].

0 denotes: < 2·5% difference with liver plasma membrane.

− : decrease
+ : increase
} 2·5–7·5% in value; more than 7·5%: double sign.

observed in the enzyme activities of these hepatoma plasma membranes [21].

The RNA content is generally increased in hepatoma plasma membranes, from about 8 μg RNA per mg liver membrane protein to 13–20 μg RNA per mg hepatoma membrane protein [18]. However, this may not be an intrinsic property of these membranes, but rather stem from the few ribosome-dotted microsomal vesicles which seem to be structurally connected with the hepatoma plasma membranes, but lacking in the case of liver [18a]. The actual role of RNA in pure plasma membrane, such as of liver, is unknown; according to some investigators [22], RNA may contribute to the negative surface charge of certain mammalian cells.

SIALIC ACID

Another compound that may contribute to the negative cell-surface charge is neuraminic (mainly *N*-acetyl) or sialic acid. It may do so in the case of free cells, whether normal (erythrocytes) or neoplastic (ascites tumor cells), but generally not in that of cells from solid tissues such as liver and hepatoma [18a, 23]. Thus, some 70% of the surface sialic acid of rat liver and hepatoma cells is released by neuraminidase action without affecting the negative surface charge [18], which could mean that sialic acid is located somewhat within the membrane element (at least 10 Å below the surface of shear).

Studies by Cook *et al.* [24] on mouse sarcoma cells growing in solid or in ascites form have shown that the higher negative surface charge of the ascites tumor cells was reduced by neuraminidase, whereas by similar treatment the solid sarcoma cells exhibited no change in electrophoretic mobility in spite of the release of sialic acid. Since the solid and ascites forms of this tumor were easily interconvertible, this result demonstrates the *plasticity of the cell surface*, i.e., its capacity to reversibly modulate its chemical expression in the face of altered environmental conditions.

It also follows that in considering the function of membrane components in processes occurring at the cell periphery, the mere amount of these compounds in the plasma membrane, or even their sensitivity (accessibility) to added hydrolytic enzymes, is less relevant than their *actual location on, or distribution within the membrane element*. As we shall presently see, this is a general principle.

With this reservation in mind, the sialic acid contents of normal and neoplastic plasma membranes should be considered. No general differences have been observed [18, 25–27]: the sialic acid content of the neoplastic membranes may either be higher or smaller than, or similar to that of the corresponding normal membranes (Table 2). The diminished sialic acid content [29] of cells transformed by DNA viruses and of their plasma membranes, has been correlated with the decreased intracellular activity of certain sialyl transferase enzymes [30]. The decrease of sialic acid in these cells appears to be an absolute one in number of glycoprotein molecules per mg cell protein, not in a shortening of glycopeptide chains [31] as is the case with the carbohydrate complement of glycosphingolipids (see below). It is also of interest that an inverse relation between sialic acid content and saturation density of a number of

Table 2. Sialic acid content of plasma membranes

Tissue	nmoles per mg protein	Change	Authors
Mouse liver	30 ± 1		Emmelot, Bos [18]
Mouse hepatoma—147042	36 ± 1	+	Emmelot, Bos [18]
—4189	27 ± 0	0	Emmelot, Bos [18]
—143066	28 ± 2	0	Emmelot, Bos [18]
Rat liver	33 ± 2		Emmelot, Bos [18]
Rat hepatoma—484	45 ± 2	+	Emmelot, Bos [18]
Rat hepatoma—484A	61 ± 1	+ +	Emmelot, Bos [18]
Rat hepatoma-ascites	28·8	?	Shimizu, Funakoshi [25]
Rat liver cells (cultured)	68 ± 17		Perdue *et al.* [26]
—, transformed by MSV	63 ± 7	0	Perdue *et al.* [26]
BHK cells	34·5		Makita, Seyama [27]
BHK-Py (polyoma)	13·7	− −	Makita, Seyama [27]
Chick fibroblasts	106 ± 3		Perdue *et al.* [28]
—, transformed by avian sarcoma virus	79 ± 3	−	Perdue *et al.* [28]
−, infected by avian leukosis virus	114 ± 3	0	Perdue *et al.* [28]

normal, SV40-transformed and revertant cells has been observed [32]. This might indicate that a decrease of surface sialic acid could be related to loss of contact inhibition. The observed decreases of the sialic acid containing glycosphingolipids in transformed cells (compare below) is in accord with this view.

Parenthetically, if levels of plasma-membrane components are governed by the activity of intracellular enzymes, intracellular membranes could be equally affected by changes in these activities; this could be of interest in the case of the initiation of DNA synthesis which occurs after DNA is attached to the nuclear inner membrane, since membrane changes might affect this attachment. An example of such a coordinated change is the increased cholesterol content of plasma membrane and endoplasmic reticulum membrane [16], which is the site of cholesterol biosynthesis, in the hepatoma cell.

The location of sialic acid in the liver plasma membrane is restricted to the outward oriented (extracellular) side of the membrane element [33]. This provides a clear example of *the molecular asymmetry* of this membrane. The outward orientation suggests some functional interaction of sialic acid with the environment. Indeed, it has been found that sialic acid is engaged in contact phenomena (metastatic attachment [34], aggregation of cells [35], desmosomal contacts [33]) and transport processes (potassium ions [36], protein [37], glucose following insulin action [38]) and that sialic acid may act as a virus receptor [39].

Lastly, sialic acid located at the tumor-cell periphery may mask, or shield off underlying tumor-associated transplantation antigens, or "repulse" immunologically competent lymphocytes [40]. By prior removal of sialic acid through neuraminidase action, the antigenicity of tumor cells appears to be strongly enhanced and this may afford a promising lead to the immunotherapy of cancer.

CHANGES IN CARBOHYDRATE EXPRESSION OF THE TUMOR-CELL SURFACE

Sialic acid and other carbohydrates (fucose, mannose, glucose, galactase, *N*-acetylglucosamine, *N*-acetylgalactosamine) are present in plasma-membrane gangliosides (sialic acid containing glycosphingolipids), other glycolipids (neutral, asialoglycosphingolipids), and in the glycoprotein that covers mammalian cells, either as an integrated part of the plasma membrane element or extending from the outer leaflet. In the latter case, this glycocalyx is sometimes of considerable extension.

It has become clear that biological specificity may reside in surface carbohydrate sequences, for instance of blood groups, mating substances of micro-organisms, cellular aggregation factors —ref. in [41]—and certain immunologically reactive tumor-cell surface components such as the carcinoembryonic antigen of human colon tumors [42].

Three types of changes in the carbohydrate expression of the tumor-cell surface have thus far been detected. These results have mainly been obtained on normal and transformed cells maintained *in vitro*, and their relevance to tumor-cell growth *in vivo* remains to be (further) established. However, in all cases there is a marked correlation between the changes and the phenotypically altered pattern of transformed cell growth *in vitro*, including normalization in cases of revertants and cells transformed by virus mutants growing at non-permissive temperature. Direct correlations with transplantability are also available.

Type I change: glycoprotein

Buck, Glick and Warren [43] found that a higher molecular weight fucose-containing glycopeptide appeared or increased on the surface of cells transformed by polyoma, MSV or RSV. Investigation with a temperature-sensitive RSV mutant demonstrated that this surface change on transformed cells is controlled by the viral gene expression [44].

Current studies in our laboratory [45] on established lines of malignant and non-malignant lymphoblasts, 3T3, spontaneously-transformed and SV40-transformed 3T3 cells, and liver and Novikoff hepatoma cells cultured *in vitro*, have shown a similar change in fucose-containing glycopeptide. This phenomenon of an *increased glycosylation restricted to certain of the surface glycoproteins* released from tumor cells by trypsin, parallels the finding by Chiarnyi and Urbano [46] of an increased glycosylation of a particular glycoprotein shed from polyoma transformed BHK cells in culture.

The most recent results of Warren *et al.* [47] show that the difference in fucose-containing glycopeptides between normal and transformed cells is due to an extra complement of sialic acid in the transformed material. This causes the shift to a seemingly higher molecular weight; the phenomenon has been confirmed for all the aforementioned cells studied by us [45]. Warren *et al.* also found a sialyl transferase, which specifically adds sialic acid to the desialylated fucose-containing glycopeptide, to be markedly increased in the transformed cells. (This con-

trast to the decreased activity of other sialyl transferases in transformed cells [30]. Intracellular heterogeneity in activity of other sugar transferases in transformed cells is also known, e.g. decreased activity of collagen; glycosyl transferase, but increased glycoprotein; glycosyl transferase [48].)

Some of the aforementioned sialyl transferase activity was located in the plasma membranes [47], and a sialyltransferase has also been observed in rat-liver plasma membranes [49], next to sialidase (neuraminidase) [50] acting on gangliosides. These results might be of interest in view of the finding that cell-surface glycosyl transferases may be involved in the mutual attachment of cells (see below).

Type II change: neutral glycosphingolipids and sialic-acid containing glycosphingolipids (gangliosides)

Studies by Hakomori [51, 52], Brady [53] and their co-workers, and others [54–56] on asialo- and sialo-glycosphingolipids, which are considered to reside mainly in plasma membranes of cells of extra-neural origin [50, 57], have shown that carbohydrate sequences of these molecules in transformed (tumorigenic) cells are incomplete as compared with normal cells. (In these experiments whole cells were used.) The carbohydrate-transferring enzymes by which these sugar chains should be elongated appear to be less active in the transformed cells (Fig. 1) [51–53, 58]. In respect of the title compounds, there is *decreased glycosyl extension* in transformed cells, but this may apply to various molecules and non-uniformly; clonal variation in glycolipid patterns have been demonstrated [55, 56].

Table 3 illustrates our results [16] on *isolated hepatoma plasma membranes*. These results show

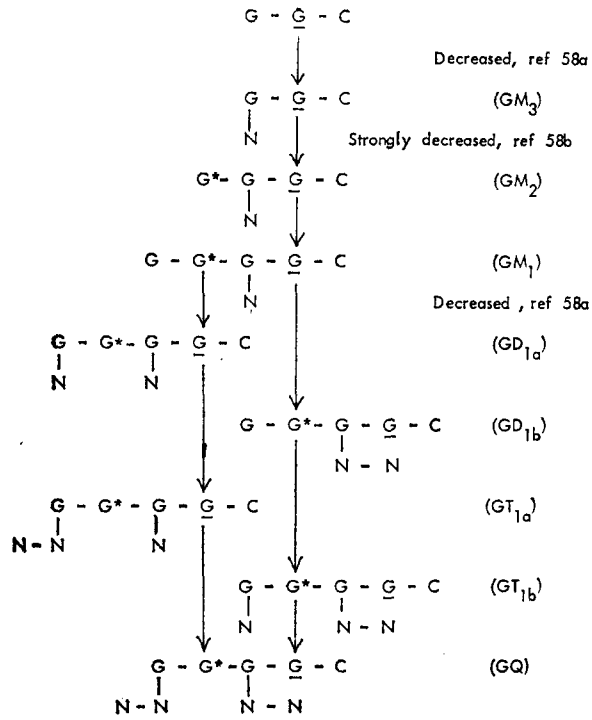


Fig. 1. Gangliosides; some decreases in enzyme activities involved in their synthesis.

G = galactose, G = glucose, C = ceramide, N = neuraminic (sialic) acid, G* = *N*-acetylgalactosamine G—G—C = lactosylceramide.

Between parentheses: GM, GD, GT and GQ = mono-, di-, tri- and tetra-sialogangliosides.

Decreased formation of hexosides from lactosylceramide: Kijimoto and Hakomori (1971) in ref. 51.

non-uniformity of ganglioside patterns for different hepatomas, total amounts of gangliosides being some 2 times higher in hepatoma than in liver plasma membranes on a dry weight basis.

In the case of the two "minimal-deviation" mouse hepatomas the monosialoganglioside GM_1 markedly increased, an observation in line with results of studies on Morris hepatomas (whole tissue) [52, 59]. The latter and certain

Table 3. Gangliosides in isolated plasma membranes

Ganglioside	Rat		Mouse		
	Liver	Hepatoma—484A	Liver	Hepatoma—147042	Hepatoma—143066
GM_3	+++	+++++	±	±	±
GM_2	±	+	+	+	−
GM_1	+	+	±	++++	+++
GD_3	±	±	−	+	±
GD_2	±	±	−	−	+
GD_{1a}	+	+	+++++	+	±
GD_{1b}	+	−	++	±	+
GT_{1b}	−	±	−	±	+++
GQ_1	++	−	++	−	+

Relative estimates following chromatographic separation using *n*-propanol–concentrated, ammonia–water (621), and resorcinol staining.

clones of transformed cells [55, 60] accumulated disialogangliosides. By contrast in the plasma membranes of the rapidly growing rat hepatoma the monoganglioside GM_3 (= sialyl lactosylceramide, which is also the main ganglioside in rat-liver plasma membranes) accumulated. Tetrasialogangliosides were deleted from the membranes of two hepatomas (484A and 147042) but were definitely present, though decreased, in those of mouse hepatoma—143066. An increase in trisialogangliosides in the two former but not in the latter membrane species is also indicated. According to Hakomori and Brady *c.s.* tri- and tetragangliosides were deleted from Morris hepatomas and virally-transformed cells; the latter also lacked GD_{1a}. In conclusion, the various results demonstrate that no detailed common change in ganglioside patterns between normal and transformed or tumor cells does exist.

Among the changes found by Hakomori and Murakami [51] in virus-transformed cells is a decrease in hematoside (GM_3, sialyl lactosylceramide) and an increase in lactosylceramide content. In spite of the decrease in hematoside, relatively more of it is actually exposed on the surface of transformed than of normal cells as demonstrated by immunological means. However, further hematoside could be uncovered by trypsin action on the normal, but not on the transformed-cell surface.

This has been described as *a loss of protein cover on the tumor cell surface*—a recurrent theme since exactly similar results have been obtained on the Forssman antigen [27, 62], which is also a glycolipid, and a surface antigen of SV40-transformed cells [63]. These antigens are exposed on transformed fibroblasts but cryptic on normal fibroblasts where they can be unmasked by trypsin. Another point in case are the receptors of the plant agglutinins (see below).

Lactosylceramide or cytolipin H, studied by Rapport [64], has been reported to act as a human tumor surface-associated antigen [65] capable of provoking an antibody response in the patient. The agglutination factor is also present in the sera of pregnant women, pointing to the embryonic expression of the antigen [65].

Embryonic expressions on tumor cell surfaces—probably a reflection of a more general pattern of embryonic protein expressions in the tumor cell—have been demonstrated as antigens on the surface of various virally-induced tumor cells [66], chemically-induced hepatomas [67], and human colon tumors where it is glycoprotein [42]. These carcinoembryonic antigens are absent from, cryptic or sometimes present in only minute amounts on the surface of normal adult cells.

Cell-surface expressions during the cell cycle

It is of interest that the two types of changes (I and II) encountered in tumor-cell surfaces, i.e. the increased glycosylation (sialylation) of a particular glycoprotein(s) and the decreased glycosylation of glycosphingolipids, have also been demonstrated in growing normal as opposed to resting cells [51–53, 68]. Quantitatively or qualitatively, however, marked differences between growing normal and transformed cells do exist in these respects. Moreover, the changes remain apparent in the transformed cells when they come to rest in a crowded culture of high density, contrary to the situation in normal cells. Thus, there is a cell-density (contact inhibition of growth) dependent shift in glycosphingolipid synthesis in normal, but not in transformed cells where the situation is fixed, apparently as a result of decreases in synthetic enzymes, so that shorter carbohydrate chains prevail (and accumulate).

These findings on normal cells illustrate an important aspect of cell-surface behavior, namely that the surface's chemical expression changes with the metabolic state of the cell, and, accordingly, that there is *physiological control of membrane structure*. More in particular, various examples of such changes as a function of the cell-cycle phase are now known, and among these is the unmasking on the fibroblast surface of some otherwise cryptic carbohydrate sites at mitosis detectable by the plant agglutinins [69, 69b]. In the non-mitotic cell these sites are covered by a trypsin-sensitive layer. In fresh early embryonic cells these sites seem to be constantly exposed, only to become gradually masked by a trypsin-sensitive layer as maturation proceeds [70].

Type III change: plant agglutinin receptors

This brings us to the third and well-known type of change in the carbohydrate expression of transformed- and tumor-cell surfaces, studied by Burger [71] and by Inbar and Sachs [72] using wheat germ agglutinin (WGA) and concanavalin A (con A) which preferentially agglutinate DNA-virus and chemically-or radiation-transformed cells by binding to certain carbohydrates of the cell surface.

Support for the view that a change in these receptors (which could very well be a reflection of the type II change mentioned above)* is causally related to the phenotypically altered

*A human-tumor glycosphingolipid prevented the agglutination of transformed cells by WGA [73].

Table 4. Changes in carbohydrate expression of tumor-cell surface—Type III: "Membrane lectinology"

1. The most agglutinable cells are the least contact inhibited (WGA) [74]; degree of loss of contact inhibition of growth parallels transplantability [75].
2. Revertants [76] and mutant-virus infected or transformed cells growing at non-permissive temperature [77], bear a normal expression.
3. Monovalent con A restores contact inhibition in transformed cells [78].
4. Agglutinability (con A) relates to transplantability [80].
5. Immunization with haptenic dimer of N-Ac-glucosamine protects against tumor growth and induction [81].
6. Trypsin unmasks agglutinin receptors on normal cells and induces DNA synthesis and cell proliferation in confluent monolayer [71, 79].

pattern of transformed cell growth—and transplantability and, thus, malignancy—has been presented, and is summarized in Table 4 [ref. 74–81]. It should be noted that Moore and Temin [82] found no evidence for a generally increased agglutination of oncornavirus transformed cells. By contrast Kapeller and Dojanski [83] obtained positive results [3], whereas most recently Burger and Martin [84] showed that hyaluronic acid may mask the agglutinin receptors of RSV-transformed fibroblasts while hyaluronidase-treated untransformed cells remained relatively unsusceptible to agglutination. Another example of such masking is presented below. Reports [e.g. ref. 85] and future findings of a lack of correlation between agglutinability by WGA or con A and transformation or malignancy should be viewed in this light. It may also be argued that certain tumors may show increased agglutination with agglutinins other than WGA and con A [ref. 13, 86]. Variations in carbohydrate expressions of tumor (transformed) cells may be significant not only in respect of glycolipids (compare above) but also of glycoproteins [87].

It is of much interest that the carbohydrate expression which allows agglutination of transformed cells by WGA and con A, is similar to that prevailing on the normal cell surface during mitosis [69, 69b]. Also in other respects does the mitotic normal cell surface resemble the transformed (tumor) cell surface: normal cells in mitosis (a) detach from the substrate and from surrounding cells, (b) do not show contact inhibition of movement when encountering another cell [88], i.e. do not make stable local contact, (c) are "leaky" (release of proteases and glycosidases) and (d) show an increased sialic acid complement in glycoprotein [69b].

On this basis the following hypothesis may be constructed (Fig. 2). The mitotic surface expression of the normal cell changes shortly after mitosis (in early G_1), either by synthesis of a protein cover or by modulation of existing structure, to the condition which allows the proper intercellular contact leading to the cessation of DNA synthesis (= functional contact). After the establishment of physical contact apparently some time (about 24 hr) is needed before DNA synthesis stops [89]. The term "functional contact" is meant to cover both the physical contact and the resulting time-dependent process, which is signaled by contact and results in the cessation (=abolition of initiation) of DNA synthesis.* Since in a confluent cell layer of fibroblasts inhibition of protein synthesis by e.g. cycloheximide causes the cells to become highly agglutinable by con A [90]†, it may be concluded that there is a high turnover of membrane molecules ("protein cover") which have to maintain the cell surface in the state required for functional contact.

*Loss of functional contact may therefore occur in various different ways.

†Such cells might be expected to start synthesizing DNA, but since the latter process is dependent on protein synthesis, which is blocked, the effects are dissociated.

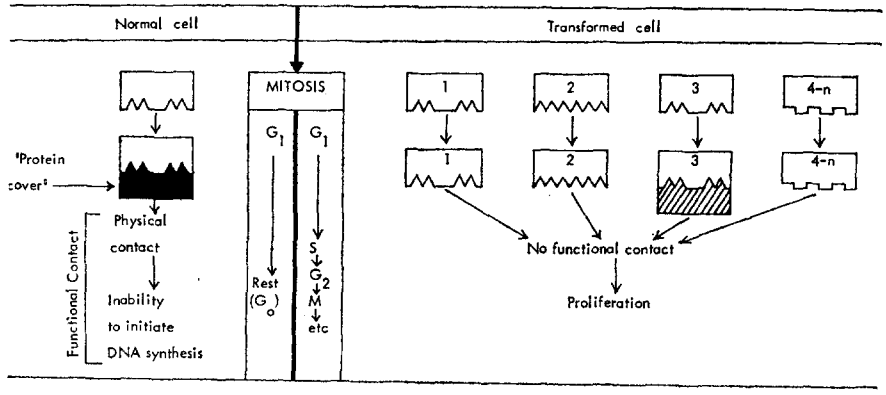


Fig. 2. Schematic representation of surface changes.

In contrast to the dynamic state of the normal cell surface, the tumor cell surface seems to be frozen or blocked in (at least that part of) the mitotic expression which is unable to provide for the proper kind of intercellular contact as defined above. According to our view it might not so much matter what actually is differently expressed at the tumor cell as compared with the normal cell surface (thus accounting for the heterogeneity observed, and undoubtedly still in store!), as that the crucial aberration is thought to be that the tumor cell surface is incomplete, or unable to modulate, or—as this is a possibility which should not be dismissed—that the proper cover is constantly being formed but shed off or broken down, for example, by proteolytic enzymes released by the tumor cells [91, 170]. In this connection it is of much interest that trypsin- and cathepsin-like activities are increased in virally-transformed cells [91a], and that also growth factors with proteo (and estero) lytic activity are known to exist.

A main point which I am trying to make is that although individual molecular expressions of carbohydrates on different tumor-cell surfaces could differ (indicated in Fig. 2 by the various surface structures), still a uniform difference, namely in growth control, might ensue by lack of a proper configuration.

Conversely, this point is illustrated by the finding that DNA synthesis and proliferation in a monolayer of resting normal cells can be induced "aspecifically", namely by many different chemical agents and conditions, which, however, all share the property that they interact in some way or another with the cell surface. Wounding [5], serum [92], trypsin [79, 93], hyaluronidase [94], colchicine [95] and presumably also the detergents lysolecithin and digitonin [94, 96] by breaking contact enlarge the area of free cell surface, or at least may change intercellular contact layers. Phorbolester (a co-carcinogen) is known to activate certain plasma-membrane enzymes [97], but this may result from the growth response (cf, ref. 98) rather than providing a growth stimulus. Phorbolester, like the mitotic inhibitors, inhibits the $(Na^+ - K^+)$-ATPase and adenyl cyclase of isolated liver plasma membranes (unpublished); the former compound has also been shown to prevent the rise of c-AMP in epidermis following injection of isoproterenol [166]. Insulin [99, 100] also acts at the plasma membrane level, inhibiting adenyl cyclase [101] and activating cyclic-AMP phosphodiesterase [102] of rat-liver plasma membranes, thus clarifying its role as an established c-AMP antagonist. Neuraminidase [102a] and urea [102b] are further examples of agents causing membrane perturbation and transient growth in resting fibroblasts; like trypsin, they induce cell overlap and exposition of agglutinin receptor sites.

When fixed, the changes in the cell surface produced by any of these agents, which normally induce DNA synthesis and at least one round of mitoses (except, of course, the mitotic inhibitors which produce DNA synthesis only [95]), would result in more permanent, "uncontrolled" growth.

Further support for the above view can be derived from the finding that agglutination by con A, which in the studies of Inbar and Sachs correlates with transplantability [80], can be due to 3 types of changes in the cell surface [80, 103].

Topography of agglutinin receptors

The changes in receptor sites were described by Sachs *et al.* (Fig. 3) as:

(a) *Exposure of sites.* More of the same binding sites on a tumor cell of (about) normal size;

(b) *Concentration.* Normal amount of binding sites on a greatly reduced tumor cell surface, due to the smaller size of the cells;

(c) *Rearrangement.* Clustering of sites, unchanged in amount, thereby promoting agglutination of tumor cells of (about) normal size. (The surface distribution of ferritin-conjugated con A on SV40-transformed 3T3 fibroblasts has revealed the clustering of the con A binding sites) [104, 69a]; trypsin had a similar effect on normal cells [104a]. This could be a dynamic process due to the (increased) movability of the sites on the tumor cell surface.

The experiments involved the measurement of agglutination titers, cell size, and the amount of radioactive agglutin bound per cell and per unit area of cell surface.

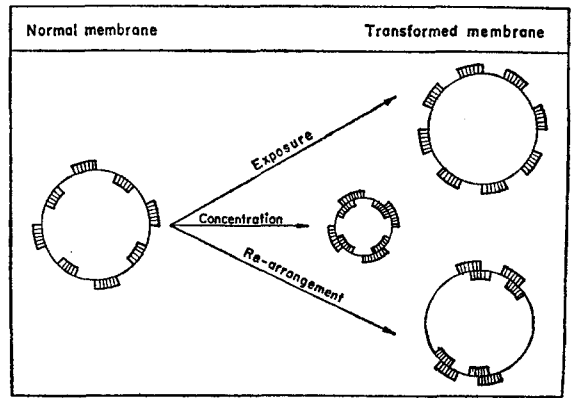


Fig. 3. Changes in cell-surface agglutinin receptor sites (from [103]).

From these studies it appeared that the type of surface change may be conditioned by the type of serum used for culturing the cells. This brings us to the crucial problem as to what extent culturing *per se* or particular culturing conditions may affect the surface expression of cells; such changes have been demonstrated for glycolipids [52, 56].

In this context, the experiments of my colleague, Dr. L. Smets [105], are relevant. He found highly malignant lymphosarcoma cells grown *in vivo* show only weak agglutination by con A, which could be considerably enhanced by either trypsin or simple hypotonic treatment of the cells. When these cells are cultured *in vitro*, they gradually become highly agglutinable but lose their transplantability in unconditioned syngeneic mice, whereas they produce lethal tumors in irradiated mice. It is evident at least that *in vivo* another cell-surface configuration is manifested than that shown by *in vitro* cells; perhaps *in vivo* another cell coat is made or *in vitro* part of the cell coat is shed off. Since the lack of agglutination of the *in vivo* grown cells was not due to absorbed antibodies, it can be concluded that a coat is present *in vivo* which shields off both tumor transplantation antigen and con A receptors. It is of further interest that the *in vivo* grown lymphosarcoma cells can also be made highly agglutinable by their prior treatment with neuraminidase [106] (compare above), which raises the question of whether the con A receptor site may be part of the tumor transplantation antigen.

As yet no studies have been reported on the increased agglutinability of *in vivo* grown tumor cells as compared with their normal counterparts. Very recently C. Borek [107] mentioned that cultured Morris hepatoma cells showed increased agglutination (WGA and con A) as compared with cultured rat liver cells. Our unpublished experiments [108] with rat liver and hepatoma-484A cells, grown *in vivo*, and their isolated plasma membranes have shown an increased agglutination of the hepatoma cells and membranes by con A. The size of the hepatoma cells used in this study was much smaller than that of the liver cells, and per unit surface area, hepatoma cells bound 30–80% more con A than did liver cells. Thus, the hepatoma cells resembled in this respect SV40-transformed 3T3 cells.

GLYCOSYL TRANSFERASES ON THE CELL SURFACE AS A POSSIBLE MEANS OF ESTABLISHING INITIAL INTERCELL CONTACT

Complex carbohydrates appear to be involved in the intercellular adhesion of various cells, and more in particular, these cells are unable to adhere following β-galactosidase treatment [109], suggesting the involvement of galactose residues in adherence. Chicken embryo neural retina cells [109], blood platelets [110], and normal and transformed fibroblasts [111, 111a] contain *on* their surface various or multifunctional glycoprotein: glycosyl transferases. These surface enzymes—of which some show an increased activity in the transformed state [111a]—and in particular a galactosyl transferase have been suggested [109–111, 112] to act in the initial attachment and contact of cells. The experiments of Roth and White [111] (Fig. 4) strongly suggest that the distance between the latter enzyme and its glycoprotein (or glycolipid) acceptor on the normal fibroblast appears to be such that the galactosylation reaction cannot proceed on the same cell; the reaction is cell-density dependent and only reaction between separate cells seems possible. This intercellular reaction, which represents a "*trans*"-glycosylation, could be a way of initiating contact ("adhesive recognition"). By contrast, enzyme and acceptor on the transformed fibroblast appear to be close enough to allow the reaction to proceed on the same cell surface (because of the smaller cell size, or movability of sites?). The resulting '*cis*"-glycosylation may counteract intercellular contact.

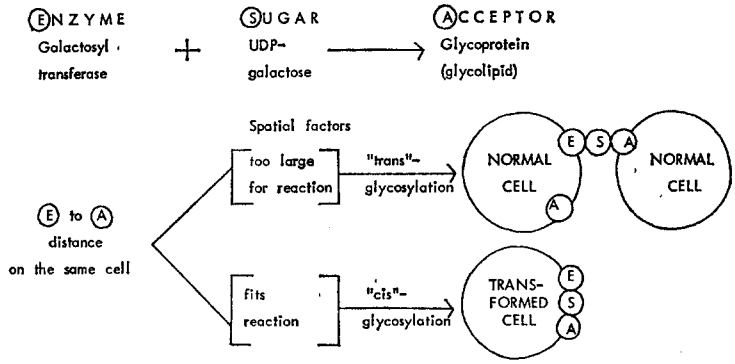


Fig. 4. Cell-surface galactosyl transferase in recognition or adhesive contact.

THE ROLE OF THE CELL SURFACE MEMBRANE IN CONTROLLING DNA SYNTHESIS

It is generally assumed that intercell contact is essential for growth control, at least *in vitro*. Cell-surface changes, especially in carbohydrate expression, could be causally connected with uncontrolled growth or at least underlie the phenotypically altered growth pattern of transformed (tumor) cells *in vitro*, by not allowing proper functional contact. As yet, the only consistent change is the increased sialyl extension on the surface glycoprotein of transformed and malignant cells.

Now, if surface processes are coupled to growth control, there must be some reaction (feedback) loop extending from a surface process into the cell reaching the nucleus for control of DNA synthesis. Two questions then arise.

First, can such a reaction be demonstrated more directly and independently? The answer is in the affirmative. Phytohaemagglutinin binding to the lymphocyte surface leads to DNA synthesis and replication of these cells. Insulin, required for DNA synthesis in many cells, has been shown [100] to exert those of its metabolic effects, which occur prior to DNA synthesis, also when bound to inert material (Sepharose beads) that is unable to enter the cell; thus, it is highly likely that insulin may act from the outside on the cell surface to induce DNA synthesis and cell proliferation. Lastly, insoluble trypsin (bound to beads) is capable of inducing transient growth in resting fibroblasts, i.e. by interacting with the cell surface solely [167].

Obviously, the second and most important question concerns the molecular mechanisms involved. Various answers have been or may be proposed. Based on purely mechanical terms it is thought that intimate intercellular contact shields off surface receptors for growth-inducing stimuli or reduces the free cell surface to such an extent that these stimuli (serum) cannot sufficiently enter [113, 114]. In this connection, the possibility that contact leads to a membrane change, e.g. in conformation, whereby membrane processes (permeability, enzyme activities) are affected, should also be considered.

Thirdly, by adherence to each other and to the substratum—another important parameter which actually deserves separate consideration (e.g. ref. 115)—the flexibility of the cell is so impaired that it cannot stretch and complete the G_1-phase of the cell cycle leading into the S-phase.

According to the present state of investigation, it seems likely that by intercellular contact a chemical signal is released that acts as an intermediate in the process leading to the quenching of DNA synthesis. This signal could be cyclic AMP (c-AMP) formed from ATP by a universally occurring plasma-membrane enzyme, adenylate cyclase, which has receptors for hormones and is thus, and by other factors, controlled [116]. The location of this enzyme in the surface membrane of many cell types, and the observed effect of c-AMP on cell metabolism make this system a likely candidate for mediating the growth response of cells to environmental changes.

THE ROLE OF CYCLIC AMP IN GROWTH AND DIFFERENTIATION

That c-AMP may control cell proliferation and differentiation is indicated by a number of recent findings; the dibutyryl derivate (dbc-AMP) is used to allow a more rapid penetration into the cells.

(a) Addition of dbc-AMP, or of an activator of adenylate cyclase (prostaglandin E_1 = PGE_1), inhibits cell motility [117] and growth [118, 118a], and increases cell-substrate adherence [118b]. The c-AMP level seems to mediate the contact inhibition of movement and growth [117].

(b) The intercellular c-AMP concentration is higher in resting than in growing cells [119–121], and the level depends on the cell population doubling time [119, 122], rapidly growing cells having low levels and cells growing more slowly having higher levels of c-AMP. That the c-AMP level is the cause rather than the result of the differences in growth (and morphology) is indicated by the following results.

(c) Agents which increase the intercellular c-AMP concentration by activating the plasma-membrane adenylate cyclase or inhibiting the phosphodiesterase which hydrolyses c-AMP, such as epinephrine (adrenalin) [123], isoproterenol [124], prostaglandins [125] and theophyllin [125, 126], inhibit the growth of various types of cells.

(d) Agents which decrease the c-AMP concentration, as demonstrated [127, 128] for trypsin, serum and insulin, induce growth in a confluent monolayer of resting cells. Cells resting in G_1 (confluent monolayer, or subconfluent in the absence

of serum) accumulate c-AMP [129], and within a few minutes after addition of serum the level of c-AMP is drastically reduced [130] (serum contains c-AMP phosphodiesterase activity [131]). The serum response, i.e. DNA synthesis and cell proliferation, is inhibited by addition of dbc-AMP [130, 131a]; the trypsin response is inhibited likewise [131a].

(e) The role of c-AMP in cytodifferentiation is indicated by a number of findings [132] (and below).

Thus, it appears that adenylate cyclase activators and/or phosphodiesterase inhibitors or the addition of dbc-AMP increase the intracellular c-AMP level and cause inhibition of cell proliferation, whereas a decrease of c-AMP initiates growth of both fibroblasts and epithelial cells (epidermis).

The results suggest that contact inhibition of growth is due to accumulation of c-AMP, and that the balance between cell proliferation and differentiation may be regulated by the c-AMP concentration. Thus, the tentative conclusion may be drawn that cell contact (or at least increase in cell density) leads to an increase in intracellular c-AMP (the parameters being synthesis and degradation of this compound) culminating in inhibition of initiation of DNA synthesis. Experiments suggest that the contact inhibitions of movement and growth are controlled by a common regulatory mechanism. Undulating membranes ("ruffling") with mutual affinity form an apposed resting contact zone. In this contact zone, the membrane conformation may be such (changed) that the net output of the adenylate cyclase system is augmented. With increasing contact, allowing less space to the cells, the whole cell surface might thus be changed.

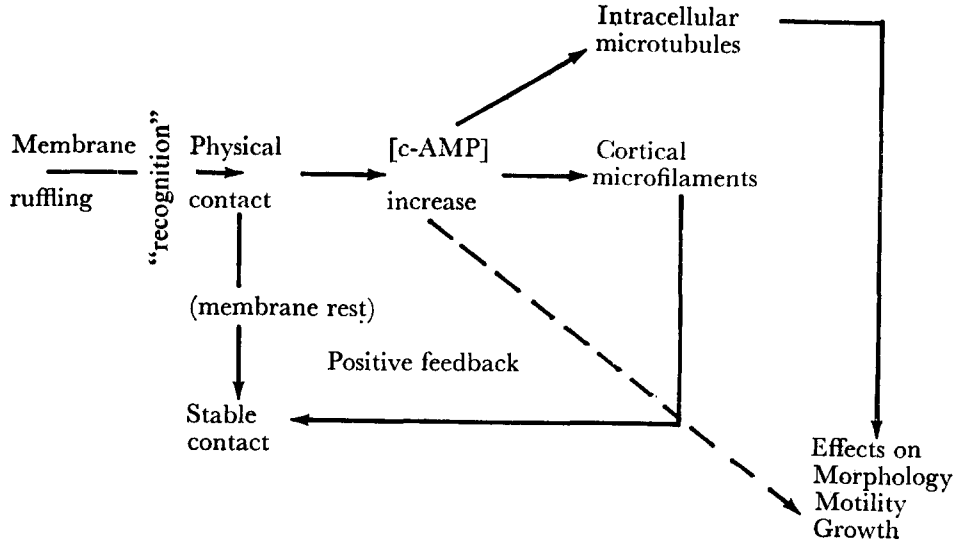
Among the many functions exerted by c-AMP is its role in the assembly and/or contractile function of microfilaments (and microtubules) [133]. Bundles of 70 Å microfilaments are located in the peripheral cytoplasm (ectoplasm) [134–136] and they are less abundant in growing than in resting cells [134]. It appears that these microfilaments are involved in the cortical organization for stable intercellular contact, since as these microfilaments are destroyed by colchicine, colcemid or Vinblastin (by binding the filament subunits), cell contacts are dissolved and DNA synthesis is initiated [95]. The disappearance of this cortical array of microfilaments also allows (ameboid) movement of the cell membranes [95, 137]. The morphological transition ("differentiation") of cultured Chinese hamster ovarian [138] and sarcoma cells [139] (to fibroblast-like cells), and neuroblastoma cells [140] (axon formation) induced by dbc-AMP is also prevented by colcemid or Vinblastin, pointing to the involvement of microfilaments (tubules) in these processes. Changes in the surface membrane organization and an increase in number of microtubules and their organization into parallel array induced by dbc-AMP, and their abolishment by colcemid (or cytocholasin B) have very recently been demonstrated [141].

The microfilaments also promote cell-substrate adherence, and it may appear that morphology, motility, and growth pattern are a function of the microfilament (tubule) system as regulated by c-AMP. It might, therefore, be concluded that c-AMP by inducing formation of these contractile elements and/or promoting their activity (by phosphorylation) imposes a rigid cytoskeleton upon the cell that counteracts membrane flexibility and cell stretching, and thus inhibits DNA synthesis. In fact, this is still a rather mechanical consideration since the underlying biochemical events other than the involvement of c-AMP are not known (cf. ref. 156a).

Is a change in c-AMP metabolism involved in oncogenesis?

According to the present view, a chemical signal, c-AMP, is emitted from the cell surface upon cell-to-cell contact to initiate a time-dependent myriad of branched biochemical events (analogous, if not similar to the pleiotypic response of Hershko *et al.* [100]) leading to the inhibition of initiation of DNA synthesis (c-AMP is a pleiotypic mediator). Alternatively, it could be argued that a certain c-AMP level is required for contacts to be made. Both views can be incorporated into a process that envisages a positive feedback of c-AMP on stable contact by way of the ectoplasmic organization of microfilaments (*as shown at top of next page*). Such a system may be defective at various sites (i.e. affected by the carcinogenic stimulus, if not by chromosomal changes in "normal" cells in culture), and the question to be considered now is whether c-AMP metabolism is changed in neoplasia.

The metabolism of c-AMP involves its formation by adenylate cyclase, breakdown by phosphodiesterase, and assimilation by protein kinases. Tumor and transformed cells generally contain smaller amounts of c-AMP than do normal cells [119, 120, 122, 127]. When the former cells stop growing in a dense culture, the level of c-AMP does not increase (as in normal



cells at lower densities) but rather decreases [119]. In view of the question of cause or effect, it is of interest that the decrease in c-AMP preceded the establishment of the virally-induced transformed state [142] so that the conclusion can be drawn that an early event in transformation leads to that decrease and that this results in the peculiar phenotype of the transformed cells. A marked fall in the capacity to form c-AMP from ATP by isolated rat-hepatoma-484A as compared with rat-liver plasma membranes, and a smaller decrease in the case of the plasma membranes isolated from a differentiated and slow growing mouse hepatoma have been demonstrated [143]. Reduced enzyme activities in virally-transformed cells and other tumor cells are also known [123, 125, 144], the activity sometimes being exceedingly low (cultured Morris hepatoma [120]). The basal activity and/or the hormone stimulation of the enzyme may be suppressed in neoplastic plasma membranes.

Of much interest are the findings made in several laboratories of a phenotypic reversion ("reverse transformation", "differentiation") of the abnormal growth pattern of transformed fibroblasts [139, 145], Chinese hamster ovarian cells [138, 146] and mouse neuroblastoma cells [140, 147, 148], including restoration of contact inhibition of growth, modification of cell shape and orientation, and disappearance of agglutinin receptors and induction of differentiation traits by the addition of dbc-AMP, or activators (prostaglandins) of the adenylate cyclase system. (For inhibition of phosphodiesterase and induction of "differentiation", see ref. 149; theophylline inhibits the growth of virally-transformed cells [144].) Only mouse neuroblastoma cells did differentiate irreversibly [140]. Human nasopharyngeal-carcinoma (epithelial) cells [150] and human astrocytoma cells [151] also responded to dbc-AMP with reduction in growth and alteration of cell size and shape. ACTH, which activates adrenal adenylate cyclase and increases the intracellular c-AMP concentration in this tissue, inhibited the replication of functional adrenal tumor cells almost completely, and induced differentiation [152].

Parenthetically, the paradoxal situation exists that c-AMP may stimulate DNA synthesis and replication of certain cells and tissues (perithereal and thymic lymphocytes [153], salivary glands [154], adrenal [152], cf. erythropoiesis [132, 154a]). Such cells replicate one or more rounds to develop into a differentiated functional state. However, in the cases studied, the homologous tumor cells (human adrenal [152], human leukemic lymphocytes [155], compare also a mouse lymphoma [156] reacting to PHA) were more sensitive than the normal cells to the inhibitory effect of c-AMP on DNA synthesis (directly shown or by implication) that appears only at higher c-AMP concentrations in the normal cells. Very recently, three regulatory sites of c-AMP in the cell cycle of normal cells have been observed: inhibition in early G_1 and in G_2, leading to inhibition of DNA synthesis and mitosis and in certain cells a speeding-up of the S-phase depending on the time elapsed between plating of the cells and addition of dbc-AMP [156a]. It may appear that reaction to c-AMP is governed by the particular gene program of the cell.

A CANCER HYPOTHESIS

When viewed in the above context, the results suggest that transformation lowers the net output of the adenylate cyclase system to respond to increases in cell density. The ob-

served decrease in the cortical organization of microfilaments in transformed cells [134, 157] is the consequence of this view.

Tumor (transformed) cells are unable to make *functional* contacts, which normally lead to an increase of intracellular c-AMP that prevents the initiation of DNA synthesis, because (a) no *stable* contacts are formed or (b) if such contacts are made, contact is not accompanied by a sufficient rise in the c-AMP concentration either as a result of decreased activity (amount) of the adenylate cyclase system in the tumor-cell membrane or of an increased phosphodiesterase activity, or both. Another possibility is that the changed architecture and chemical expression of the neoplastic plasma membranes prevent the conformational change of these membranes on contact so that the cyclase system is not sufficiently activated (or its hormone receptors exposed). Malignant cells could, therefore, be deficient in c-AMP—to various extents according to their degree of differentiation or growth rate—but still capable to respond to c-AMP: they have retained the cellular outfit for reversion to a phenotypically (more) normal state. However, it may appear that, although dbc-AMP universally slows down transformed (tumor)-cell growth, the saturation density of transformed cells may not always (or perhaps only in a few cases) be decreased [117]. This may mean either that other factors than those connected with c-AMP metabolism are involved or that intracellular processes have escaped from control by c-AMP in neoplasia. Only a future research may answer the question whether such assimilation processes in which c-AMP participates are affected by the neoplastic change; one such an example has very recently become available [158]. In view of the relation between Ca^{2+} and c-AMP [153, 159], and the finding that fibroblasts require Ca^{2+} for growth whereas RSV-transformed cells do not [160], the involvement of Ca^{2+} in the processes under consideration, including microtubular organization [133], also merits further attention.

Very recent results have shown [168, 169] that addition of dbc-AMP to transformed cells caused arrest of these cells in G_2, and, therefore, dbc-AMP does not restore the loss of contact inhibition of growth or reverse transformation, since in that case the transformed cells should have been arrested in G_1. The same applies to the chemical protease inhibitors studied by Schnebli and Burger [170] since these compounds also arrest transformed cells in G_2 (J. Collard, unpublished).* It should, however, be pointed out that these results do not invalidate the regulatory role of c-AMP in early G_1 processes [130, 131a, 156a] nor the hypothesis that proteolytic activity of the transformed cell (surface) may afford a continuous growth stimulus [170].

*Using SV40–3T3 cells.

EPILOGUE

This presentation is mainly concerned with the role of cell-to-cell contacts ("horizontal" interactions) in growth control, and therefore does not exhaust the problem since the impact of cell-substratum ("vertical") interactions, and medium, predominantly serum factors ("radial" interactions), are not or not explicitly dealt with.

Serum contains various growth stimulatory factors and it may be significant in the present context that the phosphodiesterase activity and the insulin(like factors) present in serum, are c-AMP antagonists. As concerns these two factors, it is readily apparent from their changed c-AMP metabolism that transformed and tumor cells show no or only a decreased serum requirement. Furthermore, insulin may replace serum in many respects [99, 100], and it may also be significant that the nerve-growth factor shares many properties with (pro) insulin [161]. It should be investigated as to whether serum acts by affecting the cell periphery, e.g., by binding to or by containing enzymes which act upon the membrane.

Growth of cells in suspension (soft agar) has been viewed as a characteristic property of transformed cells; however, it has recently been shown that non-transformed cells may grow likewise provided that a high concentration of insulin is present [162]. (Does the cell periphery contain insulinase activity? At least the liver plasma membrane contains glucagonase activity [101].)

Growth of fresh normal cells has been considered to be anchorage dependent [163], a solid substrate being required for these otherwise rounded-off cells in G_1 to stretch and to progress to the initiations of DNA synthesis. This "vertical" interaction has recently been reinterpreted following the finding [164] that calf serum contains an inhibitor of the initiation of DNA synthesis for normal cells in suspension, and that transformed cells were not sensitive to this inhibitor. In the presence of swine serum, which contained little of this inhibitor, normal cells grew in soft agar.

Notwithstanding this, cell-substratum (or $cell_x$-$cell_y$ layer) interactions mediated by the cell surface, may be highly important. It is likely that cell-substratum interactions may also affect cell-to-cell interactions. The membrane spread-

ing of a normal cell on a substrate leads to a large increase in the cell surface exposed to the medium, which could affect membrane conformation, permeability, enzyme activities and chemical expression. Changes in surface expression of transformed (neoplastic) cells could change the affinity of these cells for and decrease their dependence on substratum.

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Wilms' Tumour Treated at the Rotterdam Radiotherapy Institute (1964–1971)

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Abstract—*Between* 1964 *and* 1971, 43 *cases of Wilms' tumour were treated at the Rotterdam Radiotherapy Institute. Twenty-five received preoperative irradiation, followed immediately by nephrectomy and treated by actinomycin-D only in case of the presence of "bad risk" factors. Ten patients were operated, followed by postoperative irradiation and routine actinomycin-D. Six cases presented with diffuse pulmonary metastases and were treated palliatively by pulmonary irradiation, followed by nephrectomy and chemotherapy. Two cases of bilateral Wilms' tumour or a tumour in a horseshoe kidney were palliatively treated.*

Although the incidence of pulmonary metastases and incomplete operation was higher in the group which received no preoperative irradiation, final prognosis was not significantly affected by the first treatment modality if pulmonary metastases were detected early and treated efficiently. The main prognostic feature appeared to be age: those younger than 3 *years fared significantly better than the older age group.*

INTRODUCTION

In 1964 Rotterdam urologists, pediatricians, and radiotherapists agreed to a uniform protocol for treatment of Wilms' tumour. It was decided to give preoperative irradiation immediately followed by nephrectomy. Additional actinomycin-D was reserved for those patients where microscopic examination of the operative specimen established the presence of "bad risk" factors.

MATERIAL AND METHODS

The diagnosis Wilms' tumour was established by routine clinical examination consisting of physical examination, intravenous pyelography, chest X-rays including tomography, routine laboratory tests, and VMA test in order to exclude the diagnosis neuroblastoma. Routine preoperative irradiation consisted of a 2500 rad midplane dose to the region of the affected kidney, administered by linear accelerator over a period of 3–4 weeks in 5 weekly fractions.

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Immediately after the irradiation course nephrectomy was performed. Postoperative actinomycin-D, 3 standard courses at monthly intervals, was given only in case of the following histological criteria, considered to be indicators for a "bad risk" situation: invasion of the tumour into or through the capsule of the kidney, invasion of blood vessels, undifferentiated growth. In case of incomplete operation, i.e. incomplete removal of the primary as confirmed by microscopic examination of the operation specimen, a postoperative irradiation 2000 rad midplane dose was administered to the operation site. As incomplete removal always implies invasion through the capsule, indicating a "bad risk" factor, these patients also received chemotherapy. Apart from these protocol cases a few children were treated by nephrectomy followed by postoperative irradiation (the same dose and technique as in the case of preoperative irradiation) followed in all instances by actinomycin-D therapy. The cases were not treated according to protocol either because Wilms' tumour was not suspected by the treating urologist or because preoperative irradiation was "forgotten".

In the same period, patients presenting with pulmonary metastases at diagnosis were treated with pulmonary irradiation (1500 rad mid-plane dose in 3 weeks to the whole thorax, with a booster dose up to 4000 rad to two, maximal three, large persistent metastases). After subsequent nephrectomy, routine actinomycin-D chemotherapy was added. If metastases developed during the follow-up period, the same irradiation and chemotherapy scheme was applied.

A total of 43 children was treated between 1964 and 1971. Table 1 gives an impression of the distribution of cases.

Table 1. *R.R.T.I.* 1964/71 *Wilms' tumours*

Nephrectomy + Postop. Irradiation + act. D	:	10
Preop. Irradiation + Nephrectomy ± act. D	:	25
Pulmonary Metastases at 1st Treatment	:	6
Bilat. Tumour/tumour in horse-shoe kidney	:	2
Total		43

RESULTS

Of the 25 children treated by preoperative irradiation the actuarial survival was 80% after 5 years.

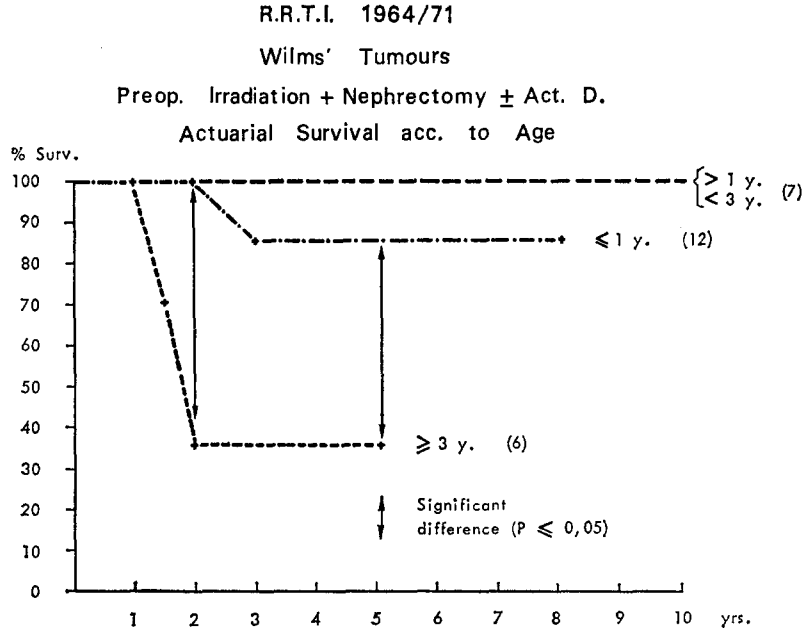
Graph 1 presents prognosis of children less than 1 year old, of children between 1 and 3 years of age, and of those older than 3 years.

As the survival of the first two groups was more or less similar, whereas the difference in survival between those older than 3 years and those younger than 3 years was statistically significant, further analysis was performed according to age less than 3 years and to age 3 years and older (Graph 2).

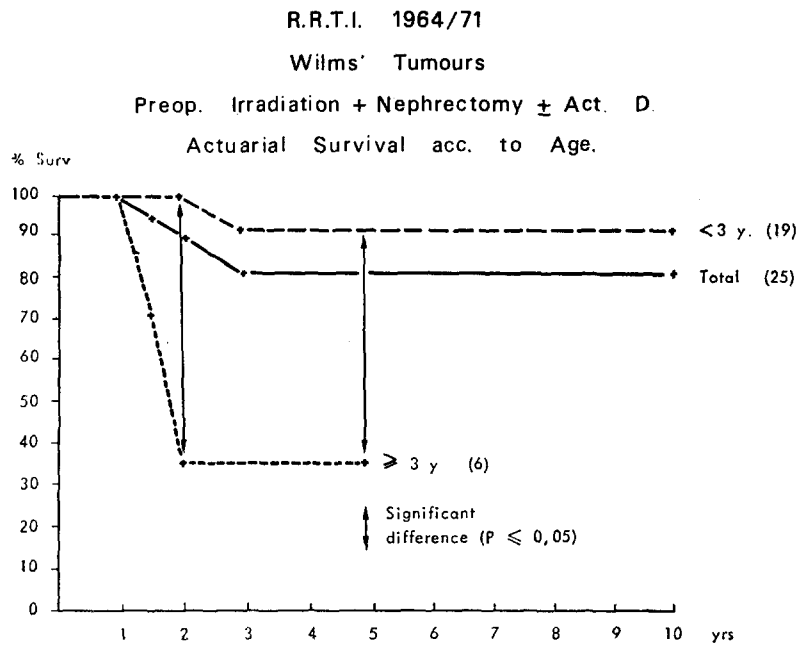
Of the 10 patients treated by nephrectomy followed by postoperative irradiation and actinomycin-D, the actuarial 5-year survival was 70%. Those under the age of 3 years showed an excellent prognosis (100% survival), whereas those of 3 years and older had only a 40% chance to survive after that period (Graph 3).

Graph 4 shows the survival rate of all patients, according to age group.

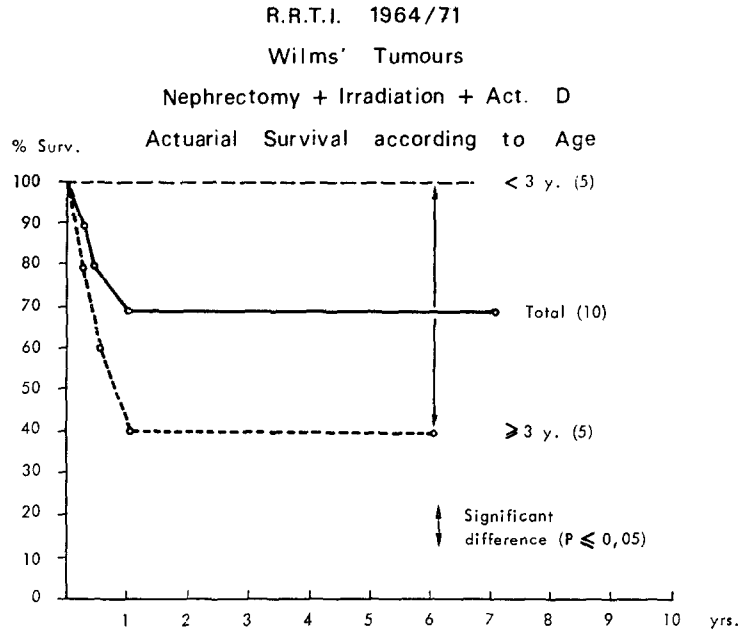
Table 2 compares the incidence of incomplete operation and of pulmonary metastases in the treatment groups "preoperative irradiation" and "postoperative irradiation". The incidence of incomplete removal—considered to be a "bad risk" factor in the group "preoperative irradiation"—is significantly higher in the group which received postoperative irradiation, and the occurrence of pulmonary metastases shows the same tendency. Table 3 presents the occurrence of pulmonary metastases after radical and incomplete operation and in relation to the administration of actinomycin-D, in the age group below 1 year, the group between 1 and 3 years of age, and in the group older than 3 years. There is a higher risk of incomplete operation in the older age group and also a higher risk of pulmonary metastases, in spite of the more frequent administration of actinomycin-D in that group.



Graph 1.



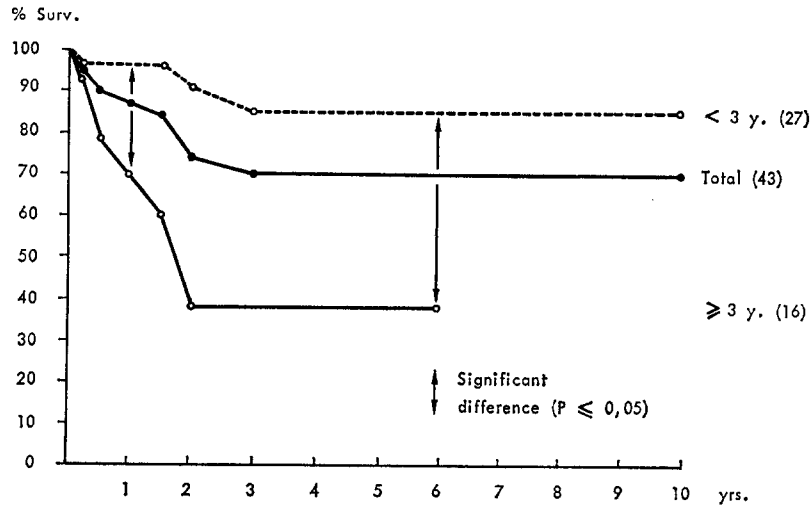
Graph 2.



Graph 3.

If all ages are considered together, there is a tendency towards a higher incidence of pulmonary metastases after incomplete operation (3 out of 5 v. 5 out of 20 after radical operation). actinomycin-D was only given to "poor risk" cases, i.e. "bad" histology of the preoperative group, and to children of the group with no preoperative irradiation: 6 out of 12 cases developed pulmonary metastases. Only 2 out of 13 "good risk" patients, who received no actinomycin-D, showed pulmonary metastases.

The actuarial survival of patients who developed pulmonary metastases during the follow-up period, and who were immediately treated by pulmonary irradiation—in a stage of localised metastasis (1–3 lesions in not more than two lung regions)—is presented in Graph 5. Here again those younger than 3 years of age



Graph 4.

Table 2. *R.R.T.I.* 1964/71 *Wilms' tumours. Preop. irradiation* ± *Act. D, postop. irradiation* + *Act. D in relation to incomplete removal and to pulm. mets.*

	Total	Incomplete oper.		Pulm. mets.	
Preop. Irrad. + or − Act. D	25	5	20% ↑	8	33%
Postop. Irrad. + Act. D	10	8	80% ↓	5	50%
		(↕ $P \leqq 005$)			

Table 3. *R.R.T.I.* 1964/71 *Wilms' tumours. Preop. irradiationn* + *ephrectomy*

	Operation		Act. D	
	Radical	Incomplete	+	−
≦ 1 yr *N* : 12	10 (1 → pulm. M)	2 (1 → pulm. M)	4 (1 → pulm. M)	8 (1 → pulm. M)
> 1 yr < 3 yr *N* : 7	7 (2 → pulm. M)	0	3 (1 → pulm. M)	4 (1 → pulm. M)
< 3 yr *N* : 19	17 (3 → pulm. M)	2 (1 → pulm. M)	7 (2 → pulm. M)	12 (2 → pulm. M)
≧ 3 yr *N* : 6	3 (2 → pulm. M)	3 (2 → pulm. M)	5 (4 → pulm. M)	1
All ages *N* : 25	20 (5 → pulm. M)	5 (3 → pulm. M)	12 (6 → pulm. M)	13 (2 → pulm. M)

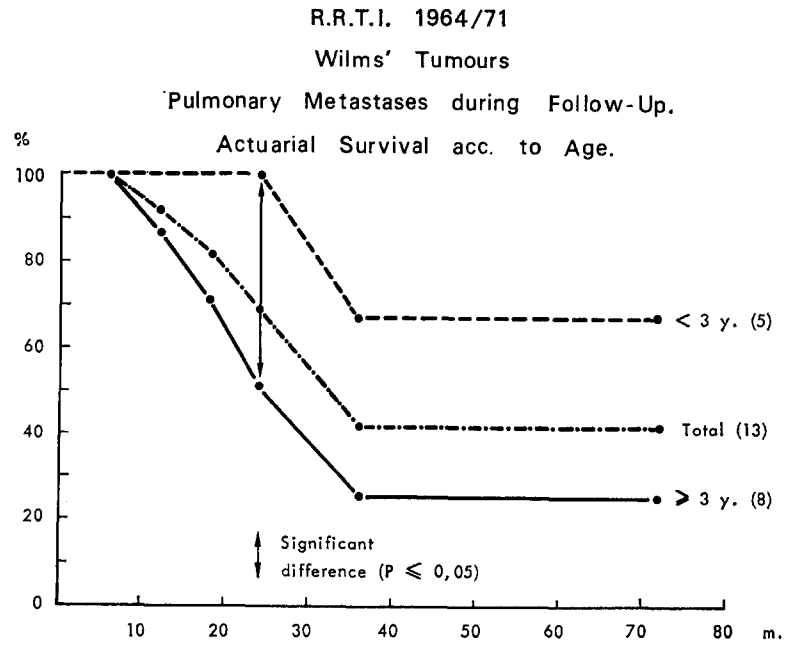
fare significantly better than those over 3 years.

Of 6 patients presented with diffuse pulmonary metastases at first treatment, only 2 of them could be cured (Graph 6). Graph 7 demonstrates the average age of patients belonging to the various treatment groups, according to complete or incomplete operation, the incidence of pulmonary metastases and the administration of actinomycin-D.

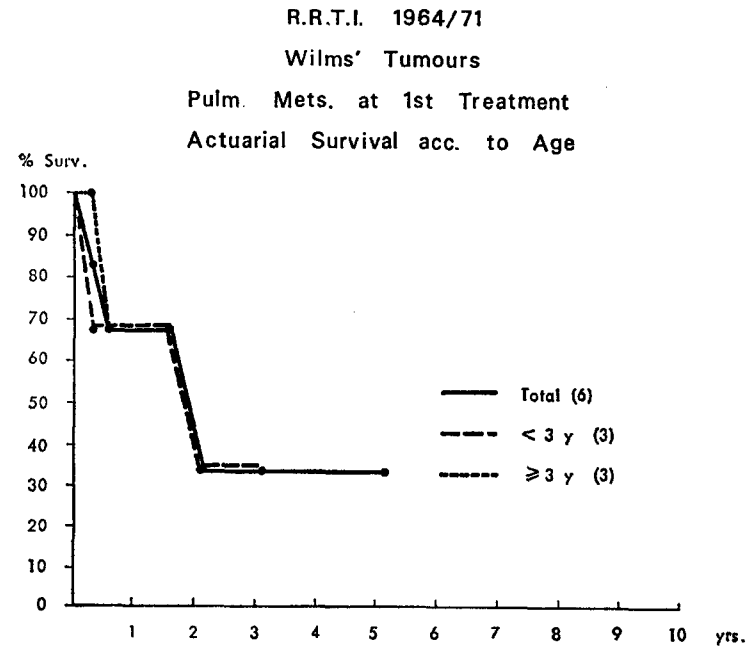
In all treatment groups there was a tendency towards a higher age in the incompletely operated children, in those with pulmonary metastases and in those who received actinomycin-D.

DISCUSSION

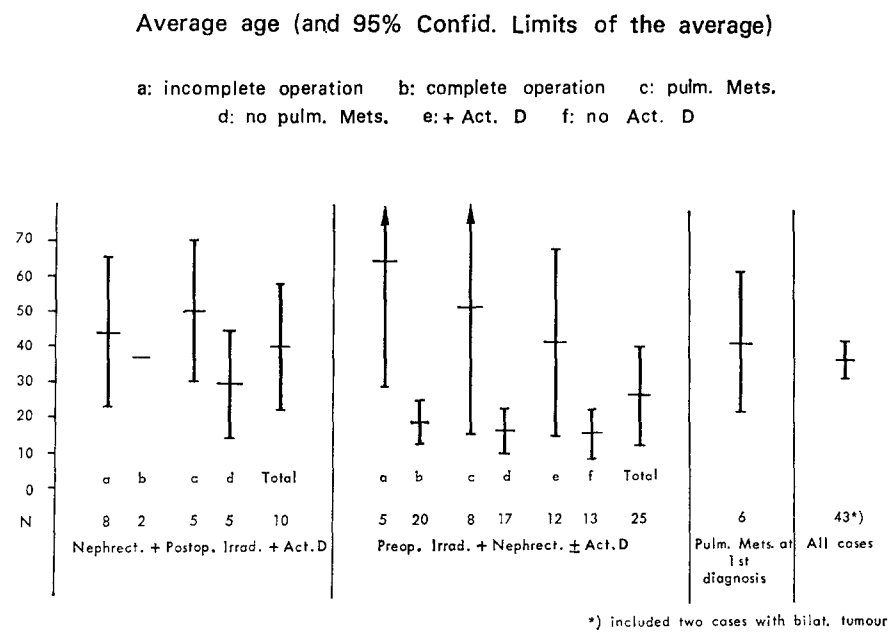
The analysis of the Rotterdam material shows that prognosis is poor though not hopeless if the patient presents with diffuse



Graph 5.



Graph 6.



Graph 7.

pulmonary metastases at first diagnosis: 2 out of 6 patients were cured after irradiation of both lungs and subsequent administration of chemotherapy. Though the incidence of incomplete operation and pulmonary metastases is higher after operation followed by postoperative irradiation and actinomycin-D, as compared with the group of preoperative irradiation with or without subsequent actinomycin-D, survival is not significantly different after these two treatment modalities. As pulmonary metastasis which develops during the follow-up can be treated adequately, prognosis remains mainly determined by age, which seems the most important indicator for prognosis: 70% cure in those younger than 3 years and 25% in those older than 3 years (Graph 5).

Table 4. *Wilms' tumour: pulmonary metastases/incidence and survival*

Author	Treatment of primary	Incidence of metastases	Treatment of metastases	≧ 2 yr Survival after treatment of metastases
Tournade, 1972 [1]	Nephrect. + X-ray postop. ± preop. X-ray − act. D	45%	all modalities − act. D	7%
	id. + act. D	33%	all mod. + act. D	23%
Hussey, 1971 [2]	Nephrect. + X-ray + chemoth.	31% (N = 29)	—	—
	id. − chemoth.	75% (N = 8)	—	—
Vietti, 1970 [3]	—	— (N = 14)	X-ray + chemoth.	(8/14) 57%
Farber, 1966 [4]	Nephrect. + X-ray + act. D	30% (N = 53)	X-ray − act. D	(10/16) 60%
Stone, 1969 [5]	+ act. D	46% (N = 13)	—	—
	− act. D	25% (N = 16)	—	—
van der Werf, 1972	nephrect. + X-ray + act. D	50% (N = 10)	X-ray + chemoth.	45% after 5 yr act. surv.
	X-ray + nephrect. ± act. D	32% (N = 25)		70% after 2 yr act. surv.

From our data it is evident that Actinomycin-D, given routinely in the postoperative irradiation group and in "bad risk" cases of the preoperative irradiation group, does not prevent pulmonary metastases in a substantial proportion of the cases (Table 2 and 4), whereas in the case of preoperative irradiation and the absence of "bad risk" factors the incidence of pulmonary metastases is low, even without the administration of actinomycin-D (Table 3). If the average age of patients in the various subgroups is compared (Graph 7), it becomes evident that in all treatment categories, pulmonary metastases and incomplete operation are related to higher age.

Also the administration of actinomycin-D in the preoperative irradiation group appears to be connected with higher age, demonstrating the relationship of "bad risk" factors with higher age.

As the majority of cases was treated by preoperative irradiation, presentation of data according to stage is not possible as usually the growth shrank significantly during preoperative irradiation, in two cases even only extensive fibrosis could be detected in the operation specimen. Nevertheless, if the overall results are compared with those of other centres, it is apparent that preoperative irradiation offers as good a prognosis as postoperative irradiation and routine chemotherapy (Table 4). Apparently special attention during treatment and keen follow-up with appropriate measures if pulmonary metastases develop are more important than the type of treatment itself (Table 5).

Table 5. *Wilms' tumours. Survival after various types of treatment*

Author		Type of treatment	Number of patients	% Survival (2 yr)	Details
Kenny, 1969	[6]	Nephrect. + X-ray	19	35	
		id. + chemoth.	15	60	
Fernbach, 1966	[7]	Nephrect. + X-ray	14	43	not all
		id. + act. D	13	92	patients passed Collins period
Stone, 1969	[5]	Nephrect. + X-ray + or − act. D	28	54	
			11	64	age < 2 yr
			17	47	age ≧ 2 yr
Hussey, 1971	[2]	Nephrect. + X-ray	42	64	
Maier, 1967	[8]	Nephrect. + X-ray + act. D	51	44	
			12	75	age < 2 yr
			29	31	age ≧ 2 yr
Tournade, 1972	[1]	Nephrect. ± act. D	205	55(5yr)	
			75	68	age < 2 yr
			130	48	age ≧ 2 yr
		+ act. D	129	60	
				70	age < 2 yr
				57	age ≧ 2 yr
		− act. D	76	50	
				65	age < 2 yr
				36	age ≧ 2 yr
Hilton, 1970	[9]	Nephrect. + X-ray ± preop. X-ray ± act. D	58	64	
van der Werf, 1972		Nephr. + X-ray + act. D	10	70	
		X-ray + Nephr. ± act. D	25	80	
		Nephr. + X-ray + act. D	5	100	age < 3 yr
			5	40	age ≧ 3 yr
		X-ray + Nephr. ± act. D	19	90	age < 3 yr
			6	35	age ≧ 3 yr

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The Cytotoxicity of Chlorambucil and its Associated Effects on NAD Metabolism*

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Abstract—*Chlorambucil has been reacted with two strains of Yoshida ascites sarcoma cells* in vitro *and* in vivo, *and the resultant changes in NAD† and NAD-metabolising enzymes related to the quantitative cytotoxicity of the drug at the various doses used. There was no change in the NAD content at any dose up to the* LD_{100} *figure* in vivo *or* in vitro, *though supra-lethal concentrations did produce a fall in NAD content* in vitro. *Curative doses of drug led to increase in the specific activities of NAD glycohydrolase and ATP:NMN adenylyl transferase* in vivo, *but these were not reflected in changes in the NAD level. Supra-lethal concentrations of the drug produced only small decreases in the levels of these enzymes* in vitro. *It is concluded that the cytostatic effects of chlorambucil are not mediated through an effect on the intracellular NAD concentration, though at supra-lethal concentrations of the drug a loss of NAD does occur, which may be attributed to non-specific damage to the plasma membrane.*

INTRODUCTION

THERE have been many reports of an impaired metabolism of pyridine nucleotides, and of the existence of abnormally low levels of these co-factors in tumour cells [1, 2, 3]. However, some normal tissues, (e.g. brain and spleen) also contain low levels of NAD [4], while increased levels have been reported in the leucocytes of patients with chronic myeloid and acute leukaemias [5]. The NAD-biosynthetic enzyme ATP:NMN adenylyl transferase (EC2.7.7.1) is present in only small amounts in a wide range of human and experimental tumours [6], but may be only partly responsible for the reduced synthesis of NAD in these tissues in response to nicotinamide challenge [7]. The NAD content of the minimum deviation hepatomas is significantly reduced, but the activity of ATP:NMN adenylyl transferase is only slightly impaired. Nichizuka and Hayaishi [8] have suggested that a decreased activity of nicotinic acid mononucleotide pyrophosphorylase (EC2.4.2.11) together with an increased rate of degradation of the mononucleotide may account for this situation. The activity of NAD glycohydrolase (EC 3.2.2.5) in tumours is within the range reported for normal tissues [9].

Under certain conditions, alkylating agents have been shown to reduce the intracellular NAD content of tumour cells, producing a depression of glycolytic activity and subsequent cell death [10, 11]. Some investigators have indicated that the cytotoxicity of an alkylating agent is proportional to the NAD-depletion which it induces: a considerable reduction in NAD occurring in tumour cells which are killed by alkylating agents, while no change is seen in alkylating agent-resistant tumours [12, 13]. There are conflicting views concerning the mechanisms which may be responsible for these changes. Holzer and his collaborators

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†*Abbreviations:*

ADH	—	alcohol dehydrogenase
ATP	—	adenosine triphosphate
NAD	—	nicotinamide adenine dinucleotide
NADase	—	NAD glycohydrolase
NMN	—	nicotinamide mononucleotide

have attributed the alkylating agent-induced reduction of NAD content to reduced biosynthesis of the co-enzyme [10, 14], while Green and Bodansky [15] consider that increased levels of NAD glycohydrolase (produced in response to the alkylating agent) lead to a more extensive catabolism of NAD.

Whatever mechanism is responsible for these alterations in NAD metabolism, however, there is no general agreement that alkylating agents manifest their cytotoxic effects through an initial lowering of NAD content. The concept has been challenged by Fusenig and Obrecht [16], and by Liss and Palme [17]. The former workers showed that Trenimon was cytotoxic at doses which did not deplete tumour NAD, while the latter found that DNA synthesis could be inhibited by alkylating agents at doses which were without effect on the NAD level or on the rate of glycolysis. There is now good evidence that cross linking of DNA by alkylating agents may represent the primary biochemical lesion responsible for the cytotoxicity of these compounds [18], and that other biochemical changes may be secondary effects, or effects which may be observed only at drug levels considerably in excess of those required to insert DNA cross-links [19].

In an attempt to resolve some of these conflicting theories, we wish to report the results of some further experiments designed to elucidate whether the effects of alkylating agents on pyridine nucleotides, and on the metabolism of these compounds, are indeed causally related to the cytotoxicity of these agents. The drug-sensitive strain of the Yoshida ascites sarcoma and a derived sub-line made resistant to alkylating agents by long-term exposure to low doses of Melphalan provided a useful experimental system in which to investigate this problem.

The effects of the alkylating agent chlorambucil on the NAD metabolism of the two tumour strains were observed following both *in vivo* and *in vitro* exposure to the drug. These studies were extended further to examine the relationship between membrane damage and loss of NAD in the erythrocyte following chlorambucil treatment.

MATERIAL AND METHODS

Chlorambucil (Leukeran) $(ClCH_2CH_2)_2NC_6H_4(CH_2)_3COOH$ was synthesized in the Chester Beatty Research Institute. Alcohol dehydrogenase, ATP, and pyridine nucleotides were obtained from Boehringer Corporation (London) Ltd., TC199 from Glaxo, and Horse Serum No. 2 from Burroughs Wellcome. Other chemicals were purchased from May and Baker Ltd., British Drug Houses, or Hopkin and Williams Ltd., reagent grades being used where available.

Details of the animal experimentation and isolation of the tumour cells have been published previously [20, 21]. For the *in vivo* experiments, animals undergoing drug treatment were injected with chlorambucil (8 mg/kg) 4 days after tumour implantation. The drug was dissolved in a minimum of 2% ethanolic HCl (not greater than 0·1 ml), and made up to the required volume (usually 10 ml) with a solution containing 2% K_2HPO_4 in 50% v/v propylene glycol. Control animals were injected with solvent alone.

In vitro *drug treatment of cells*

(a) *Ascites tumour cells.* Isolated tumour cells were suspended to a concentration of 2×10^7/ml, in equal volumes of horse serum and TC199; 10 ml incubates were used for each assay. The cell suspensions were incubated with chlorambucil at 37°C for the stated period, after which the cells were spun down, washed once with TC199, and the cell pellet extracted with the appropriate reagent for assay.

(b) *Human erythrocytes.* Blood was removed by cubital venipuncture, the cells spun down at 350 **g** for 10 min and, after removal of serum and buffy coat, the red cells were resuspended in Waymouth medium pH 7·4 to the original blood volume; 0·5 ml aliquots of the erythrocyte suspension were added to a further 9·4 ml Waymouth Medium, and duplicate samples incubated for 1 hr at 37°C with varying concentrations of chlorambucil in ethanol (0·1 ml). The cells were then centrifuged (350 **g**, 10 min, 0°C) and extracted at 0°C with 5 ml 0·02N H_2SO_4 in 0·1M Na_2SO_4 for NAD analysis. The degree of cell lysis incurred by the drug was determined by measurement of the extinction at 540 nm of samples of the suspension medium.

Bioassay of chlorambucil in ascites tumour cells

Sterile solutions and equipment were used throughout. The isolated tumour cells were incubated with chlorambucil as follows: for sensitive cells at concentrations between 0·25 and 6·3 μM, and for resistant cells at concentrations between 0·25 μM and 0·2 mM. Solvent-treated controls of each cell type were included. Following 1 hr incubation with the drug, 5 ml aliquots were removed, centrifuged at 350 **g** for 2 min, washed with TC199, and resuspended in 10 ml TC199. one-half milliliter of sus-

pension (1×10^6 cells) was injected into the right inguinal region of healthy rats (5 animals at each point). After 12 days the animals were killed by cervical dislocation, and the tumours dissected out and weighed. It was previously demonstrated that the tumour weight was directly proportional to the number of viable cells injected (between 10^4 and 10^7 cells).

Estimation of NAD

NAD was extracted by the method of Burch *et al.* [22]. To a pellet containing 2×10^8 cells was added 5 ml ice-cold 0·02N H_2SO_4 in 0·1 M Na_2SO_4. The cells were disrupted by sonic oscillation (MSE Ultrasonicator 20kG/sec.) for 1 min, and then heated at 60°C for 30 min to inactivate NADase. Protein was removed by centrifugation at 1,000 **g** for 15 min. NAD in the supernatant was measured using alcohol dehydrogenase (ADH) according to the method of Racker [23] as modified by Greengard *et al.* [24]. The NAD content of erythrocytes was measured by the fluorimetric procedure of Lowry *et al.* [25] using an Aminco Bowman spectrofluorimeter.

Methods for the measurement of oxygen consumption and CO_2 evolution have been described previously [26].

NAD glycohydrolase activity was measured by a modification of the method of Kaplan [27]. A pellet containing 2×10^8 washed cells was suspended in 10 ml 0·05 M phosphate buffer pH 7·0, and the cells ruptured by sonic oscillation of the total homogenate were pre-incubated at 17°C, and to each sample was added 1·65 μmoles NAD in 1·5 ml 0·05 M phosphate buffer pH 7·0. Samples were incubated for 10 min, and the reaction terminated by addition of 1·5 ml 10% TCA. Following centrifugation (1000 **g** for 15 min), the NAD remaining in 0·1 ml of the acid supernatant was measured by the ADH method.

ATP:NMN adenylyl transferase activity was measured by a modification of the method of Greenbaum [28]. A pellet containing 6×10^8 washed ascites cells in 2·7 ml ice-cold 0·25 M sucrose was ruptured using an Ultra-Turrax (Janke and Kungel); 0·9 ml of the resultant cell lysate was mixed with 0·1 ml sodium deoxycholate (80 mg/ml).

Duplicate 0·1 ml aliquots of the treated lysate were incubated for 1 hr at 37°C with 0·15 ml of an incubation mixture having the following composition:

glycylglycine buffer pH 7·6 50 mM;
NMN 2 mM; ATP 4 mM;
$MgCl_2$ 4 mM; nicotinamide 200 mM.

The reaction was terminated by addition of 0·2 ml 20% TCA. After centrifugation (1000 **g**, 15 min) to remove denatured protein, the NAD in the supernatant was measured by the ADH method.

Protein was estimated by the method of Lowry [29].

RESULTS

The results obtained from *in vivo* and *in vitro* studies will be presented separately.

In vivo *experiments*

Animals carrying the drug-sensitive strain of the Yoshida ascites tumour can be cured of their disease by a single subcutaneous dose of chlorambucil (8 mg/kg), while the drug-resistant sub-line is refractory to such treatment. The effects of this dose of chlorambucil on the NAD content of the two tumour strains are summarised in Table 1. In the drug-sensitive tumour, a progressive increase in the NAD level occurred, which was accompanied by a rise in the protein content of the cells as they enlarged [21]. Expressed on a protein basis, the NAD content of the treated cells remained unchanged. This treatment also led to an increase in the oxygen uptake, and in the aerobic evolution of CO_2 in the drug-sensitive cells [26]. Treatment of the drug-resistant tumour line produced no changes in any of these properties.

Table 1. NAD content of Yoshida ascites sarcoma cells following the administration of chlorambucil (8 mg/kg) to tumour-bearing rats

Time after drug admin. (hr)	μ moles NAD/10^9 cells Control Mean	S.E.	Treated Mean	S.E.
Sensitive				
0	0·61	0·05		
24	0·49	0·17	0·84	0·08
48	0·60	0·21	1·16	0·13
72	0·57	0·14	1·27	0·08
Resistant				
0	0·56	0·05		
24	0·44	0·11	0·40	0·06
48	0·43	0·06	0·46	0·12
72	0·45	0·19	0·39	0·15

The values shown are the means of duplicate estimations on 3–6 animals at each time point.

The effects of chlorambucil *in vivo* on the activity of NMN adenylyl transferase in the two tumour strains are summarised in Table 2. In the sensitive tumour, enzyme activity increased proportionately to the drug-induced

Table 2. ATP:NMN adenylyl transferase activity in Yoshida ascites sarcoma cells removed from animals which had previously received chlorambucil (8 mg/kg) at the times shown

Time after drug admin.	μ moles NAD synthesized /hr/10^9 cells		mμ moles NAD synthesized /hr/mg protein	
Sensitive	Mean	S.E.	Mean	S.E.
0	3·1	0·30	18·1	1·8
24	5·2	0·23	17·5	1·1
48	8·1	0·16	19·8	0·6
Resistant				
0	2·1	0·15	13·2	0·75
24	2·0	0·06	12·6	0·80
48	1·8	0·09	13·0	0·73

Each value is the mean of duplicate estimations on 5–9 animals.

elevation in the total protein, so that the specific activity of this enzyme remained unaltered. No changes in NMN adenylyl transferase were induced in the drug-resistant cells.

In the case of NADase, chlorambucil treatment did produce an increase in the specific activity of the enzyme in the sensitive cells (Table 3), no change occurring in the drug-resistant cells.

Location of NAD glycohydrolase

In Ehrlich ascites cells modifications of this enzyme have been found predominantly in the nuclear and microsomal fractions [30], while in the erythrocyte the enzyme is associated exclusively with the outer surface of the plasma membrane [31]. In the following experiment, the enzyme activity in intact Yoshida ascites cells and cell lysates was compared: cells were examined from both treated and untreated animals. The results shown in Table 4 indicate that intact cells and cell lysates degrade NAD at a comparable rate, while treatment of the host animals with chlorambucil did not alter this finding. Since NAD is unable to enter the cell [32], this observation suggests that NAD glycohydrolase is associated with the outer surface of the plasma membrane of Yoshida cells, and that no change in distribution occurs as a result of *in vivo* drug-treatment.

In vitro *experiments*

The toxicity of chlorambucil to the two tumour cell strains is shown graphically in Fig. 1. The LD_{100} for the sensitive and resistant tumour was 0·002 and 0·1 mM respectively demonstrating a 50-fold difference in sensitivity between the two tumour strains. It is notable that once a threshold cytotoxic concentration of drug had been achieved, further increases in concentration produced similar effects on the survival of each cell strain. No change was observed in the intracellular NAD content of

Table 3. NAD glycohydrolase activity in Yoshida ascites sarcoma cells removed from animals which had previously received chlorambucil (8 mg/kg) at the times shown

Hr. after drug admin.	μ moles NAD degraded /min/10^9 cells				mμ moles NAD degraded /min/mg protein			
	Treated		Control		Treated		Control	
	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.
Sensitive								
0	—	—	3·9	0·1	—	—	24·4	0·2
24	6·9	0·7	3·8	0·1	31·0	0·4	25·5	0·4
48	11·3	0·9	4·0	0·2	35·5	2·2	24·5	0·4
72	12·3	0·7	—	—	39·3	1·0	—	—
Resistant								
0	—	—	2·1	0·2	—	—	14·5	0·9
24	2.6	0·8	2·1	0·3	15·7	0·5	13·5	0·9
48	2·9	0·2	2·3	0·4	17·5	0·8	14·1	0·5
72	2·2	0·2	2·4	0·2	16·5	1·2	16·4	0·5

Each point is the mean of duplicate estimations in 3–6 animals.

Table 4. Degradation of NAD by intact cells and by cell lysates

Cell type	μ moles NAD degraded/min/10^9 cells Intact Mean	Intact S.E.	Lysate Mean	Lysate S.E.
Sensitive untreated	4·3	0·1	4·4	0·1
Sensitive, 48 hr after treatment	9·5	0·5	9·5	0·5
Resistant untreated	2·8	0·2	3·1	0·2
Resistant, 48 hr after treatment	2·5	0·3	2·6	0·2

Each point represents the mean of duplicate estimations in 3–6 animals.

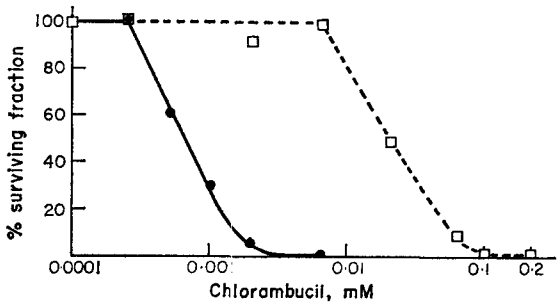

Fig. 1. The relationship between chlorambucil concentration and subsequent viability of drug-sensitive and -resistant Yoshida ascites cells following their in vitro *exposure for 1 hr.*

□– – –□ resistant strain

●——● sensitive strain

Each point represents the mean weight of tumours from 5 animals. Scatter ≯ 10%.

the tumour cells following their exposure to chlorambucil within the cytotoxic range of concentrations (i.e. up to 0·1 mM). The effects of higher concentrations of chlorambucil on the NAD content of the two cell strains are shown in Fig. 2, and it will be noticed that for each strain the NAD level was reduced only by supralethal concentrations of drug. This occurred at a rate only marginally less in the drug-resistant strain than in the sensitive strain. It should also be noted that the NAD content

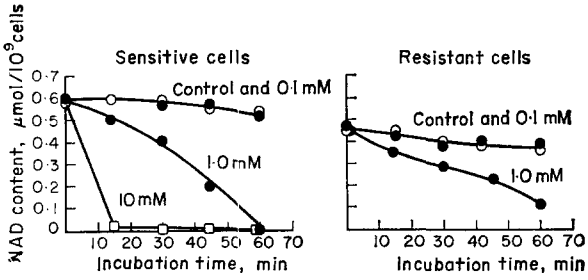

Fig. 2. Comparison of the effects of incubation with chlorambucil on the NAD levels of drug-sensitive and -resistant Yoshida ascites cells. Concentrations of chlorambucil are indicated. Each value represents the mean of duplicate incubations.

of resistant cells was approximately 75% of that of sensitive cells. NAD itself if not modified by chlorambucil and incubation of the nucleotide with the drug resulted in no loss of coenzyme activity. As shown in Fig. 3 oxygen uptake and aerobic CO_2 evolution remained unaffected by a chlorambucil concentration of

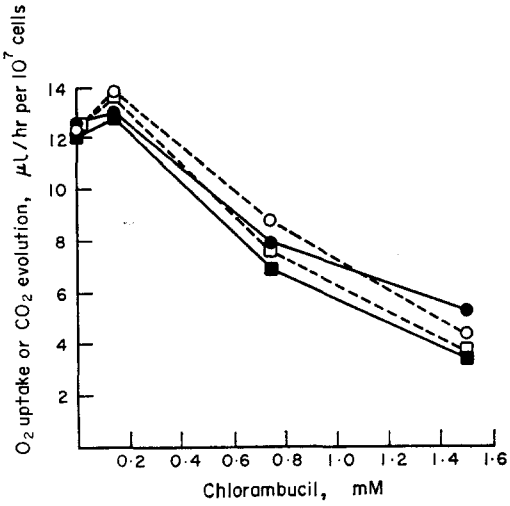

Fig. 3. Effects of treatment in vitro *with various doses of chlorambucil on the O_2 uptake and aerobic CO_2 evolution of Yoshida ascites cells.*

●——● O_2 uptake (S)

○– – –○ O_2 uptake (R)

■——■ CO_2 evolution (S)

□– – –□ CO_2 evolution (R)

0·2 mM, although incubation at higher drug concentrations resulted in progressive inhibition. These results are described in greater detail elsewhere [26]. ATP:NMN adenylyl transferase activity was inhibited when cells of either strain were incubated with a supralethal (2 mM) concentration of chlorambucil (see Fig. 4a). The enzyme activity in both sensitive and resistant cells was inhibited to a similar extent. The activity of this enzyme in the Yoshida ascites cells, as in other tumour

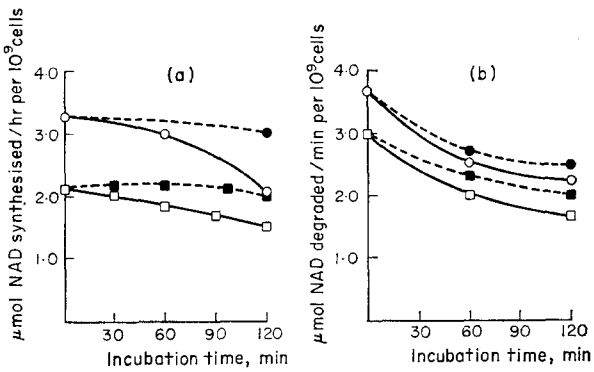

Fig. 4. Effect of incubation for 2 hr with 2·0 mM chlorambucil on (a) *NMN adenylyl transferase and* (b) *NAD glycohydrolase in drug sensitive and resistant strains of the Yoshida ascites sarcoma*

● - - - ● sensitive control
○ —— ○ sensitive treated
■ - - - ■ resistant control
□ —— □ resistant treated

Duplicate incubations were carried out at each point. Scatter at each point ≯ 11 %.

tissues, is low in comparison, for example, with that of liver (liver, 5 μmoles, NAD synthesized/hr/g tissue; Yoshida cells, 0·002 μmoles). ATP:NMN adenylyl transferase activity (calculated on a per-cell basis and on a protein basis) was lower in the resistant than in the sensitive cells. The addition of sodium deoxycholate was an obligatory requirement for the detection of enzyme activity in these cells.

Following exposure to 2 mM chlorambucil, the activity of NAD glycohydrolase was also inhibited, to an extent comparable with that of the biosynthetic enzyme. Activity in sensitive and resistant cells was similarly affected. Since NAD pyrophosphatase activity could not be detected in these cells, the degradation of NAD was attributed entirely to NAD glycohydrolase. The decreased activity of NADase in the treated cells could not be restored by dialysis, and was therefore not due to inhibition by nicotinamide (accumulating as a result of the extensive NAD catabolism). A similar degree of inhibition was observed when a purified preparation of NADase from calf spleen was incubated with the same concentration of chlorambucil.

It should be emphasized that a 2 mM concentration of drug produced only relatively small effects on NMN adenylyl transferase and NAD glycohydrolase activities, whereas NAD was deleted completely during the 2 hr exposure of cells to the drug. It appears likely therefore that some additional factor is responsible for this rapid loss of the co-enzyme.

Effects of chlorambucil on erythrocyte NAD

The deletion of NAD in tumour cells *in vitro* following high doses of chlorambucil might be attributable to membrane damage. This would lead to leakage of the co-enzyme, allowing contact with membrane-associated NADase. However, it was difficult to substantiate this speculation since an electron-microscopic examination of chlorambucil-treated tumour cells failed to reveal any gross structural damage.

The erythrocyte may be a more convenient cell in which to investigate such problems. Froese *et al.* [33] have shown that chlorambucil combines chemically with erythrocyte membranes producing lysis and leakage of haemaglobin. In Fig. 5 are shown the extent of haemolysis, and the associated fall in NAD

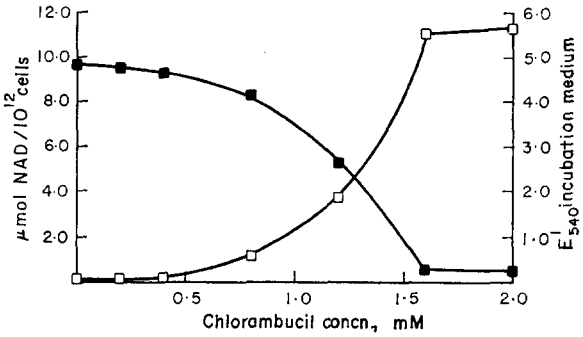

Fig. 5. Effect of increasing dose of chlorambucil on the leakage of haemoglobin and depletion of NAD in human erythrocytes.

■ —— ■ *NAD level in erythrocytes*
□ —— □ E^1_{540} *of incubation medium due to haemoglobin leakage. For details of experimental method see text.*

content, of a suspension of human erythrocytes incubated with varying concentrations of chlorambucil. Loss of NAD occurred coincident with and proportional to, the amount of haemaglobin appearing in the suspension medium. No morphologic changes were detectable by electron microscopy in the membrane structure of treated erythrocytes, though "ghosts" were detected in the preparation.

DISCUSSION

Chlorambucil was without effect on the NAD content of drug-sensitive or -resistant lines of the Yoshida ascites sarcoma *in vitro* at concentrations which were lethal to the cells (0·02 mM for S; 0·1 mM for R). A supralethal concentration of drug (1 mM) did reduce the cellular NAD, but the effect was comparable in each cell line. A delayed fall in the NAD of drug-resistant cells similar to that described

by Scriba *et al.* [34] was not observed in this work. The fall in NAD was accompanied by a reduction in the rate of oxygen uptake and aerobic CO_2 evolution, and in the activity of NAD -synthetic and -degradative enzymes. However, the rapid decrease in NAD could not be accounted for by a depression in the rate of co-enzyme synthesis. By analogy with the studies in erythrocytes, it seems possible that the fall in NAD results primarily from a drug-induced modification of the cell membrane. The NADase activity of the Yoshida ascites cell is extremely high, and once in contact with intracellular NAD could produce extremely rapid deletion of the cytoplasmic co-factor. In these cells the enzyme is associated mostly with the outer surface of the plasma membrane and is thus separated from its substrate. It has long been recognised that pyridine nucleotides undergo rapid degradation when the cellular structure is disorganised, and Froese and his collaborators [33] have demonstrated that binding of chlorambucil to the membrane of erythrocytes causes cell lysis. The present experiments have shown that the degree of lysis is paralleled by a corresponding loss in NAD. Furthermore, chlorambucil forms a transient association with a lipid fraction of the Yoshida ascites tumour, and Hill *et al.* [35] have speculated that this may reflect binding to membrane phospholipid. By producing even a slight alteration in the structure of the plasma membrane, chlorambucil may affect compartmentation between NADase and its substrate, leading to a depletion of NAD.

When the sensitive tumour was treated with a curative dose of chlorambucil *in vivo*, the NAD level of the cells increased, accompanied by a comparable rise in the total protein content. Oxygen uptake, aerobic CO_2 evolution, and NMN adenylyl transferase activity increased concomitantly with the rise in protein. There was, however, an increase in the specific activity of NADase in the drug-sensitive cells. None of these changes were observed in cells which were resistant to chlorambucil.

The results reported here are not in accord with those of Green and Dobrjansky [36], who demonstrated a direct relationship between NAD and NADase levels, and the cytotoxicity of BCNU to Ehrlich ascites cells in mice. It should be noted, however, that while BCNU contains a chloroethyl group at each end of the molecule, it does not contain the characteristic mustard group, nor does it possess all of the properties typical of a nitrogen mustard.

Our results confirm those of Aoshima *et al.* who demonstrated that the cytotoxicity of a number of cytostatic agents to Yoshida ascites tumour cells in culture could be correlated with changes in NADase activity, but not with NAD content [37].

In summary, the present work has shown that no change occurs in the levels of NAD in Yoshida ascites cells following interaction with chlorambucil *in vitro* at drug concentrations up to the LD_{100} value. When the drug is administered at curative doses to tumour-bearing animals, there is again no change in the specific level of NAD, though the specific activity of NADase does increase. These observations support the view that the toxicity of chlorambucil to drug-resistant Yoshida ascites cells is not dependent on or mediated through depression of intracellular NAD levels.

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Content of Estrogen Receptors in Human Breast Cancers*

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Abstract—*The estrogen binding capacity of 251 human breast cancers has been determined. The cytosol of the tumours was incubated with labelled estradiol-17-β and subjected to sucrose-gradient-density centrifugation. The difference in the radioactivity in the 8S area between samples incubated in the absence and presence of an estrogen antagonist was taken as a measure of the estrogen receptor content.*

The receptor content of the tumours varied over a wide range. Measurable amounts were present in 184 cases (73·1%). The content was 2–3 times higher in tumours from patients above 50 years than in those below this age. No relationship was found between the receptor content and the histological type of the tumours with the exception that tumours of the scirrhus type contained less receptors than other types of tumour. The receptor concentration was the same in different clinical stages of the disease suggesting that the receptor content of human breast cancers does not tend to decline with progression of the tumours.

INTRODUCTION

RECENT data, both from studies on experimental animals [1, 2] and on humans [3–5], indicate that the response of breast cancers to endocrine treatment depends on the presence in the tumour tissue of specific estrogen receptors in the cytosol. Determination of estrogen receptors in tumour tissue is therefore a potentially useful method in predicting the response of patients to endocrine therapy. An evaluation of the clinical usefulness of such measurements must be based on correlation of biochemical and clinical data on a large number of patients. However, the available data on human tumours are, as yet, limited.

In the present study the estrogen receptor content has been measured in tumour tissue from 251 patients with breast cancer. A preliminary report on part of this work has previously appeared [6].

MATERIAL AND METHODS

The tumours examined were obtained from patients operated at The Norwegian Radium Hospital. Most patients were in stages I and II and were subjected to mastectomy with or without block dissection of the axillary glands. During the operation samples from the primary tumour and sometimes from axillary lymph metastases were taken for histological classification [7] and chemical analysis. If the samples were not analyzed immediately, they were promptly frozen in liquid nitrogen and stored at −20°C.

The specimens were analyzed within one week by the procedure developed by Jensen *et al.* [4] with minor modifications. High speed supernatants (400 μl) were incubated at 0°C for 30 min with [6,7-^{3}H]-estradiol-17-β (spec.

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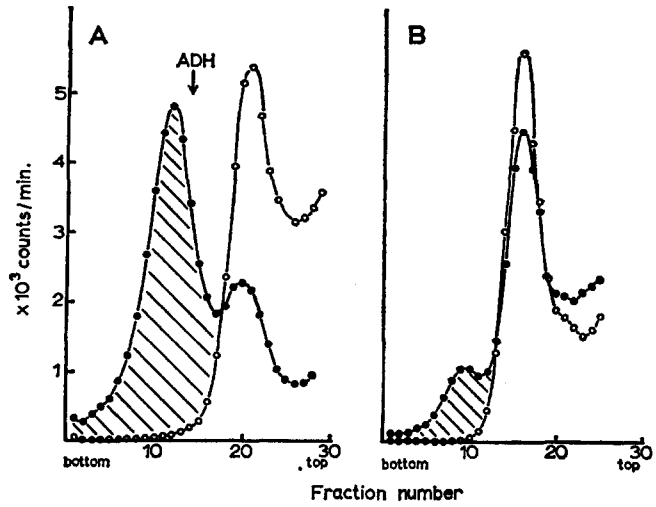

Fig. 1. Typical sedimentation patterns of estrogen-binding receptor protein in two cases of human breast cancer. Cytosol prepared from two different breast cancers (A and B) were incubated with 5 μM estradiol-17-β in the presence and absence of the estrogen antagonist Parke–Davis CN-55, 945-27 and the samples were submitted to centrifugation in a 5–20% (w/v) sucrose gradient. The approximate S values of the peaks were calculated according to Martin and Ames [17] to be 8·5 S and 4·5 S. The arrow in (A) represents the sedimentation rate of the marker, yeast alcohol dehydrogenase (7·6 S). (●—●) no estrogen antagonist; (○—○) estrogen antagonist present. The total radioactivity in the hatched areas is taken as a measure of the estrogen-binding capacity of the tumour. Note that the estrogen antagonist abolished binding in the 8 S area while concomitantly the unspecific binding to serum albumin in the 4·5 S area increased.

act. 40 Ci/mmole) in a final concentration of 5 μM. In each case a control was preincubated for 15 min with an estrogen antagonist (Parke–Davis CN-55, 945-27) in a final concentration of 5 μM. After incubation the samples were subjected to sucrose-gradient-density centrifugation at 120·000 *g* for 17 hr. Fractions (7 drops) were collected directly on Gelman glass fiber filters (type A) and the dried filters were counted in a Beckman LS-130 scintillation system [8]. The amount of specific estradiol-binding component was calculated from the difference between the radioactivity in the 8S area observed in the presence and absence of the estrogen antagonist (Fig. 1). The lowest amount of specific receptor protein that could be measured with confidence corresponded to 1×10^{-14} moles of estradiol bound per mg of cytosol protein. The protein content was determined by the method of Lowry *et al.* [9].

The [6,7-^{3}H]-estradiol-17-β was obtained from New England Nuclear Corporation, Boston. The estrogen antagonist Parke–Davis CN-55, 945-27 (1-[2-(*p*-[α-(*p*-methoxy-phenyl)-β-nitrostyryl] phenoxy) ethyl]-pyrolidine, monocitrate) was a gift from Parke–Davis & Co., Detroit.

RESULTS AND DISCUSSION

In the present study estradiol-binding capacity was definitely established (Table 1) in 184

Table 1. Estrogen receptor content in tumours of different histological types

Histological type	Total number of tumours	Average receptor concn. $\times 10^{14}$ moles/mg of cytosol protein	No. with measurable receptor content*
Adenocarcinoma	47	10·5 ± 2·1†	39 (83·0)‡
Medullary carcinoma	75	13·5 ± 2·2	51 (68·0)
Carcinoma simplex	78	12·6 ± 1·8	63 (80·8)
Scirrhus	21	3·6 ± 0·9	15 (71·5)
Other types	30	11·1 ± 2·8	16 (53·3)
Total	251	11·5	184 (73·1)

*Tumours containing more than 1×10^{-14} moles [^{3}H]-estradiol-17-β bound per mg of cytosol protein.

†Standard error of the mean.

‡Percentage of total number of tumours in each histological group.

out of the 251 mammary cancers studied, i.e. in 73·1%, while in 9 cases of normal breast tissue no specific binding was found. The average receptor content in the 184 "positive" cases was $15{\cdot}7 \times 10^{-14}$ moles of estradiol bound per mg of cytosol protein. Recently, Feherty *et al.* [10] using a charcoal adsorption assay found high affinity estradiol receptors in 37 out of 53 cancers examined, i.e. in a percentage similar as to the present study. However, in other previous studies on relatively small numbers of patients estrogen receptors were found with considerably lower frequency (36–45%) in the tumours examined [4, 5, 11–15].

Previous authors [10, 13] have concluded, on the basis of a much smaller number of patients, that the estrogen receptor content of breast cancer is not clearly related to their histology. This is confirmed by the results in Table 1 with the exception of the scirrhus tumours where the receptor content was only about 1/3 of that in the other types. Since the measurable receptor content is related to the age (see below), it is of interest that the age distribution in the case of the scirrhus cancers was not different from that in the other histological types. Possibly, the low receptor content in the scirrhus cancers reflect the low proportion of tumour cells in this type.

Table 2. Estrogen receptor content in relation to age of patient

Age (yr)	No. of cases	Average receptor concn. $\times 10^{14}$ moles/mg of cytosol protein	No. with measurable receptor content*
30–39	7	1·3 ± 0·9†	2 (28·6)‡
40–49	51	3·8 ± 0·7	35 (70·0)
50–59	68	10·8 ± 2·0	51 (76·1)
60–69	59	15·9 ± 2·5	46 (79·3)
70–79	51	15·4 ± 2·2	39 (76·5)
>80	15	11·9 ± 4·1	10 (66·7)

*Tumours containing more than 1×10^{-14} moles [^{3}H]-estradiol-17-β bound per mg of cytosol protein.
†Standard error of the mean.
‡Percentage of total number of tumours in each age group.

The receptor content in relation to the age of the patients is shown in Table 2. It is apparent that the receptor content was 2–3 times lower in patients below 50 years of age than in those above this age. Also, the percentage of cases with measurable receptor content was lower in the youngest age group. The results confirm and extend our previous findings on 87 patients [6] and those of several other investigators [10, 14, 15]. Possibly, the lower estrogen-binding capacity in premenopausal women may be due to a blocking of receptor cells by endogeneous hormone [10]. This question requires further investigation.

In Table 3 data are given on the estrogen receptor content in relation to the clinical stage of the disease. The results indicate that tumours in different clinical stages have the same average receptor content and that the percentage with measurable receptor content is the same in early and late stages of the disease. In 8 cases the receptor content in the primary tumours was compared with that in the axillary lymph metastases (Table 4). In some of these cases large differences were found between the primary tumour and the metastases, but the differences showed no consistent trend. Moreover, in

Table 3. Estrogen receptor content in relation to the clinical stage of the disease

Clinical stage	Total number of tumours	Average receptor concn. $\times 10^{14}$ moles/mg of cytosol protein	No. with measurable receptor content*
I	156	11·6 ± 1·3†	115 (73·7)‡
II	71	11·9 ± 1·9	53 (74·7)
III and IV	22	9·6 ± 3·8	15 (68·2)

*Tumours containing more than 1×10^{-14} moles [^{3}H]-estradiol-17-β bound per mg of cytosol protein.
†Standard error of the mean.
‡Percentage of total number of tumours in each clinical stage.

Table 4. Estrogen receptor content in primary tumours and in axillary lymph metastases

Patient	Receptor concn. $\times 10^{14}$ moles/mg cytosol protein: Primary tumours	Metastases
2338/65	75	25
1325/72	53	51
1075/71	12	21
872/71	17	9·7
1190/71	6·7	26
920/55	2·7	8·2
1839/71	0	0
4031/71	0	0

two cases of recurrent tumours no striking changes in the binding capacity occurred over a period of two years. Altogether the results are in agreement with those of earlier investigators [5, 13, 14], indicating that the receptor content is not clearly related to the clinical stage at the time of operation. The possibility that upon progression tumours tend to lose their estrogen-binding capacity is not supported by these data. Rather, they suggest that the receptor content of breast cancers tend to remain fairly constant with progression of the disease.

Limited data are available on the correlation between the receptor content of tumours and the success or failure of endocrine treatment. The studies of Jensen *et al.* [4] and of Maass *et al.* [5] demonstrate that when estrogen receptors could not be detected in the tumour tissue, failure of endocrine treatment was to be expected. In contrast, most, but not all, patients whose tumour tissue showed the presence of receptors responded to different types of endocrine treatment.

Since only about 1/3 of breast cancer patients respond favourably to endocrine treatment [16], it is possible that sensitivity to endocrine treatment requires the presence in the tumours of a certain minimum concentration of receptor sites. Further data are needed to establish whether a definite quantitative relationship exists between the receptor content of breast tumours and their hormone responsiveness and whether measurements of estrogen receptors in mammary cancers can safely be used to predict the individual response to endocrine treatment.

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Ultrastructure of Pulmonary Adenomas Induced by Intratracheal Instillation of Diethylnitrosamine in Syrian Golden Hamsters*

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Abstract—*Lung tumours induced in Syrian golden hamsters by multiple intratracheal instillations of diethylnitrosamine (DENA) were studied with semithin and electron microscopic techniques. Two distinctive types of epithelial cells could be identified, (a) small, cytologically fairly undifferentiated cells and (b) larger well-differentiated cells closely resembling normal type II pneumocytes. No squamous epithelium was found in the tumours.*

INTRODUCTION

DIETHYLNITROSAMINE (DENA) has repeatedly been shown to be carcinogenic for the respiratory system of Syrian golden hamsters [1–9]. The neoplastic response in the nasal cavity, larynx, and trachea was generally found to be much higher than that in the lower respiratory tract [3, 4, 7–11]. A high incidence of lower respiratory tract tumours, however, was observed in hamsters when subcutaneous injection of DENA was followed by intratracheal instillation of ferric oxide [8, 10] which has never been found to possess carcinogenic activity of its own despite extensive testing [10, 12]. Moreover, Feron *et al.* [13] have recently shown that repeated intratracheal instillations of relatively high doses of DENA alone and with ferric oxide resulted in considerable numbers of tumours localized in the bronchiolo-alveolar region of Syrian golden hamsters. Nearly all of these peripheral neoplasms were classified as benign growths consisting of adenomatous structures lined by stratified epithelium resembling squamous epithelium upon light microscopy. In an attempt to determine the type and orgin of this neoplastic epithelium, a few of such DENA-induced lung tumours were examined by electron microscopy. The present paper describes this ultrastructural study.

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MATERIAL AND METHODS

Tumour material was obtained from 1 male and 1 female Syrian golden hamster which had received intratracheal instillations of 0·1 ml 0·5% diethylnitrosamine (EGA-Chemie K.G., Keppler and Reif, Steinheim/Albuch, W-Germany) in 0·9% NaCl (sterile, non-pyrogenic, Baxter Laboratories, A. Christiaens N.V. Brussels, Belgium) once every 2 weeks for a period of 1 yr starting at an age of 11 weeks. Both hamsters were 75 weeks old when they were killed by intra-abdominal injection of Brietal sodium (Lilly Co., Indianapolis, Ind., U.S.A.). The origin of the animals, their housing and feeding were the same as described previously [13].

Immediately after death samples of several

pulmonary tumours were cut into small tissue blocks of about 1 mm^3 in volume and immersed for 2 hr in ice-cold 2% glutaraldehyde buffered to pH 7·4 with 0·2 M phosphate–sucrose buffer at 0°C. The specimens were postfixed in 1% OsO_4, dehydrated in graded alcohols and embedded in Epon. One micron sections were stained with silvermethenamine and methylene blue, and examined by phase-contrast microscopy. Thin sections (500 Å) were stained with uranylacetate and lead citrate [14], and viewed with a Siemens Elmiskop I electron microscope at 60 kV.

RESULTS

Each of the two hamsters examined showed 2 pulmonary tumours measuring 0·2 to 0·7 cm in diameter. In addition, multiple tracheal papillomas were found in both animals. Gross and light microscopic appearance of both the pulmonary and tracheal neoplasms were the same as described previously [3, 4, 7–10, 13].

Phase-contrast microscopy

The pulmonary tumours consisted of fairly solid masses of connected cells often arranged in sheets around spaces indicating an adenomatous growth pattern (Fig. 1). The majority of the tumour cells was uniform in size and shape and contained a relatively large nucleus, whereas the cytoplasm was scanty. Connections seen between the cells resembled intercellular bridges (Fig. 2). Scattered among this uniform type of cells, larger cells containing relatively small nuclei and a considerable amount of cytoplasm appeared to be present mainly at the luminal border of the adenomatous structures (Figs. 1, 2). The abundant vesicular cytoplasm of these larger cells contained granular structures, the nature of which was obscure under the light microscope. Bronchiolar elements could not be discerned.

Electron microscopy

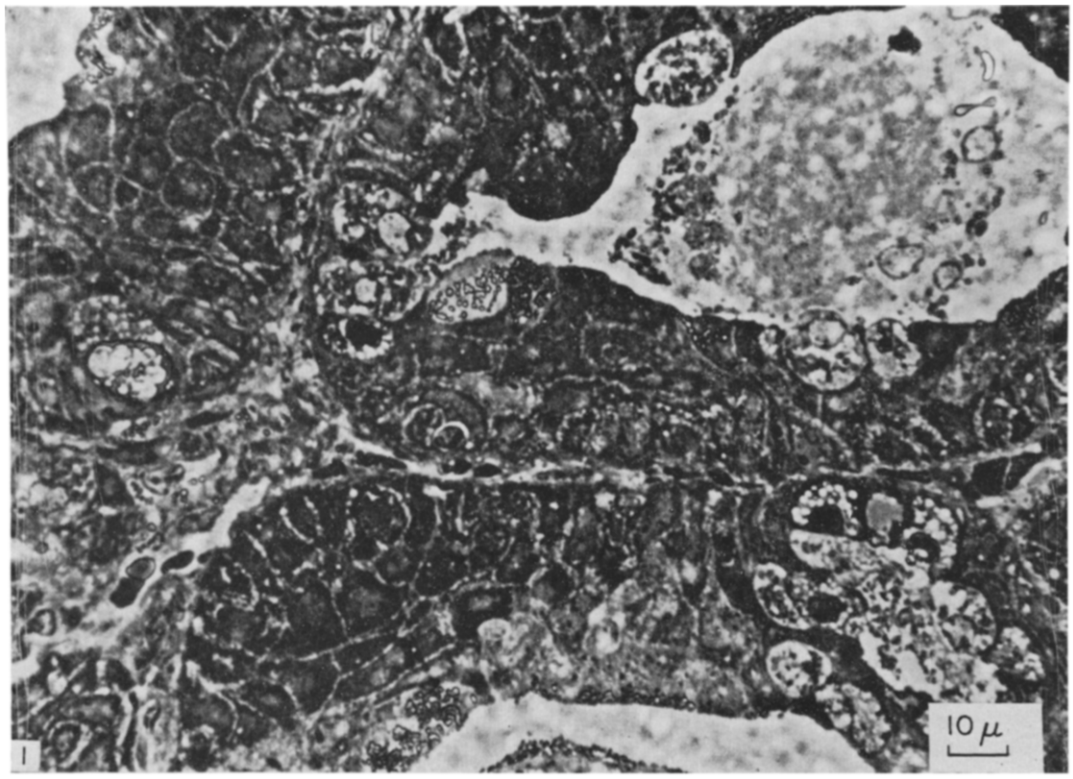
The smaller type of cells contained many normal-appearing mitochondria, some lysosomes, but otherwise few cytoplasmic organelles. These cells were joined together by complex cytoplasmic interdigitations. There were numerous desmosomes, but no tonofilaments were observed. The free surface of the cells lining a lumen was provided with rather short, blunt microvilli which were located at irregular distances from each other (Fig. 3).

The larger type of cells contained less electron dense cytoplasm than did the smaller cells. Cytoplasmic interdigitations between the two cell types were lacking, but desmosomes did occur (Fig. 4). The larger cells also showed short microvilli at their luminal surface (Fig. 5). Many lysosomal structures and a few myelin figures were noticed in their cytoplasm. Moreover, these cells contained electron dense lamellar bodies, often showing lamellae nicely running parallel (Figs. 4, 5). These lamellar bodies, which are highly characteristic for type II pneumocytes, occasionally showed signs of disintegration. The endoplasmic reticulum was slightly widened. Other cytoplasmic organelles were unremarkable.

DISCUSSION

The lung tumours induced in hamsters by intratracheal instillations of DENA have been found, by light microscopy, to consist of adenomatous structures lined by stratified epithelium resembling squamous epithelium [13]. The present ultrastructural study, however, revealed no squamous epithelium in this type of pulmonary tumours. Even semi-thin plastic sections examined by phase-contrast microscopy did not provide sufficient detail to establish the absence of squamous cells, which demonstrates that electron microscopic techniques should be used if cell types in lung tumours are to be identified correctly.

Two distinctive types of epithelial cells could be recognized: (a) small polygonal cytologically undifferentiated cells and (b) larger well-differentiated cells resembling normal type II pneumocytes. By far the greatest part of the tumour cells were of the first type. These little-differentiated cells, which were relatively deficient in cytoplasmic organelles except for mitochondria, might have originated from type I pneumocytes. On the other hand, the presence of numerous desmosomes and complex interdigitations between the cells might indicate that the smaller tumour cells arose from basal cells of the tracheobronchial epithelium and retained some of their ultrastructural characteristics. The tracheal epithelium is deliberately mentioned as a possible site of origin, because the occurrence of the lung tumours could be due to transplantation of the tracheal papillary tumours into the lower respiratory tract as a consequence of the instillation procedure [13]. This type of DENA-induced tracheal neoplasms has often been described as squamous cell tumours [1–9]. However, a recent study of these tumours performed with semi-thin techniques revealed no metaplastic alterations to cornified or uncornified squamous cells [15]. Consequently, the absence of squamous epi-



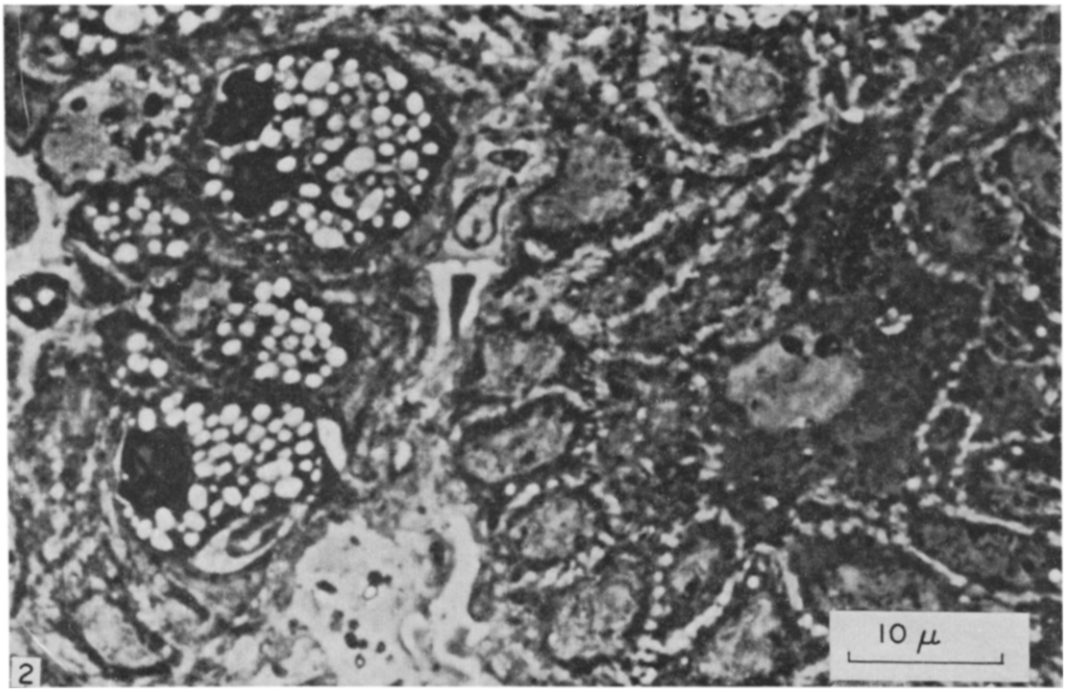


Fig. 1. *Adenomatous part of pulmonary tumour. A few small groups of relatively large cells containing vesicular cytoplasm occur among sheets of smaller dark cells (phase-contrast picture, silvermethenamine,* × 800).

Fig. 2. *Detail of pulmonary tumour. To the left* 3 *large cells containing abundant cytoplasm and to the right smaller cells containing scanty cytoplasm. Connections seen between the smaller cells are suggestive of intercellular bridges (phase-contrast picture, silvermethenamine,* × 2000).

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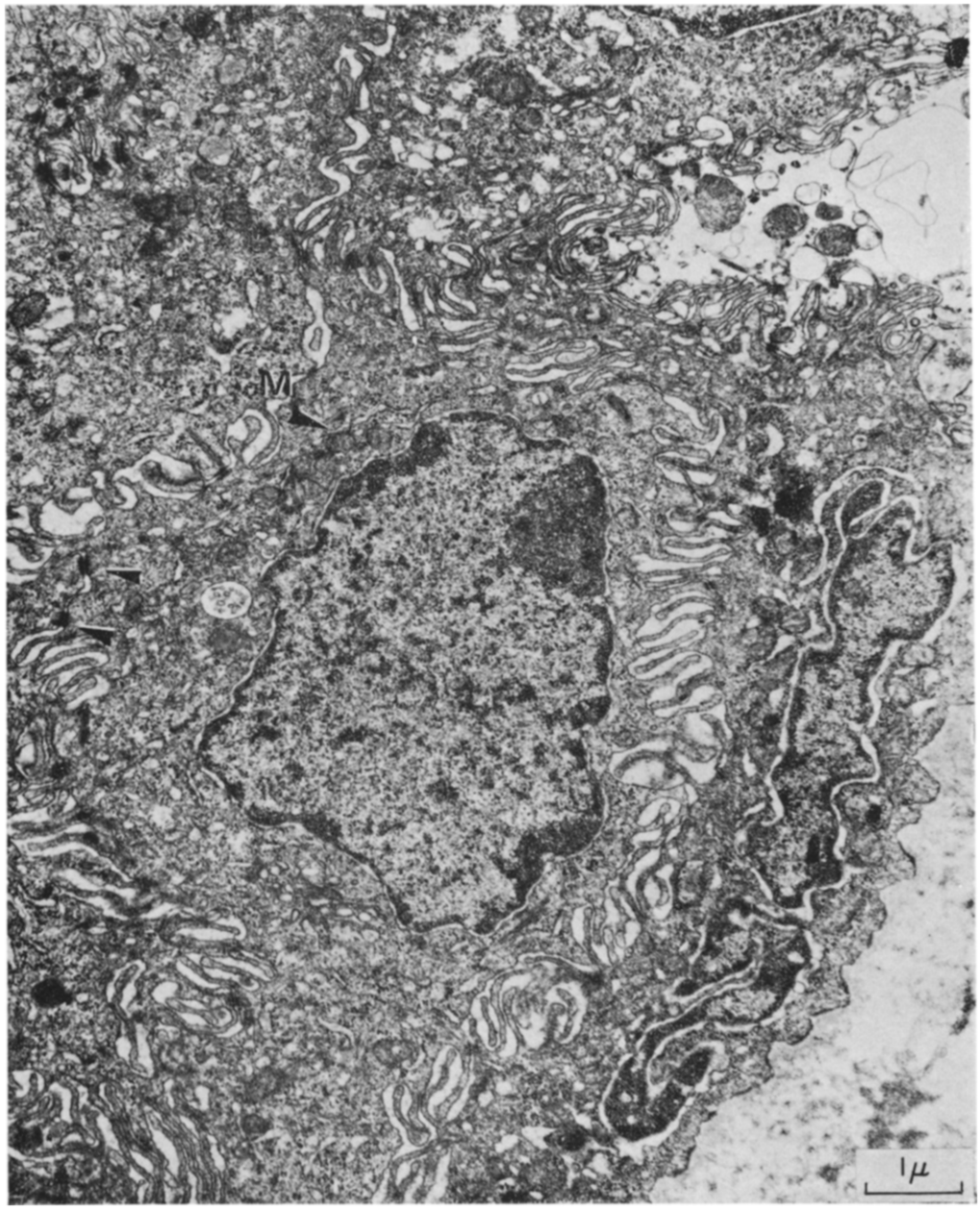

Fig. 3. *Small type of tumour cell. It has a prominent nucleus and only a small amount of cytoplasm, which contains several mitochondria* (*M*). *Complex cytoplasmic interdigitations and many desmosomes* (*arrows*) *characterize the intercellular relationship between these cells* (*uranyl acetate and lead citrate*, × 13800).

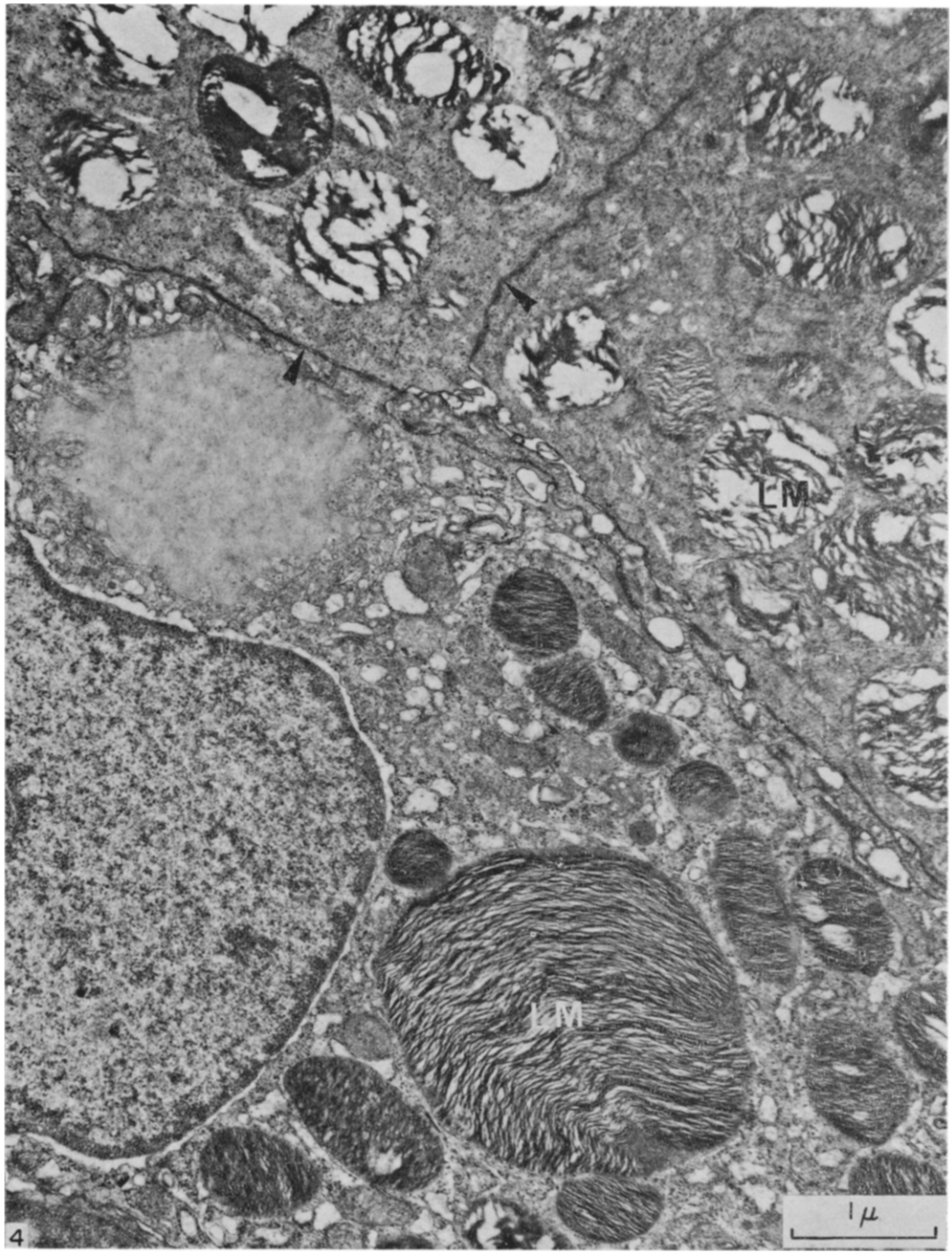

Fig. 4. Tumour cells resembling normal type II pneumocytes. The cytoplasm contains many lysosomal structures and lamellar bodies (LM). The cells appear to be held together by desmosomes (arrows) and straight lengths of closely apposed cell membranes (uranyl acetate and lead citrate, × 15900).

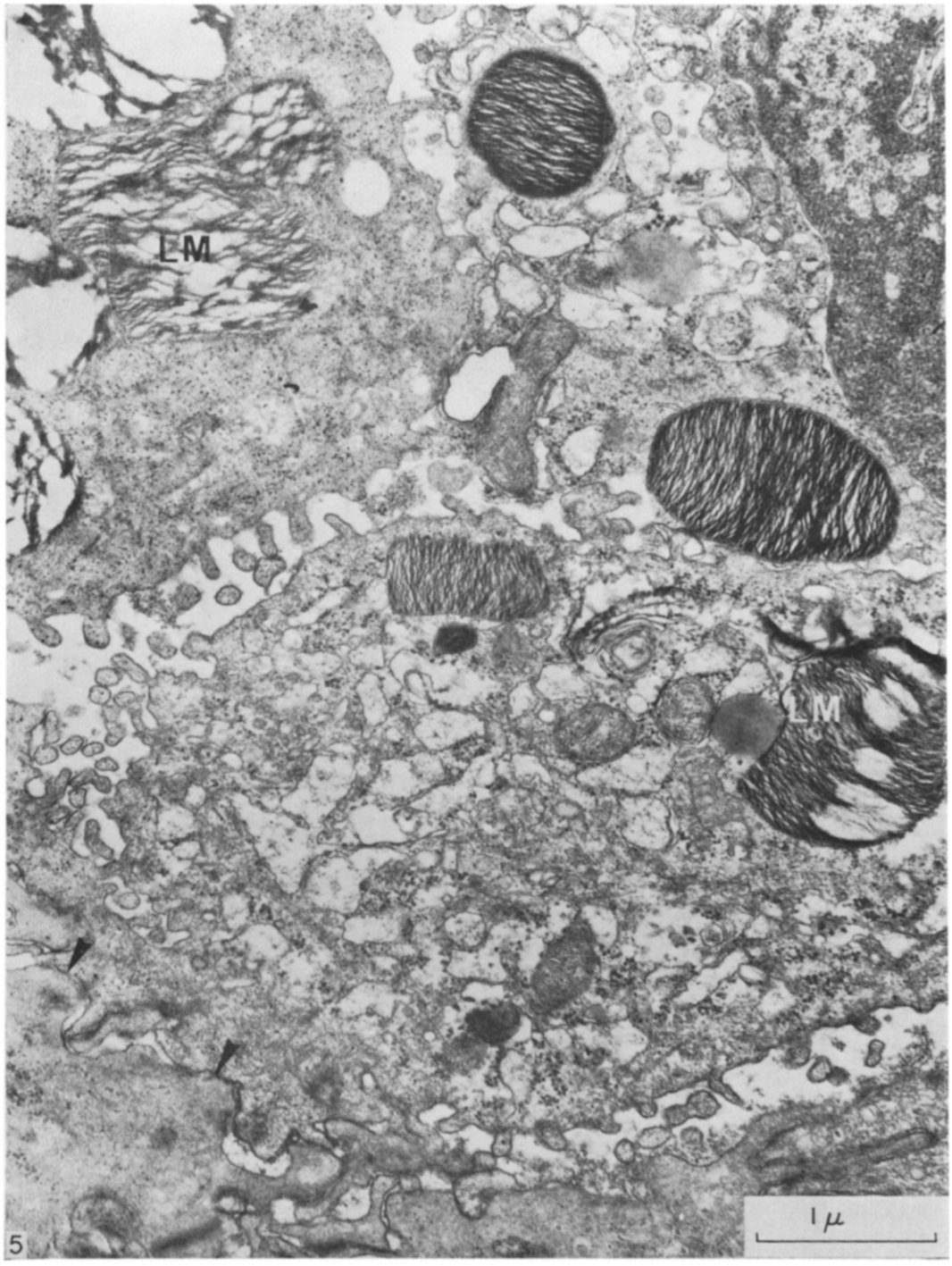

Fig. 5. Tumour cells resembling type II pneumocytes. Note the short blunt microvilli at the free surface, lamellar bodies (LM), desmosomes (arrows) and lysosomes (uranyl acetate and lead citrate, ×26500).

thelium in the present lung tumours does not exclude their origin from tracheal neoplasms.

The second type of cells occurring in the lung tumours closely resembled type II pneumocytes, though they did not always rest on a basal membrane. On the other hand, they did form junctions with each other and with the smaller type of cells. It is not clear whether the larger cells are an essential part of the tumour or represent a cellular (alveolar cell) reaction to the presence in the lung tissue of the true neoplastic cells, viz., the small undifferentiated cells. The latter possibility seems to be preferable, since hyperplasia of type II pneumocytes is known to occur under various pathological conditions [16–18]. In addition, great alveolar cell tumours reported so far in several animal species [19–23] and in man [24–26] indeed appeared to consist only of well-recognizable type II pneumocytes invariably attached to a basal lamina.

Further discussion on the cellular composition and the histogenesis of the tumours described here would lead to rather useless speculations. For the present, it is justified to state that the tumours do not contain squamous epithelium but are comprised of fairly undifferentiated epithelial cells and larger cells closely resembling type II pneumocytes. Several experiments are currently being performed to obtain more information on the ultrastructural and biological characteristics of both the DENA-induced pulmonary and tracheal neoplasms in Syrian golden hamsters.

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Qualitative Restriction of Transcription in Human Leukemic Lymphocytes*

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Abstract—*RNA from human normal lymphocytes and Burkitt lymphoma cells were compared with respect to the number of kinds of hybridizable RNA complementary to normal human DNA, and to the degree of similarity between normal and lymphoma RNA. Both saturation and competition experiments were performed with RNA uniformly labeled* in vitro *with dimethyl-H³-sulfate and human placental DNA. Normal lymphocytes possess more kinds of RNA that hybridize with normal human DNA than the Burkitt lymphoma cells. Competition experiments revealed that some kinds of hybridizable species of RNA present in normal lymphocytes are not present in the leukemic cells.*

INTRODUCTION

RECENTLY it has been shown that rapidly-labeled RNA species, which cannot be detected in the normal homologous tissue or cells, are detected in transplantable mammary carcinomas and in chronic lymphocytic leukemia cells [1, 2]. However, other reports suggest that rat liver hepatomas lack some of the hybridizable RNA species present in normal rat liver [3]. Does the change from normal to neoplastic state involve repression or derepression of transcription? In this present work a comparison of RNA transcripts of normal and Burkitt lymphoma cells was made utilizing the hybridization techniques of saturation and competition.

MATERIAL AND METHODS

Cells

The P3JHR-1K Burkitt lymphoma cells cloned from the P3 (Jijoye) line in 1967 by Hinuma and Grace [4] and normal human lymphocytes [Fletcher, Roswell Park Memorial Institute (R.P.M.I.) No. 7241] were supplied to the authors in gram amounts by Dr. George Moore and Dr. Jun Minowada of the Roswell Park Memorial Institute. The P3JHR-1K and the normal lymphocytes are grown continuously in stationary and spinner cultures in R.P.M.I. medium #1640 plus 10% (v/v) heat-inactivated fetal calf serum in the absence of antibiotics.

PREPARATION OF NUCLEIC ACIDS

DNA was prepared from human placentae using the chloroform-isoamyl-alcohol technique [5], combined with pronase digestion [6]. The 260/230 mμ and 260/280 mμ absorption ratios were always above 2, indicating little protein was present. If they were not, the RNA preparations were treated with pronase again.

RNA was prepared from whole normal and lymphoma cells using a stepwise hot phenol extraction procedure [6]. Polysaccharides were removed [7, 8] and the RNA was further treated with DNase, then pronased and extracted twice with phenol to remove proteins. The RNA was methylated *in vitro* with carrier-free dimethyl-H³-sulfate according to Smith *et al.* [19], followed by an additional DNase treatment, a pronase treatment (500 μg/ml in 0·13 M Tris-HCl, pH 8·6 for 90 min at 37°C) and two additional phenol extractions. The purified lymphoma and normal RNA were then passed through Sephadex G-25 in 0·15 M NaCl to remove small molecular weight contaminants. The RNA concentrations were determined by measuring the absorbance of duplicate aliquots at 260 mμ and specific activities determined by counting in a scintillation spectrometer.

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RNA–DNA HYBRIDIZATION TECHNIQUE

DNA filters were prepared by the method of Gillespie and Spiegelman [10]. The annealing procedure consisted of incubating 3 mm nitrocellulose filters (type B-6 coarse, Schleicher and Schuell) containing 0·5 μg denatured DNA with RNA dissolved in 2 × SSC (1 × SSC is 0·15 M NaCl, 0·015 M sodium citrate) plus 50% formamide in a total volume of 50 μl. Annealing was carried out in small Beem capsules at 37°C for 48 hr with shaking. The procedures for washing and counting filters have been previously described [6]. RNA labeled with H^3-dimethylsulfate will not lose activity below 40°C [11], and annealing at 37°C in 50% formamide-2 × SSC (pH 7·3) is equivalent to annealing at 72°C in 3 × SSC [12].

RESULTS

Saturation of DNA

In order to determine the relative number of kinds of hybridizable RNA complementary to normal human DNA in normal and lymphoma lymphocytes, three series of saturation experiments were performed with the labeled RNA. Increasing amounts of normal RNA (9,109 DPM/μg) and lymphoma RNA (11,423 DPM/μg) were incubated with DNA filters containing 0·5 μg of DNA for 48 hr in 50% formamide in 2 × SSC at 37°C with shaking. Normal lymphocyte RNA hybridizes at saturation with 1·2% of the DNA at an RNA : DNA ratio of 1600 while the lymphoma RNA transcripts hybridize at saturation with 0·7% of the DNA at an RNA : DNA ratio of 2000 (Fig. 1). In each series of experiments the background levels for blank control filters and those with *E. coli* DNA were less than 1% of the counts hybridized. The saturation curves clearly demonstrate the presence of more kinds of hybridizable RNA complementary to normal human DNA in the normal lymphocytes than are present in the Burkitt lymphoma cells.

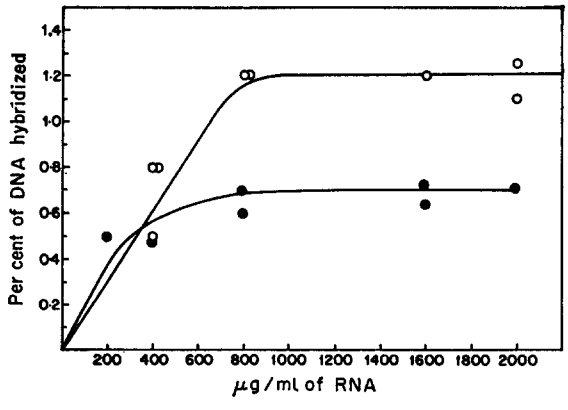

Fig. 1. Saturation curves: RNA from normal human lymphocytes (○) and leukemic lymphoma lymphocytes (●) was labeled in vitro *with H^3-dimethyl-sulfate and incubated for 48 hr in 0·3 M NaCl-0·030 M trisodium citrate (pH 7·5), 50% formamide at 37°C with nitrocellulose filters containing 0·5 μg denatured human placental DNA in a volume of 50 μl.*

COMPARISON OF RNA POPULATIONS FROM NORMAL AND LYMPHOMA LYMPHOCYTES

In order to determine the degree of similarity between the species of normal and lymphoma RNA three series of homologous and heterologous competition experiments were performed. In these experiments saturating concentrations of labeled RNA were incubated with small filters containing 0·5 μg of denatured placental DNA, together with increasing concentrations of unlabeled RNA. Competition of unlabeled RNA from normal or leukemic cells with labeled RNA from normal lymphocytes shows slight differences and reveals that the tumor cells lack certain species of RNA present in the normal cells (Fig. 2). When unlabeled RNA from normal or tumor cells are competed against labeled RNA from tumor cells, the results show that normal cells contain all the kinds of hybridizable RNA present in the tumor cells (Fig. 3). The results of these competition experiments suggest that the leukemic lymphocytes do not possess all the kinds of hybridizable RNA complementary to normal human DNA that are present in the normal lymphocytes.

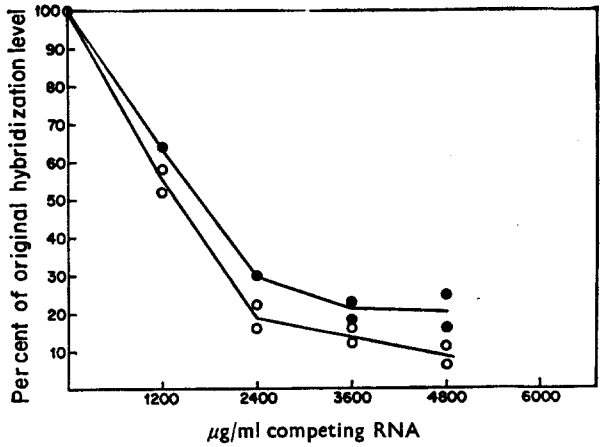

Fig. 2. Competition experiments with labeled RNA from normal human lymphocytes. Increasing amounts of unlabeled RNA from normal human lymphocytes (○) and leukemic lymphoma lymphocytes (●) were added to a saturating concentration (1200 μg/ml) of labeled RNA from normal human lymphocytes.

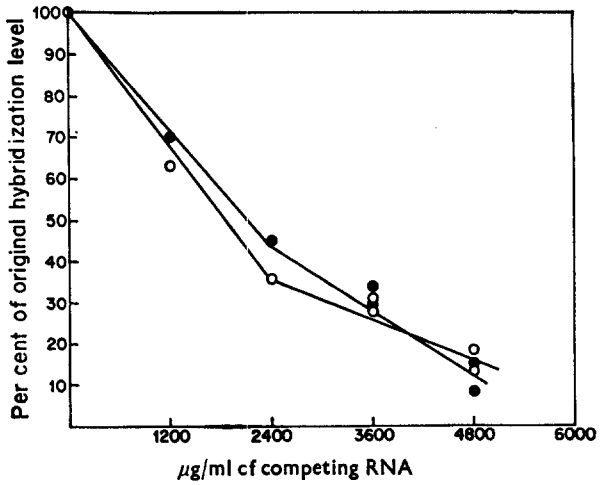

Fig. 3. Competition experiments with labeled RNA from leukemic lymphoma lymphocytes. Increasing amounts of unlabeled RNA from normal human lymphocytes (○) and leukemic lymphoma lymphocytes (●) were added to a saturating concentration (1200 μg/ml) of labeled RNA from leukemic lymphoma lymphocytes.

DISCUSSION

The present study has utilized the techniques of DNA–RNA saturation and competition to examine both the number of kinds of hybridizable RNA present in normal and in Burkitt lymphoma lymphocytes which are complementary to normal human DNA, and the similarity between the species of normal and lymphoma RNA. Saturation experiments demonstrated the presence of more kinds of hybridizable RNA complementary to normal human DNA in the normal lymphocytes (1·2% hybridization) than in the leukemic cells (0·7% hybridization). Saturation was attained at an RNA: DNA ratio of 1600 for the normal cells and 2000 for the Burkitt lymphoma cells. Neiman and Henry [1] report a similar percentage of hybridization for pulse-labeled RNA from normal and chronic lymphocyte leukemic cells obtained directly from patients. It must be remembered that the increased redundancy in mammalian DNA allows only the most repetitive, transcribable genome sequences to be detected by the filter hybridization technique [13] and that our conclusions deal only with RNA transcribed from repetitive DNA.

The hybridization–competition experiments utilizing normal and lymphoma cell RNA indicate that Burkitt lymphoma cells lack some of the kinds of hybridizable RNA species present in normal cells, while normal cells contain all the hybridizable kinds of RNA found in the lymphoma cells. Although the differences observed in the competition experiments were small, they were detected in each of the three competition experiments. However, it must be remembered that the recorded qualitative differences may only reflect a quantitative difference in some of the species of RNA that were transcribed in few copies. In comparing the experimental system chosen for this study and the use of normal and chronic lymphocytic leukemic cells isolated directly from human patients, the latter best reflects the *in vivo* situation. The lymphoma cells used in this study have been in constant cultivation since 1964, and although chromosome number and karyotype compare favorably with normal lymphocytes [14] these cells have most probably undergone subtle changes which may have been reflected ultimately in their gene transcripts. However, the differences between our results and those of Neiman and Henry [1] may also be because H^3-dimethylsulfate labels all the hybridizable RNA in the cells while pulse labeling detects only RNA synthesized during the period of exposure of the cells to the labeled precursor. Another possible source for the difference is that higher ratios of labeled RNA to DNA were utilized in our hybridization experiments.

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Histopathology and Cytogenetics of Tumors Induced by the Application of 7, 12-Dimethylbenz[a]anthracene (DMBA) in Mouse Cervix*

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Abstract—*DMBA (7,12-dimethylbenz[a]anthracene)-coated threads were placed in the vaginal fornix of 4–5 week-old ICR mice. Malignant tumors developed in 56% of the 126 animals after an average latent period of 22·8 ± 12·4 weeks. The tumors included squamous cell carcinomas (37%), fibrosarcomas (34%), leimyosarcomas (5%), lymphosarcomas (4%), mixed tumors (16%) and double tumors (4%). No tumors appeared in 54 sham operated control mice. Cytogenetic analysis of three fibrosarcomas, two squamous carcinomas and one lymphosarcoma revealed a wide range of karyotypic composition. The two fibrosarcomas were predominantly diploid, while the third had a bimodal distribution in the tetraploid and octaploid range. One squamous carcinoma was tetraploid and the other aneuploid with 41 chromosomes in modal cell line. The lymphosarcoma had a heterogeneous cell population with a majority of the metaphase plates containing four metacentric chromosomes. The variation in the cytogenetic pattern of these tumors was in contrast with that of thymic lymphosarcomas induced in mice by the same carcinogen.*

INTRODUCTION

DIMETHYLBENZ[A]ANTHRACENE (DMBA) has been found to produce many different types of neoplastic lesions in experimental animals. Oral administration of DMBA produces mammary adenocarcinomas and fibroadenomas in rats [1, 2]. Intravenously it produces granulocytic leukemia, mammary, and sebaceous gland carcinomas in rats [3, 4]. Subcutaneous injection of this carcinogenic chemical into newborn mice yields a high frequency of lymphatic leukemias usually originating in the thymus [5, 6]. More recently, the application of DMBA in the uterine cervix and horns of mice induced a high incidence of squamous cell carcinomas [7, 8]. Of the various types of DMBA-induced malignant tumors, lymphatic leukemias of mice have been thoroughly investigated for the cytogenetic alterations during the transformation of normal cell populations into malignant ones [6, 9, 10, 11, 12]. The DMBA-produced lymphatic leukemias of mice are particularly interesting because a large majority of them shows a consistency in their chromosomal pattern, i.e. 41 chromosomes in the modal cell lines instead of the normal 40 [6, 12, 13]. Of the 35 DMBA-induced lymphatic leukemias in mice, 80% had 41 chromosomes in their predominant cell types [6, 12, 13]. The main aim of this investigation was to determine whether chromosomal pattern of malignant tumors induced by the application of DMBA to the genital tract of female mice was comparable to that of lymphatic leukemias induced by the same carcinogen. In addition, because of the great variety and arrangement of histological structures in the female genital tract as compared with lymphoid tissues, the frequency and types of tumors

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produced were examined prior to the cytogenetic analysis.

MATERIAL AND METHODS

Animals

Female random-bred ICR/Ha mice obtained from Health Research Laboratories, West Seneca, New York, were used throughout this study. Mice were received at the age of 3–4 weeks and were used for experiments at the age of 6 weeks.

Carcinogen

The 7, 12 - dimethylbenz [a] anthracene (DMBA), purchased from Fisher Scientific Company, was prepared for application to the cervix of mice according to the methods of Meisels [8]. Triple overhand knots were tied in the ends of pieces of surgical silk thread (size 000) about 15 cm in length. The knots were then dipped in DMBA which had been melted in a beaker over an alcohol flame. The DMBA pearl was allowed to cool and harden on the knots of thread. Usually, portions of the threads distal to the knot also became coated with the DMBA. The average amount of DMBA per knot was $1{\cdot}35 \pm 0{\cdot}69$ mg in a sample of ten threads.

Application of DMBA

Throughout the operation the mice were kept anaesthetized with ethyl ether. A midline laparotomy 1·0 to 1·5 cm. in length was performed. A blunted sewing needle carrying the DMBA coated thread was passed through the vagina, into the fornix and through the wall of the fornix to its external surface. Gentle traction on the thread pulled the pearl of DMBA into the fornix. Several overhand knots were then applied on the outside of vaginal wall to hold the DMBA pearl in place. Excess thread was cut and the incision was closed using 11 mm Michel surgical clips. Control mice were subjected to identical procedure except the threads were not coated with the carcinogen.

Tumors

The mice were checked daily and weighed weekly. Those showing signs of tumor growth such as swellings in the lower quadrants of the abdomen or the vaginal canal, a red and swollen vaginal opening, an exudate from the vagina, a rough hair coat or general malaise, were autopsied. The suspected tumor tissues and other organs of these mice were sectioned and stained with Hematoxylin and eosin routinely. Some animals, both in control and treated groups, showed excessive aggregation of pelvic fat.

Chromosome preparations

Direct squash technique. Mice suspected of carrying tumors were injected intraperitoneally with 20 μg/g body weight of colchicine prepared as a 0·05% solution in 0.85% NaCl.

Each animal was exposed to colchicine for 4 hr and killed with ethyl ether. The tumor was removed, weighed and divided into two parts. One part was fixed in 3:1 absolute alcohol–glacial acetic acid mixture for paraffin sectioning. The other part was processed for chromosome analysis by direct-squash method. The preparations were examined with a Carl Zeiss phase-contrast photomicroscope. A great deal of difficulty was encountered in obtaining satisfactory preparations by this method. A total of 24 tumors were processed and only seven produced acceptable preparations. Of these, five gave satisfactory metaphase counts of karyotypes and six were suitable for frequency of polypoid metaphase plates determined by using a 40X objective.

Two groups of six untreated control mice each were used. Tissues from the vagina and cervix of these were processed from young (6–7 weeks) and old (50–51 weeks) mice. In order to accumulate a large number of metaphase plates the colchicine dose was increased to 70 μg/g body weight and time to 6 hr. However, the results especially in old mice were not perfectly satisfactory due to the lack of sufficient number of mitotic cells.

Tissue culture method. One tumor produced satisfactory chromosome preparations by this method. Very small pieces of the tumor were washed thoroughly in phosphate-buffered saline and treated with 0·12% trypsin solution. The supernatant was inactivated with heat-inactivated fetal calf serum containing 0.5% neomycin. Centrifugation at 1000 rev/min for 15 min formed a button of cells which was suspended in the culture medium containing Medium 199, 10% fetal calf serum, and 0·5% neomycin. The cultures were set up in several 16 ml Saniglass bottles containing approximately 500,000 cells/ml. The culture medium was changed daily. When the cells had almost formed a monolayer, cultures were harvested for chromosome preparation. The cells were exposed to colchicine (10 μg/ml) for 6 hr and treated with 1% sodium citrate at 37°C for

15 min for hypotonic treatment. Air-dried preparations from these cultured cells were stained with Giemsa.

RESULTS

Incidence and type of tumor

Of the total of 126 animals which survived after the insertion of DMBA-coated threads, 71 (56%) developed tumors (Table 1). The average latent period for all tumors was 22·8 ± 12·4 (S.D.) weeks with a range of 4–48 weeks. None of the 54 control animals developed any tumors. At necroscopy, only 58% of the mice in both treated and control groups had their threads in place. However, the frequency of the tumors did not appear to have been affected because about 55% of the tumors developed in those DMBA-treated mice which had lost the thread. Squamous cell carcinomas were the most common type of tumors (37%), while 34% were fibrosarcomas. The remainder were leiomyosarcomas, mixed tumors, double tumors, and lymphosarcomas. It is likely that some of the squamous carcinomas may have originated from the vaginal wall rather than from the cervix.

Histopathology of tumors

Squamous cell carcinomas. This group exhibited a great variety of histological appearances depending upon the degree of differentiation of the cells. Because of these variations the tumors were subdivided into highly keratinized (Fig. 1), lightly keratinized (Fig. 2), and non-keratinized (Fig. 3) types. Approximately half of the squamous carcinomas belonged to the highly keratinized category. The frequency of mitoses was extremely variable throughout the individual tumors and within the entire group. The amount of connective tissue stroma was usually very much reduced and the pearl formation varied considerably.

Fibrosarcomas. The histological appearance of these tumors varied according to the proportion of cellular and intercellular elements. In well-differentiated forms abundant collagen fibers separated the cellular elements while the undifferentiated ones were usually packed into bundles without any directional pattern (Fig. 4). Mitotic figures were frequent. Multinucleated cells, giant cells, and pleomorphic cells were occasionally found. Many of the tumors formed spherical masses with necrotic centres and exhibited a gradual degeneration of elements from the periphery towards the center of the mass.

Leiomyosarcomas. This group constituted only about 4% of all the tumors recovered. These tumors ranged from well-differentiated forms to highly undifferentiated. The latter were relatively more difficult to distinguish from the fibrosarcomas, but usually contained hyperchromatic nuclei and lesser amounts of connective tissue stroma (Fig. 5).

Mixed and double tumors. Ten DMBA-treated animals developed mixed tumors containing two different types of tissues. The bulk of the tumor growth was usually formed by a primary type of tissue and the secondary tissue elements were present in lesser amounts. Four of these neoplasms combined smooth muscle cells and fibroblasts. The others showed various combinations of squamous carcinomas with leiomyosarcoma, rhabdomyosarcoma, and fibrosarcoma. An example of squamous carcinoma–fibrosarcoma is shown in Fig. 6. Three mice developed double tumors originating from two separate tissues which remained histologically independent from each other. The combinations of tumors in these animals were fibrosarcoma–rhabdomyosarcoma, squamous carcinoma–leiomyosarcoma, and squamous carcinoma–fibrosarcoma.

Table 1. *Incidence of malignant tumors induced by implantation of the* 7,12-*dimethylbenz*[*a*]*anthracene* (*DMBA*) *pearl in the genital tract of female ICR mice*

Treatment	Total No. of mice	No. with tumors	Average latent period (weeks) ± S.D.	Types of tumors (%)
DMBA pearl	126	71 (56%)	22·8 ± 12·4	Squamous carcinomas — 37 Fibrosarcomas — 34 Mixed tumors — 16 Leiomyosarcomas — 5 Double tumors — 4 Lymphosarcomas — 4
Thread only control*	54	0		

*Examined 50 weeks after placing the thread.

Lymphosarcomas. Three mice developed lymphosarcomas which were initially diagnosed due to the enlarged lymphoid organs. Histological examination of the spleen, thymus, lymph node and other tissues confirmed the diagnosis. All affected organs were characterized by an abundance of large lymphocytes and mitoses. Two mice showed infiltration of the uterine tissue (Fig. 7) and one revealed infiltration of the kidney (Fig. 8).

Cytogenetic studies

Controls. The squash preparations of tissues from cervical and vaginal regions were attempted from two groups of mice: young, 6–7 weeks of age, and old, 50–51 weeks. Satisfactory metaphase plates for a detailed analysis of the karyotype were available only from younger mice and showed the expected normal counts of 40 chromosomes of acrocentric shapes in the majority of the cells (Table 2). Older mice did not yield satisfactory preparations for metaphase analyses due to a relatively low mitotic activity. The available metaphase plates were scored in both groups for their ploidy using approximate counts. No polyploid metaphases were observed in either group.

Tumors. Three fibrosarcomas, two squamous cell carcinomas and one lymphosarcoma yielded suitable preparations for analyses of metaphase plates.

Fibrosarcomas. Two of the fibrosarcomas (Nos. 39 and 100) were examined by direct-squash method while the third (No. 99) was grown in tissue culture for four days. Both tumors, Nos. 39 and 100, showed a normal count of 40 chromosomes in the modal cell lines (Fig. 9), (Table 3). The chromosomes did not reveal any structural anomalies. In addition some metaphase plates gave counts between the diploid and octaploid range suggesting an increase in polyploidy. The third fibrosarcoma (No. 99) examined after a short-term growth in culture medium, showed a wide range of chromosome numbers from 38 to 223 (Fig. 13). Sixty-three metaphase plates were used in these counts and a majority of the cells were concentrated in the tetraploid and octaploid range (Fig. 14). Only a few cells had diploid or near-diploid chromosome numbers. Although it appears highly unlikely that the polyploidy was induced by the *in vitro* environment, this possibility must be considered with all cytogenetic analyses on cultured materials.

Table 2. Chromosome analysis of the cervical tissue from untreated female ICR mice

Number of mice	Age group (weeks)	Total metaphase plates analyzed	Number of cells with indicated chromosome numbers 38	39	40	Polyploid ratio* (polyploids/total metaphases)
6	6–7	27	2	3	22	0/292
6†	50–51	—	—	—	—	0/75

*Determined by scanning the squash preparations with 10 × and 40 × objectives.

†This group did not give satisfactory preparations for chromosome analysis due to lack of sufficient number of dividing cells.

Table 3. Chromosome analysis of fibrosarcomas and squamous carcinomas developing after implantation of the 7,12-dimethylbenz[a]anthracene (DMBA) pearl in the genital tract of female ICR mice

Mouse number	Type of tumor	Total metaphase plates analyzed	Number of cells with indicated chromosome number 38	39	40	41	42	43	80	Others
39	Fibrosarcoma	21	3	5	8	—	—	—	1	1(49) 1(53) 1(71) 1(78) 1(156)
100	Fibrosarcoma	85	1	5	74	2	—	2	—	1(50)
226	Squamous carcinoma	44	1	2	3	34	3	1	—	—
56	Squamous carcinoma	5	—	—	—	—	—	—	5	—

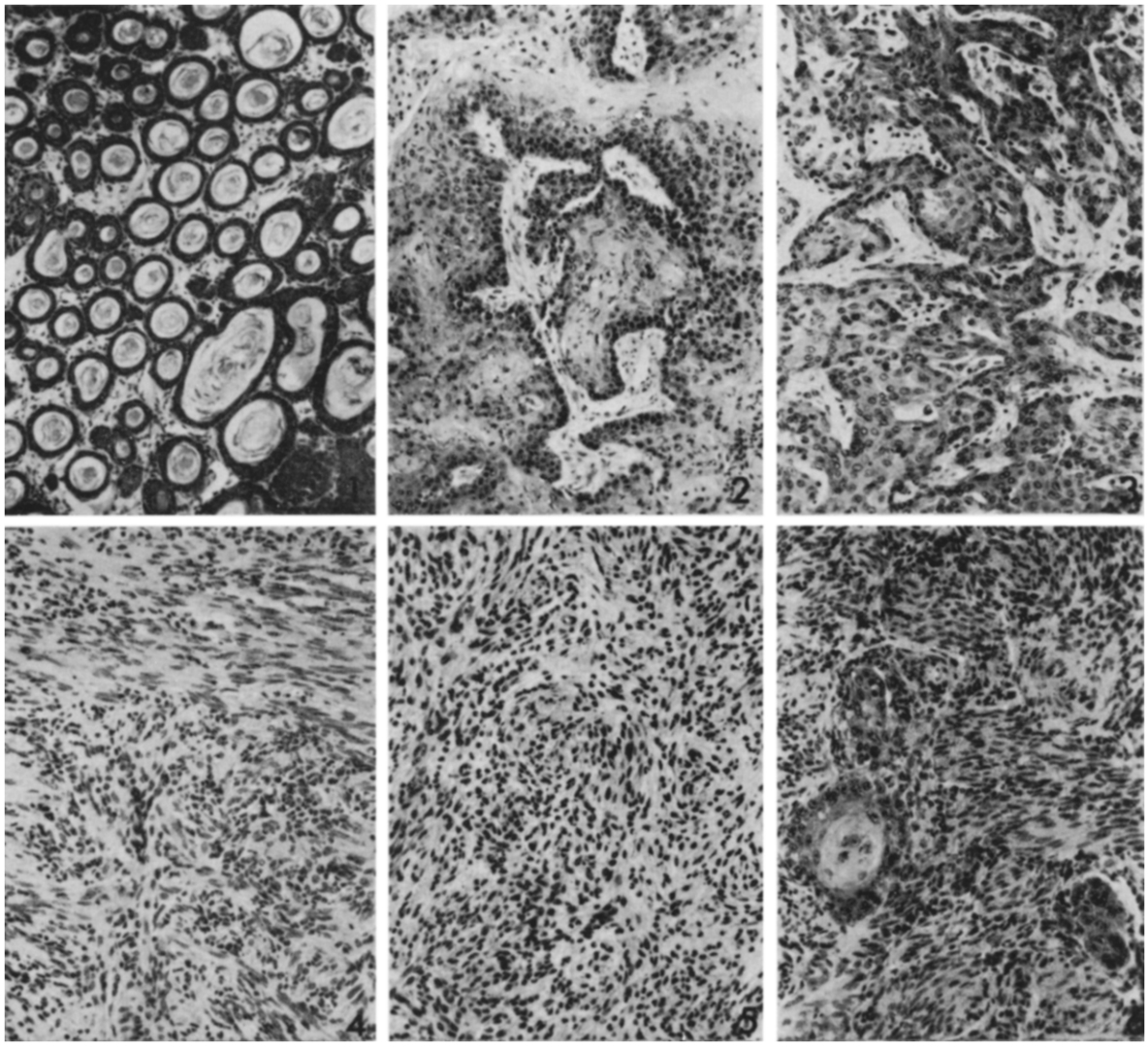

Fig. 1. *A highly keratinized squamous carcinoma from mouse No.* 202, *showing numerous pearl formations. H. & E.* × 70.

Fig. 2. *A lightly keratinized squamous carcinoma from mouse No.* 89. *H. & E.* × 125.

Fig. 3. *A non-keratinized squamous carcinoma from mouse No.* 122. *H. & E.* × 150.

Fig. 4. *A fibrosarcoma from mouse No.* 100. *H. & E.* × 135.

Fig. 5. *A leiomyosarcoma from mouse No.* 35. *H. & E.* × 125.

Fig. 6. *A mixed tumor containing tissue elements of fibrosarcoma and squamous carcinoma from mouse No.* 211. *H. & E.* × 150.

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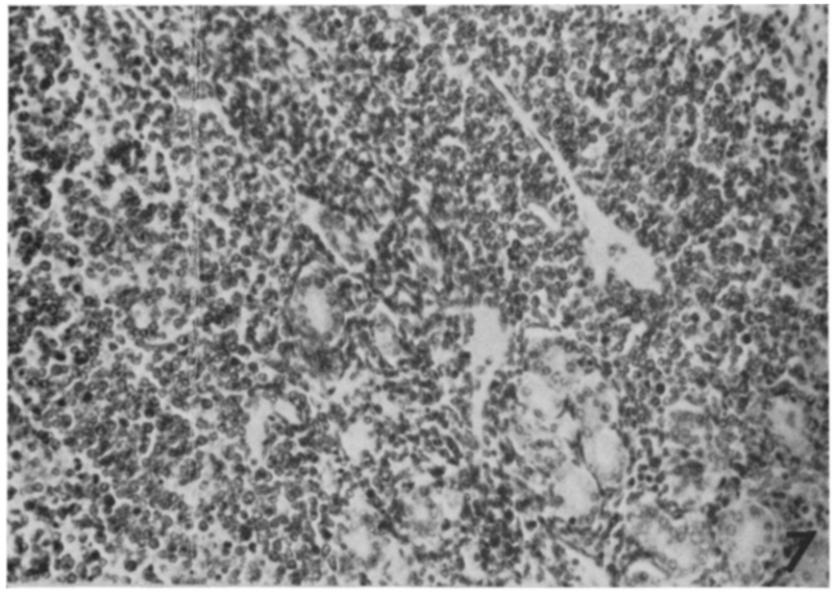

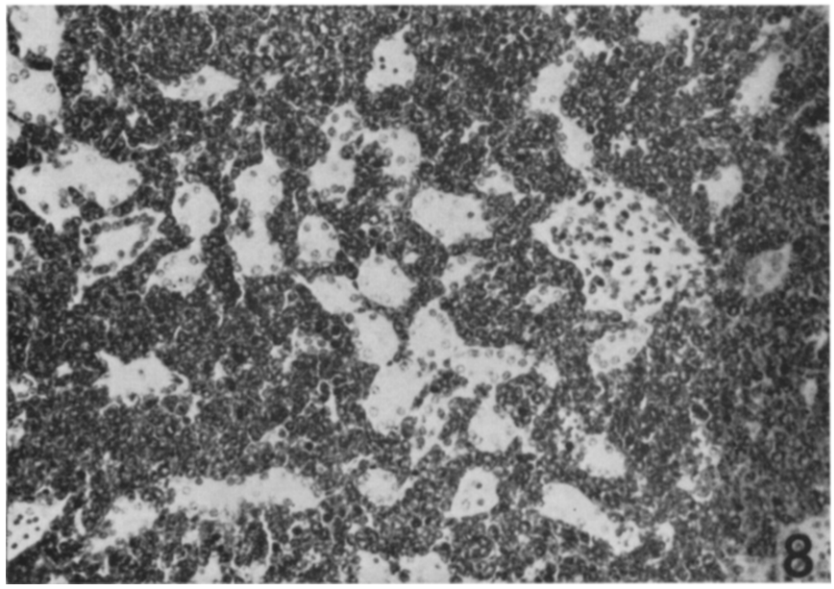

Fig. 7. *Lymphocytic infiltration of the endometrium in the uterine horn of mouse No.* 238 *with a lymphosarcoma. H. & E.* × 200.

Fig. 8. *Lymphocytic infiltration of the kidney in mouse No.* 238 *with a lymphosarcoma. H. & E.* × 200.

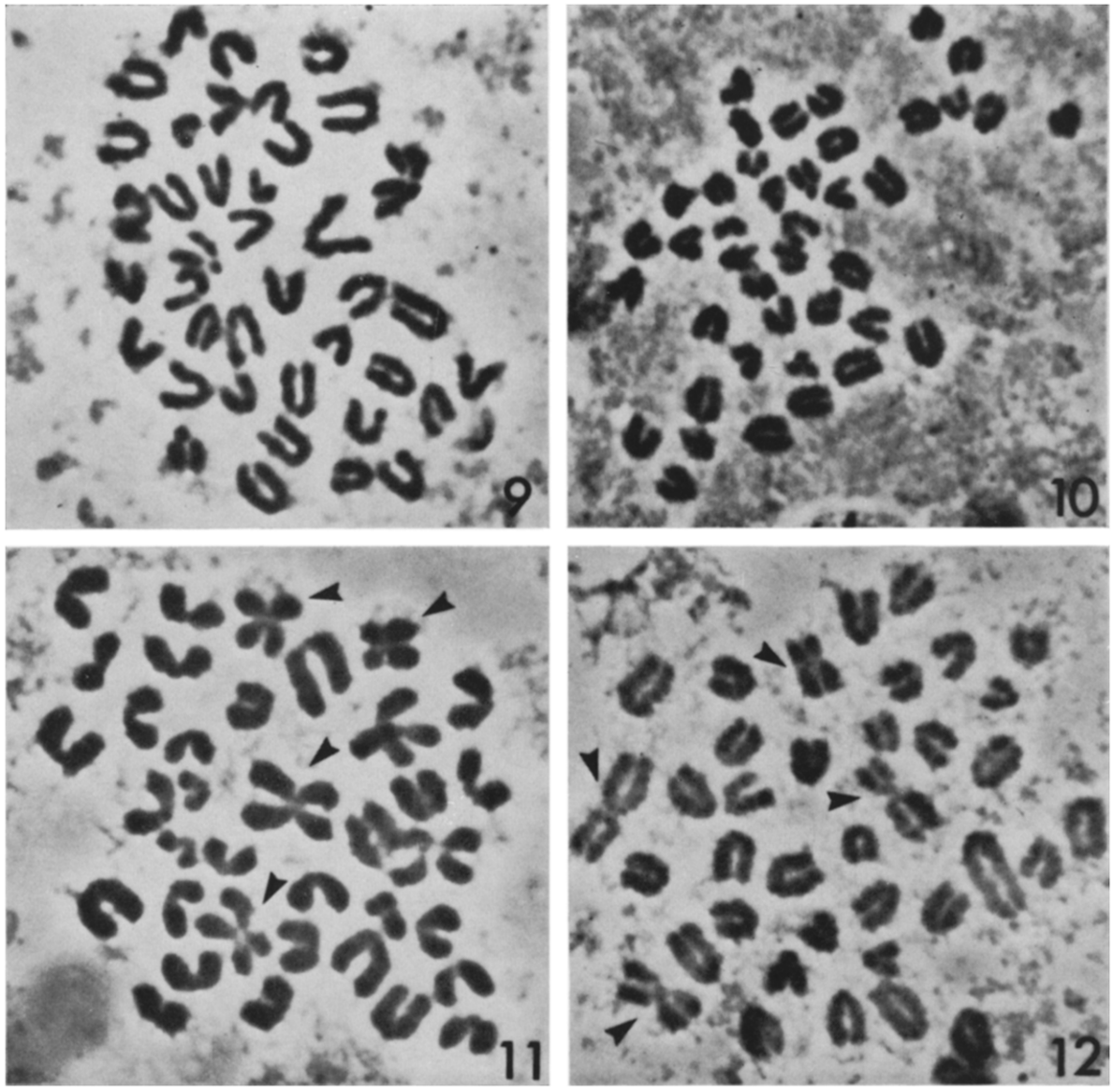

Fig. 9. *A metaphase plate with* 40 *acrocentric chromosomes from the fibrosarcoma recovered from mouse No.* 100. *Direct-squash method. Orcein stain.* × 7,000.

Fig. 10. *A metaphase plate with* 41 *acrocentric chromosomes from the squamous carcinoma recovered from mouse No.* 226. *Direct-squash method. Orcein stain.* × 6,400.

Fig. 11. *A metaphase plate showing* 38 *chromosomes including* 4 *metacentrics (arrows) from the thymus of mouse No.* 231 *with lymphosarcoma. Direct-squash method. Orcein stain.* × 6,400.

Fig. 12. *A metaphase plate showing* 37 *chromosomes including* 4 *metacentrics (arrows) from a mesenteric lymph node of mouse No.* 231 *with lymphosarcoma. Direct-squash method. Orcein stain.* × 6,400.

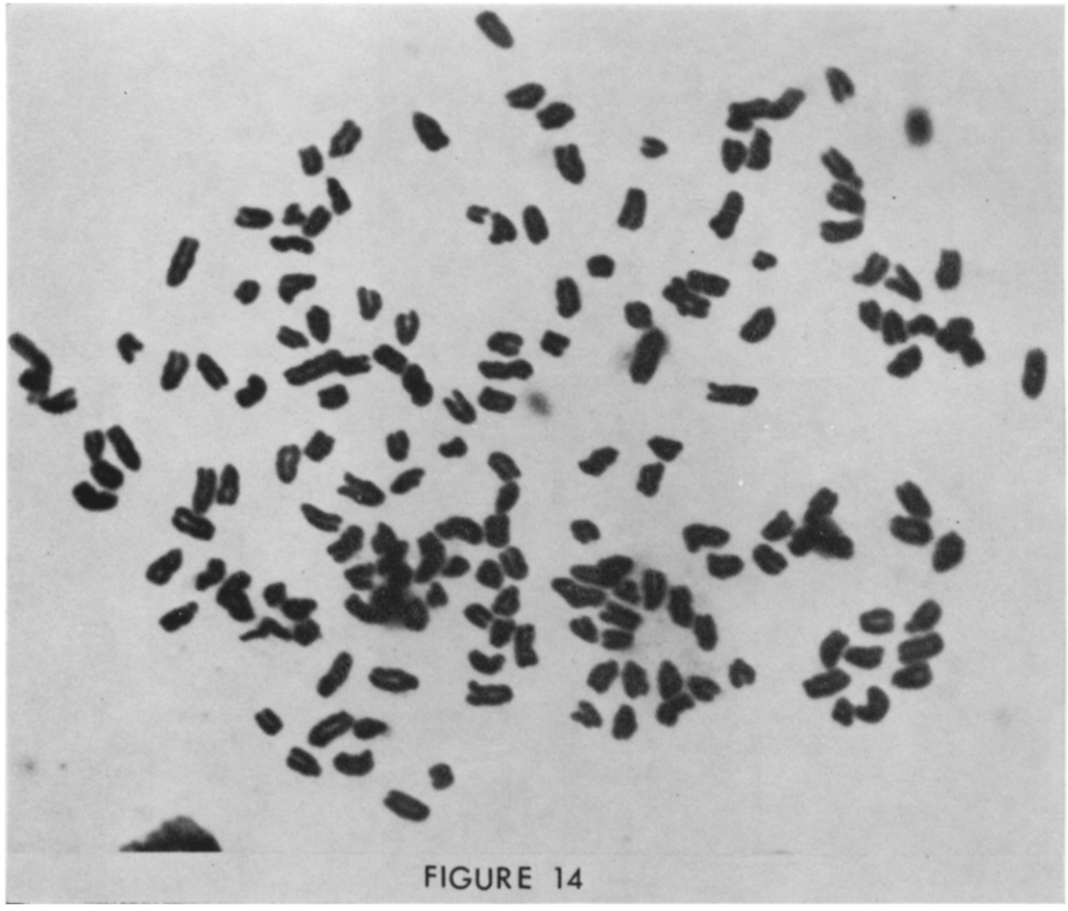

Fig. 14. *A metaphase showing* 162 *chromosomes from the fibrosarcoma of mouse No.* 99. *Air-dried preparation. Giesma stain.* × 6,400.

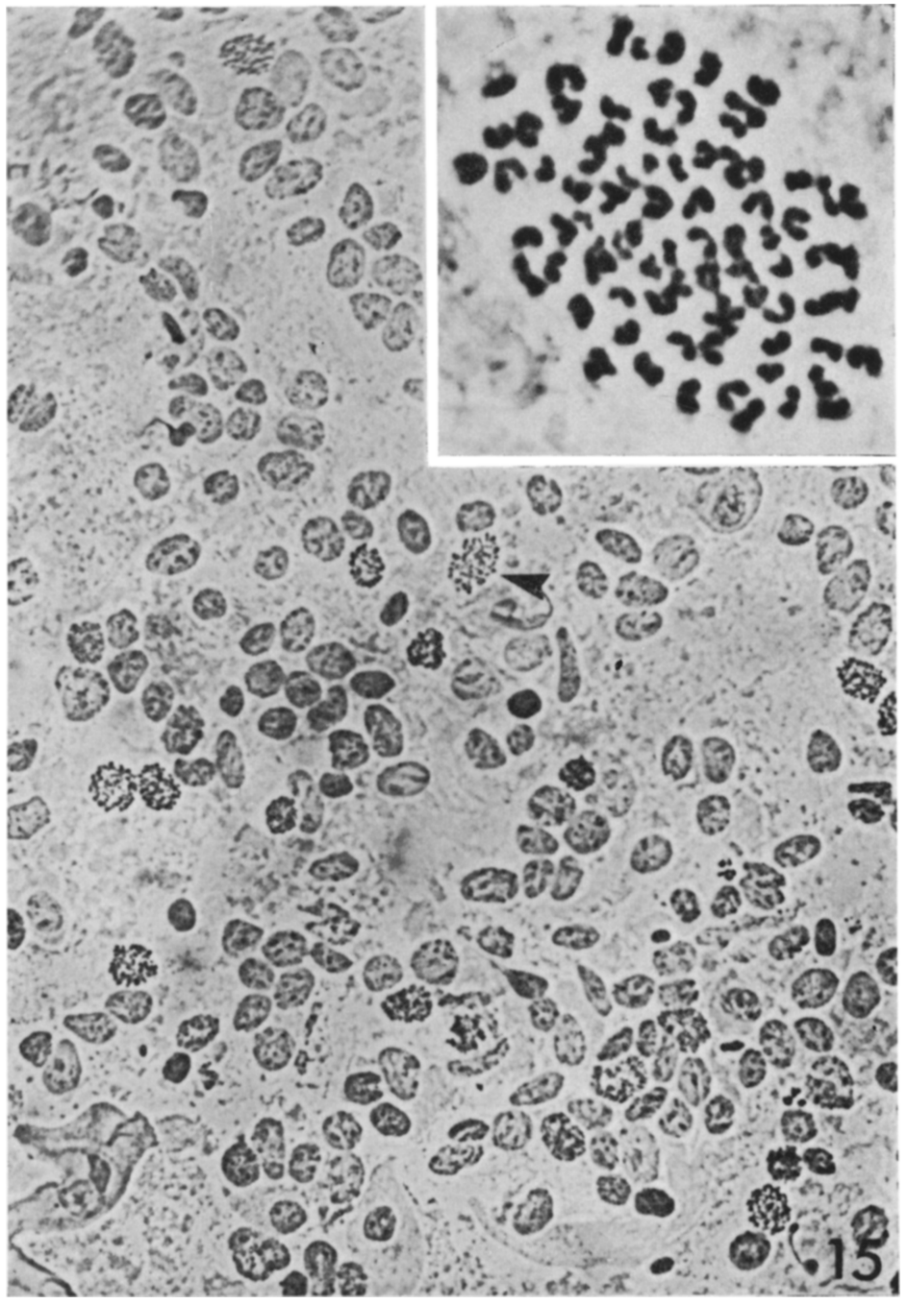

Fig. 15. *A low magnification photograph of squash preparation from a squamous carcinoma of mouse No.* 56. *Note the large number of metaphase plates. The metaphase plate indicated by an arrow contains* 80 *chromosomes* (*inset*). *Direct-squash method. Orcein stain.* × 1,000; *inset* × 6,400.

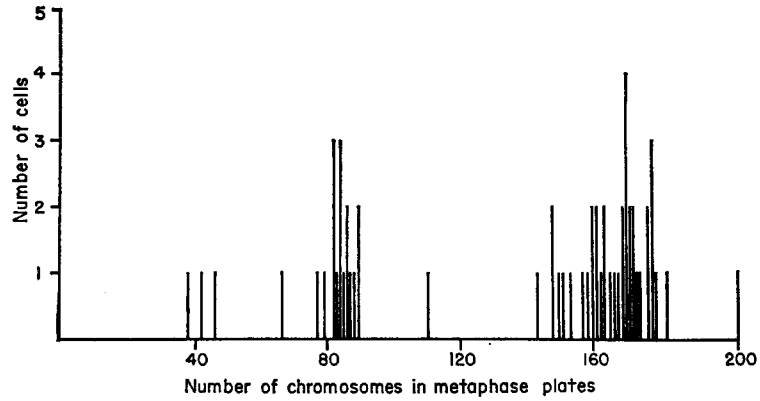

Fig. 13. *A histogram showing the distribution of chromosome numbers from* 63 *metaphase plates from the fibrosarcoma (mouse No.* 99) *cultured for* 4 *days.*

Squamous cell carcinomas. Both squamous carcinomas (Nos. 226 and 56) revealed abnormal chromosome composition in the tumor cells examined by squash method (Table 3). The carcinoma No. 226 showed an aneuploid modal cell line with 41 chromosomes (Fig. 10). The additional chromosome was indistinguishable from the rest due to acrocentric appearance of all chromosomes. The second tumor (No. 56) showed a polyploid cell population. Due to the high number of chromosomes, only five metaphase plates gave accurate counts and all had 80 (tetraploid) chromosomes. Figure 15 shows a high frequency of polyploid metaphase plates in the squash preparations from this specimen.

Lymphosarcoma. The data obtained from the lymphosarcoma of mouse No. 231 revealed a large number of metaphase plates with abnormal karyotypes (Table 4). In addition to a great deal of numerical heterogeneity, the metaphase plates from the various tissues showed a highly altered structure of chromosomes. A majority of the cells in all the three tissues examined showed four metacentric chromosomes (Figs. 11 and 12). This suggests the possibility of origin of these cells from a single parent cell.

Frequency of polyploid metaphase plates. In order to determine the frequency of polyploidy, approximate counts with a 40X objective were obtained from six tumors induced by DMBA (Table 5). Three of these showed a relatively large number of metaphase plates with polyploid chromosome constitution, two had a low frequency of polyploid cells and one did not show any polyploid cells. In addition, tumor No. 99 examined by tissue culture method showed a high degree of polyploidy. It is interesting to note that tumor No. 226, which lacked polyploid cells, had aneuploid chromosome composition of 41 in the modal cell line (Table 3). These observations suggest a great

Table 4. *Chromosome analysis of lymphoid organs from DMBA-treated mouse No.* 231 *with a lymphosarcoma*

Tissue	Total metaphase plates analyzed	Number of metaphase plates with indicated chromosome complements and no structural anomalies							Number of metaphase plates with indicated number of metacentric chromosomes					
		36	37	38	39	40	41	42	1	2	3	4	5	8
Thymus	36	1	1	2	2	4	2	1	1(37)* 1(39) 1(40)	2(37) 2(38) 1(39) 1(40)	2(37) 1(38) 1(39)	3(36) 2(37) 2(38)	1(37) 1(57)	1(74)
Spleen	32	2	1	2	2	18	1	1	—	—	1(38)	1(35) 1(36) 2(37)	—	—
Mesenteric Nodes	15	—	1	1	—	1	—	—	—	1(37) 1(38) 1(39)	—	2(36) 3(37) 3(38) 1(53)	—	—
Total	83	3	3	5	4	23	3	2	3	9	5	20	2	1

*The figures in parentheses represent the total number of chromosomes in the plates.

Table 5. Frequency of polyploid metaphase plates in DMBA-induced squamous carcinomas, fibrosarcomas, and mixed sarcomas in female ICR mice

Mouse number	Type of tumor	Total metaphases scored	Polyploid metaphases (%)
56	Squamous carcinoma	172	76
226	Squamous carcinoma	95	0
39	Fibrosarcoma	111	17
28	Fibrosarcoma	54	20
100	Fibrosarcoma	100	5
43	Fibrosarcoma–leoimyosarcoma	26	4

tendency of DMBA-induced squamous carcinomas and fibrosarcomas towards polyploidy.

DISCUSSION

In the present study, 56% of the mice exposed to DMBA by the placement of a carcinogen-coated thread into the vaginal fornix developed malignant tumors. The production of experimentally-induced carcinoma of the cervix in animals by this method has received a great deal of attention. Direct application of carcinogenic agents [7, 8, 14, 15, 16, 17, 26] and systemic administration of estrogenic hormones [18, 19] have proven to be the most satisfactory methods of induction of cervical tumors. The direct application of carcinogens has involved painting the cervix with carcinogens such as benzopyrene [20] and methylcholanthrene [17], coating the exposed cervix with carcinogens by means of a wire loop applicator [21, 22], inserting pellets containing carcinogen into the vagina [21] or placing carcinogen impregnated threads into the region of the cervix [7, 8, 15, 16]. Murphy [17] compared several techniques of cervical carcinoma production using methylcholanthrene and found an increased yield of tumors and a shorter latent period with the coated thread method. A majority of the tumors were invasive squamous carcinomas with a variable degree of cellular differentiation and keratin formation. A similar experiment using methylcholanthrene and beeswax-impregnated threads produced a 100% yield of cancerous lesions [16]. Using the same technique, Graham [15] found epidermization occurring in both the treated and control groups of mice bearing only the thread. More recently, a 50% yield of squamous carcinomas has been reported using methylcholanthrene-coated threads placed in the uterine cervix of mice [7]. Similarly, Meisels [8] has reported a high yield of tumors after the insertion of 7,12-dimethylbenzanthracene (DMBA)-coated threads into the cervix of mice. The high yield of tumors in the present study is consistent with these reports.

Squamous carcinomas and fibrosarcomas were the most common types of tumors. The less frequent types included leiomyosarcomas, lymphosarcomas, mixed tumors, and double tumors. The occurrence of a wide variety of neoplasms in the present study, and previous reports on the presence of lesions in the lungs [8], the production of fibrosarcomas in rats [14], and the several neoplasms associated with the injection of DMBA into neonatal mice [23] indicate that this carcinogen has the ability to become dispersed throughout the body and affect a large spectrum of tissues and organs.

Cytogenetic examination of the DMBA-induced tumors revealed a wide distribution in the chromosome complements of the neoplastic cells. Tumors with diploid, aneuploid, tetraploid, and octaploid chromosome constitution were produced. Also, a tendency towards polyploidy was exhibited by a majority of the tumors examined. Tumors induced by injection of methylcholanthrene and dibenzpyrene have also revealed a wide variation in chromosome constitution [24, 25]. Generally, chemically-induced tumors of experimental animals have an abnormal chromosome composition [11, 24, 25]. Most spontaneously occurring tumors possess a normal diploid chromosome constitution with the exception of such cases as human myeloid leukemia [26] and the spontaneous leukemia of AKR mice [27]. In a comparative study of chemical-, radiation- and virus-induced murine leukemogenesis, most of the leukemic cell populations were characterized by an abnormal complement of 41 chromosomes in the thymus, spleen and bone marrow [13]. Kurita *et al.* [28] have reported that the chromosomes of DMBA-induced leukemic mice were predominantly normal although they published no data to substantiate their claim. Recently, we have found that 8 out of 9 mice with DMBA-induced leukemia had aneuploid constitutions

of 41 chromosomes in the lymphoid tissues examined [13]. These findings were consistent with previous reports of an extremely high frequency of karyotypes with 41 chromosomes in the modal cell lines in a majority of chemically-induced lymphatic leukemias of mice [6, 12]. The lymphosarcoma reported in this study is interesting in consideration of these investigations. This tumor showed a highly altered karyotypic pattern with a preponderance of abnormal metacentric chromosomes ranging from 1 to 4 in hypodiploid cells.

The present observations on various types of DMBA-induced malignant tumors along with the results of others [24, 25] seem to indicate that there is no consistency in the chromosome pattern of these experimental tumors in contrast to the DMBA-induced lymphatic leukemia of mice [6, 12, 13] and the "Philadelphia" chromosome of human myeloid leukemia [26]. The numerical chromosome anomaly of the additional acrocentric chromosome found in a majority of the mouse leukemias [6, 12, 13] may not necessarily represent the same genetic alteration. A wide spectrum of chromosomal abnormalities have been reported in dysplasias, carcinomas-*in-situ*, and invasive squamous carcinomas of the human cervix [29, 30, 31]. However, Manocha [32] has shown that most of the human basal cell carcinomas contain a diploid deoxyribonucleic acid (DNA) content. Hauschka [33] has suggested that "through its growth pressure against the host, a tumor quickly initiates variegation in its internal and external milieu" as a result of tissue necrosis, infection, anoxia, and lack of nutrition. These "physiological gradients influence the selection of random karyotypic mutants" and are responsible for the karyotypically heterogeneous cell populations which occur in many neoplasms. It is possible that each malignant cell population will have a different chromosome composition as a result of its unique internal tissue environment.

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Method for Tissue Culture Evaluation of the Cytoxic Activity of Drugs Active Through the Formation of Metabolites*

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Abstract—*A "tissue culture" method for testing the cytotoxic activity of compounds active through the formation of metabolites is described and cyclophosphamide (CPA), which is known to exert its effect only after hepatic biotransformation, has been chosen as "model" substance.*

A monolayer of cultured KB cells is treated for one hour with CPA at 37°C, in the presence of different amounts of microsomal enzymes (105,000 ×g fraction) and their co-factors and at the end of the incubation the medium is removed and changed.

A dose-effect relation is observed by measuring the inhibition of growth or the destruction of the KB cells after 24 hr.

The significance of this procedure for screening purposes or for studying the possible cytotoxicity of unstable metabolites is outlined.

INTRODUCTION

A CONSIDERABLE limitation of the *in vitro* methods for testing the cytotoxic activity of drugs on cultured cells is represented by the fact that compounds, which are active through the formation of metabolites, are effective only if tested *in vivo*.

It is, indeed, well known that cyclophosphamide (CPA), which is widely used in cancer therapy, does not show any cytotoxic activity *in vitro*, as it must be transformed into active alkylating metabolites by microsomal enzymes located in the liver [1, 2, 3, 4].

Previous studies showed a cytotoxic activity of CPA *in vitro* by incubating cancer cells with serum of animals pretreated with CPA or with CPA previously incubated with liver slices and homogenates [1, 5].

Connors *et al.* [6] showed that Walker tumor cells exposed to CPA in the presence of the liver microsomal fraction (105·000 ×*g*) are less effective than cancer cells exposed only to CPA in inducing tumors when injected *in vivo*.

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This report describes a procedure in which cultured cancer cells are incubated with CPA in the presence of liver microsomal enzymes to achieve metabolism of CPA and, thus, a marked cytotoxic activity *in vitro*.

MATERIAL AND METHODS

(a) *Preparation of sterile microsomes*

The microsomal fraction obtained from the livers of male CD_1 mice (24 ± 2 g body weight) was utilized. The mice were killed by decapitation, the livers were excised [at 10:100 a.m. to avoid the daily rhythms of microsomal enzymes [7, 8]] under sterile conditions, cooled, and homogenized in 4 volumes of Dulbecco's phosphate buffer saline, pH 7·4, 0°C, (PBS) with a motor-driven Potter–Elvehjen homogenizer.

The homogenate was centrifuged in a refrigerated Sorvall centrifuge, model RC2-B, at 9,000 ×*g* for 20 min.

The supernatant, containing the microsomal and soluble fractions, was removed and centrifuged in a refrigerated Beckman ultracentrifuge, model L, at 105,000 ×*g* for 1 hr. The microsomal pellet was washed by resuspending in PBS with a Teflon pestle and recentrifuged at 105,000 ×*g* for 1 hr.

The volume of the final microsomal suspension was adjusted to contain microsomes from 1 g liver wet weight per ml of PBS.

All the operations were carried out at a temperature of 0–4°C and great care was taken to work in completely sterile conditions. Sterility was controlled in Standard NKS membranes (Sartorius–Membranfilter, GmbH, Göttingen, Germany) over a 48 hr period of time.

(b) *Preparation of KB cell monolayers*

KB cells were grown as monolayers in roller-tubes in Eagle medium with penicillin and streptomycin added and supplemented with 10% calf serum.

About 10^5 cells in 1 ml of medium were seeded in each culture tube and incubated at 37°C in a roller apparatus at 6 rev/hr to obtain a monolayer of KB cells.

After 48 hr, tubes were randomly chosen and grouped. One of the groups was trypsinized (0·25% DIFCO trypsin in PBS) and counted by means of a Coulter Counter, which is able to count a concentration of cells as low as 10^3/ml.

The cell number at the beginning of the experiment (T_0) was considered the Base Line.

The tubes of the remaining groups were treated by changing the growth medium as described under experimental design.

(c) *Experimental design*

To the test tubes containing the monolayer of KB cells, microsomes (M), cyclophosphamide* (CPA), and co-factors (CF) were added in the following amounts:

1. 1 ml of PBS containing microsomes equivalent to 25–50–100–125–250–500–750 mg of wet liver;
2. 1 ml of PBS containing graded concentrations of CPA corresponding to a concentration of 10, 100, 250, 500, 750 and 1000 μg/ml incubation medium;
3. 1 ml of PBS containing the following CF: 10 mg of glucose-6-phosphate (disodium salt), 1·2 mg NADP (sodium salt), 2·0 mg $MgCl_2 \cdot 6H_2O$, 5·0 μl glucose-6-phosphate dehydrogenase (0·5 units).

The final volume was therefore 3 ml. The solutions of cyclophosphamide and co-factors were sterilized by filtration through a cellulose membrane filter of 0·2 mμ pore, size type MF 14 (Sartorius–Membranfilter, GmbH, Göttingen, Germany).

*Cyclophosphamide was kindly supplied by Zilliken, (Italy).

The test tubes were covered with an aluminum cap that allows oxygenation of the mixture, placed on a roller whose speed was increased to 1 rev/min in a thermostatic chamber at 37°C, and incubated for 1 hr. After incubation the mixture was removed with a sterile syringe, the test tubes were gently rinsed with PBS and to each test tube 1 ml of Eagle medium containing antibiotics (100 units penicillin/ml and 50 μg streptomycin/ml) and 10% calf serum was added.

The tubes were then placed in a tissue culture roller at 37°C. After 24 hr (T_{24h}) the cellular debris was removed by washing with PBS, and trypsin was added to remove the viable cells from the glass wall. The cell suspension was then counted by means of a Coulter Counter.

The data were calculated as described by Morasca and Leonardi [9] by measuring the inhibition of growth or the destruction of the KB cells.

The percentage of growth was evaluated with respect to the increase of the number of control cells during the 24 hr of incubation. The percentage of destruction was evaluated with respect to the Base Line value. The experiments were repeated twice and each time each group consisted of six samples. Since the results were essentially the same, the tables report only the results of one experiment.

The significance of the differences between control and treated groups was established by use of analysis of variance on the absolute values.

RESULTS

Table 1 shows that microsomal enzymes of the liver alone, or microsomes with added co-factors (CF), as well as cyclophosphamide (CPA) alone (even at a concentration of 1000 μg/ml) do not significantly affect the growth of KB cells *in vitro*. However, when CPA was added to liver microsomes and CF, it exerted a statistically significant inhibition of KB cell growth at a concentration of 100 μg/ml. With higher concentration of CPA the growth inhibition was complete and a destruction of KB cells appeared. At a concentration of 1000 μg/ml, CPA, in the presence of liver microsomes and CF, showed a complete destruction of cancer cells. A moderate, not significant inhibition of growth was present also when CPA (1000 μg/ml) was incubated with liver microsomes without added CF. This is due to the fact that liver microsomes may retain attached a minimum amount of CF. Table 2

Table 1. *Cytotoxic activity on cultured KB cells induced by different concentrations of cyclophosphamide (CPA) in the presence of liver microsomes (M) and co-factors (CF)*

CPA treatment	(μg/ml)	Inhibition of* growth (%)	Destruction† (%)
Untreated		+16	0
M + CF (Controls)		0	0
CPA	1000	0	0
CF + CPA	1000	1	0
M + CPA	1000	44	0
M + CF + CPA	10	42	0
M + CF + CPA	100	57‡	0
M + CF + CPA	250	100‡	15‡
M + CF + CPA	500	100‡	69§
M + CF + CPA	750	100‡	74§
M + CF + CPA	1000	100‡	89§

The figures are the average of six samples:

*Relative to control values at T_{24hr} ($327 \pm 31 \times 10^3$ cells); † relative to Base Line values at T_0($197 \pm 6 \times 10^3$) cells).

‡$P < 0{\cdot}05$ §$P < 0{\cdot}01$ after analysis of variance.

M = Microsomes ($105{,}000 \times g$ liver fraction) equivalent to 750 mg of wet liver.

Table 2. *Cytotoxic activity on cultured KB cells induced by cyclophosphamide (CPA) in the presence of co-factors (CF) and of differing amounts of liver microsomes (M)*

CPA treatment (1,000 μg/ml)	Inhibition* (%)	Destruction† (%)
Untreated	0	0
M 750 + CF (Controls)	0	0
CPA	2	0
CF + CPA	1	0
M 50 + CPA	0	0
M 750 + CPA	44	0
M 25 + CF + CPA	42	0
M 50 + CF + CPA	82‡	0
M 100 + CF + CPA	100§	25‡
M 125 + CF + CPA	100§	67§
M 250 + CF + CPA	100§	72§
M 500 + CF + CPA	100§	91§
M 750 + CF + CPA	100§	98§

The figures are the average of six samples.

*Relative to control values at T_{24hr} ($282 \pm 12 \times 10^3$ cells); †relative to Base Line values at T_0 ($189 \pm 11 \times 10^3$ cells).

‡$P < 0{\cdot}05$ §$P < 0{\cdot}01$ after analysis of variance.

M = Microsomes ($105{,}000 \times g$ liver fraction) equivalent to 25, 50, 100, 125, 250, 500, 750 mg of wet liver.

shows that the effect of a given concentration of CPA (1000 μg/ml) is proportional to the amount of liver microsomal fraction. In fact, by increasing liver microsomes in the presence of the same concentration of CPA, the growth inhibition increased gradually and resulted in a complete destruction of cancer cells.

DISCUSSION

This report describes a new experimental procedure for an evaluation of the cytotoxicity exerted by drugs requiring an *in vivo* biotransformation to become active.

Liver microsomal preparations were utilized as a model of enzymes involved in the metabolism of drugs. Methods for preparing microsomes under sterile conditions, as well as experimental procedures which allow the enzymatic activity of these microsomes in the presence of cultured cancer cells, have been described.

Among the drugs which are transformed into active metabolites *in vivo*, cyclophosphamide (CPA) was selected as a model substance. The results obtained show that CPA exerts a clear cytotoxic effect *in vitro*. This effect is related to the concentration of CPA when high amounts of liver microsomes (corresponding to 750 mg of wet liver) are employed or it is proportional to the amount of liver microsomal fraction when a high concentration of CPA (1000 μg/ml) is utilized.

The described method may prove useful in assessing the antitumoral effect of chemicals *in vitro*.

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Standards for the Assessment of Estrogen Receptors in Human Breast Cancer

Report of a workshop on September 29, 1972, at the Antoni van Leeuwenhoek-Huis, Amsterdam

E.O.R.T.C. BREAST CANCER COOPERATIVE GROUP

Abstract—*The participants agreed on*: 1 *the potential value of assaying estrogen receptor content in human mammary carcinomas for therapeutic guidance*, 2. *the urgency of fully establishing this relation and* 3. *the necessity of a joint effort towards achieving this goal within the earliest time possible. The charcoal adsorption technique was adopted as a first standard assay. A common protocol form will be used for clinical evaluation. All data will be pooled at the E.O.R.T.C. data bank, Institut Bordet, Brussels. Cooperation in the project is highly welcomed.*

EXPERIMENTAL PROCEDURES

1. *Tissue collection and storage*

The tumor is put into an ice-cooled container immediately after removal. It is freed from surrounding fat and connective tissue and rinsed with ice-cold saline. Aliquots taken for histological examination and for receptor assay should be of similar composition. The tumor should be either processed within 2 hr after the operation or stored in liquid nitrogen which is preferred over storage at −70°C. For transportation, the tissue should be kept in dry ice.

2. *Preparation of extract*

It is essential to homogenize at low temperature to avoid denaturation of the heat-labile receptor. The homogenate should contain less than 2% unbroken cells. The (minced) frozen tissue can be pulverized in a porcelain mortar chilled in liquid nitrogen, or preferably in a "microdismembrator" (Braun, Melsungen, Germany). In this device, the tissue mince is placed on the wall of a 7 ml teflon cylinder, free of a 10 mm tungsten–carbide ball at the bottom. The container is then capped, immersed in liquid nitrogen for 5 min, subsequently fixed in the instrument and vibrated for 45 sec at maximum frequency. If small chunks of tissue remained unpulverized, chilling and vibrating are repeated. Chilled stainless steel spatulas or spoons are used for transferring the tissue powder to glass containers. Four volumes of a 0·01 M Tris/HCl, 0·001 M EDTA,

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Reprints are available from Dr. J. C. Heuson, chairman of the E.O.R.T.C. Breast Cancer Cooperative Group, Institut Bordet, Brussels, Belgium.

0·003 M NaN_3, pH 7·5 buffer are added to the powder, the mixture is stirred in the cold for 10 min and then spun at 0–2°C for 60 min at 100,000 × *g* (e.g. Spinco SW 50·1, 2 ml cellulose nitrate reduction tubes). The clear supernatant without the fatty layer on top is collected by piercing the tube wall above the sediment. It is immediately used for receptor assay in order to minimize decay of free receptor. Protein concentration should not be less than 4 mg/ml.

3. *Charcoal adsorption technique*

Tritium labelled estradiol (6,7 ^{3}H or 2,4,6, 7 ^{3}H) should be of sufficiently high specific activity and at least 95% pure. Charcoal suspension consists of 0·5% Norit A (sieved through 25 μm nylon netting), 0·05% dextran T 70, 0·1% gelatine in extraction buffer. The gelatine is completely dissolved by heating before addition of the charcoal. The suspension is stirred for 24 hr at 0–4°C before use. Aliquots of the extract are pipetted into glass tubes and ^{3}H estradiol solutions are added to obtain at least 5 final concentrations between 10^{-10} and 10^{-8} M. Controls are prepared with buffer instead of extract. Tubes are sealed and incubated either for 2 hr at 20°C or overnight at 0–4°C. After incubation, an equal volume of charcoal suspension is added and the tubes are shaken for 90 min at 0–4°C. The charcoal is (completely !) removed by centrifugation and (triplicate) aliquots of the supernatant are transferred for scintillation counting. Duplicate assays should be run for each estradiol concentration. Values (minus buffer/charcoal-control) are plotted according to Scatchard [1]. A K_d in the order of 10^{-10} M must be obtained from the slope of the plot; the concentration of estradiol binding sites is read at the intercept with the *x*-axis. If not sufficient material is available for a full scale assay, incubation with a single, saturating concentration of estradiol (e.g. 1×10^{-8} M) may be used. Controls are run with extract heated for 1 hr at 45°C or preincubated with a 10^3-fold excess of Upjohn 11.100 A before addition of estradiol.

4. *Optional technique*

Agargel electrophoresis [2] is recommended as an accurate method of high sensitivity, but requires some equipment not normally available in most laboratories. It will be indispensable when the study is extended to the assay of androgen receptors, which can not be distinguished from SHBG by the charcoal technique.

5. *Auxiliary assays*

(a) In tissue extracts:
Total protein is assayed by Lowry's method [3] using human serum albumin as a standard.
Human serum albumin is assayed immunologically by single diffusion [4, 5].
Endogenous estrogen to assess occupied receptor should be measured by radioimmunoassay.

(b) In homogenate:
DNA assay, e.g. by the method of Giles and Myers [6].

6. *Expression of receptor content*

Receptor content is expressed as moles of estradiol bound per mg extracted tissue protein and per mg DNA. The soluble tissue protein concentration is obtained from: total protein concentration—albumin concentration × 1·67, arbitrarily assuming a 60% albumin concentration in the contaminating plasma proteins. (Since the actual albumin concentration might range up to 80%, the calculated receptor content can be too high. Therefore, the reference to DNA concentration is a useful second parameter).

RULES OF OPERATION

1. *Quality control*

Each participating institution will mail twice a year thoroughly minced aliquots of one or several tumors to all members for full scale assay in order to check on within-group reproducibility. Results shall be reported to the sender for listing and distribution.

2. *Clinical protocol and data compilation*

E.O.R.T.C. forms will be used for clinical evaluation and the data will be filed at the E.O.R.T.C., Institut Bordet, Brussels, under the supervision of Dr. J. C. Heuson.

3. *Publication of results*

Results will be published according to the by-laws of the group.

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Letter to the Editor

Effect of Polymethacrylic Acid (PMAA)* on Tumour Metastasis

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THE LUNG carcinoma of Lewis is described as a spontaneous anaplastic carcinoma that arose in 1951 in the lung of a C57BL mouse [1].

Female and male C57BL/10J.Sel inbred mice were transplanted intramuscularly in the right posterior leg with 10^5 cancer cells (viable as tested by dye exclusion test) of Lewis Carcinoma suspended by mechanical homogenization in phosphate buffer, pH 6·9. At autopsy 25 days after tumour implantation, the tumour mass present in the leg was dissected and weighed. The lung of each mouse was fixed in 40% formaldehyde solution. The metastatic nodules on the surface of the lungs were then counted according to Wexler [2].

The transplantable osteosarcoma C22LR [3] is inoculated into a testicle of (C57BL/Rij × CBA/Rij) F_1 hybrid mice by injection of 2×10^5 tumour cells in 0·01 ml Hanks balanced salt solution. The site of puncture is sealed by thermo-cauterization. Eighteen days later many mice show lymph node metastases in the hilus of the kidney or along the abdominal aorta. The evaluation of the presence of metastases is done by inspection at autopsy and occasionally checked by histological study. The percentage of animals showing metastases was calculated without considering the number of metastases present.

PMAA (mol. weight: 750,000) was administered as a gel solution in water after mixing it for several hours with a magnetic stirrer.

The effects on lung metastases from Lewis carcinoma are presented in Table 1.

It is evident that i.p. or i.v. daily administration of 5–40 mg PMAA/kg is able to decrease the frequency of lung metastases even at doses which are unable to affect the growth of the primary tumor. The subcutaneous and the oral route of administration are instead completely inactive.

The incidence of metastases from osteosarcoma C22LR is presented in Table 2. The data indicate a significant decrease in the frequency of lymph node metastasis but not of lung metastasis due to a brief treatment with PMAA. Data not reported here in detail show

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*PMAA is a polymerization product of methacrylic acid in the presence of benzylperoxide. (Kindly provided by Prof. P. de Somer, Rega Institute, Louvain) EORTC No. 1631.

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Table 1. *Effect of PMAA at different treatment schedule on lung metastases from Lewis carcinoma* (10^5 *cells*/0·1 *ml*) *administered intramuscularly*

Route of administration	Treatment schedule	Total No. of doses	Dose (mg/kg)	Tumor weight (g ± S.E.)		No. lung metast./Mouse	
				Controls	Treated	Controls	Treated
i.p.	qd, 3–24	22	20	5.9 ± 0.2	4·0 ± 0·4*	23 ± 2	4 ± 1*
			10		5·1 ± 0·2		8 ± 2*
			5		5·4 ± 0·2		9 ± 2
	q2d, 3–23	11	10	6.3 ± 0.4	6·0 ± 0·4	15 ± 3	3 ± 0·9*
			5		5·8 ± 0·7		9 ± 4
	q5d, 0–20	5	40	6.8 ± 0.3	6·5 ± 0·1	18 ± 2	9 ± 1·2*
i.v.	q5d, 0–20	5	40		7·0 ± 0·3		†6 ± 1*
s.c.	q5d, 0–20	5	40		7·3 ± 0·2		15 ± 2
o.s.	q5d, 0–20	5	40		7·1 ± 0·3		15 ± 1

Each group consists of 6–9 animals. All the animals were sacrificed 25 days after tumour implantation.
i.p. = intraperitoneally; i.v. = intravenously; s.c. = subcutaneously; o.s. = orally.
*Student's *t*-test $P < 0{\cdot}05$.
†3/9 animals were free of lung metastases at autopsy.

Table 2. *Effect of PMAA on lymphnode metastases from C22LR osteosarcoma* (2×10^5 *cells*/0·01 *ml*) *transplanted into a testicle*

	No. of mice	% of animals showing lymph-node metastasis	% of animals showing lung metastasis
Control	200	77	7
5 × 40 mg/kg PMAA i.p. on days 0 through 4	27	52*	7

*χ^2 test: $P < 0{\cdot}05$.

also that a 5-day treatment with 40 mg/kg i.p. of PMAA does not affect the subcutaneous growth of osteosarcoma C22LR.

PMAA can therefore be added to the list of polymers effective in decreasing the appearance of metastases in suitable experimental models [4, 5, 6].

As far as the mechanism of PMAA it may be relevant to recall that this polymer is a moderately effective inducer of interferon [7], it mobilizes lymphocytes [8] as well as lymphoma cells [9]. Furthermore, a weak heparine-like action has also been described for this agent [9]. Whether the property of PMAA on the frequency of tumor metastases is related to one or more of the above-mentioned effects, remains to be established. Further studies are in progress.

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Letter to the Editor

Dose–Response Correlation for the Induction of Respiratory-Tract Tumours in Syrian Golden Hamsters by Intratracheal Instillations of Benzo(a)pyrene*

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A SUCCESSFUL method of inducing bronchogenic cancer and other respiratory-tract tumours in hamsters is repeated intratracheal instillation of a benzo(a)pyrene-ferric oxide mixture suspended in 0·9% NaCl solution [1]. Using this model system a positive dose–effect relationship has been demonstrated [2].

We have recently shown that the above method is also effective when benzo(a)pyrene (BP) is given alone [3]. Obviously, ferric oxide particles are not indispensable for the induction of respiratory-tract tumours by BP. Whether a positive dose–response relationship can also be obtained using BP alone was examined in the work described in the present report.

For this study 180 young adult male Syrian golden hamsters were divided into 6 groups, each consisting of 30 animals. They received weekly one intratracheal instillation during a period of 52 weeks. Each instillation in the 6 groups of animals consisted of 0·2 ml 0·9% NaCl solution, in which 0, 0·0625, 0·125, 0·25, 0·5 or 1·0 mg BP had been suspended. The animals were checked daily and weighed individually once monthly. The experiment was terminated after an experimental period of 78 weeks. Autopsies were performed on all animals, except a few lost through cannibalism. For killing the hamsters, and removing and processing the lungs, the techniques described by Saffiotti *et al.* [1] were used. Other details concerning the animals, materials, and procedures used were the same as those previously reported [3].

From week 25 onwards the average body weights of the animals in the various test groups were slightly lower than those of the controls, but in this respect no clear dose–response relationship was apparent. The animals of all 6 groups survived well up to week 52 (Table 1). Subsequently mortality increased, more rapidly for the 3 highest dose groups than for the other groups.

The tumour incidences for all groups are given in Table 2, and the probabilities for observation of respiratory-tract tumours at death of the animals have been plotted in Fig. 1. A positive dose–response correlation is demonstrated by the increasing numbers of hamsters bearing respiratory-tract tumours with increasing doses of BP, and by a distinct shortening of the latent period for these neoplasms with increasing quantities of BP administered. In addition, carcinomas of the respiratory tract were found only at the 2 highest dose levels (Table 3).

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Table 1. Cumulative mortality of male Syrian golden hamsters following 52 weekly intratracheal instillations of different doses of BP. Each dose group initially consisted of 30 animals

Group	Total dose administered (mg BP)	No. of deaths at the end of week						
		4	13	26	39	52	65	78
1	0	0	0	1	1	2	2	5
2	3·25	0	1	1	1	2	3	5
3	6·5	0	0	0	1	2	2	3
4	13	0	0	0	0	3	6	7
5	26	0	1	2	2	2	8	11
6	52	0	0	0	0	4	15	22

Table 2. Tumour induction in male Syrian golden hamsters following 52 weekly intratracheal instillations of different doses of BP. Each dose group initially consisted of 30 animals

Group	Total dose administered (mg BP)	Effective* no. of animals	No. of hamsters with tumours of				Total no. of respiratory tract tumours	No. of animals with non-respiratory-tract tumours†
			Respiratory tract (total %)	Trachea	Bronchi	Bronchiolo-alveolar region		
1	0	29	0 (0)	0	0	0	0	2
2	3·25	30	3 (10)	3	0	1	4	1
3	6·5	30	4 (13)	1	0	4	5	1
4	13	30	9 (30)	5	0	6	12	6
5	26	29	25 (86)	17	6	17	44	2
6	52	28	26 (93)	17	11	19	58	2

*A few animals were lost because of advanced autolysis or cannibalism.

†The non-respiratory tract tumours observed in the various groups included 10 adrenal cortical adenomas, 1 adrenal cortical carcinoma, 1 thyroid adenoma and 2 leukemias.

Table 3. Type and incidence of respiratory-tract tumours in male Syrian golden hamsters following 52 weekly intratracheal instillations of different doses of BP. Each dose group initially consisted of 30 animals

Sites and types of tumours	Incidences of tumours					
	Group 1 0 mg BP	Group 2 3·25 mg BP	Group 3 6·5 mg BP	Group 4 13 mg BP	Group 5 26 mg BP	Group 6 52 mg BP
Number of animals examined*	29	30	30	30	29	28
Trachea						
Polyp	0	0	0	0	2	0
Papilloma	0	3	1	5	9	6
Squamous cell carcinoma	0	0	0	0	5	11
Adeno-squamous carcinoma	0	0	0	0	1	1
Fibrosarcoma	0	0	0	0	1	0
Bronchi						
Polyp	0	0	0	0	2	1
Papilloma	0	0	0	0	1	2
Squamous cell carcinoma	0	0	0	0	2	4
Adeno-squamous carcinoma	0	0	0	0	1	2
Adenocarcinoma	0	0	0	0	0	4
Anaplastic carcinoma	0	0	0	0	0	1
Bronchioles and alveoli						
Papillary adenoma	0	0	2	1	6	3
Acinar adenoma	0	0	1	1	4	2
Adeno-squamous adenoma	0	1	1	5	7	11
Squamous cell carcinoma	0	0	0	0	2	4
Adeno-squamous carcinoma	0	0	0	0	0	3
Adenocarcinoma	0	0	0	0	1	1
Anaplastic carcinoma	0	0	0	0	0	2

*A few animals were lost through cannibalism or autolysis.

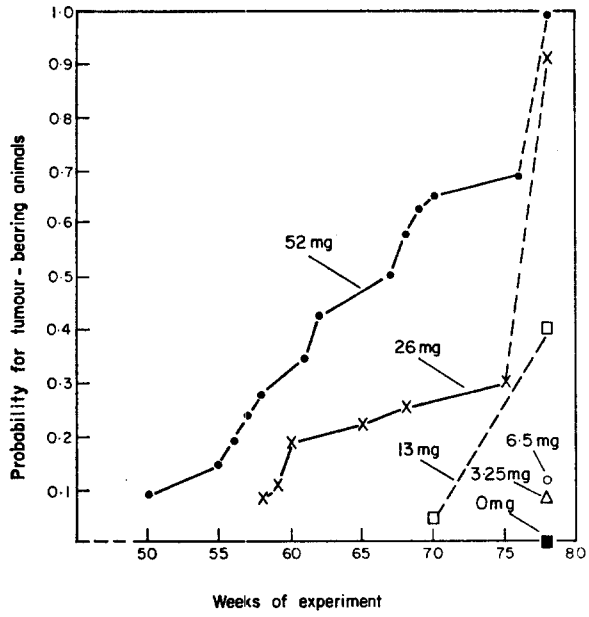

Fig. 1. Probabilities for the observation of a respiratory-tract tumour at death of male Syrian golden hamsters following 52 weekly intratracheal instillations of different doses of BP. The probabilities were calculated as described by Saffiotti et al. [2]. *Total dose of BP: 0 mg (■), 3·25 mg (△), 6·5 mg (○), 13 mg (□), 26 mg (×) and 52 mg (●). The last part of the graphs are dotted lines because the values at week 78, when all animals still alive were killed, were not calculated on the basis of the number of animals that died during that week with an observed respiratory-tract tumour, but on the basis of the number of animals that survived up to week 78 and showed a tumour of the respiratory tract.*

Tumours were classified as bronchial tumours only when their origin from bronchial epithelium was clearly recognizable. Several of the carcinomas destroying large portions of the lung parenchyma, here classified as tumours of the bronchiolo-alveolar region, actually might have been derived from the epithelium of larger bronchi. Gross and microscopic examination did not reveal tumours of the nasal cavity and larynx.

The most frequent non-neoplastic lesions observed in the respiratory tract included hyperplasia and squamous metaplasia of the tracheobronchial lining epithelium, peribronchiolar adenomatoid lesions and several types of alveolar metaplasia (squamous, mucous and mixed forms). Extent and frequency of these alterations clearly increased with increasing dose levels of BP.

The morphological aspects of the tumours and other changes induced in the respiratory tract were essentially those previously described in detail [1, 3, 4].

It can be concluded from this experiment that a positive dose–response relationship exists for the induction of respiratory-tract tumours in Syrian golden hamsters by repeated intratracheal instillations of BP particles suspended in 0·9% NaCl solution. In addition, the present observations confirm our previous finding that, using the above model system, ferric oxide particles are not indispensable for the induction of respiratory-tract tumours by BP [3].

As to the type and incidence of the observed respiratory-tract tumours, there were no essential differences between BP + ferric oxide studies in hamsters [1, 2, 4] and our BP study; however, the latent period of the tumours was clearly longer in hamsters receiving BP alone.

Our results differ from those reported by Kuschner [5], who did not observe respiratory-tract tumours in hamsters following multiple intratracheal injections of large BP doses given without a carrier dust. The reason for this discrepancy is not clear. As regards our technique, a high reproducibility of the results is suggested by the preliminary data of another similar experiment [6]. Therefore, the present data furnish a base-line for studying co-carcinogenic factors in respiratory-tract carcinogenesis.

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Letter to the Editor

DNA–Daunorubicin Complex: Preliminary Trials In Human Leukaemia*

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WE REPORT here the results of preliminary clinical trials carried out with the daunorubicin–DNA complex (DRC–DNA), a "lysosomotropic" form of daunorubicin shown experimentally to be less toxic and more effective than the free drug in the treatment of L 1210 leukaemia in mice [1].

pyrogen removal. The DRC solution was mixed sterilely with the DNA solution in a ratio of 1–100 (v/v) under vigorous stirring.

The complex was used within 24 hr after preparation. It was given to the patients by intravenous infusion at a maximum rate of 100 ml/hr (corresponding to 20 mg/hr DRC).

Table 1. *Effects of treatment on leukaemic blood cells*

Patients						
No.	Sex	Age	Diagnosis	Initial values of blood blasts (per mm³)	Total dosis mg/kg DRC–DNA	Blood blast cells after treatment (per mm³)
1	F	19	A.M.L.*	69,000	4	25,300
2	M	5½	A.M.L.*	85,000	4	177
3	F	41	A.M.L.*	64,500	4	3,100
4	M	40	A.M.L.*	279,000	6	105,000
5	M	68	A.M.L.	319,000	6	2,420
			6 weeks later	247,500	14	782
6	M	62	Lymphosarc. A.L.L.	429	4	0
7		71	A.M.L.*	130,000	AMC–DNA 1,5	0

*Myeloblastic transformation of chronic myelogenous leukemia.

Daunorubicin (Specia, Brussels) was dissolved in sterile pyrogen-free 0·15 M NaCl to a concentration of 20 mg/ml. Calf thymus DNA (Type V, Sigma, St. Louis) was dissolved in the same medium to a concentration of 2·34 mg/ml, and autoclaved for 15 min at 120°C. This solution was then filtered in batches of 500 ml on a EKS II Seitz Filter (14 cm in dia.) for

In Table 1 is shown the effect of DRC–DNA (1–2 mg/kg per injection) on 6 terminal patients, 4 of whom were entirely resistant to free DRC (cases 1–4). Another patient was treated with the DNA complex of adriamycin (Farmitalia, Milan). This complex (AMC–DNA) has the same properties as DRC–DNA and has an even better therapeutic index on leukaemic mice (Trouet and De Smedt-Malengreaux, unpublished results).

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Marked reduction of leukaemic blood cells was obtained consistently, even in the 4 resistant cases. The effects were long-acting, with lowest blast cell counts occurring as late as 3–9 days after the last injection, thanks presumably to the prolonged circulating time of the complex. Medullary remissions were not observed, but the dosage was kept very low in these preliminary trials.

The patients usually tolerated injection of the complex with little or no manifestation of discomfort, objective or subjective. A febrile reaction was occasionally observed. Allergic reactions due to sensitization to DNA were not observed and immunofluorescent tests to detect anti-DNA antibodies remained negative.

Toxic reactions such as stomatitis or oesophagitis were not observed, even in patient 5, aged 69, who received a total dosis of 20 mg/kg. There were no signs of cardiac toxicity.

Blood granulocytes and platelets generally fell with the blast cells, but to a lesser extent. No major problem arising from haemorrhagic manifestations or infections was encountered.

Based on these findings, a scheme of combination therapy was elaborated. It takes advantage of the cell synchronization obtained by means of vincristine and ARA-C and of the long-acting effect on DRC–DNA.

It consists in a first course of two injections of vincristine (1 mg/m^2 each, 12 hr interval) followed after 24 hr by two injections of DRC–DNA (60 mg/m^2 each, 24 hr interval). In further courses, given at 8 day intervals until complete remission, vincristine is replaced by one injection of ARA-C (200 mg/m^2, i.v.) followed by two injections of DRC–DNA as above. Essentially the same scheme is followed with AMC–DNA.

Up to to-day, seven patients, all fresh previously untreated case of A.M.L., have undergone this kind of combination therapy. One achieved complete remission after two courses and two others after three. The other patients are still under treatment, having received no more than two or three courses. At least two of them are on the verge of a complete remission.

Further studies on a larger scale and under various conditions are certainly necessary before we can evaluate the exact place and value of this new drug, given alone or in combination with others in the treatment of acute leukaemia. The promising results obtained to date and especially the observed activity at low cost of toxicity, definitely justify trials on a larger scale and by other groups. They also validate the concept of lysosomotropic drug [1] and recommend further research inspired by this concept.

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Foreword

THE REMARKABLE progress made in treating advanced or generalized human cancer is mainly the result of the search for new compounds blocking selectively the multiplication of neoplastic cells.

Additive therapy to treat hemorrhages and infections, the two major complications of malignant diseases and chemotherapeutic treatment, has also contributed to better results. Indeed, systemic local infection is often the cause of the patient's death.

Many infections are preventable or curable if adequate care of these complications is taken. In particular, the realization of a protected environment combined with sterilisation of the digestive tract seem sometimes appropriate, as well as empiric therapy in high risk patients. However, controlled clinical studies are lacking in most instances, and are badly needed.

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Optimal Antibiotic Therapy for Infections in Patients with Cancer: Prospects for the Future

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CONSIDERABLE clinical effort and large amounts of money have been spent over the recent years to improve the quality and the duration of life of patients with disseminated malignant tumors.

In some areas, such as lymphoblastic leukemia in children, these efforts have been partly successful, owing probably to the use of aggressive chemotherapeutic regimens. This form of therapy has been supported by extensive laboratory investigations and its efficacy has been tested in large cooperative clinical trials.

It is unclear, however, to what extent supportive therapy has played a role in the improved survival rate of patients treated with cytostatic therapy; undoubtedly, some forms of the supportive care, such as platelet transfusions, are important [1], but the role of many others remains to be investigated. This is particularly true with respect to the prevention and therapy of infectious complications which occur so often in patients with cancer and represent, in this clinical setting, the major cause of death [2].

The prevention of serious infections through the realization of a "sterile" environment and the use of granulocyte transfusions as an adjunct to antibiotics have been investigated in granulocytopenic patients with cancer and encouraging, though not dramatic, effects have been reported. Certainly, more work is needed in these areas; however, it might be suspected that techniques such as complete bacteriologic isolation and granulocytes transfusions will be beneficial to only a relatively small number of patients who will be admitted to a few centers equipped with elaborated facilities.

Up to now, the adequate administration of effective antimicrobial agents remains the only form of antibacterial therapy that has proven itself to be essential in the management of severe infections.

Since the principles of diagnosis and treatment of infections that occur in patients with cancer are basically similar to those used for other forms of infectious illness, it might be suspected that the administration of antibiotics that are active on the offending microorganism will also be of critical importance in the therapy of infections in patients with compromized host defences.

That antimicrobial therapy must be instituted early in severe infections has been already stressed by Finland, well before the advent of antimicrobial drugs, in patients with pneumonia treated with anti-pneumococcal sera [3]. Later, several experimental and clinical studies have re-emphasized the need for early therapy in potentially lethal sepsis: many infectious diseases may cause, within the infected host, changes that are no longer reversible after a critical period even if large amounts of antibiotics, to which the offending microorganism is exquisitely sensitive, are administered.

In the patient with extensive cancer, who frequently is receiving therapies that further impair his capacities for antibacterial defence, the need for early antimicrobial therapy when sepsis is suspected might be even more important than in other clinical conditions [4]. First, in the compromized host, bacterial infection may be fulminant and kill the patient within a few hours; second, the discovery of an infectious process may be particularly difficult in patients with depressed host

resistance, especially those with cancer—in this type of patient the unusual clinical and laboratory signs of infection (fever, chills, leukocytosis, etc.) may be absent and, moreover, neoplastic disease may mimic infection.

The latter observation is important to justify the administration to patients with cancer, when severe sepsis is suspected, of antibiotics that will be chosen empirically, without waiting for the isolation of the offending microorganism. Obviously, empiric therapy will not be used when gram-stained smears, cultures, or epidemiological data strongly suggest a precise bacteriological etiology. In the other circumstances, when severe sepsis is suspected without knowing the nature of the offending microorganism, empiric therapy will be undertaken only after extensive culturing.

Most of the infections occurring in patients with extensive neoplastic diseases are "opportunistic" infections in the sense that many are caused by microorganisms belonging to the normal flora of the host and are generally regarded, in other circumstances, as nonpathogenic. These infections are caused by a wide spectrum of bacterial, fungal, viral, and parasitic organisms leading to a variety of clinical pictures, which are not, however, entirely diagnostic of the bacteriologic etiology [5].

Among the opportunistic pathogens that cause severe infection in patients with cancer, enterobacteriacae and *Pseudomonas aeruginosa* are the most frequent, and therefore empiric antimicrobial therapy should be directed primarily against these pathogens. The increased frequency of infections caused by these gram-negative rods is not restricted to patients with cancer; it has been observed in most hospitals in the world since the introduction of antibacterial drugs and has progressively increased in spite of the use of new antibiotics active on gram-negative rods [6].

Which antibiotics are to be administered empirically to the patient with cancer and suspected sepsis is to be discussed at this meeting. Although the administration of several antibiotics at the same time is not in accordance with the usual principles of antimicrobial therapy, it may be suspected that a combination of drugs will be recommended as the optimal therapy since no single antibiotic has a broad enough spectrum to be recommended safely alone.

Whatever this combination of antibiotics might be today, more studies will be necessary in the future to compare various antibacterial regimens and to investigate new drugs. Therefore, an essential goal of this meeting should be the definition of a basis for the evaluation of the results of antimicrobial therapy in the compromized host with extensive cancer.

According to Freid and Vosti [7], patients with ultimately fatal underlying diseases such as metastatic cancer, chronic leukemia, or lymphoma and those with rapidly fatal diseases such as acute leukemia represent, respectively, a homogenous population with respect to the outcome of gram-negative bacteremia.

Should other parameters such as the source of the infection or the nature of the offending microorganism be taken into consideration when the effectiveness of antimicrobial regimens are being evaluated? Should infections other than bacteremia and "infections" without demonstrated pathogenic microorganisms be used to test new antibiotic regimens? To what extent should we pay attention to neutropenia, cytostatic drugs, corticoids, and other forms of therapy? Finally, we have to decide what are the criteria for cure and failure of antimicrobial treatment in patients with cancer and what is the significance of superinfection for this evaluation. It should also be asked whether there are simple quantitative methods to determine the antibacterial activity in tissues and in body fluids which could be predictive of clinical effectiveness.

None of these questions has received a clear-cut answer up to now. Obviously, the optimal evaluation of a therapy should include consideration for all the cited factors and, possibly, for many others. However, it would be impossible for one investigator alone, to study over a short period of time a sufficient number of absolutely comparable patients. Thus, the conclusion is very clear: an effort to establish cooperative studies of the evaluation of various therapeutic regimens in patients with cancer and infection should be undertaken. The U.S. Veterans Administration—Armed Forces Cooperative studies on the treatment of tuberculosis proved that with proper leadership and organization and with active, voluntary and well-co-ordinated participation of several groups of investigators using carefully prepared protocols, it is possible to answer important therapeutic questions [8]. Another example of cooperative trial in the field of infectious diseases is the one started in 1971 by the EORTC in order to study in patients with acute leukemia the respective merits of reverse isolation coupled with gastro-intestinal decontamination [9]. Its organization and protocol might be the stimulus and the model for new studies of the preventive and therapeutic measures to be used in patients with cancer and infection.

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Changing Patterns of Infection in Cancer Patients

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Abstract—*Overwhelming infection is a frequent occurrence in cancer patients and death often supervenes before the infecting organism can be identified. The changing spectrum of organisms isolated from infected neutropenic leukaemia patients at Hammersmith Hospital is reported and compared with the organisms isolated in other series. The reasons for this changing pattern are discussed and lead to the rational basis for the choice of a combination of four antibiotics—carbenicillin, cephalothin, gentamicin and clindamycin—as initial empirical therapy for presumptive infection.*

INTRODUCTION

PYREXIAL episodes are not uncommon in cancer patients, especially those with leukaemia and neutropenia, and overwhelming infection is a frequent cause of death [1–4]. Patients often die before the infecting organism can be identified bacteriologically. As the classical causes of infection, such as *Staph. aureus* and *Ps. aeruginosa*, appear to be occurring relatively less frequently, it becomes increasingly important to be aware of the nature and antibiotic sensitivities of all potential pathogens in order to be better able to make a rational choice of antimicrobial agents for initial therapy.

We report here the nature of the infecting organisms isolated in Hammersmith Hospital from neutropenic patients with malignant blood diseases over a period of 33 months, and discuss possible reasons for the changing pattern of infection which we have observed.

MATERIAL AND METHODS

The patients had leukaemia, lymphoma or aplastic anaemia as a primary diagnosis. In general, patients with a pyrexia of 38°C or over for 48 hr, in the absence of obvious non-bacterial causes such as coryza or drug therapy, were investigated. Clinical judgement in each case was, however, taken into account. Thus, in in a very ill febrile patient a 48-hr wait was considered unreasonable and bacteriological specimens were taken earlier.

Blood cultures were taken on three occasions and urine, sputum, and stool were cultured, as were swabs from nose, throat, vagina, skin and any possibly relevant lesions, e.g. furuncles, mouth ulcers, i.v. sites. All pathogens isolated were identified by standard methods [5].

The observations were made over two separate but consecutive periods of time. During the first period, from April 1970 till October 1971, patients were nursed in open wards of the "Nightingale" type with 20 or more beds. In the second period, from October 1971 till December 1972, patients were nursed in a new purpose-built ward, with single rooms, protective isolation facilities and bacteriological control of environmental contamination.

RESULTS

Table 1 shows the organisms isolated from patients during the first period of observation. *Ps. aeruginosa* accounted for 11 (55%) of the 20 infections, *Staph, aureus* and klebsiella for 3 (15%) each, and *E. coli* for 2 (10%).

Table 2 shows corresponding data for the second period. *E. coli* was responsible for 7 (28%) of the 25 infections, *Staph. aureus*, *Clostridium welchii* and *Ps. aeruginosa* for 3 (12%) each, and klebsiella for 2 (8%). Other organisms were isolated from the remaining 7 (28%) infections.

A summary of the chemotherapeutic regimens received by patients in relation to their infecting agents is shown in Table 3. No definite

relationship can be seen between the infecting organisms and the remission induction chemotherapeutic regimens, but it appears that patients with pseudomonas infections received fewer chemotherapeutic agents.

Table 1. *Organisms isolated from patients* 1970–1

Organism	No. of infections	% of total
Ps. aeruginosa	11	55
Staph. aureus	3	15
Klebsiella	3	15
E. coli	2	10
Ch. aureum	1	5
Total	20	100

Table 2. *Organisms isolated from patients* 1971–2

Organism	No. of infections	% of total
E. coli	7	28
Staph. aureus	3	12
C. welchii	3	12
Ps. aeruginosa	3	12
Klebsiella	2	8
Streptococcus (haem.)	1	4
Staph. albus	1	4
Micrococcus	1	4
Diptheroids	1	4
Pneumocystis	1	4
Proteus	1	4
Bacillus	1	4
Total	25	100

Table 3. *Chemotherapeutic regimens and infective agents*

Organism	Chemotherapy A	B	C	D	E	F	G	H	I	J
Ps. aeruginosa	X	X	X	X	X	X				
Ps. aeruginosa	X	X		X						
Ps. aeruginosa			X			X				
Ps. aeruginosa + klebsiella	X	X	X		X					
Ps. aeruginosa + klebsiella + *E. coli*	X	X	X	X						
Ps. aeruginosa + *E. coli*			X							
Ps. aeruginosa					X					
Ps. aeruginosa + *St. aureus*	X	X	X	X		X		X		
Ps. aeruginosa			X		X	X				
Ps. aeruginosa			X							
Ps. aeruginosa	X	X	X	X	X	X		X		
Ps. aeruginosa	X	X	X	X	X	X		X		
Ps. aeruginosa			X							
Ps. aeruginosa	X	X	X	X						
St. aureus	X	X	X	X	X	X		X		
Ch. aureum	X			X						
Klebsiella	X	X	X	X						
St. aureus	X	X								
St. albus										X
Cl. welchii	X	X	X	X	X	X		X		
E. coli			X		X				X	
Pneumocystis	X	X	X	X	X	X			X	
St. aureus	X	X	X	X	X	X			X	
E. coli + *Cl. welchii*	X	X	X	X						
Klebsiella	X	X	X	X						
E. coli	X	X	X	X	X	X		X		
E. coli	X	X	X	X	X	X		X		
Streptococcus			X							
E. coli + klebsiella			X							
Cl. welchii + micrococcus	X	X	X	X	X	X		X		
St. aureus	X	X	X	X	X	X		X		
St. aureus	X	X	X	X	X	X		X		
Diptheroid	X	X	X	X	X	X		X		
Proteus	X	X	X	X						
E. coli	X	X								
Bacillus	X	X								
E. coli	X	X								

A = Cytosine arabinoside. B = Daunorubicin. C = Prednisolone.
D = Thioguanine. E = Cyclophosphamide. F = Vincristine.
G = 6-mercaptopurine. H = Methotrexate. I = Chlorambucil.
J = Busulphan.

Table 4. Relative frequencies of occurrence of pathogenes in various series

Organisms	Frequency of isolation (%)													
Ps. aeruginosa	55	12	31	17	11	21	25	27	29	26	24	14	8	6
Esch. coli	10	28	20	11	32	22.5	6	15	10	13	10	19	21	25
Klebsiella/Enterobacter	15	8	11	2	5		3	12	14	15	9	15	16	17
Proteus		4	2.5	4	5	7	5		4	1	3	17	12	11
Staph. aureus	15	12	13	28	30	15.5	3	19	7	13	27	10	5	11
Staph. albus/Micrococcus		8	4		7	4		8	21	1		2		
Streptococcus (haem.)		4	2.5	4	2	6			1	1	7		5	2
Strep. faecalis						3			4	5	1	9	16	3
Clostridia		12	7	6	2	3			1					
Other bacteria		8	4	4	6	18	4	11	9	9	7	13	17	25
Candida				19			28	8		15	10			
Other fungi	5		2.5	5			25			1	2	1		
Pneumocystis		4	2.5				1							
DATES	1970–1	1971–2	1970–2	1953–6	1955–6	1958–67	1962–3	1966–70	1967–9	1969–71	1955–7	1969–70	1971	1969–70
PLACES	Hammersmith, London		U.S.A.	U.S.A.		Australia	U.S.A.	London	France	U.S.A.	U.S.A.	U.S.A.	U.S.A.	U.S.A.
PATIENTS	Leukaemia			LEUKAEMIA							Cancer	Hospital		Community
REFERENCES	3			11	12	13	11	14	15	16	17	18	19	18

DISCUSSION

During the two periods of observation in our study the organisms isolated occurred with very different frequencies. *Ps. aeruginosa* accounted for 55% of the first group but only 12% of the second, while *E. coli* increased in incidence from 10 to 28%. Gram-negative bacilli, though still remaining the most frequent pathogens, dropped in incidence from 80 to 52%. The overall frequencies for the two periods together are similar to those in other series (Table 4), but it is interesting to observe that the frequency of pseudomonas infections appears to increase between 1950 and 1970 as also does that of infections due to the klebsiella/enterobacter group, whilst that of *Staph. aureus* infections remains relatively constant or declines slightly. However, we and others [6] have observed a decline in pseudomonas infections over the past year, and it is to be hoped that this trend will continue to be observed elsewhere. One conclusion does, however, clearly emerge. The spectrum of infection not only varies from country to country and from one hospital to another but it also changes with time. Thus, it would seem difficult and perhaps dangerous to predict future patterns of infection.

The reasons for the changes we have observed are not easy to explain and several factors may be responsible. At the beginning of the second period a new ward, specially designed for the management of leukaemia patients, was opened and the second group of patients was treated in this ward. Its single rooms are equipped with modern protective isolation facilities, air-conditioning with filtered air and various features (such as heated sink traps) [7] designed to reduce environmental contamination. Regular bacteriological surveillance of patients and environment has been maintained and there has been little evidence of cross-infection within the ward nor has there been any evidence of heavy contamination of the environment. Many of the patients in the second group received oral non-absorbable antibiotics to suppress endogenous intestinal flora during their remission induction chemotherapy and the effects of these antibiotics on the bowel flora were monitored. In no case was *E. coli* persistently isolated from the faeces of these patients, making the increased frequency of septicaemia due to this organism difficult to explain on the basis of systemic invasion by intestinal commensal organisms.

Goldschmidt and Bodey [8] have suggested that chemotherapeutic agents used for remission induction may give *Ps. aeruginosa* a selective advantage over other bowel organisms thus leading to a predominance of pseudomonas infections in leukaemia patients. From Table 3, no relationship can be seen between chemotherapeutic regimens and the nature of the infecting organism. If anything, the patients with pseudomonas infections received *fewer* chemotherapeutic agents than the others. The shift towards *E. coli* cannot be explained on the basis of this theory.

Perhaps the reasons for the changes are less tangible. Over the years we have become more conscious of the dangers of infection with *Ps. aeruginosa*. We have improved our wards, techniques, isolation facilities, and hygienic standards and have perhaps produced an environment less favourable to this interesting and troublesome organism. Whatever the reasons it is clear that leukaemic and neutropenic patients are still prone to infection by a variety of organisms and we must be prepared to treat any such infection promptly—before culture or antibiotic sensitivity results can be obtained.

The organisms isolated from leukaemia patients are no different in their sensitivity patterns from the organisms infecting other patients in the hospital. Thus, the sensitivities of potential pathogens are predictable and may be obtained from any reliable textbook on the subject [9]. The sensitivities of the major groups of organisms to four antibiotics are outlined in Fig. 1.

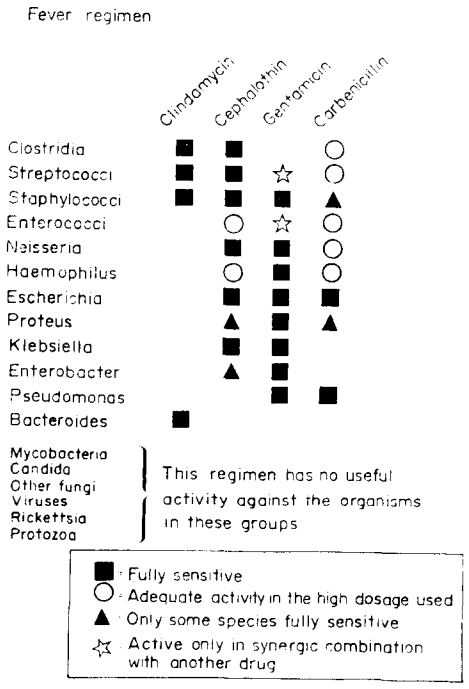

Fig. 1. Sensitivities of potentially pathogenic bacteria to a combination of four antibiotics.

Various authors have suggested antibiotic combinations of one sort or another as initial treatment for presumptive infection in patients with reduced resistance to infection. Schimpff *et al.* [10] have reported on the use of a carbenicillin–gentamicin combination which was effective in over 60% of patients with pseudomonas infection, but less so in those with klebsiella and *E. coli* infections. Levine *et al.* [4] suggest two different empirical antibiotic regimens, *viz*, gentamicin with or without a cephalosporin or penicellinase-resistant penicillin and carbenicillin with a cephalosporin. Tattersall *et al.* [3] have previously reported on the use of a combination of five antibiotics as initial therapy for febrile patients with leukaemia and neutropenia. This was the combination used during the first period of the study, and the antibiotics were gentamicin, cephalothin, carbenicillin, clindamycin and methicillin (Table 5).

Because *Staph. aureus* was isolated in only 15% of the infections during that period, and because the other antibiotics provide adequate cover for *Staph. aureus*, methicillin was omitted from the combination used in the second period (Table 5). At that time we considered whether any of the other agents could be omitted but decided that it would be unsafe to do so as the removal of any other antibiotic would leave gaps in the spectrum of cover (Fig. 1). Gentamicin is the agent with the broadest spectrum in the combination, but it should not be used alone. Therefore, cephalothin is added to give extra cover against staphylococci, streptococci, clostridia, enterococci, escherichia, and klebsiella. Carbenicillin is added for pseudomonas and clindamycin for bacteroides, clostridia, streptococci, and staphylococci.

Table 5. Antibiotic combinations used

Period	Drug	Dosage (per sq. m. per day)	Route
November 1970– October 1971	Methicillin	8 g	i.v.
	Carbenicillin	20 g	i.v.
	Cephalothin	4 g	i.v.
	Gentamicin	140 mg	i.v.
	Clindamycin*	1200 mg	p.o.
	Probenecid	1250 mg	p.o.
October 1971– December 1972	Carbenicillin	20 g	i.v.
	Cephalothin	4 g	i.v.
	Gentamicin	140 mg	i.v.
	Clindamycin*	1200 mg	p.o.
	Probenecid	1250 mg	p.o.

i.v. = intravenous. p.o. = oral.

*If patient unable to take clindamycin orally, substitute: Lincomycin 1500 mg per sq. m. per day.

The results of using this combination are reported elsewhere in this Symposium (Tattersall *et al.*). We stress that when infection is suspected, empirical antibiotic therapy must be started immediately after taking the appropriate diagnostic specimens. Delay in initiating therapy reduces the chance of a successful outcome, and the broader the spectrum of cover in the early stages, the better. Once culture results and antibiotic sensitivities become known, or if the patient fails to respond to the initial therapy, appropriate modifications to the regimen may be made.

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Antibiotic Combinations for Gram-Negative Infections in Patients with Cancer*

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Abstract—*Various antimicrobial regimens that have been recently employed at the Institut Jules Bordet and by other investigators have been reviewed.*

In addition, a series of 40 *patients with cancer and gram-negative infections who have been treated with carbenicillin and cephalothin is presented.*

As could have been expected from in vitro *studies, the clinical results were encouraging* (80% *of satisfactory responses in septicemic patients) and comparable to those obtained with the combination carbenicillin plus gentamicin.*

The need for further clinical and laboratory studies of antibiotic regimens to be used empirically in patients with poor host-defence mechanisms against infection has been stressed.

INTRODUCTION

SINCE effective therapy is now available for some malignant diseases, successful management of infections in patients with cancer has become increasingly important.

In this type of patients, who have impaired host-defence mechanisms against infection as a result of their malignant disease or its treatment, a high frequency of bacterial infections is usually observed.

Although the cancer patient is susceptible to a wide variety of microorganisms, the majority of infections in these patients is caused by gram-negative bacilli. During the period from January 1970 to June 1972, 64% of the episodes of bacteremia observed at our institution were caused by gram negative bacilli. Among these microorganisms, *Escherchia coli*, Klebsiella–Enterobacter species and *Pseudomonas aeruginosa* were most frequently isolated [1].

The mortality associated with severe gram-negative infections in patients who present rapidly or ultimately fatal diseases is extremely high [2, 3, 4]. Death frequently occurs, especially in granulocytopenic patients, within a short time of the initial positive blood culture.

These observations suggest the need for early therapy with antibiotics for the patient with cancer who is suspected to present a serious infection. Treatment should be initiated, after extensive culturing, once a presumptive diagnosis of sepsis is made, without waiting for the isolation, identification and study of the *in vitro* sensitivity of the offending pathogen.

The choice of the optimal antimicrobial therapy is usually based on the known susceptibility of the various bacteria that are encountered in similar clinical conditions within a particular hospital. However, only clinical trials with antibiotics active against Enterobacteriacae and *Pseudomonas aeruginosa* can indicate which antimicrobial regimen might represent the optimal treatment.

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Bodey *et al.* found a response rate of 59% when carbenicillin alone was used as an initial treatment in patients with cancer for infections caused by gram-negative rods; in their study, however, infections caused by *Pseudomonas aeruginosa* were cured in 91% of 23 episodes [5]. These investigators evaluated also the therapy with gentamicin in a similar clinical setting: 56% of the patients responded well [6]. However, only a minority of infections that occurred in patients with less than 100 neutrophils per mm^3 responded favorably.

Carbenicillin and gentamicin are synergistic against many Enterobacteriacae and *Pseudomonas aeruginosa* [7]; therefore their use in combination has been advocated for empiric treatment for febrile patients with cancer and granulocytopenia [8]. A response rate of 64% has been found by Schimpff and coworkers and 56% by Bodey *et al.* [8, 9].

More recently, carbenicillin has been used with cephalothin or kanamycin; granulocytopenic patients who had infections caused by gram-negative rods responded to these regimens in 62% and 48% of the cases respectively [10]. Initial therapy with a combination of 5 antibiotics (methicillin, carbenicillin, cephalothin, gentamicin, clindamycin) in patients with leukemia and infections caused by gram-negative rods has resulted in a therapeutic success in 7 [58%] of 12 patients [11].

From these studies, which are summarized in Table 1, it is difficult to determine the optimal initial therapy for infections in patients with cancer; although all the patients studied presented a neoplastic underlying disease and severe infection caused by gram-negative rods, many other variables may have influenced the clinical outcome. Several combinations of antibiotics have been tested during the past 3 years in our hospital as empiric therapies for gram-negative infections occurring in patients with cancer [12, 13, 14]. The combination of carbenicillin and gentamicin (carb–gen) has been found effective as well as the association of carbenicillin and cephalothin (carb–ceph). In the present study, we present data about the effectiveness of these 2 antimicrobial regimens.

MATERIAL AND METHODS

Bacteria

All the bacteria tested in the present study were pathogens isolated from hospitalized patients at the Institut Jules Bordet, which is the clinical center for cancer therapy of Brussels University. They included 140 *Escherichia coli*, 90 Klebsiella–Enterobacter strains, 91 Proteus or Providence and 64 *Pseudomonas aeruginosa*. These strains were tested simultaneously for their susceptibility to gentamicin, carbenicillin, cephalothin, carb–gen (33:1; weight:weight proportion) and carb–ceph (4:1; weight:weight proportion). The sensitivity of the bacteria studied here to these antibiotics alone or in combination was determined by the inocula-replicating method [15]. Muller Hinton agar (Difco) and overnight cultures in Muller Hinton broth diluted to 10^{-4} were used throughout.

A combination was considered to be synergistic when it was at least 4 times as effective as either drug alone.

Patients

The combination of carb–gen has been given to 57 patients; all of them received 30 g of carbenicillin daily as 3 intravenous injections of 10 g in 5% dextrose in water over a period of 30 min; 34 received 3–4 mg/kg per day gentamicin and 23 received 6–8 mg/kg per day. Gentamicin was given in 3 divided doses intramuscularly except in a few thrombocytopenic patients in whom the intravenous route has been used.

Carb–ceph has been given to 40 patients; 30 g of carbenicillin and 9 g of cephalothin were given in all of them as 3 daily intravenous

Table 1. *Results of empiric therapy in cancer patients presenting bacterial infections caused by gram-negative rods*

	Number of patients	Percentage clinical success
Carbenicillin + Gentamicin [8]	39	64
Carbenicillin + Gentamicin [9]	32	56
Carbenicillin + Cephalothin [10]	16	62
Carbenicillin [5]	56	59
Gentamicin [6]	76	56
Carbenicillin + Kanamycin [10]	25	48
Methicillin + Carbenicillin + Cephalothin + Gentamicin + Clindamycin [11]	15	53

injections performed successively over a period of 30 min for each of them.

No other antibiotics were given to these patients at the same time, and therapy was started in all of them as soon as a presumptive diagnosis of gram-negative infection had been made.

The general characteristics of the patients included in these 2 series are summarized in Table 2.

The frequency of severe neutropenia (less than 1500 granulocytes per mm^3) and the frequency of acute leukemia, chronic leukemia or lymphoma, and metastatic carcinoma were similar in both groups. The distribution of the various pathogenic organisms responsible for the infectious episodes in this series was also similar in both groups.

It should be pointed out, however, that more septicemias were documented in the carb–ceph group and more pulmonary infections in the other. Although no significant differences in the outcome of patients with gram-negative septicemias or pneumonias could be found in previous studies from this laboratory, the higher number of septicemic patients in the carb–ceph group have influenced the results [16].

A patient was considered to be "cured" when the clinical signs of infection disappeared and the patient survived or died from causes unrelated to the infection. The asymptomatic persistance of the offending microorganism at tracheotomy sites or wounds, and asymptomatic colonization was not considered necessarily to indicate a failure of antimicrobial therapy.

Bacterial colonization was defined as the appearance or significant increase in number of a potential pathogen in cultures of sputum, urine, or wounds after antibiotic therapy was begun. Superinfection was defined as a new clinical infection caused by the colonizing agents.

A patient was considered to represent a failure of the antibacterial therapy if he died as a result of his infection or superinfection or if the antibiotics had to be changed because of a lack of therapeutic response.

RESULTS

Antimicrobial activity in vitro. Table 3 shows the proportion of strains killed by concentrations of carbenicillin plus gentamicin (carb–gen) and carbenicillin plus cephalothin (carb–ceph) readily attainable in the blood stream after treatment with usual doses. Carb–ceph was active on less strains of Klebsiella–Enterobacter and *Pseudomonas aeruginosa* than carb–gen and this difference is statistically significant.

These data do not bear upon the issue of synergism but demonstrate that carb–gen provides a broader coverage against gram-negative rods isolated from patients with serious infections than carb–ceph. The respective advantages of carb–gen and carb–ceph over each drug alone is also ilustrated in Table 3; since these aspects have been discussed already in previous studies from this laboratory [7, 17], they will not be further considered here.

Table 4 shows the frequency with which synergism, as defined in the Material and

Table 2. Characteristics of the population studied

	Carbenicillin + Gentamicin				Carbenicillin + Cephalothin	
	Gentamicin 3–4 mg/kg No.	Gentamicin 6–8 mg/kg No.	Total No.	%	Total No.	%
Number	34	23	57		40	
Ratio male/female	1·6	2·8	2·2		1·7	
Mean age (years)	58	59	58		57	
Leucopenia (1500 granulocytes per mm^3)	11	3	14	24	8	20
Acute leukemia	5	2	7	12	5	12
Lymphoma–Chronic leukemia	6	2	8	14	4	10
Metastatic carcinoma	23	19	42	74	31	77
Septicemia	6	5	11	19	20	50
Pulmonary infection	18	10	28	49	9	22
Urinary infection	4	2	8	14	2	5
Wound infection	6	6	12	21	9	22
Escherichia coli	3	7	10	17	10	25
Klebsiella–Enterobacter	8	1	9	16	10	25
Proteus–Providence	9	5	14	24	8	20
Pseudomonas aeruginosa	14	10	24	42	12	30

Table 3. Percentage of strains inhibited by carbenicillin, gentamicin, and cephalothin alone or in combination

Antibiotics concentration (ug/ml)	Car 25	Gen 0·7	Cep 6	Car–Gen 25–0·7	Car–Cep 25–6	Car 100	Gen 3	Cep 25	Car–Gen 100–3	Car–Cep 100–25
Escherichia coli (140)	74	92	72	99	96	79	97	82	100	99
Klebsiella–Enterobacter (90)	19	85	67	97*	78*	47	89	71	99	81
Proteus–Providence (91)	79	86	87	100	100	81	92	91	100	110
Pseudomonas aeruginosa (64)	70	12	5	95†	81†	92	72	6	100	95
Total (385)	36	76	64	97	90	74	90	67	99	94

*$p < 0{\cdot}01$ †$p < 0{\cdot}02$

Table 4. Frequency of synergism between carbenicillin and gentamicin or cephalothin against gram-negative rods isolated from patients with severe hospital infections

	Number of strains	Percentage of strains on which antibiotics were synergistic: Carbenicillin + Gentamicin	Carbenicillin + Cephalothin
Escherichia coli	140	40.5	73·2
Klebsiella–Enterobacter	90	52·6	58·6
Proteus–Providence	91	42·8	76·1
Pseudomonas aeruginosa	64	70·1	42·2
Total	385	50·3	60·4

Methods section, has been found here. Carb–gen was synergistic on more strains of *Pseudomonas aeruginosa* than carb–ceph; the latter combination was more frequently synergistic on *E. coli* and Proteus strains. Both combinations were synergistic on similar numbers of strains of Klebsiella–Enterobacter.

Compared effectiveness of carb–gen and carb–ceph

The efficacy of carb–gen has been previously reported from this laboratory [14, 18] and will not be discussed in detail here. As shown in Table 5, the combination carb–ceph was used empirically in 40 patients with a proven diagnosis of gram-negative infection. In 20 of them septicemia could be documented and 16 (80%) of these bacteremic patients, recovered. The 4 patients who did not respond to therapy with carb–ceph were all severely granulocytopenic and all had terminal acute leukemia not responding to cytostatic chemotherapy. Three other granulocytopenic patients with septicemia caused by gram-negative rods survived as well as 2 granulocytopenic patients who presented a wound infection and a pyelonephritis respectively. Four non-granulocytopenic patients failed to respond to carb–ceph treatment. One had a large perinephritic abscess resulting from the perforation of a colonic carcinoma into the retroperitoneal space. He ultimately recovered after several surgical procedures. Another patient, who presented a severe bronchopneumonia due to a Klebsiella which was resistant to both carbenicillin and cephalothin, died before therapy could be changed according to the *in vitro* studies.

Two other patients responded well initially to carb–ceph; one presented subsequently a severe hemorrhage for which no obvious etiology could have been found, except a prolongation of the one-stage prothrombine time which appeared a few days after the onset of antimicrobial therapy with carb–ceph. Another patient presented during therapy with carb–ceph a severe hypokaliemia and died suddenly before adequate amounts of potassium could have been administered. These 2 complications were considered as possibly related to the administration of high doses of carbenicillin and the course of these patients was considered as unsuccessful. Owing to the relatively small

Table 5. Clinical results with the combination carbenicillin–gentamicin and carbenicillin–cephalothin

	Carbenicillin + Gentamicin			Carbenicillin + Cephalothin		
		Clinical success			Clinical success	
	No.	No.	%	No.	No.	%
Acute leukemia	7	4	57	5	1	20
Septicemia	11	9	82	20	16	80
Leukopenia	14	10	71	9	5	55
Total	57	46	81	40	32	80

number of patients in each group, no significant influence of the site of the infection or of the type of microorganism could be detected here. It should be noticed, however, that leucopenic patients, and those who had pulmonary infections or infections caused by the Proteus–Providence group microorganisms had the lowest rate of cure in this series, as shown in Table 6.

The total rate of failure was thus 20% in this series and this result compares favorably with the results obtained with other regimens in this institution and elsewhere as indicated in Tables 1 and 7.

It should be observed that the rate of cure obtained with carb–ceph is identical to that observed in this hospital with carb–gen.

That the antibacterial effectiveness of these 2 regimens was similar is further substantiated by the observation that the bactericidal activities of the treated patients was similar whether carb–gen or carb–ceph had been given. The mean peak level was 1/16 and 1/32 for carb–gen and carb–ceph respectively and the corresponding mean through levels were 1/4 and 1/8.

The significance of *in vitro* synergism between antibiotics has been recently studied in our laboratory; it has been found that the rate of response was significantly better in the patients who received a combination of antibiotics that were synergistic on the offending microorganisms when compared to the patients receiving additive or antagonistic combinations of antibiotics [16]. This has been verified in the present study, since we have observed that the number of clinical failures was significantly higher among the patients infected with microorganisms on which no synergistic action had been detected *in vitro*.

Adverse reactions to the combination carb–ceph occurred with a similar frequency in both series as indicated in Table 8. Hypokalemia was a relatively frequent complication in this series and occurred in 9 of 97 patients. It has been attributed to the administration of large doses of carbenicillin which acts as a non reabsorbable anion on the kidney and promotes the excretion of large amounts of sodium [19]. In patients whose general pool of potassium is altered and/or who are treated with digitalis, the administration of high doses of carbenicillin should be cautiously undertaken with frequent monitoring of the potassium serum level. Bleeding, presumably caused by a prolongation of the prothrombin time, has been reported in association with therapy using carbenicillin [20]. This happened in one patient in this series, in whom no other cause for the hemorrhage could be found.

However, since hypokalemia and bleeding are common complications in severely ill patients with cancer, the reality of the association between therapy with carbenicillin and

Table 6. Clinical results with the combination carbenicillin–cephalothin

	Number of patients	Clinical success	
		Number	Percentage
Septicemia	20	16	80
Wound infection	9	8	89
Pulmonary infection	9	6	66
Urinary infection	2	2	100
Leucopenia	9	5	55
Escherichia coli	10	8	80
Klebsiella–Enterobacter	10	9	90
Proteus–Providence	8	5	62
Pseudomonas aeruginosa	12	10	89
Total	40	32	80

Table 7. Results obtained with various antimicrobial regimens in gram-negative infections at the Institut Jules Bordet

Antimicrobial regimens*	Total number of patients		Neutropenic patients	
	No.	% success	No.	% success
Carbenicillin or Gentamicin (14)	45	53	9	22
Carbenicillin + Polymyxin B (12)	21	62	7	43
Ampicillin or Cephalothin + Gentamicin (13)	55	62	7	71
Carbenicillin + Gentamicin	57	81	14	71
Carbenicillin + Cephalothin	40	80	9	55

*The differences between carb–gen or carb–ceph and gentamicin or carbenicillin alone ($P < 0{\cdot}01$) and between these combinations and ampicillin or cephalothin plus gentamicin ($P < 0{\cdot}05$) were statistically significant.

Table 8. Frequency of adverse reactions to carb–gen and carb–ceph

Adverse reactions	Carbenicillin + Gentamicin (57)	Carbenicillin + Cephalothin (40)
Rash	1	0
Drug fever	0	1
Elevated SGOT	8	5
Azotemia	7	1*
Hemorrhage	0	1
Edema	0	1
Hypokalemia	4	5
Phlebitis	2	3
Superinfection	5	2
Total	27 (47%)	19 (47%)

*Therapy with carb–ceph was given to 3 other patients who presented an elevated creatinine serum level (2·1–3·4); no further deterioration of the renal function has been observed.

these manifestations must be investigated in further studies.

Use of large doses of gentamicin. Two different dosages of gentamicin were used in this study: 34 patients received, in addition to carbenicillin, 3–4 mg/kg per day of gentamicin and 23 were treated with 6–8 mg/kg per day.

Clinical results were similar as shown in Table 9: the rate of clinical success was 79 and 83% with the lower and the higher dose respectively.

There were too few patients with acute leukemia in these series to allow valid comparison; but when patients with metastatic cancer are considered, the respective cure rate was 79 and 90%. This difference is not statistically significant; it suggests, however, the need for further studies comparing usual and higher doses of gentamicin in the treatment of severe infections in patients with cancer.

The tolerance of larger doses of gentamicin was further investigated in the 23 patients who have been treated with carb–gen and in 23 additional patients who received gentamicin alone. All received gentamicin at doses varying between 6·2 and 8·4 mg/kg per day and the mean duration of therapy was 9·0 (4–15) days.

Renal function was monitored by serum creatinine levels that were determined each other day; serum urea levels and urinalysis were also available. Special tests to detect ototoxicity of gentamicin were performed in a few patients only, and the results were always within normal limits. No gross alteration has been observed by clinical evaluation of the 8th nerve function.

The mean peak level of gentamicin in the serum of these patients who received a mean dose of 7·8 mg kg/per day was 8·6 μg/ml (3·0–18·0); the mean through level was 2·0 μg/ml (0·0–4·5).

The mean levels of blood urea and creatinine

Table 9. Comparative effectiveness of carb–gen in relationship to the dose of gentamicin

	Carbenicillin + Gentamicin (3–4 mg/kg/day)	Carbenicillin + Gentamicin (6–8 mg/kg/day)
Number of patients	34	23
Clinical success	27 (79·4%)	19 (82·6%)
Number of patients presenting metastatic carcinoma	29	21
Clinical success	23 (79·3%)	19 (90·4%)
Superinfection	4 (11·7%)	1 (4·3%)

did not rise during the administration of gentamicin, however, in 7 (16%) patients a rise of the creatinine level was observed, suggesting some impairment of the renal function.

Three of these patients had a severe underlying pyelonephritis that has been documented at post-mortem examination.

Another patient had received prior to this episode, several treatments with kanamycin and gentamicin; his creatinine level was 1·7 mg% initially, rose during therapy with gentamicin and came to normal values when treatment was discontinued.

In the 3 remaining patients, the rise of creatinine during therapy with gentamicin was minimal and the abnormal values returned to normal after discontinuation of therapy with gentamicin.

Data obtained from microscopic examination of the urine contribute little information here. Changes that could have been attributed quite definitely to the administration of gentamicin were not observed.

DISCUSSION

Antimicrobial therapy in serious infections presumed to be caused by gram-negative rods is usually started before bacterial identification and sensitivity tests of the offending microorganisms have been performed. The extra day or two afforded by empiric therapy may be important for the outcome of patients with severe gram-negative sepsis [8, 11]. Therefore, the bacteriological and clinical evaluation of potential empiric therapies is important.

The combination of carb–ceph, studied here from the bacteriological point of view, had a broad spectrum of antimicrobial activity including most pathogenic gram-positive and gram-negative microorganisms. At levels that are easily reachable in the blood of treated patients, the combination was inhibitory against about 90% of the gram-negative rods isolated in our hospital from patients with cancer and infection.

The combination carb–gen which has been recommended as a valuable empiric treatment in febrile granulocytopenic patients with cancer, has been studied here in a comparative manner with the combination carb–ceph. The combination carb–ceph was less active *in vitro* than carb–gen on Klebsiella–Enterobacter and *Pseudomonas aeruginosa* strains.

Synergism between antibiotics may have some clinical importance in the treatment of severe gram-negative infections [16] and has been found to be present here for a substantial proportion of gram-negative strains. As pointed out already in previous studies from this laboratory, carb–gen is more frequently synergistic against *Pseudomonas aeruginosa* than other combinations such as carb–ceph [7], however, carb–ceph seems to be more often synergistic on *E. coli* and Proteus strains.

In these studies, we confirmed previously published data showing that synergistic combinations of antibiotics are more effective clinically than non-synergistic combination [16]; however, both regimens, carb–gen and carb–ceph, were equally effective here. The cure rate was 80% and compares favorably with other regimens studied in our laboratory [12, 13, 14] or elsewhere [5, 6, 8, 9, 10, 11]. The carb–ceph combination might have the theoretical advantage over carb–gen of being less toxic, even in patients with impaired renal function and of providing an adequate therapy for staphylococcal infections. However, only further controlled studies could establish which therapy is optimal for patients with cancer presumed to present a severe gram-negative sepsis. In particular, more studies in neutropenic patients are needed. Recent studies [11] have suggested that empiric therapy in the compromized host should be undertaken with a combination of 5 antibiotics (methicillin, carbenicillin, cephalothin, gentamicin, and lin-

comycin) to cover the majority of possible pathogens. The combination of carb–ceph–gen has been studied comparatively to carb–ceph in our hospital. Another reason to associate cephalothin and gentamicin is their efficacy against methicillin-resistant staphylococci [21]. The routine addition to this triple regimen of methicillin and lincomycin seems less mandatory.

The simultaneous administration of several antibiotics together is not in accordance with the classical principles of antibiotic therapy. It is, however, justified by the frequently fulminant course of bacterial infection in patients whose natural resistance to infection has been lessened by a disease or a certain therapy and by the wide variety of the pathogens that may be involved in the infections of these patients.

However, our studies do not indicate the need for multiple antibiotic therapy of any serious infection, even in a debilitated host. Positive bacteriological diagnoses should permit adequate *in vitro* sensitivity studies and prompt curtailment of the superfluous antibiotics in many patients in whom adequate specimens for bacteriological and virological cultures have been obtained prior to the empiric therapy with a combination of drugs.

The optimal dosage of gentamicin in severe infections outside the urinary tract is still controversed; however, Jackson and Riff showed clearly that the concentration of gentamicin in the serum was directly related to the eradication of bacteremia and survival of the patient [22]: a level of 4 ug/ml or more eliminated the bacteremic state in all except leukemic patients, in whom neutropenia and less effectively phagocyting leukemic leukocytes presumably explain the frequent failure of gentamicin therapy. There is some rationale, thus, to use high doses of gentamicin in order to attain adequate blood levels. Gentamicin at doses ranging from 6 to 8 mg/kg/day was well tolerated in patients without prior renal insufficiency. Results with high doses of gentamicin were not significantly better than those observed with lower doses; however, in our study gentamicin has been used in combination with carbenicillin and potentiation of one drug by the other occurs frequently and can be of considerable importance even when relatively low doses of gentamicin are used. This may make the necessity for high doses of gentamicin less mandatory when a synergistic drug is administered at the same time.

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Empirical Antibiotic Therapy in Febrile Patients with Neutropenia and Malignant Disease

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Abstract—*Forty-three episodes of presumed infection in patients with malignant disease have been treated with an empirical combination of four antibiotics. The outcome was satisfactory in* 12 (54·5%) *of the twenty-two in which infection was confirmed by blood culture, and in* 75% *of those in which antibiotics were administered for more than* 48 *hours.*

In spite of advances in anti-leukaemic chemotherapy and supportive care during the last decade, infection remains the leading cause of death in patients with malignant blood disease [1, 2, 3]. Infection is also a major cause of morbidity and mortality in patients with other cancers and optimal management of presumed infection in these patients might improve the prognosis of their primary disease by making possible more vigorous antineoplastic chemotherapy [4]. Several factors predispose patients with malignant disease to infection. Many of the therapeutic agents used to treat the primary disease depress the bone marrow and impair the cellular and humoral responses to antigenic challenge. In addition, many of the cytotoxic drugs used in the chemotherapy of cancer cause mucosal ulceration and favour the systemic spread of the intestinal microbial flora. The patient with neutropenia, either because of primary bone marrow disease or following therapy for malignancy, is particularly at risk from infection since circulating neutrophils constitute a defence against bacterial septicaemia and may enhance the effectiveness of many antibacterial drugs [5, 6]. Because of the hazards of neutropenia, the use of cytotoxic drugs in the treatment of malignant disease is frequently limited by neutrophil count irrespective of tumour response.

During the past few years, the susceptibility to infection of patients with malignant disease has become an increasingly important aspect of cancer treatment. The more effective cytotoxic drugs which have been developed have produced only modest improvement in survival for many types of cancer, partly because of the increased susceptibility to infection caused by their use. Several approaches to this problem have been made pending the development of more selective antitumour drugs. The two most promising have been the use of patient isolation with antimicrobial decontamination before and during cytotoxic therapy [7–13], and the empirical use of antibiotic combinations as initial treatment for patients with presumed infection [6, 14–16]. During the past two and a half years it has been our policy to treat presumed infection in febrile neutropenic patients with a combination of antibiotics. Subsequently, if organisms are cultured the irrelevant antibiotics are stopped and appropriate additional antibiotics substituted if indicated by the *in vitro* sensitivities of the pathogen.

We have reported the results of this approach in the management of 27 episodes of presumed infection in neutropenic patients [16]. Encouraged by those results, we now record the results of treating a further 43 febrile episodes. The success rate in 22 cases of documented infection was 54·5%.

MATERIAL AND METHODS

The patients had leukaemia, malignant lymphoma, or lupus erythematosus, and were eligible for empirical combination antibiotic therapy if they were febrile (temperature ≧ 38°C) for 48 hr in the absence of obvious non-bacterial causes such as coryza or drug therapy and were neutropenic (less than 1000 neutrophils per μl). In seriously ill patients, the 48 hour delay was waived and therapy was begun immediately after bacteriological specimens had been obtained. Urine, sputum and stool were cultured, as were swabs taken from nose, throat, vagina and any possibly relevant lesions, e.g., furuncles, mouth ulcers, i.v. sites, etc. Blood cultures were taken on three occasions before starting the antibiotics. In most cases a chest X-ray, and routine haematological and biochemical tests were also done.

An intravenous infusion was set up and the antibiotics administered as rapid infusions every 6 hr, the infusion being continued with suitable fluids in the intervals. The antibiotics used are listed in Table 1. The parenteral drugs other than gentamicin were dissolved in 100 ml of sterile water shortly before their

Table 1

Antibiotic	Combinations used Dosage per m^2 per 24 hr
Carbenicillin	20 g
Cephalothin	4 g
Gentamicin	140 mg
Clindamycin*	1200 mg
Probenecid	1250 mg
Patients with significant penicillin allergy	
Cephalothin	4 g
Gentamicin	140 mg
Polymyxin	$1{\cdot}5 \times 10^6$ i.u.
Clindamycin*	1200 mg

*If the patient is unable to take clindamycin by mouth, substitute lincomycin 1500 mg.

administration. Gentamicin was given as a separate "bolus" injection into the tubing of the intravenous apparatus after completing each infusion of the other antibiotics [17]. The dosages of carbenicillin and gentamicin were reduced where indicated in patients with impaired renal function [18, 19], but administration every 6 hours was continued. Probenecid (1,250 mg/m^2 per day) and potassium chloride (1,800 mg per day) were commenced immediately if the patient could take oral drugs. Intravenous potassium 120 mEq/24 hr was given if oral potassium could not be taken.

Antibiotic therapy was modified as required when sensitivity patterns were determined. If no pathogen was isolated, the drugs were continued for at least 5 days. If no clinical improvement occurred within 48 hr of starting the antibiotics, and infection still seemed clinically probable, amphotericin B (40 mg/m^2, alternative days, i.v.) was added to the treatment regimen. Blood counts and liver function were monitored frequently, and the blood urea and electrolytes were estimated daily.

A positive microbiological diagnosis was not established in all patients who received the antibiotic combination treatment, and for purposes of evaluation, "infections" were classified into 3 groups:

"Documented infection", where proof of infection was obtained from the blood culture.

"Probable infection", where clinical signs and progress were consistent with an infection, but where cultures were negative.

"Infection doubtful", where infection seemed improbable on review of clinical signs and progress.

The results of antibiotic therapy were assessed by progress of clinical signs and fever. In a case of treatment failure, persistence of infection was presumed or proved on the basis of culture of organisms and clinical deterioration. Patients were considered to have responded to antibiotic therapy if they become afebrile and all signs of infection disappeared.

RESULTS

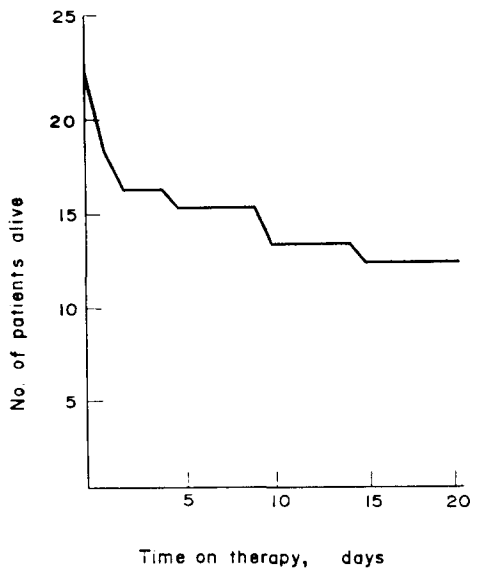
Table 2 shows the distribution of infections in 43 febrile episodes treated with the combination of antibiotics. In the 22 cases of documented infection, 12 had a satisfactory response (54·5%). None of those responding was treated with amphotericin, thus indicating that their response to initial treatment was rapid. Ten patients died with signs of persisting infection while receiving antibiotic treatment: 4 of these died within 24 hr of starting the treatment, and a further 2 within the next 24 hr of treatment. Thus, of the 16 patients surviving

Table 2

Infection category	Outcome (All pyrexial episodes treated) Total	Success	% Success
Documented	22	12	54.5
Probable	15	15	100
Doubtful	6		
Total	43	27	61

48 hr 12 (75%) were cured. This data, shown in Table 3, indicates that infection in neutropenic patients is often very rapidly fatal. All patients with probable infection improved initially and the fever lysis in all these patients

Table 3



seemed to be related to the administration of antibiotics. In 6 of the 43 febrile episodes (14%) an infection was subsequently judged improbable, and although no clear cause for the fever was established, in 2 patients with high peripheral blood blast cell counts, fever may have been due to the activity of their leukaemia *per se*.

Table 4 summarises the clinical and laboratory data of the 22 documented infections (25 organisms). *E. coli* was isolated on 7 occasions (28%) and 4 of these patients survived (57%). *Staph. aureus* was cultured from 3 patients (2 survived). *Clostridium welchii* was cultured from 3 different patients (1 survived), as was pseudomonas (1 survived). One of the 2 patients with klebsiella infection died (within 24 hr) but the other from whom *E. coli* was also isolated survived. Table 5 presents the data on the organisms isolated and the outcome of therapy. Recovery from a documented infection in these patients was not related to the peripheral blood neutrophil count, and the factor most obviously related to treatment failure was delay in initiating antibiotic therapy.

Table 4. *Case details*

Case No.	Primary diagnosis	Initial white cell count: Total /μl	Initial white cell count: Neutrophil /μl	Organism	Outcome	White cell count when antibiotics stopped or at death: Total /μl	White cell count when antibiotics stopped or at death: Neutrophil /μl	Antibiotic treatment duration (days)
1	A.M.L.	1100	800	*E. coli*	Success	1000	800	9
2	S.L.E.	1400	50	β Haem. Strep.	Success	1400	500	6
3	S.L.E.*	1100	0	Klebsiella + *E. coli*	Success	1000	100	10
4	A.M.L.	6000	300	*Cl. welchii* + Micrococcus	Success	2200	130	6
5	A.M.L.	2100	100	Anaerobic spore-bearing diptheroids	Success	1200	10	6
6	A.M.L.	300	0	*S. aureus*	Success	1200	900	6
7	A.M.L.	1600	50	*S. aureus*	Success	700	0	6
8	A.M.L.	1700	0	Proteus	Success	1900	40	5
9	A.M.L.	1600	50	*E. coli*	Success	1800	150	5
10	A.M.L.	700	350	Bacillus	Success	500	200	6
11	A.M.L.	1000	50	Pseudomonas	Success	2000	50	10
12	A.M.L.	1300	500	*E. coli*	Success	700	100	6
13	A.M.L.	1600	0	Pseudomonas	Failure	4700	470	15
14	CGL.B.T.	13000	1000	Pseudomonas	Failure	2000	60	10
15	CGL.B.T.	9400	100	*S. albus*	Failure	7500	375	2
16	A.M.Mol.	140	0	*Cl. welchii*	Failure	700	0	2
17	C.L.L.	1500	100	*E. coli*	Failure			1
18	A.M.L.	900	0	Pneumocystis	Failure	33000	0	15
19	A.M.L.	980	0	*S. aureus*	Failure	1000	100	1
20	A.M.L.	2200	350	*Cl. welchii* + *E. coli*	Failure			5
21	A.M.L.	1200	600	Klebsiella	Failure	1300	400	1
22	A.M.L.	500	0	*E. coli*	Failure	1500	0	2

*Died while on fever regimen with no evidence of active infection.

Table 5. *Organisms isolated*

Organism	No. of cases	Successfully treated	% Success	Organism % of total isolated
E. coli	7	4	57	28
Pseudomonas	3	1	33	12
S. aureus	3	2	67	12
Cl. welchii	3	1	33	12
Klebsiella	2	1	50	8
S. albus (inc. Micrococcus)	2	1	50	8
β Haem. Strep.	1	1	100	4
Pneumocystis	1	0	0	4
Proteus	1	1	100	4
Other bacteria	2	2	100	8
Total	25	14		

COMPLICATIONS OF TREATMENT

In 2 patients signs of ototoxicity developed while receiving the antibiotics, and in each gentamicin was omitted from the regimen with subsequent improvement in hearing. In one patient (with no history of penicillin allergy) a widespread macular eruption was thought to be due to carbenicillin allergy, and the rash faded after this drug was stopped.

Weight gain and oedema were frequently observed during treatment. The sodium content of the antibiotics used is considerable (cephalothin 2·4 mEq Na/g and carbenicillin 4·5 mEq Na/g). In every case in which diuretics were used, the oedema cleared. Hypokalaemia was frequently seen in spite of the routine administration of potassium supplements. In two patients the serum potassium fell to 2 mEq/l, and muscle weakness in these patients was marked.

DISCUSSION

Fever is common in patients with leukaemia and cancer, but it is not always due to infection. Infections remain the commonest cause of death in leukaemia, and in patients with other cancers, particularly those receiving chemotherapy, infection is a major cause of morbidity and mortality. Gram-negative bacteria, especially *Ps. aeruginosa*, are very common causes of infection in these patients and death may be very rapid, frequently occurring before the causative organism has been identified (Table 3). We believe that antibiotic therapy in patients with malignant disease must be instituted as soon as infection is suspected. The antibiotics used should provide good cover against gram-negative bacteria in particular, and ideally treatment should include agents active against the whole range of possible organisms.

Empirical antibiotic treatment for presumed infection in patients with malignant disease has been reported using combinations such as carbenicillin and gentamicin [6, 7], cephalothin and gentamicin [20], carbenicillin and polymyxin [15] and with these treatments survival rates of about 30% have been recorded. Using a combination of five antibiotics—carbenicillin, gentamicin, clindamycin, cephalothin and methicillin—our success rate was 53% in 15 neutropenic patients with documented infection [16]. On the basis of that study, in which *Staph. aureus* was isolated from only 15% of the patients, and because staphylococci are well-covered by the other agents, we decided to omit methicillin from the present regimen, and continue the trial. The success rate using this four antibiotic combination was 54·5 per cent in 22 cases of documented septicaemia.

There has been a change in the spectrum of organisms isolated in patients in these two groups and, although gram-negative organisms remain the most important pathogen, *Pseudomonas aeruginosa*, which constituted 53% of the organisms isolated in the first group [16], was cultured from only 12% of the second group. Several factors may be responsible for this change and they are discussed in detail in the paper by Gaya *et al.* in this symposium. However, the increased frequency of *E. coli* as a cause of septicaemia in the second group of patients cannot easily be explained on the basis of systemic invasion by normal or previously isolated gut organisms [3].

That the 12 documented successfully treated septicaemias occurred in only 6 patients is of considerable interest. Was there a particular feature of these patients' diseases or their antileukaemic treatments which conferred an ability to survive repeated septicaemic episodes? The serum lysozyme in these patients was not high, nor were their ages, neutrophil counts, or disease states strikingly different from those of the total series. However, 5 of the 6 patients who survived 12 documented septicaemic episodes received oral anti-microbial gut prophylaxis, but only 5 of the 10 patients who died of infection were similarly treated in the period immediately preceding their septicaemia. It is possible, but unlikely, that this difference is due to the absorption of the oral antibiotics which influenced the course of the systemic infection.

Any consideration of empirical antibiotic treatment in patients with malignant blood diseases or solid tumours must be based on a knowledge of the likely micro-organisms which may be responsible. Table 6 shows the 45

causes of 37 infective episodes in patients with neutropenia and/or malignant blood disease seen in our Unit during the period 1970–1972. In this group pseudomonas has been isolated in 32% and *E. coli* in 17%. Thus, initial antibiotic treatment in presumptive infection must include agents active against both organisms.

Table 6. *Organisms isolated from the blood*

Organism	Total group No.	Success	% Success	Organism % of total
Pseudomonas	14	7	50	31
E. coli	9	4	44	20
St. aureus	6	3	50	13
Klebsiella	5	2	40	11
Cl. welchii	3	1	33	7
Diptheroid	1	1		
Micrococcus	1	1		
Proteus	1	1		
Pneumocystis	1	0		
St. albus	1	0		
Strep. Hbem.	1	1		
Ch. aureum	1	0		
Bacillus	1	1		

Various authors have suggested antibiotic combinations as initial treatment for presumed infection in patients with haematological malignancy. Schimpff *et al.* [13] used carbenicillin and gentamicin in 48 patients with subsequently documented infection. Although effective in over 60% of patients with pseudomonas infection, this combination was less effective in those with klebsiella and *E. coli* infections. Levine *et al.* [2], in a recent review of infectious complications of haematological malignancy, have suggested two empirical antibiotic regimens determined by the patient's neutrophil count: in those with adequate neutrophils ($> 100/\mu l$) they recommend gentamicin ± a cephalosporin- or penicillinase-resistant penicillin. In those patients with neutropenia ($< 500/\mu l$), they suggest carbenicillin and a cephalosporin. Their rationale for these suggestions is based on two observations; firstly, that gentamicin is a highly effective antibiotic only in the presence of adequate circulating granulocytes, and secondly, that the combination of gentamicin and carbenicillin, while optimal treatment for confirmed pseudomonas infection, has less effect in klebsiella and *E. coli* infection than gentamicin alone [5, 6]. Thus they maintain that the combination of gentamicin and carbenicillin should not be administered except in confirmed pseudomonas infection, and that it is inappropriate to administer gentamicin to granulocytopenic patients. Results have been reported of treatment of gram-negative infection with gentamicin and cephalothin [20] but not for carbenicillin and a cephalosporin in patients with neutropenia. Thus, although many authors agree that therapy for presumed infection in patients with neutropenia and malignant disease should be started as soon as appropriate cultures have been taken, the choice of antibiotics has not been clearly defined.

Presumed infection in a combined series totalling 70 episodes was subsequently documented on only 37 occasions (52·9%), and thought unlikely on 10 (15%). In the remaining cases, failure lay in our inability to document the infection and not so much in its management. Several methods for rapidly establishing the existence of infection in patients at risk have been suggested. The nitroblue tetrazolium (NBT) test, although a reliable index of bacterial infection in haematologically normal adults, has not been very helpful in deciding which of these febrile patients was infected [21, 22]. Radiometric methods for rapidly establishing the presence of bacteraemia are being investigated, but at present they appear to be of little value (H. Gaya, unpublished). Assays of endotoxin in the plasma of febrile patients may help clinicians to decide whether to treat febrile neutropenic patients with antibiotics [23].

The toxicity of the fever regimen in our patients has not been marked, but a consistent side effect has been the development of low serum potassium levels in the majority of patients [24]. The mechanisms of hypokalaemia have been investigated by radioisotopic measurement of total body and intracellular potassium changes in patients on the antibiotic regimen, and it is clear that two factors contribute. Potassium diuresis occurs and is probably a result of both the large sodium load and the antibiotics in the renal tubular fluid inhibiting potassium reabsorption. A potassium redistribution also occurs, since the red cell potassium concentration increases as the serum potassium level falls [25]. It is now our practice to supplement the potassium intake of all patients in the fever regimen.

The results of this trial of multiple antibiotic therapy in 22 documented infections shows a success rate of 54·5% which compares very favourably with other reported regimens, and is apparently as satisfactory as using "appropriate" antibiotics and white cell

transfusions [26]. If deaths during the first 48 hours of treatment are excluded the success rate is 75%. It is possible that other antibiotics substituted in the regimen may enhance its effects, and there is clearly a need for collaborative studies in this area.

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Administration of Carbenicillin and Ticarcillin—Pharmaceutical Aspects

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Abstract—*Ticarcillin is a new injectable semi-synthetic penicillin for infections caused by* Pseudomonas aeruginosa *and indole-positive* Proteus *spp. Its properties are compared with those of carbenicillin.*

In Pseudomonas *infection a regimen of* 5 *g carbenicillin in* 100 *ml of water, infused over* 30 *min at* 4*-hour intervals, would seem to meet the necessary criteria for both height and duration of serum levels. On the bases of the* in vitro *antibacterial activities and pharmacological data, equivalent effectiveness would be obtained using a lower dosage of ticarcillin.*

THE TREATMENT of infection in patients with leukaemia, and those undergoing radiotherapy or treatment with cytotoxic agents, poses many problems. The increasing incidence in such patients in recent years of infections caused by Gram-negative bacilli, including *Escherichia coli*, *Klebsiella*, *Enterobacter*, *Proteus* and *Pseudomonas*, has made the situation even more difficult. *Pseudomonas*, in particular, is an organism against which few antibiotics are active.

Because of the overwhelming importance assumed by β-lactamase (penicillinase)-producing *Staphylococcus aureus* as a cause of hospital-acquired infection in the late 1950's and 1960's, the preparation of derivatives active against this organism was one of the priorities of penicillin research. This work led to the introduction of penicillins stable to staphylococcal β-lactamase, firstly methicillin [1] and more recently the isoxazolyl compounds, cloxacillin [2] and flucloxacillin [3]. However, with the emergence of *Pseudomonas aeruginosa* as an increasingly important cause of severe infections, particularly in aged, debilitated, immunodeficient, burned and post-surgical patients, and in those with chronic underlying diseases, increasing research effort in this area became a matter of urgency.

Carbenicillin, α-carboxybenzylpenicillin (BRL 2064), was the first available penicillin with therapeutic activity against *Pseudomonas aeruginosa* and indole-positive *Proteus* [4–6]. It is a non-toxic agent which can be given in high intravenous dosage [5, 7, 8]. Carbenicillin has been accepted as a first-line drug in the treatment of *Pseudomonas* infections, and laboratory and clinical experience with it has been presented at four international symposia [9–12]. An aspect of particular interest is the synergy which is found between carbenicillin and gentamicin against many strains of *Pseudomonas aeruginosa* [4, 13–17]. This synergy is very marked with some strains, less marked with others, but concurrent use of these two bactericidal antibiotics can provide extremely effective therapy for serious *Pseudomonas* infections [17–20].

Continued work in this field has resulted in the synthesis of a further compound with promising antipseudomonal activity, namely, α-carboxy-3-thienylmethylpenicillin (BRL 2288), which has recently been given the approved name ticarcillin. This penicillin is at present undergoing clinical trial and is not yet generally available, but its antibacterial properties have been reported from several centres [21–25]. Briefly, ticarcillin is at least twofold more active than carbenicillin against *Pseudomonas aeruginosa* and also somewhat more active against *Enterobacter* species. It is as active as, or slightly more active than, ampicillin and carbenicillin against *Esch. coli*, and all three agents have similar activity against strains of *Proteus mirabilis* which do not produce β-lactamase. Like carbenicillin, ticarcillin is active against a high proportion of strains of indole-positive *Proteus* species (*morganii*, *rettgeri*, *vulgaris*). Most strains of *Klebsiella aerogenes* and *Serratia marcescens* are relatively resistant to both carbenicillin and ticarcillin. An interesting point is that meningococci, gonococci and *Haemophilus influenzae* are at least as sensitive to ticarcillin as to ampicillin [23, 26].

Studies on the human [27] and animal [28] pharmacology of ticarcillin have also been published. In *Pseudomonas* infections in mice, the mean CD_{50} values with ticarcillin were generally about half those with carbenicillin [28]. As in the case of carbenicillin, synergy against *Pseudomonas* is often seen when ticarcillin is used in conjunction with either gentamicin or polymyxin B, both *in vitro* [23], and in experimental infections in animals [28].

Various physical and pharmaceutical properties of carbenicillin have been reported previously [29–33]. The present paper is the first account of these aspects of ticarcillin. The characteristics of the two compounds will be compared, and some of the factors relevant to their intravenous usage will be discussed.

STRUCTURES

The structures of carbenicillin and ticarcillin are shown in Fig. 1. In both cases the available compound is a mixture of the D- and L-isomers.

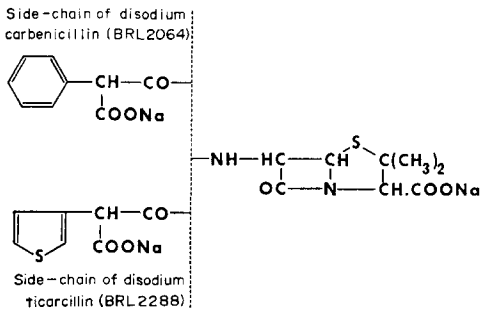

Fig. 1. Structures of the disodium salts of carbenicillin and ticarcillin.

It has been established that the presence of a polar grouping on the α-carbon atom of the side-chain in penicillin derivatives is associated with activity against Gram-negative bacteria. In both carbenicillin and ticarcillin the α-substituent is a carboxyl group, which is apparently the key to their activity against *Pseudomonas* and their relative stability to the β-lactamases produced by indole-positive species of *Proteus*. The chemical difference between the two is that ticarcillin has a 3-thienyl group (thiophene ring) in place of the phenyl group (benzene ring) of carbenicillin.

PHYSICAL PROPERTIES

Some of the more important physical characteristics of the two compounds are summarized in Table 1. Their properties are, in fact, closely comparable. Both penicillins are used as disodium salts, which are very hygroscopic and freely soluble in water. The sodium content is worth bearing in mind in the treatment of patients in whom sodium restriction is indicated. Neither agent is sufficiently stable at gastric acid pH to be administered orally. The vials of dry powder should be stored in the refrigerator, at which temperature little or no decomposition takes place during storage for 12 months or more.

STABILITY IN AQUEOUS SOLUTION

Tables 2 and 3 give figures for the stability of disodium carbenicillin and disodium ticarcillin, respectively, in aqueous solution at the concentrations used for intramuscular injection. Determinations of antibiotic were carried out by plate assay using *Pseudomonas aeruginosa*. Though carbenicillin is somewhat more stable than ticarcillin, in neither case is there much loss of activity in three days at 5°C. We nevertheless recommend that intramuscular solutions should be used within 30 min of preparation, because small amounts of polymer are formed by penicillins and cephalosporins in solution especially at high concentration. The clinical significance, if any, of polymer formation is unknown, but polymers could be involved in the occurrence of delayed skin rashes.

We have also studied the stability of buffered and unbuffered 10% solutions of disodium carbenicillin over a period of eight days

Table 1. Physical characteristics of disodium carbenicillin (BRL 2064) and disodium ticarcillin (BRL 2288)

	Molecular weight	Sodium content (per weight equivalent to 1 g free acid)	Solubility in water (20°C)	Isotonic solution (volume of water per g)	pH (10% w/v solution in CO_2-free water)	Stability at pH2 and 37°C (half-life in 0·1% w/v solution)
Disodium Carbenicillin	422·4	5·4 m eqw	1 in 1·2	28·5 ml	6·0–8·0	approx. 30 min
Disodium Ticarcillin	428·4	5·2 m eqw	> 1 in 1	28·0 ml	6·0–8·0	approx. 45 min

Table 2. Stability of disodium carbenicillin in concentrated aqueous solution (amount of disodium salt equivalent to 1 g free acid, dissolved in 2 ml water)

Days stored	Residual potency % at 5°C	Residual potency % at 23°C
1	98	94
3	100	81
6	99	

Table 3. Stability of disodium ticarcillin in concentrated aqueous solution (amount of disodium salt equivalent to 500 mg free acid, dissolved in 1 ml water)

Days stored	5°C pH	5°C Residual potency %	25°C pH	25°C Residual potency %
0	6·9		7·0	
1	6·6	97	5·8	93
3	6·3	95	5·6	73
7	5·8	91		

at 23°C, and the results obtained by microbiological assay are summarized in Table 4. Differences were negligible at 1 day and small at 3 days. At 8 days the unbuffered solution at pH 7·3 retained the highest degree of activity, while the solution in pH 6·3 citrate buffer was the least stable and those in pH 7·0 and pH 8·0 phosphate buffer were intermediate.

STABILITY IN INTRAVENOUS SOLUTIONS

Results of our studies of ticarcillin and carbenicillin stabilities in a range of intravenous solutions are presented in Tables 5 and 6, respectively. The antibiotics were assayed microbiologically, and the figures for residual potency are average values obtained from a number of separate determinations.

Both compounds are least stable in sodium lactate solution, but generally speaking they have good stabilities in the fluids tested.

Table 4. Effect of buffer solutions on the stability of 10% w/v disodium carbenicillin at 23°C

Days stored at 23°C	Residual potency (%) No buffer pH 7·3	Citrate buffer pH 6·3	Phosphate buffer pH 7·0	Phosphate buffer pH 8·0
1	102	99	101	100
3	99	97	99	98
8	93	80	88	86

Table 5. Stability of disodium tacarcillin, equivalent to 2·0% w/v free acid, in four intravenous fluids at 25°C

	pH values Initial	4 hr	24 hr	Residual potency (%) 4 hr	24 hr
Sodium chloride 0·9%	6·85	6·7	6·1	98	94
Dextrose 5%	6·9	6·6	5·95	99	97
Dextrose 4·3% with sodium chloride 0·18%	6·8	6·6	6·0	101	101
Sodium lactate M/6	5·0	5·0	5·1	101	89

Table 6. Stability of disodium carbenicillin in intravenous fluids at 23°C

	Concentration*	pH values Initial	6 hr	24 hr	Residual potency (%) 6 hr	24 hr
Sodium chloride 0·9%	2·0% w/v	6·5	6·4	5·9	96	96
Dextrose 5%	2·0% w/v	6·7	6·4	6·3	97	95
Dextrose 4·3% with sodium chloride 0·18%	2·0% w/v	6·5	6·3	6·0	99	96
Sodium lactate M/6	2·0% w/v	6·7	6·7	6·5	94	88
Ringer's solution	0·2% w/v				97	96
Sodium bicarbonate 1·4%	3·0% w/v	8·1	8·1	8·1	96	94
Dextran 40 (Rheomacrodex) 10% in sodium chloride 0·9%	2·0% w/v	6·55	6·35	6·0	96	92
Dextran 40 (Rheomacrodex) 10% in dextrose 5%	2·0% w/v	6·6	6·5	6·1	97	91

*Expressed in terms of the free acid.

Stability is therefore not a critical factor, and the method of intravenous administration is determined solely by the necessity of achieving very high serum levels in the treatment of infections caused by *Pseudomonas aeruginosa*.

INCOMPATIBILITIES AND DRUG INTERACTIONS

With the increasing complexity of modern therapeutics, problems of drug interaction, both chemical and pharmacological are becoming more frequent. I shall confine myself to the question of physical or chemical incompatibility—particularly in intravenous infusions, where the majority of drug mixtures occur. Unfortunately, only a limited amount of factual information is available on the compatibility and stability of drug mixtures, and provision of such data is urgently required. Experimental assessment of all the possible combinations of therapeutic agents (and of the variations of concentration and conditions which are encountered) is clearly impossible, but it is important that systematic investigation of the more commonly encountered mixtures should be carried out.

In addition, I believe that where possible we should exploit ways of preventing or minimising drug contact, when there is reason to doubt compatibility or where relevant information is not available. This is often possible, even when a patient is receiving several drugs, by making use of an infusion set which incorporates a Y-tube, a burette, or a special drug injection site, or by injecting the agents alternately into the drip tubing. Those agents which can not be administered by intravenous bolus or those, like heparin, which are often required by continuous infusion, may cause special difficulties.

Penicillin/aminoglycoside interactions

Interactions between penicillins and aminoglycosides are of particular interest and importance because of the frequency with which antibiotics of these two groups are used in combination for severe infections. The use of benzylpenicillin or ampicillin with streptomycin or kanamycin against *Streptococcus faecalis* (the enteroccus); of carbenicillin with gentamicin against *Pseudomonas aeruginosa*; and of methicillin or cloxacillin with kanamycin or gentamicin against *Staphylococcus aureus*, are all examples of potentially synergistic combinations.

The first indication of adverse interactions between penicillins and aminoglycosides was the report in 1967 of mutual inactivation between methicillin and kanamycin when mixed at intramuscular concentrations [34]. This was not followed up at the time, but subsequent clinical observations suggested that gentamicin may have been inactivated *in vivo* by concurrently administered carbenicillin [35, 36]. This raises the intriguing general question of interactions between antibiotics in the body, which needs to be further explored. Slow *in vitro* inactivation of gentamicin was demonstrated by the same workers: using typical therapeutic concentrations of the two antibiotics, the gentamicin half-life was about 40 hr at 35°C [35]. It should be noted, however, that a very high ratio of penicillin to aminoglycoside is necessary to bring about significant inactivation of the latter [36–38]. Two points are therefore worth stressing:

1. The clinical significance of penicillin/aminoglycoside interactions would seem to be confined to patients in renal failure, where excretion would be delayed, dosage would be less frequent, and the agents would be in contact in the body for relatively prolonged periods. Gentamicin inactivation is too slow to be of clinical importance in patients with normal renal function.
2. For all practical purposes, the *in vivo* effect is limited to penicillins which are given in very high intravenous dosage, that is, essentially to carbenicillin (which is used at a dosage of 30 g per day) and occasionally benzylpenicillin (which may sometimes be used at dosages of 60 mega-units per day or more). Other semi-synthetic penicillins are rarely given in dosages of this order. Ticarcillin will also be used in fairly high dosage and, in the absence of information to the contrary, it is advisable to assume that it may be capable of interacting with gentamicin, and possibly with other aminoglycosides.

Very marked inactivation of gentamicin by carbenicillin or ampicillin has been reported at the concentrations likely to be added to intravenous infusion bottles [38]. Gentamicin 160 μg/ml was mixed with carbenicillin 20 mg/ml in 0·9% sodium chloride, sodium chloride/dextrose and 5% dextrose solutions at room temperature, and 50% of the gentamicin activity was lost in 8–12 hr. In the presence of 8 mg/ml ampicillin, 50% of the gentamicin was lost in 2 hr and no gentamicin activity remained at 24 hr. We ourselves have found that interactions may take place within 15–30 min in mixed intramuscular solutions of penicillins and aminoglycosides, often

with significant loss of activity of one or both components [39].

To summarize, therefore, there is no contraindication to concurrent therapy with gentamicin and carbenicillin, provided that they are not mixed in the syringe or in the infusion bottle. In patients with renal impairment, however, it would be advisable to monitor gentamicin serum levels, to ensure that they are adequate.

Penicillins with heparin

In collaboration with colleagues in hospital pharmacy, we are at present engaged in an investigation of possible interactions between penicillins and heparin in intravenous solutions at room temperature. So far, it has been found that 10 mega units per 500 ml of benzylpenicillin and 5g/500 ml of ampicillin or methicillin have no effect on the stability or anticoagulant activity of heparin sodium (10,000 U/500 ml). Nor was the stability of the penicillins adversely affected by the presence of heparin. Study of individual stabilities, however, led to some interesting findings. Heparin remained stable and active for 24 hr in normal saline, but there was a rapid inactivation of 40–55% in solutions containing dextrose or lactate. Ampicillin was shown to be unstable in dextrose solutions, and it would be preferable for it not to be added to intravenous dextrose, unless the solution can be infused within 1–2 hr (preferably within 1 hr) of preparation.

THERAPEUTIC USE OF CARBENICILLIN AND TICARCILLIN

Antibacterial activity

Sutherland *et al.* measured the activity of ticarcillin and carbenicillin against 74 clinical isolates of *Pseudomonas aeruginosa*, and found that ticarcillin was at least twice as active as carbenicillin [23]. Their results were as follows:

	Cumulative % strains inhibited by:				
	12·5 μg/ml	25 μg/ml	50 μg/ml	125 μg/ml	250 μg/ml
Ticarcillin	26	80	95	100	100
Carbenicillin	8	20	79	95	100

It can be seen that the minimum inhibitory concentration for the majority of strains is in the 12·5–25 μg/ml range for ticarcillin and in the 25–50 μg/ml range for carbenicillin. This agrees broadly with the findings of Adler *et al.* in 162 strains [21]. Neu and Winshell also found ticarcillin to be consistently twofold more active than carbenicillin against *Pseudomonas*, and reported that 89% of strains were inhibited by 100 μg/ml or less of ticarcillin and 72% by 50 μg/ml or less [22]. Bodey and Deerhake stated that 80% of *Pseudomonas* isolates were sensitive to 50 μg/ml or less of ticarcillin, compared with under 30% for carbenicillin [24].

Very high intravenous dosage of carbenicillin is needed to achieve the requisite serum and tissue levels, and the increased activity of ticarcillin should therefore be of clinical importance. Bearing in mind that a higher concentration than the MIC may be required to produce a good bactericidal effect; that, like carbenicillin and benzylpenicillin, ticarcillin is reversibly bound to serum albumin to an extent of approximately 50 per cent; and that the concentration at the site of infection may sometimes be lower than that in the blood, it appears that therapeutically we need to achieve serum levels in excess of 125 μg/ml ticarcillin, and with carbenicillin even higher.

Principles of intravenous administration

A substantial body of opinion has always advocated that during penicillin therapy, continuously inhibitory levels of the antibiotic should, as far as possible, be maintained at the site of infection. An opposing viewpoint is that intermittent bolus administration, giving high peak concentrations alternating with periods where serum levels may be sub-inhibitory, could well be more effective. The theory behind this is based on the well-known fact that penicillins act only against growing bacterial cells. It is suggested that bacteria which have escaped the effect of one dose by being a resting phase should be allowed to resume active growth in the absence of penicillin, so that they will then be susceptible to the next dose. This is an attractive, though perhaps oversimplified, concept, and the truth may lie somewhere between the two approaches. As far as intravenous therapy is concerned, the danger of continuous, slow infusion is that serum, and hence tissue, levels of penicillin may be inadequate. With intermittent, rapid infusion, on the other hand, it is obviously vital that the penicillin-free intervals should not be so prolonged as to allow a significant amount of bacterial growth and multiplication to take place, otherwise the patient's condition would deteriorate. One of the key factors here is the time taken for bacterial cells

which have been exposed to penicillin to "recover" and move once more into a phase of normal growth and division.

Investigations of the antibacterial effect of penicillins *in vitro*, carried out by my colleagues in the microbiology department at Beecham Research Laboratories, have thrown some light on this question of bacterial recovery times [40]. The data available so far suggest a marked difference between Gram-positive bacteria and Gram-negative bacilli in their response to removal of penicillin by penicillinase after varying periods of exposure. It has been found that exposure of *Staphylococcus aureus*, *Bacillus subtilis* and *Streptococcus faecalis* to benzylpenicillin or ampicillin is followed by an interval during which the viable count ceases to fall and remains almost constant, before growth is resumed at a normal rate. This "recovery period" is commonly 1½–2 hr, but may be as long as 4½ hr, depending upon the concentration of penicillin and the exposure time. In contrast, exposure of *Escherichia coli* or *Proteus mirabilis* to ampicillin, and *Pseudomonas aeruginosa* to carbenicillin, is followed by resumption of growth as soon as the penicillin is removed.

In vivo studies with the mouse thigh lesion test, using *Pseudomonas aeruginosa* strain 44 as the infecting organism and carbenicillin as therapy are also in progress [40]. The work is concerned with the relationship between carbenicillin dosage, concentrations of antibiotic *in vivo*, antibacterial effect and overall therapeutic response. Although it would be dangerous to draw too close a parallel between experimental and human infections, particularly on the evidence of a single model, a few relevant points have emerged:

1. If the dosage interval was lengthened beyond the optimum, resumption of bacterial growth between doses coincided with the fall in serum concentrations of carbenicillin to low levels, and took place without any recovery period. This agrees with the *in vitro* findings and indicates the importance of the dosage interval.
2. Serum levels which correspond closely with the MIC *in vitro* resulted in a rapid bactericidal effect *in vivo* over the first few hours.
3. Quite low serum concentrations of carbenicillin often had a marked bactericidal effect, but bacterial growth resumed soon after dosage ceased, and the overall therapeutic effect was not comparable to that given by higher dosage. In contrast, therapeutically effective regimes brought about a steady fall in the number of viable bacteria, and this continued after the end of treatment (presumably due to normal body defence mechanisms).
4. Provided that the dosage interval did not allow significant resumption of bacterial growth, it appeared that less frequent higher dosage had a better therapeutic effect than more frequent lower dosage.

Serum concentrations after intravenous infusion

My colleague Dr. Lees has recently carried out a cross-over study to determine the serum levels achieved in healthy adult male volunteers who received 5 g carbenicillin and ticarcillin, either by rapid intravenous infusion over 30 min or by slow infusion over 4 hr, both with and without concurrent oral probenecid [41]. The complete data are to be published in due course, but it would be useful to summarise the essential findings.

(*a*) *Carbenicillin.* (*Fig.* 2) With infusion of 5 g over 30 min, mean peak serum concentrations of approximately 500 μg/ml were obtained at the end of the infusion and concentrations remained above 100 μg/ml for the first 3 hr. Probenecid led to some prolongation of serum levels, but did not enhance the peak value.

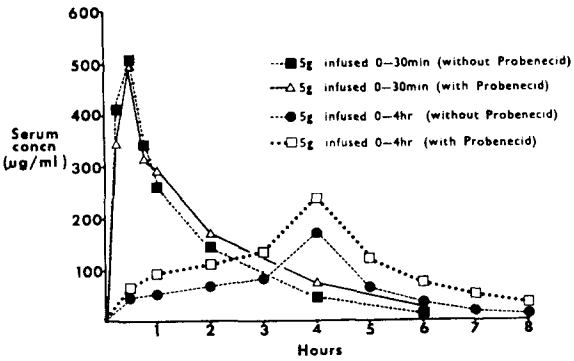

Fig. 2. *Intravenous infusion of carbenicillin.*

With infusion of 5 g over 4 hr, peak concentrations were again reached at the end of the infusion period, and averaged 170 μg/ml. The mean peak serum concentration was 230 μg/ml with concurrent probenecid, and excretion was also delayed. In the probenecid study, serum levels were in excess of 100 μg/ml between 2 and 5 hr.

(*b*) *Ticarcillin.* (*Fig.* 3). By rapid infusion, mean peak serum concentrations of 350–400 μg/ml were achieved at 30 min, falling to below 100 μg/ml at about 2½ hr. As with carbenicillin, probenecid had little effect on the peak concentration, though there was slight prolongation of levels from 2½ hr onwards.

Slow infusion of ticarcillin over a 4-hr period gave peak serum concentrations of 250–300 μg/

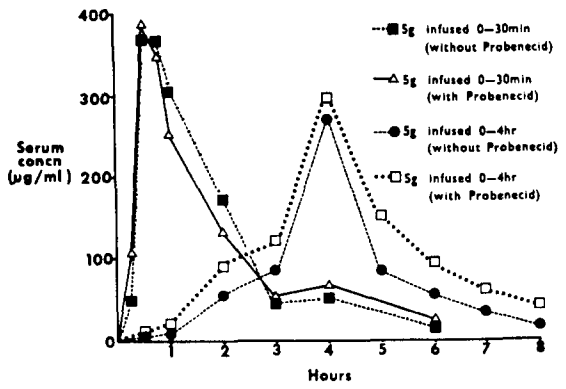

Fig. 3. Intravenous infusion of ticarcillin.

ml at 4 hr. These were somewhat higher than the corresponding carbenicillin levels. There was some enhancement of levels with probenecid, both at peak and at earlier and later measurements. For subjects who received probenecid, levels were above 100 µg/ml between approximately 2½ and 5½ hr.

Conclusions

From the data presented, it is doubtful whether serum and tissue levels of carbenicillin adequate for less sensitive strains of *Pseudomonas* would be achieved by infusion of the antibiotic over a 4 hour period. However, a regime of 5 g in 100 ml of water, infused over 30 min at four-hour intervals, would seem to meet the necessary criteria for both height and duration of levels.

Some clinicians, whilst using the same total daily amount of carbenicillin, modify the regime to 10 g given at 8-hourly intervals. The first 5 g is infused over 30 min, rapidly achieving peak serum concentrations of some 500 µg/ml, and the second 5 g is infused more slowly over a further 90 min, to sustain these peak levels. This form of administration might be expected to result in a rather longer period of relatively low concentrations, before the next intravenous bolus is given. Infusion of the second 5 g over 3–4 hr instead of over 90 min might overcome this. In any case, clinical results using this regime appear to have been quite satisfactory, which is the most important criterion.

Rapid infusion of 10 g carbenicillin gives very high peak serum concentrations, but if this is administered at 8-hour intervals the question arises as to whether concentrations would be sustained at a sufficiently high level during this period. Klastersky and his colleagues have studied serum concentrations following 5, 10 and 15 g carbenicillin infused over 45 min [8]. Levels of antipseudomonas activity in the serum, measured 15 min after the infusion, corresponded to 1000, 250 and 125 µg/ml following the 15, 10 and 5 g doses, respectively. A level of inhibitory activity equivalent to 125 µg/ml remained at 7 hr after the onset of the 15 g infusion and at 6 hr in the case of the 10 g infusion. The authors suggest that the effectiveness of 10 g carbenicillin given three or four times daily should be assessed in severe *Pseudomonas* infections. In addition, it would be valuable to have comparative clinical data for the various alternative regimes.

On the basis of the *in vitro* antibacterial activities and comparative pharmacological data referred to, it would seem that equivalent effectiveness would be obtained using a lower dosage of ticarcillin, for example 3–4 g by rapid infusion over 30 min, six times daily, or 5 g four times daily. Alternatively, use of the same dosage as carbenicillin would give a greater therapeutic margin, which would be particularly valuable against strains of marginal sensitivity to carbenicillin. Ticarcillin promises to be a valuable addition to our limited but growing range of antipseudomonas drugs, and every effort should be made to determine the most effective means of using this new antibiotic.

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Advances in the Management of *Pseudomonas aeruginosa* Infections in Cancer Patients

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Abstract—*Prior to* 1961, *less than* 10 *per cent of patients with serious underlying diseases survived an episode of* Pseudomonas septicemia. *Polymyxin B and colistin were the first antibiotics introduced which had antipseudomonial activity* in vitro. *These were followed by gentamicin, whose therapeutic activity is related to the patient's neutrophil count. The introduction of carbenicillin has had a major impact on the management of* Pseudomonas *infections. Carbenicillin is effective even in patients with severe neutropenia. Several other interesting penicillins and new aminoglycoside antibiotics have been synthetized which have antipseudomonial activity and are currently under investigation.*

Pseudomonas aeruginosa was first isolated and identified in 1882 and the first case of *Pseudomonas septicemia* was established in 1896. Pseudomonas infections were diagnosed infrequently prior to 1940, but since that time, they have become increasingly common in hospitalized patients. Patients with neoplastic diseases, thymic aplasia, extensive thermal burns and cystic fibrosis are especially suscept tible to these infections [1–3]. We have conducted studies on the epidemiology, natural history and treatment of *Pseudomonas* infections in cancer patients during the past decade which will be summarized in this review.

Pseudomonas aeruginosa has been recognized as a major cause of septicemia in cancer patients for many years. During the 1950's, this organism accounted for 25% of fatal infections occurring in patients with acute leukemia [4]. A decade later, 35% of fatal infections were caused by *P. aeruginosa*. In a series of 98 children who died with malignant diseases, 47% of the episodes of fatal septicemia were caused by this organism [5]. During the past several years, it has been responsible for about 25% of the episodes of gram-negative bacilli septicemia at our institution [6].

Micro-epidemics of *Pseudomonas* infections have been observed among leukemia patients suggesting that they acquire the organism from the hospital environment [1]. The carrier rate of *P. aeruginosa* in the stools has varied from 20% among burn patients to 38% among surgical patients [7, 8]. In a study of 87 leukemic patients, we found a carrier rate in the stool or throat of 25% on admission to the hospital [9]. The carrier rate increased to 47% after 4 weeks in the hospital, beyond which time there was no further increase. The frequency of *Pseudomonas* infection was 26% among carriers compared to 13% among non-carriers. Forty-five patients were studied for at least one month (Table 1). Fifty per cent of patients who had the organism isolated from more than half of their culture sets developed *Pseudomonas* infection compared to only 12% of patients who had the organism isolated from less than 25% of their culture sets.

The lung is a common site of *Pseudomonas* infection. Animal experiments indicate that *P. aeruginosa* is cleared less quickly from the lung by host defense mechanisms than other Gram-

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Table 1. *Relationship between carrier state and* Pseudomonas *infection**

Culture sets† with *Pseudomonas* sp. (%)	Number of patients		
	With infection	Without infection	Total
> 50	9	9	18
< 50	6	21	27
< 25	2	15	17
Total	15	30	45

*Considering only patients who submitted at least four culture sets and, hence, were studied for at least one month.

†Culture set = one throat swab and stool specimen.

negative bacilli [10]. *Pseudomonas aeruginosa* pneumonia usually appears on chest x-ray film as a diffuse bronchopneumonia with nodular infiltrates and small areas of radiolucency due to microabscesses [11]. The infection usually involves the lower lobes and spares the apices. Pathologically, the lesions consist of firm, well-defined necrotic nodules surrounded by hemorrhagic parenchyma or of poorly defined hemorrhagic nodular, indurated areas [12]. Foci of infection frequently are located subpleurally and may rupture, resulting in empyema. Microscopically, the lesions are characterized by central areas of coagulative necrosis surrounded by hemorrhagic necrosis and edema, associated with vasculitis of small arteries and veins.

Ecthyma gangrenosum is the hallmark of *Pseudomonas* infection (Fig. 1). These skin lesions occur in 30% of patients with septicemia [13]. Characteristically, they are found in the perineal, axillary and inframammary areas, but they may arise anywhere. Initially, the lesion appears as a red macule and then becomes pustular. Over 12–48 hr it progresses to hemorrhagic necrosis, at which time it has the characteristic bluish-purple center surrounded by an erythematous halo. *P. aeruginosa* can be cultured from these lesions and skin biopsy reveals an acute vasculitis with extensive bacterial colonization of the vessel walls usually without thrombosis. Other types of *Pseudomonas* skin lesions include hemorrhagic necrotic cellulitis, painful inflamed vesicles with an erythematous base and painful subcutaneous nodules [1].

Meningitis, endophthalmitis, pharyngitis and necrotizing enterocolitis are particularly distressing *Pseudomonas* infections which may occur in cancer patients. *Pseudomonas* meningitis may follow neurosurgical procedures, especially when intrathecal catheters are utilized. It is a common form of meningitis in neutropenic patients. *Pseudomonas* blepharitis or conjunctivitis may respond promptly to antibiotic therapy or may progress to endophthalmitis with complete destruction of the eye. Pharyngitis is associated with pseudomembrane formation and may result in extensive necrosis. Necrotizing enterocolitis is a complication of neutropenic patients which is often caused by *P. aeruginosa*. It characteristically involves the cecum but may arise elsewhere and is usually fatal.

We reviewed 67 cases of *Pseudomonas* septicemia in cancer patients [13]. Thirty-four had acute leukemia, and 16 had chronic lymphocytic leukemia or lymphoma. About 80% were receiving cancer chemotherapy and 58% were receiving adrenal corticosteroids. Seventy per cent had neutrophil counts less than 500/mm^3 and 37% had neutrophil counts less than 100/mm^3. About half of the patients were febrile for only one day prior to recovery of *P. aeruginosa* from the blood. The infection was often rapidly fatal. About half of the patients were dead within 2 days and 16% died within 12 hr (Fig. 2).

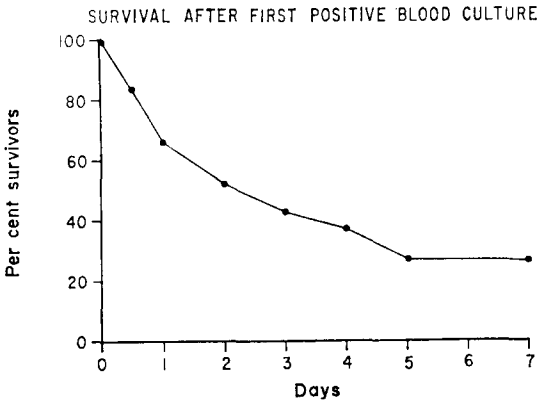

Fig. 2. *Survival from the onset of* Pseudomonas *septicemia in* 67 *cancer patients. Septicemia was dated from when the organism was first cultured from the blood.*

Prior to 1961, less than 10% of patients with serious underlying diseases survived an episode of Pseudomonas septicemia [1, 14]. The polypeptides, polymyxin B and colistin, were the first antibiotics introduced which had substantial anti-pseudomonal activity *in vitro*. All of our isolates of *P. aeruginosa* cultured from cancer patients have been sensitive to 3·12 μg/ml of polymyxin B or less (Fig. 3). Unfortunately, the polymyxins have been of little value for the treatment of serious systemic infections. Only 11 of 46 cancer patients (24%) who developed septicemia [13], and received a polymyxin survived (Table 2). Twenty-two of these patients already were receiving a polymyxin when *P. aeruginosa* was cultured from their blood for

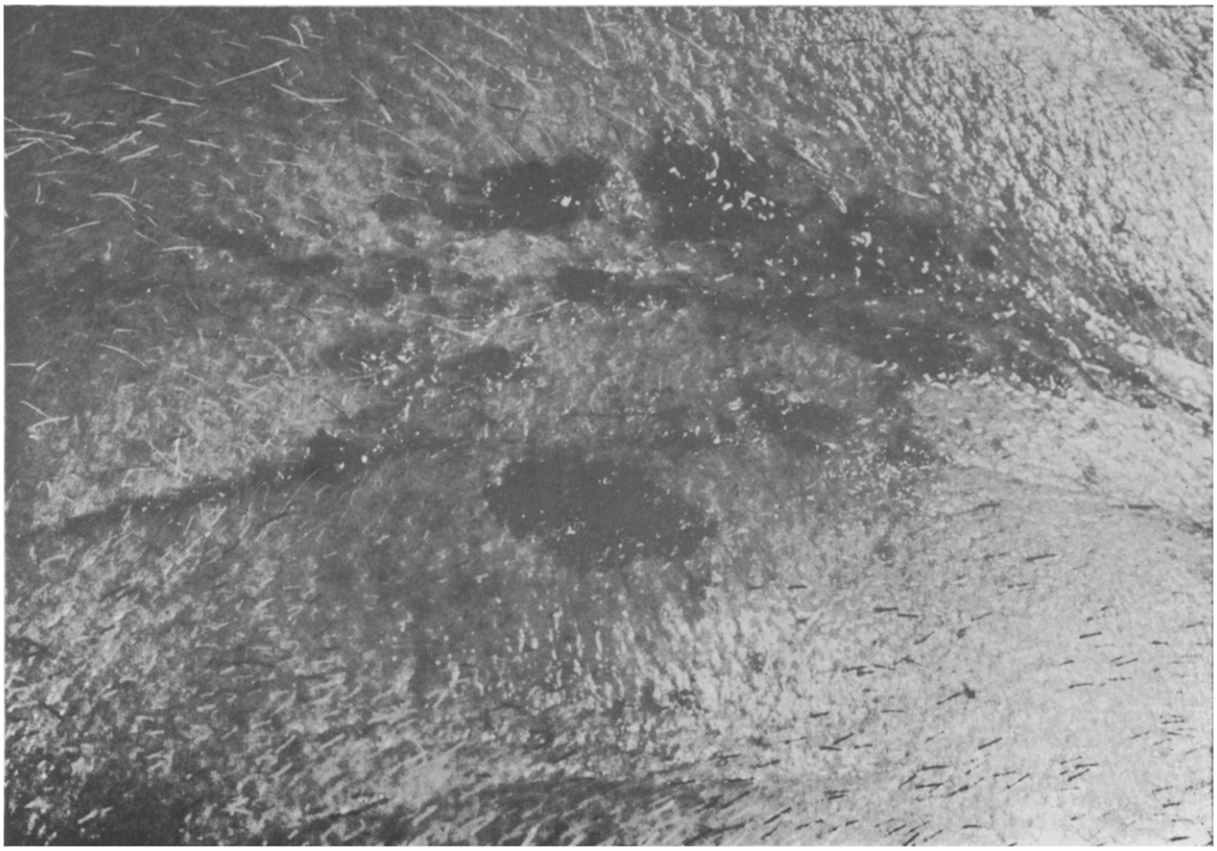

Fig. 1. Ecthyma gangrenosum.

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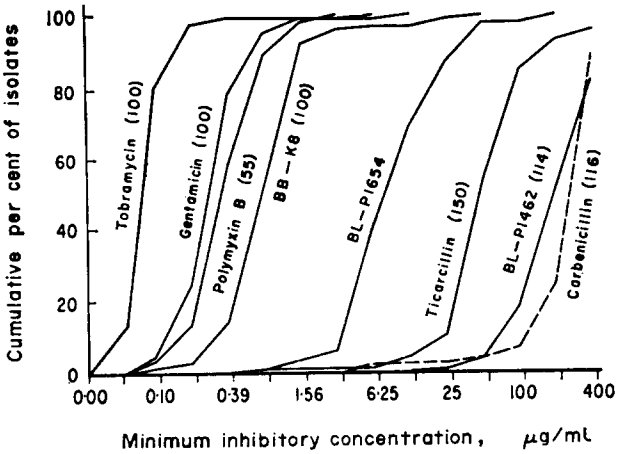

Fig. 3. In vitro *sensitivity of* Pseudomonas aeruginosa *to antibiotics. Numbers in parentheses indicate number of isolates tested. These studies were not performed simultaneously, nor were the same organisms included in all of the studies.*

Table 2. *Effect of antibiotic therapy on* Pseudomonas *infections*

Antibiotic	Total patients	Patients with septicemia	Responded* (%)
Polymyxins	46	46	34
Gentamicin	22	10	45
Carbenicillin	59	38	92
BL-P1462	8	2	75
Ticarcillin	13	4	92

*Some of these patients died of other causes during therapy, or relapsed when the drug was discontinued.

the first time. The survival rate among 21 patients who received either no or inappropriate antibiotic therapy was 14%. Hence, it is clear that polymyxin therapy has little impact on this infection in cancer patients.

Survival was related to the patients neutrophil counts (Table 3). Among the 46 patients who received polymyxins, 58% of those whose neutrophil count increased during the first week of infection survived. Only 8% of those patients whose neutrophil count decreased or remained unchanged survived.

A major disadvantage of the polymyxins is the serious nature of their toxicity. Azotemia, proteinuria and peripheral neuropathy are common side-effects. We have observed electrolyte abnormalities in over 50% of patients receiving polymyxin B [15]. The abnormalities include hyponatremia, hypokalemia, hypochloremia, and hypocalcemia. The drug probably causes a renal tubular defect since these patients excrete inappropriate amounts of electrolytes in their urine. Elderly patients, those receiving concomitant cephalothin or adrenal corticosteroids and those receiving prolonged polymyxin B therapy are most susceptible to nephritoxicity. Serious neurotoxicity and nephrotoxicity also result from colistimethate therapy [16].

Gentamicin, an aminoglycoside antibiotic, has been used extensively for the treatment of *Pseudomonas* infections. In our recent studies, about 95% of isolates of *P. aeruginosa* are sensitive *in vitro* to 1·56 μg/ml of gentamicin or less (Fig. 3) [17]. We usually administer the antibiotic at a dose of 30 mg/m^2. The drug is prepared in 100–200 ml of 5% dextrose and infused intravenously over a 2 hr period. Serum levels of about 1 μg/ml are maintained and peak levels of 4 μg/ml are achieved [18]. Twenty-two cancer patients with *Pseudomonas* infections received gentamicin therapy and 45% responded (Table 2) [19]. Ten of these patients had septicemia.

Response to gentamicin therapy was also related to the patients' neutrophil counts (Table 3). Eight of the 12 patients (67%) with initial neutrophil counts greater than 1000/mm^3 responded. Only 2 of the 10 patients (20%) with initial neutrophil counts less than 1000/mm^3 responded. Eight of the 9 patients (89%) who either

Table 3. *Relation between neutrophil count and response to antibiotics*

Antibiotic	Initial neutrophil count/mm^3 < 1000 Total patients	< 1000 % Response	> 1000 Total patients	> 1000 % Response	Response to infection: Inadequate† Total patients	Inadequate† % Response	Adequate* Total patients	Adequate* % Response
Polymyxins	—	18	—	30	—	8	—	58
Gentamicin	10	20	12	67	13	15	9	89
Carbenicillin	42	81	9	56	29	76	22	82

*Adequate = Increase of initial neutrophil count to 1000/mm^3, or no change if initial neutrophil count > 1000/mm^3.

†Inadequate = Decrease in neutrophil count or no change if initial count < 1000/mm^3.

maintained an adequate neutrophil count or who reacted to their infection with an increase in their neutrophil count responded to gentamicin. Only 2 of the 13 patients (15%) with persistent neutropenia or whose neutrophil count decreased during their infection responded. These results indicate that gentamicin is highly effective in patients with adequate bone marrow reserves, but of little value in neutropenic patients.

Adequate serum activity has been demonstrated in neutropenic patients who failed to respond to gentamicin [20]. Serum specimens have been obtained during therapy and serial twofold dilutions have been made. The infecting organism has been tested *in vitro* to determine the greatest serum dilution which is inhibitory. All organisms tested have been inhibited by 1:4 or greater dilutions of the patients' serum specimens. Organisms causing infection which were still cultured during therapy retained their sensitivity to gentamicin. None of the patients who failed to respond had localized abscesses where poor perfusion could have led to failure. Furthermore, a 50–150% increase in drug dosage did not cure these infections.

Cancer patients develop nephrotoxicity from gentamicin therapy more often than other patients. The frequency of azotemia was 30% during 81 episodes of infection in which the drug was administered for at least 7 days [19]. Some of these patients received multiple courses of gentamicin which may explain this observation. Among 27 patients who received multiple courses, azotemia was observed in 4% following the first course of gentamicin and in 37% following the second course.

The introduction of carbenicillin has had a major impact on the management of *Pseudomonas* infections. Most clinical isolates of *P. aeruginosa* have been sensitive *in vitro* to only 50–300 μg/ml of carbenicillin (Fig. 3). Consequently, large doses of drug must be administered for the treatment of systemic infections. The usual daily dose is 30 g and doses less than 20 g have been associated with suboptimal response rates. When 5 g of carbenicillin are given intravenously over a 2 hr period every 4 hr mean serum levels exceeding 140 μg/ml are maintained and a mean peak serum level of over 200 μg/ml is obtained at the end of the first hour of infusion (Fig. 4) [21].

When administered in this fashion, carbenicillin cured 44 of 59 *Pseudomonas* infections in cancer patients (Table 2) [22]. Thirty-eight of these patients had septicemia. Four additional patients were responding to therapy when they died of other causes. Six patients relapsed when carbenicillin was discontinued, but their organisms remained sensitive to the drug *in vitro*. Five patients were retreated. Two responded, 2 relapsed again and 1 failed to respond. Response rate did not correlate closely with *in vitro* sensitivity of *P. aeruginosa*. Five of 6 infections caused by organisms resistant to 200 μg/ml carbenicillin responded, as did 31 of 33 infections caused by organisms sensitive to 200 μg/ml or less.

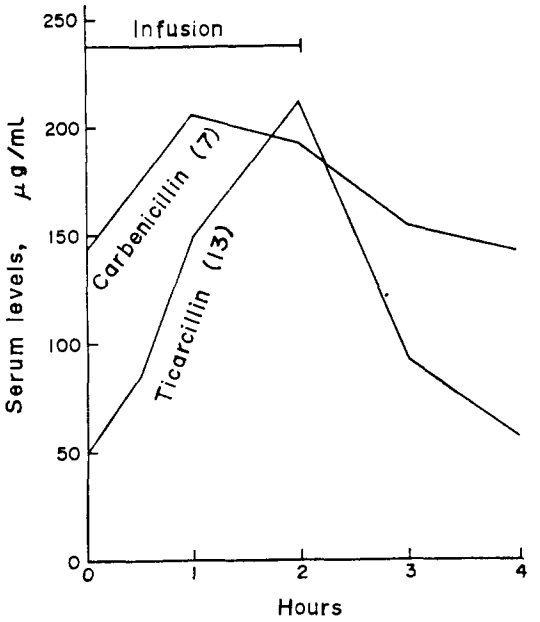

Fig. 4. Serum levels obtained with ticarcillin and carbenicillin. Numbers in parentheses indicate the number of studies conducted. Patients were receiving the drug for at least 2 days when the studies were instituted. Carbenicillin was administered at a dose of 5 g over a 2 hr period every 4 hr with probenecid. Ticarcillin was administered at a dose of 3.5 g over a 2 hr period every 4 hr without probenecid.

Unlike gentamicin, carbenicillin is effective even in patients with severe neutropenia (Table 3). The cure rate was similar regardless of the patient's initial neutrophil count. Twenty-two of the 29 patients (76%) whose neutrophil count fell during the first week of infection were cured, compared to 18 of the 22 patients (82%) whose neutrophil count increased in response to their infection.

The value of carbenicillin can be appreciated by comparing the survival of patients with *Pseudomonas* septicemia who were treated with this drug, gentamicin or a polymyxin (Fig. 5). At 10 days after onset of infection, over 80% of patients treated with carbenicillin were alive compared to 50% of patients treated with gentamicin and only 28% of patients treated with a polymyxin. Prior to the introduction of carbenicillin, 82% of deaths on our leukemia service were due to infection and 38% of these infections were caused by *P. aeruginosa*. Since carbenicillin

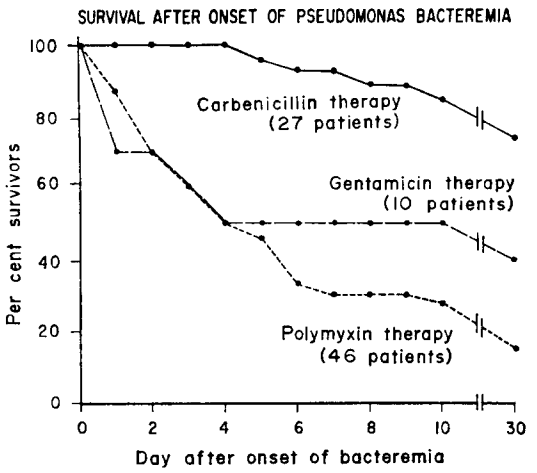

Fig. 5. Effect of antibiotic therapy on survival after onset of Pseudomonas *septicemia in cancer patients.*

has been used routinely, 68% of deaths have been due to infection and only 11% of these infections have been due to *P. aeruginosa*.

Recently, it has become popular to use the combination of carbenicillin and gentamicin for the treatment of these infections because they are synergistic *in vitro* against a substantial proportion of isolates of *P. aeruginosa* [23]. We have conducted a study in which patients with documented *Pseudomonas* and *Proteus* infections were randomized to receive either carbenicillin alone or carbenicillin plus gentamicin. A preliminary analysis of this study indicated that all 7 patients receiving carbenicillin responded compared to 13 of 16 patients receiving the combination. The frequency of superinfection was similar in both groups. Nephrotoxicity was observed in 4 patients receiving the combination but in none of the patients receiving only carbenicillin. Hence, it appears that gentamicin adds only the possibility of nephrotoxicity and no therapeutic benefit.

Since the discovery of carbenicillin, several other interesting penicillins have been synthesized which have anti-pseudomonal activity. The first of these was 6-(*D*-α sulfoamino) penicillin (BL-P 1462) (Fig. 3). About 16% of 70 isolates of *P. aeruginosa* tested were substantially more sensitive *in vitro* to this drug than to carbenicillin and 56% were slightly more sensitive [24]. Only 14% of isolates were more sensitive to carbenicillin. Six of 8 evaluable patients with *Pseudomonas* infections responded to BL-P 1462 [25]. The usual daily dose of drug was 24 g administered in divided doses every 4 hr. This drug has not been evaluated further because more active derivatives have been discovered.

Ticarcillin (BRL-2288) is another new penicillin with anti-pseudomonal activity [26]. Ticarcillin was more active than carbenicillin or BL-P 1462 *in vitro* against *P. aeruginosa* (Fig. 3). It inhibited 80% of 50 isolates at a concentration of 50 μg/ml or less whereas the other 2 drugs inhibited less than 30% of isolates at the same concentration. Twenty-six per cent of the isolates were substantially more sensitive to ticarcillin whereas none were substantially more sensitive to the other 2 penicillins.

Clinical pharmacological studies have been conducted in patients receiving ticarcillin as therapy [27]. We originally administered the drug at a dose of 5 g intravenously over a 2-hr period every 6 hr. This regimen produced peak serum levels of about 200 μg/ml but at the 6th hr the serum level was only about 20 μg/ml. Five patients with *Pseudomonas* infections were treated in this manner [28]. Only 3 responded and 1 of these relapsed when the drug was discontinued. A more effective regimen is the administration of 3·5 g of ticarcillin over a 2-hr period every 4 hr (Fig. 4). This regimen also produces a mean peak serum level of about 200 μg/ml and maintains mean serum levels of about 50 μg/ml [27]. Twelve of 13 patients with *Pseudomonas* infections who were treated in this manner responded [28].

A series of (α-substituted ureido) penicillins have been synthesized which are very active *in vitro* against *P. aeruginosa* [29]. One of these derivatives, 6(*D*-α guanylureido) penicillin (BL-P 1654), has been selected for clinical evaluation. This drug inhibited about 75% of 50 isolates of *P. aeruginosa* at a concentration of 12·5 μg/ml or less (Fig. 3). Toxicology studies in animals suggest that this penicillin may have some nephrotoxicity. Clinical pharmacological studies are currently in progress and therapeutic studies are being initiated.

Several new aminoglycoside antibiotics with anti-pseudomonal activity are currently under investigation. Tobramycin has been isolated from an antibiotic complex produced by *Streptomyces tenebrarius* [30]. Its spectrum of activity is similar to that of gentamicin, however, it is more active against *P. aeruginosa* (Fig. 3). In our study of 100 isolates, tobramycin inhibited 80% at a concentration of 0·10 μg/ml, whereas gentamicin inhibited only 4% [31]. Preliminary clinical investigations with this drug have been very encouraging. Ten of 13 cancer patients with *Pseudomonas* urinary tract infections were cured with tobramycin therapy [32].

BB-K8 is a new antibiotic which is a chemical derivative of kanamycin sulfate. It has broad-

spectrum activity against Gram-negative bacilli. Unlike kanamycin, it is also active against *P. aeruginosa* [33]. However, it is less active *in vitro* than either tobramycin or gentamicin (Fig. 3). At a concentration of 0·78 μg/ml, tobramycin inhibited 96% of 50 isolates of *P. aeruginosa*, gentamicin inhibited 56% and BB-K8 inhibited 8%. BB-K8 inhibited over 95% of isolates at a concentration 3·12 μg/ml. An important feature of this antibiotic is its activity against isolates of Gram-negative bacillli which are resistant to other aminoglycosides. The toxicity of this drug in animals is less than gentamicin so it is likely that higher doses can be given, resulting in higher serum levels [34].

Butirosin is a new aminoglycoside antibiotic complex which has broad-spectrum activity against Gram-negative bacilli [35]. It is active against *P. aeruginosa*, including clinical isolates resistant to gentamicin. Clinical studies of this drug have not been initiated at the present time.

Ten years ago when a neutropenic patient developed *Pseudomonas* infection, he almost always died. Today, the vast majority recover, and many become asymptomatic within 24–48 hr. New chemotherapeutic approaches have improved considerably the survival of patients with many malignant diseases, including acute leukemia, lymphoma, soft tissue sarcomas and breast carcinoma. The ability to control *Pseudomonas* infections has played a contributing role in this success.

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Antimicrobial Therapy as a Part of The Decontamination Procedures for Patients with Acute Leukemia*

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Abstract—*Thirty patients suffering from acute leukemia were treated with cytostatic drugs to induce remission under gnotobiotic care. Twenty-one were isolated and decontaminated by antibiotics and nine were isolated only in plastic isolation systems. When compared retrospectively with patients treated on the open ward it seemed that the remission rate of 83 per cent (complete and partial remission) achieved in the gnotobiotic patients was possibly higher. However, pneumonias have not been seen in the gnotobiotic patients whereas the patients on the open ward suffered from respiratory infections frequently. Comparing the susceptibility to infection as partially indicated by the degree of neutropenia, the rate of fever caused by infection or of unknown origin and the remission rate in both, isolated, and isolated and decontaminated patients no striking difference could be found. These results are preliminary. More data to be statistically evaluated will be obtained in a prospective study which is performed currently under the auspices of the E.O.R.T.C. Gnotobiotic Project Group.*

Some side-effects of the antimicrobial treatment with non-absorbable antibiotics seem to be of importance. The patients treated with a combination of neomycin sulfate, bacitracin, polymyxin B and nystatin had a significantly higher max ε in thrombelastographic studies. During the treatment with the antibiotics the absorption of D-*glucose was not impaired. In contrast, the absorption of* D-*xylose was decreased.*

INTRODUCTION

DURING the past few years it has been shown that the survival time of patients suffering from acute leukemia (AL) can be extended from months to some years by remission-inducing chemotherapy [1, 2]. However, the management of the therapeutic attempts of AL bears many difficulties, and the success of extending the survival is not only attributed to the chemotherapeutic trial, but also to the improved supportive care [3]. A high incidence of infections and hemorrhages is observed among other less frequent complications due to the suppression of the remaining hemopoiesis and of the immune apparatus, and the damaging effects on the mucous membranes during the chemotherapy, which may go on for several weeks. Thus, the benefit of the cytostatics is restricted to those patients who survive the phase until remission is achieved, whereas the life-expectancy of the others may be shortened due to the same therapeutic regimen.

The problem, to treat the life-threatening infections, arises from the fact that most of the microbes of the ordinary environment and also those belonging to the patients own microflora can become pathogenic in patients with AL under cytostatic therapy. The bacteria and

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fungi of the intestinal tract, the body orifices and the skin can be the causing agents of fatal septicemias. Also, rare infections due to viruses or parasites do occur. It could be shown that germfree mice bearing a spontaneous leukemia tolerated cytostatics better than their conventional counterparts. In fact, the germfree mice survived much higher doses of cyclophosphamide [4]. This led to the assumption that a germfree or gnotobiotic state is desirable in patients to be treated by high doses of cytostatic drugs. Usually, germfree patients are not available but the successful decontamination of the associated microflora in mice, rats and monkeys by non-absorbable antibiotics showed a possibility how to render patients germfree or at least free from bacteria and fungi [5]. After the development of isolation devices one expected to achieve bacteria and fungi free states in man by the use of non-absorbable antibiotics in analogy to the results in animal research.

It is the aim of this presentation to report about experiences in the treatment of AL under gnotobiotic care, that is, isolation or isolation and decontamination. It should be mentioned that our unit is undertaking a prospective study randomizing patients to different treatment groups in cooperation with other European centers and under the auspices of the EORTC Gnotobiotic Project Group. This study is not yet completed. Our own data are to be understood as preliminary.

PATIENTS AND PATIENTS' CARE

Thirty patients treated in a gnobobiotic state were studied. Twenty-one of them were isolated and decontaminated, nine were isolated only. Some comparisons with patients on the open ward were done retrospectively. The isolation took place either in a "LIFE ISLAND" or a completely tight new isolator of own construction (Ulm Bed Isolated System).

The decontamination therapy applied throughout the isolation period, (Fig. 1) consisted of daily bathing with TEGO tenside, a surface disinfecting agent combining the detergent effect of anionic and the bactericidal effect of kationic ($C_{12}H_{25}$—NH—C_2H_4—NH—C_2H_4—NH—CH_2—COOH), inhalation of neomycin sulfate, bacitracin and nystratin, the treatment of the oral cavity by hexetidine and a combination of non-absorbable antimicrobial agents: bacitracin 160 000 units, neomycin sulfate 3·0 g, polymyxin B 200 mg and nystatin 6 mio units/day. This antibiotic regimen has been given up to 9 weeks and to a total dose of 207·0 g neomycin sulfate, 13·8 g polymyxin

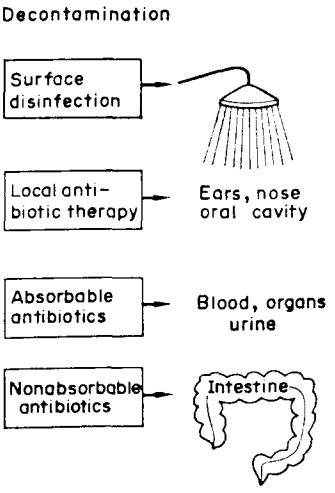

Fig. 1. Principle of decontamination procedures.

B, 11 mio units bacitracin and 414 mio units nystatin. The observation time was terminated after completion of the cytostatic therapy and achievement of remission and a rise of the granulocytes above 1500/mm^3, or when the applied therapy failed to produce remission. The duration of the gnotobiotic care varied from 20 to 105 days. The mean duration was 40 days. The total observation time comprised 1191 days.

RESULTS

The decontamination treatment resulted in a markedly decreased variety of the microflora indicating the suppression of the microorganisms. This favourable result is impaired by the fact that potential pathogens as *Klebsiella* sp. or *Candida* sp. may remain as mono- or diflora as observed in several patients. Approximately 3–4 days after the initiation of the antibiotic combination therapy the gut flora did not show any growth of microbes, but in all patients single microbial strains could be detected in the feces during the course of treatment in varying intervals. In contrast, from the oral cavity germs could be cultured almost constantly. Of 378 skin cultures 177 remained sterile (47%). Of 104 investigations of ears and nose, 52 swabs (50%) were without microbial growth. One hundred and sixty-four of 226 fecal cultures did not show any microbial growth (73%). Only in 7 fecal cultures could anaerobic bacteria be found. Although the oral cavity was treated by decontamination measures after the intensive dentist's care, only 21 (7%) of 296 specimens of the oropharynx did not grow bacteria. However, it has to be noticed that only the association with *Streptococci* and *Neisseria* spp. could be observed, whereas bacteria belonging to ordinary intestinal micro-

flora are found constantly in the throat of leukemic patients without decontamination procedures.

Due to the leukemic process and to the bone marrow toxicity of the applied cytostatic drugs neutropenia was observed frequently. The isolated and decontaminated patients had a granulocyte count below 500/mm^3 blood during 471 of 785 observation days = 60%. The isolated patients had a granulocyte count below 500/mm^3 blood during 244 of 406 days = 60%. Elevated body temperature above 38°C caused by infection or of unknown origin was seen during 167 days (21%) in the decontaminated patients and during 74 days (18%) in the group of patients isolated only. Also, there was not any difference between the rates of complete and partial remission of both patient groups. Eighty-three per cent of all gnotobiotic patients achieved partial or complete remission. Twenty-four of the 30 patients were living and well 30 days after the termination of the gnotobiotic care = 80%. Causes of death: one patient who was isolated only died in partial remission from a generalized *Herpes simplex* infection and a *Staph. aureus* septicemia. Both germs were associated with the patient from the entry to the hospital throughout the observation period. Of the decontaminated patients one died from a hemorrhage after discontinuation of gnotobiotic care, one from pulmonary infarction, one from cardiac failure probably due to Daunorubicin, one from pulmonary oedema 5 days after the discontinuation of gnotobiotic care and after coecostomy (for the treatment of earlier radiation lesion). One decontaminated patient died from an aerobacter septicemia cultured in the fecal flora and subsequently in the blood. This bacterium was resistant to all antibiotics except gentamycin sulfate. Obviously this monoassociation resulted directly from the treatment with the non-absorbable antibiotics which did not contain gentamycin. Despite parenteral treatment with gentamycin up to 5 mg/kg per 24 hr the septicemia could not be controlled.

SIDE EFFECTS

The applied antibiotics were tolerated by the patients but caused nausea frequently and vomiting occasionally. To control the effects of the antibiotics on the intestinal mucosa the absorption of disaccharides was investigated. The uptake of oral glucose was not altered by the decontamination and cytostatic chemotherapy. Throughout the period of treatment the absorption was within normal limits. An

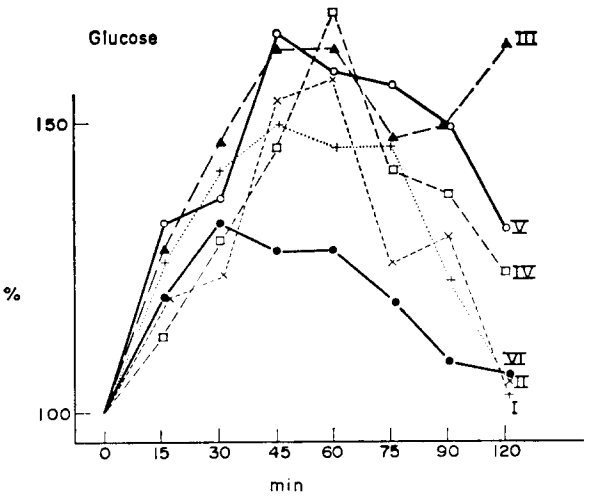

Fig. 2. Blood level of absorbed D-glucose during two hours after the oral application.

example is demonstrated in a patient with acute myeloid leukemia (Fig. 2). The test was performed repeatedly, before the decontamination (I), weekly thereafter (II, III, IV, V), and after the discontinuation of the antibiotics (VI).

In contrast, the absorption of D-xylose, also a disaccharide, was impaired by the decontamination regimen measured by the urinary output during 6 hours after the oral application. An example of the dramatic decrease of the D-xylose excretion is shown in a patient treated for an undifferentiated AL and decontaminated for 4 weeks (Fig. 3). The test was performed

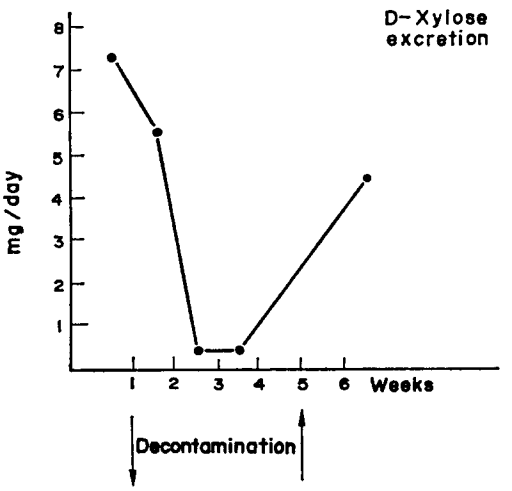

Fig. 3. D-xylose excretion in a patient with leukemia.

before, weekly during, and after the discontinuation of the non-absorbable antibiotics. The investigations indicated that the absorption of different sugars (pentoses and hexoses) by the mucosa cell are not equally affected by the used antimicrobial agents.

The clinical observation of the leukemic patients suggested that the risk of hemorrhages was less pronounced in decontaminated

patients. Investigations of a number of hemostaseological tests gave not different results comparing patients with and without antibiotics except the thrombelastographic studies. Whereas the reaction time (r) was not altered, the quality of the coagulum differed obviously expressed by a higher value of max ε seen in the decontaminated patients. (Fig. 4) These laboratory findings may correspond with the subjective impression of a less pronounced bleeding tendency in these patients.

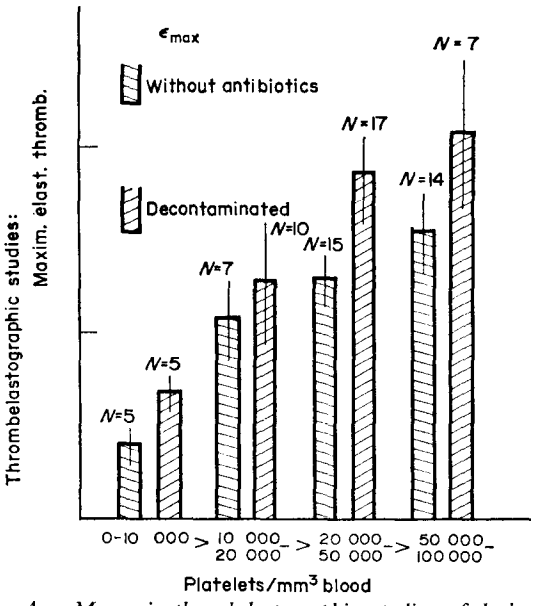

Fig. 4. Max ε in thrombelastographic studies of leukemic patients with and without therapy with nonabsorbable antibiotics correlated to different ranges of platelet counts.

DISCUSSION

Although several clinical centers have applied decontamination methods in hundreds of patients, a germfree state could not be reported. In fact, only once was it possible to achieve a state free of bacteria and fungi after the discontinuation of the antibiotics in a small infant treated in our hospital [6]. It is likely that the used antibiotics suppress the replication of bacteria but do not eliminate it entirely [7]. Because germfree states are not achieved yet the original target of complete prevention of infection could not be reached. The question remains whether at least partially a prevention can be achieved to justify the elaborate work and expensive therapeutic approach. From the literature it seems now that the antibiotic decontamination alone does not give the expected benefit. Only those patients who are isolated simultaneously have less infections than the controls [7]. On the other hand, those patients isolated adequately and treated by decontamination methods may still suffer from severe infection and eventually die from a monoassociation that we have observed. So far, the decontamination by non-absorbable antimicrobial agents may occasionally endanger an individual patient's life by influencing the microbial interaction unfavourably. Though the incidence of severe infections has been reduced in various centers the remission and survival rate have not been better in a prospective study of the NCI [7]. If that is true in general, one has to consider abandoning the decontamination therapy. Bodey and coworkers [8] also reported not a better rate of remission but a significantly longer duration of remission in decontaminated patients treated in protected environments. Assuming the statistical design of that retrospective study is appropriate, still there remains some doubt as to whether a more aggressive chemotherapy was applied to those patients without bias. It could be that the treating doctors felt safer to use higher doses of cytostatics in the patients in the prophylactic antibiotic-protected environment program, so that this decision was not made arbitrarily. This question has to be investigated in further prospective studies. In our experience there was no striking difference between the patients isolated only and those who were decontaminated simultaneously. In both groups pneumonias have not been seen whereas respiratory infections and pneumonias occurred in patients on the open ward occasionally. This could indicate that the isolation prevents aerogenic infection. Evidently the differences in the outcome of patients treated by prophylactic antibiotic programs to decontaminate the endogenous microflora and those treated under usual circumstances are not great, so that the advantages of such a treatment may only be seen in clinical studies including a large number of patients. Therefore, one has to consider what side effects are caused by such a therapy. Usually the antibiotics are tolerated moderately but can cause nausea and vomiting. Interesting is the fact that the hemostasis may be affected by the non-absorbable antibiotics used in our patients. It is not clearly understood whether the suppression of the microflora or the pharmacological action of one of the antimicrobial agents is responsible for that finding. It is known that f.i. neomycin sulfate influences the cholesterol absorption and excretion in rats and chicken in different ways: by its antimicrobial properties and also by its polybasic properties [9]. This has also to be discussed when trying to understand the different results in the absorption studies of D-glycose and D-xylose. Whether this action on the mucosa cell of the

used polybasic antibiotics is harmful for the patient or not remains uncertain.

It may be concluded from our experiences and preliminary results of the described patients study and from the reports in the literature that at present one cannot state that the decontamination by non-absorbable antibiotics during the treatment of leukemia is the supportive therapy of choice. It can only be recommended to clinical centers able to perform or to participate in prospective patient studies with large numbers of patients.

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Pseudomonas Septicemia: Incidence, Epidemiology, Prevention and Therapy in Patients with Advanced Cancer

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Abstract—*In the past decade,* Pseudomonas aeruginosa *has become a common cause of infection in cancer patients and* Pseudomomas *sepsis has become the most frequent infection in patients with acute non-lymphocytic leukemia. Although only some patients are colonized with this organism at the time of hospital admission, approximately* 50% *of ANLL patients will eventually become colonized. Once colonized, subsequent septicemia is the rule in the granulocytopenic patient. Surveillance cultures, in association with serotyping, can assist in detecting the colonized, at-risk patient and aid in establishing and maintaining preventive measures such as environmental decontamination, personnel hygiene, reverse isolation (including sterile water and cooked food), prophylactic non-absorbable antibiotics, and vaccination. Prevention of infection must take high priority because of the high morbidity and mortality of this disease. Until the advent of carbenicillin, death was the rule despite antibiotics such as polymixin B, colistin, and gentamicin which are bactericidal* in vitro *but not effective in the granulocytopenic patient. Carbenicillin with or without added gentamicin has been shown to consistently achieve an improvement rate of well over* 50%. *The combination is usually markedly synergistic and resistance, although occurring, has been relatively infrequent. At the present time, we favor this combination for the treatment of* Pseudomonas aeruginosa *infections in granulocytopenic cancer patients and favor the addition of cephalothin for initial empiric therapy before culture results are available.*

INCIDENCE AND EPIDEMIOLOGY

In the two years from July 1, 1970 to June 30, 1972, *Pseudomonas aeruginosa* has been the most frequent cause of septicemia (Table 1) at the Baltimore Cancer Research Center (BCRC). In acute non-lymphocytic leukemia (ANLL), 15 of 35 septicemias (43%) were caused by *P. aeruginosa.* As would be expected, few septicemias occurred in the patients with stages I-III Hodgkin's disease, but *P. aeruginosa* caused 3 of the 5 which did occur. In the non-leukemic patients with advanced cancer being treated by drug therapy (stage IV lymphoma and metastatic solid tumors), 7 of 43 septicemias (16%) were due to *P. aeruginosa.* That is, *Pseudomonas* is a more frequent cause of septicemia in acute leukemia patients than in most other advanced cancer patients.

We recently reviewed the infections occurring in 48 consecutive ANLL patients at the BCRC [1]. As noted above, *Pseudomonas aeruginosa* was found to be the most common cause of bacteremia and a frequent cause of non-bacteremic infections. During hospitalization, 28 of the 48 (58%) patients were found by twice weekly routine surveillance cultures (rectum, gingiva, nose, axilla, and urine) to be colonized by one or more serotypes of *Pseudomonas aeruginosa* (Table 2). Twenty of these colonized patients (20 of 28 or 72%) subsequently developed infection with the corre-

Table 1. *Septicemias in cancer patients July* 1, 1970—*June* 30, 1972

	ANLL	ALL	HD1-3	Lym	Solid	Total
Pseudomonas aeruginosa	15	1	3	4	3	26
Klebsiella spp	5	0	0	2	4	11
Escherichia coli	4	2	0	2	0	8
Staphylococcus aureus	1	2	0	3	1	7
Gp D *Streptococcus*	1	0	0	2	3	6
Candida albicans	1	2	0	1	4	8
Others	8	3	2	6	8	27
Total	35	10	5	20	23	93

Table 2. *Septicemia subsequent to colonization in* 48 *ANLL patients*

	Colonized	Septicemias
Pseudomonas aeruginosa	28	19
Klebsiella spp	42	6
Escherchia coli	42	5
Serratia sp	9	3
Staphylococcus aureus	26	2
Candida albicans	39	2
Gp D *streptococcus*	44	2
Proteus spp	41	0
Non-aeruginosa *Pseudomonas*	29	0

sponding serotype of *P. aeruginosa*; 16 of these patients (16 of 28 or 57%) developed *Pseudomonas* septicemia. In each case, infection developed during a period of profound, prolonged granulocytopenia, a major predisposing factor to infection in acute leukemia [2]. No patient who developed *Pseudomonas* sepsis had not had *P. aeruginosa* of the same serotype previously detected in the routine surveillance cultures [1].

Based on the surveillance culture data (Table 2), no other organism colonizing the ANLL patients was as invasive as *P. aeruginosa*. Most patients were colonized with *E. coli*, *Klebsiella* spp, group D *Streptococcus* spp, and *Candida albicans*, but infection with these colonizing organisms was relatively infrequent compared to *Pseudomonas aeruginosa*. Non-aeruginosa *Pseudomonas* spp and *Proteus* spp colonized multiple patients but none progressed to bacteremia [1].

Particularly distressing was the observation that two-thirds of the 28 patients colonized with *Pseudomonas aeruginosa* (Table 3) acquired that organism only after admission to the hospital [1]. Bodey found that 25% of entering leukemia patients carried *Pseudomonas aeruginosa* whereas after 2-4 weeks of hospitalization, 47% of patients were colonized, i.e. approximately half of the colonized patients apparently acquired this pathogen in the hospital [3].

Epidemiologic investigations at the BCRC have been greatly aided in recent years by a rapid and reproducible serotyping system developed by Fisher, Devlin, and Gnabasik [4]. Since 1969, BCRC isolates of *Pseudomonas* have been speciated and all *P. aeruginosa* have been serotyped [5]. Serotyping made practical serial evaluation of colonization and subsequent infection [1, 5, 6], assisted in establishing the presence and origin of certain "mini"-epidemics, and, most recently, aided in evaluating the emergence of carbenicillin- and/or gentamicin-resistant organisms [7].

In 1969–1970 we evaluated 190 cancer patients with weekly surveillance cultures (Table 4) of rectum, gingiva, axilla, nose, and urine [6]. *Pseudomonas aeruginosa* was recovered from 57 (30%) of these patients, but there was considerable variation in ultimate colonization rates between tumor types. Nearly 50% of the patients with hematologic malignancies were either colonized at admission or acquired *P. aeruginosa* during their hospitalization, whereas only 14% of lymphoma patients and 19% of advanced metastatic solid tumor patients were ultimately colonized. In that

Table 3. Pseudomonas aeruginosa *colonization and subsequent infection*

	Baseline	Acquired	Total
Patients colonized	9	22	28
Patients infected	7	15	20
Patients with bacteremia	3	13	16

Table 4. *Colonization and subsequent sepsis with* Pseudomonas aeruginosa 1968–1969

Tumor type	Cultured	Colonized	Septicemia
Hematologic	66	31 (47%)	11 (35%)
Lymphoma	43	6	
Brain	28	10 (21%)	1 (3.6%)
Solid	53	10	
Total	190	57 (30%)	12 (21%)

series, 35% of the colonized patients with a hematologic cancer developed a septicemia with *P. aeruginosa* of identical serotype to that previously detected in the surveillance cultures. In marked contrast, only one of the 26 (3·6%) colonized patients with non-hematologic cancer progressed to subsequent septicemia. Patients with hematologic malignancies, notably acute leukemia, tend to become colonized with *Pseudomonas aeruginosa* considerably more frequently than do most other patients with advanced cancer. Furthermore, subsequent *Pseudomonas* septicemia is frequent in the colonized leukemia patient but relatively uncommon in the colonized patient with lymphoma, brain tumor, or metastatic tumor. Additional investigation of these patients indicated that the major predisposing factor to subsequent septicemia, other than tumor type, was granulocytopenia. *Pseudomonas* sepsis did not occur in patients with > 1000 granulocytes/mm^3, whereas 44% of the granulocytopenic colonized patients developed sepsis [6].

We believe that these investigations indicate the usefulness of routine surveillance cultures (rectum and gingiva are generally sufficient to detect *Pseudomonas aeruginosa* colonization) in centers treating patients with leukemia. Twice or thrice weekly cultures will usually identify the colonized, at-risk patients who, should they have or develop granulocytopenia, will likely progress to *Pseudomonas aeruginosa* septicemia. Such information allows for: (1) pre-infection antibiotic susceptibility testing, (2) detection of changing patterns of colonization leading to identification of acquisition sources such as food, water, hands, or equipment, (3) identification of the at-risk patient for controlled investigations of vaccines, prophylactic non-absorbable antibiotics, etc., and (4) increased awareness of possible *Pseudonomas aeruginosa* invasion should signs of infection develop in the colonized patient.

PREVENTION

The surveillance-culturing technique has suggested that a majority of infected patients become colonized following admission to the hospital. A variety of possible routes of acquisition have been identified: contaminated hands of personnel, contaminated food, water, ice, humidifiers, respirators, etc. [6–10]. Cultures of the environment taken at routine intervals to assess for contamination (serotyping will identify environmental organisms that are concurrently colonizing patients) will aid in planning appropriate remedial programs. A constant program of personnel education, supplemented with intermittent hand cultures, helps to ensure adequate hand hygiene.

Currently a number of centers, including the BCRC [11], are evaluating the ability of reverse isolation with laminar air flow rooms and/or oral non-absorbable antibiotics [12–14]. The data available strongly suggest that *Pseudomonas aeruginosa* infections are reduced in acute leukemia patients treated in these isolation rooms with the non-absorbable antibiotics. It is not established whether the use of the non-absorbable antibiotics alone will reduce *Pseudomonas* acquisition and infection.

Another potential prophylactic measure is to vaccine the high-risk patient. An investigational polyvalent vaccine has been prepared by Parke-Davis, Co. against the original 7 serotypes of *P. aeruginosa*. Initial trials were done with burn patients and it is now under active

investigation in cancer patients [15]. Young *et al.* [16] recently completed a randomized controlled trial in over 270 advanced cancer patients and observed a definite although limited reduction in fatal *Pseudomonas* infections. Although the vaccine produced a rise in opsonizing antibody titer, infected patients had marked granulocytopenia, suggesting the importance of both antibody and neutrophils in preventing *Pseudomonas* invasion [17].

THERAPY

The significance of *Pseudomonas aeruginosa* sepsis is apparent from Fig. 1, which is a composite of survival records from various institutions. Curtin *et al.* [18] reviewed 91 *Pseudomonas* bacteremias prior to 1961 at Johns Hopkins;

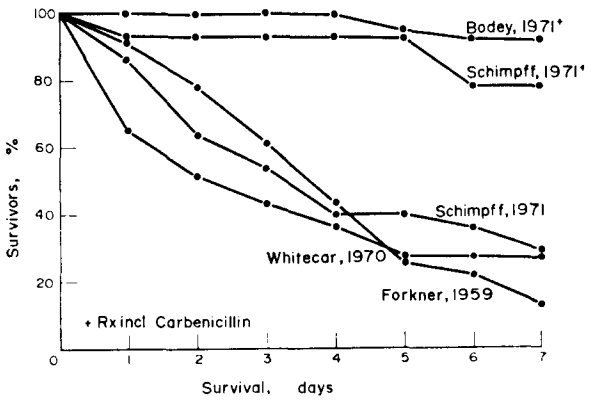

Figure 1.

the great majority died and 24 of the 28 septic leukemic patients succumbed. Forkner *et al.* [19] reviewed the NIH experience prior to 1958; all 23 septic patients died of their infection. Notably, 9 were dead by the third day (i.e., before culture results and antibiotic sensitivities were usually available) and 20 of the 23 were dead within a week of onset. Whitecar *et al.* [20] reviewed 67 episodes of *Pseudomonas* bacteremia occurring in cancer patients at M. D. Anderson Hospital from 1965–1968. Half of the patients had died within two days despite prompt antibiotic therapy, usually with a polymixin. Indeed, 22 of these 67 patients were already receiving a polymixin antibiotic at the onset of bacteremia. In the year prior to institution of the BCRC empiric antibiotic therapy protocol in 1969, half of the patients with *Pseudomonas aeruginosa* bacteremia died within three days and ultimately all but 2 of these 22 patients expired [21]. Finally, despite promptly initiated empiric antibiotic therapy, 7 of 8 granulocytopenic cancer patients treated at the BCRC with gentamicin (81 mg IM tid) as the sole antipseudomonal antibiotic had persistently positive blood cultures [21].

Since the availability of carbenicillin, there has been a marked improvement in the survival of these bacteremic patients. Using carbenicillin alone (5 g intravenously every 4 hr), Bodey *et al.* [22] observed a complete or partial response in 12 of 14 cancer patients with *Pseudomonas* septicemia despite persisting granulocytopenia. This impressive improvement in survival compared to earlier years has persisted in a follow-up report in which 19 of 24 additional septic patients improved [23]. Tattersall *et al.* [24] utilized carbenicillin in combination with 4 other antibiotics (often including gentamicin) and observed improvement in 6 of 7 neutropenic leukemia patients with *Pseudomonas* bacteremia.

At the BCRC, a program of initial empiric antibiotic therapy with carbenicillin and gentamicin was begun in 1969 [21]. Carbenicillin was chosen for its bactericidal *in vitro* activity against *Pseudomonas aeruginosa*; gentamicin was selected both for its activity against *P. aeruginosa* and for its wide spectrum against most of the other frequently encountered pathogens. In addition, the two drugs were reported to be synergistic and a suggestion was made that combination therapy might tend to retard development of resistance to *P. aeruginosa* [25]. Carbenicillin was given as 5 g intravenously every 4 hours and gentamicin as 80 mg intramuscularly every 8 hr. These antibiotics were used as initial empiric therapy for granulocytopenic febrile cancer patients and were begun immediately after cultures were obtained from blood, urine, nose, throat, stool and any other appropriate site. The patients were carefully examined and examinations were repeated at least once a day both to detect the site of infection origin and to assess the patient's clinical course.

Prompt antibiotic therapy proved to be appropriate in most instances: infection was microbiologically documented in 48 of 75 episodes and clinically proven in an additional 12 episodes (60 of 75 or 80%). *Pseudomonas aeruginosa* was etiologic in 21, other Gram-negative bacilli in 20, Gram-positive organisms in 6, and *Candida albicans* in 1; all but the *C. albicans* were sensitive to one or both antibiotics *in vitro*. Improvement occurred in 14 of the 21 (67%) *Pseudomonas aeruginosa* infections including 8 of the 13 (62%) bacteremias. With a single exception, among those patients who died of bacteremia, death occurred from 5 to more than 90 days after the onset of

bacteremia, a marked change from the previously observed responses to polymixin or gentamicin noted above. None of the patients with abacteremic *Pseudomonas* infections died of that infection (Table 5).

Unfortunately, the response of patients with other Gram-negative infections was rather poor (only 8 of 20 or 40% improved). In those infections, generally only the gentamicin showed *in vitro* activity against the invading bacteria, again suggesting that gentamicin alone, despite *in vitro* susceptibility, is inadequate therapy for Gram-negative bacillary infections in granulocytopenic cancer patients [21]. This observation is consistent with those of Klastersky [26], Bodey [27], and Tattersall [24].

Cephalothin was, therefore, added to the antibiotic regimen in the hope that it would be synergistic with gentamicin against *Klebsiella* spp and perhaps other Gram-negative bacilli as well. During the next 20 months, this combination was used 177 times with 14 of 24 (58%) episodes of *Pseudomonas aeruginosa* infection improving. This is perhaps slightly less than the 67% improvement rate reported in the first study but there has been a distinct increase in the response rate to *Klebsiella* sp and *E. coli* infections [28].

Empiric antibiotic therapy is inconsistent with many of the accepted principles of good antibiotic management of infected patients. We believe, however, that there is ample evidence (see Fig. 1) that the granulocytopenic cancer patient must be treated appropriately at the first evidence of infection, otherwise, death from bacteremia all too often occurs before blood culture results are available. The combination of carbenicillin and gentamicin as empiric therapy has been very efficacious for *Pseudomonas aeruginosa* septicemia, and despite widespread use at the BCRC, there has been relatively little development of resistant *P. aeruginosa* [7].

Standiford and Kluge [29] have performed *in vitro* synergism assays on many *P. aeruginosa* isolates from the BCRC and elsewhere. Synergism between carbenicillin and gentamicin has been detected in 82 of 85 clinical isolates when the MIC to gentamicin has been < 40 μg/ml. However when the MIC to gentamicin has been > 40 μg/ml, none of 18 strains showed clinically useful synergism, not unlike previous synergism results with penicillin and streptomycin for enterococcus [30]. This data will later be correlated with the known clinical course of each infected patient to determine if synergism was of clinical importance. In this regard, Klastersky [26] has shown that when antibiotic combinations of ampicillin and gentamicin or cephalothin and gentamicin are synergistic *in vitro* against the invading organism, the rate of therapeutic failure is less (20%) than when the combination is found to be non-synergistic (51%).

Table 5. Empiric carbenicillin–gentamicin antibiotic therapy in 75 suspected infections in granulocytopenic cancer patients

Classification	Infections	Evaluable	Improved (%)
Documented infections	48	43	25 (58)
P. aeruginosa (bacteremia)	13	13	8 (62)
P. aeruginosa (no bacteremia)	8	6	6 (100)
Other Gram-negative bacilli	20	20	8 (40)
Other organisms	7	4	3 (75)
Probable infections	12	8	8 (100)
Possible infections	12	5	5 (100)
Infection doubtful	3	0	—
Total	75	56	38 (68)

*% of evaluable infections.

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Closing Remarks

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THIS SYMPOSIUM has presented some carefully made observations and studies in a field which does not lend itself readily to clinical scientific investigation and represents, therefore, a temptation to the medical profession for an intuitive approach. We should resist this temptation by all means, including the use of comparative clinical trials, in order to minimize the area of uncertainty, which is costly to the patient, and to the community just as well. And it is perhaps appropriate to confine these closing remarks to the significance of controlled clinical trials in our health programs. Many people think that there is, at present, a crisis in health care both in Europe and in the United States. The health care industry is now funded at a level of 75 billion $ per year in the United States and at a level not too far from this amount in Western Europe. The more alert among our citizens are beginning to wonder and to demand that we, the medical profession, provide justification for our program of patient management. We are expected to exert leadership in placing our patient care program on a scientific footing.

Therefore, in addition to the classical clinical investigation which has been responsible for striking medical progress and has nearly eradicated some diseases, for instance erythroblastosis fetalis and poliomyelitis with the resulting saving of money in hospitalization, there is the opportunity of major contributions in research in clinical day to day health care and this was really the subject of to-day's meeting in the area of infectious diseases and cancer. Generally speaking, much of what we do in caring for patients is too often based on no sounder scientific data than that one can attribute to old wives' tales; being scientifically weak, it cannot help but be a financial catastrophe. Here is an example taken from the American literature quoted by Jerry Lewis: in this coming year, 600,000 Americans and 500,000 West Europeans will sustain a heart attack. Twenty years ago such patients were kept in bed 6 weeks in complete rest. In a recent study it was shown that in uncomplicated coronary occlusion 42 days hospitalization was no more beneficial than 2 weeks. Therefore, without laboratory research and just by clinical evaluation the duration of hospitalization could be reduced from 42 to 15 days by the type of study described to-day, and this represents a saving of $3000 per patient. This also demonstrates that treatment is too often by general consensus and not based upon scientific rationale. Another example is the 70,000 new cases of cancer of the breast registered each year in a 220 million people community. The initial treatment varies greatly. Had these patients been properly categorized a decade ago there would be more unanimity on what is the most effective management of these patients.

Because we, in the hospitals and in private practice, have not utilized fully the patients' material to learn to refine our patient management program, medical care patterns have changed very slowly over the years and this is due to the unavailability of solid data. A continuation of health care patterns not based on observations, but on our prior training, which was often based on tradition, is highly inefficient and should be a source of continual embarrassment to those of us in the clinical field.

I believe, therefore, that scientific clinical medicine is not only due the patient for his own sake, but that we have the obligation, as hospital and community physicians to make sure that health care money is well spent and this means the promotion of better medicine. This is why we were glad to be here and to see our medical attitudes reviewed, discussed and revised. This work on critical evaluation of

anti-infectious treatment is urgently to be continued.

Our thanks and congratulations go to the speakers and especially to those who accepted to travel a long way to bring us their experience and stimulation. But our thanks go also to Dr. Staquet and Dr. Klastersky who organized this meeting.

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EORTC Protocol for the Study of CCNU in the Treatment of Irradiated, Operated, Malignant Glioma of the Brain*

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Abstract—*The EORTC protocol for brain tumor is described. In addition to survival time, an attempt is made to determine remission rate. CCNU is compared to a group without chemotherapy in a randomized trial.*

QUANTITATIVE evaluation of therapy in malignant brain tumors is complicated by the necessity to appreciate the variation in tumor size by indirect means which are influenced by several factors other than the neoplasm, especially the edema surrounding the tumor. These difficulties, which are encountered in studies aiming to determine the rate of objective remissions, can be avoided by measuring the survival time. This last criteria is useful in patients with brain tumors where survival is short; however, it has the disadvantage of requiring hospitalization of terminal patients, both treated and controls, because of the necessity of identical supportive care. It should also be noted that tumor shrinkage and prolongation of life are not measuring necessarily the same phenomenon, and may give apparently contradictory results. For instance, BCNU is able to produce a high rate of objective remission [1, 2, 3] but does not prolong significantly the survival time when given without radiotherapy [4]. In particular cases, the period between surgical removal of the tumor and the relapse (free interval) can be measured and used for objective evaluation of therapy. This parameter has the advantage of measuring the quality of the survival and does not necessitate prolonged hospitalization, but has the drawback that recurrence is not easy to determine with accuracy in many patients.

Accepted 20 March 1973.

*EORTC Brain Tumor Cooperative Group.

1. AIM OF THE STUDY

The goal of this study is to evaluate the effect of CCNU in operated and irradiated patients with malignant glioma of the brain, using the following parameters:

(*a*) survival time from operation

(*b*) rate of objective remission

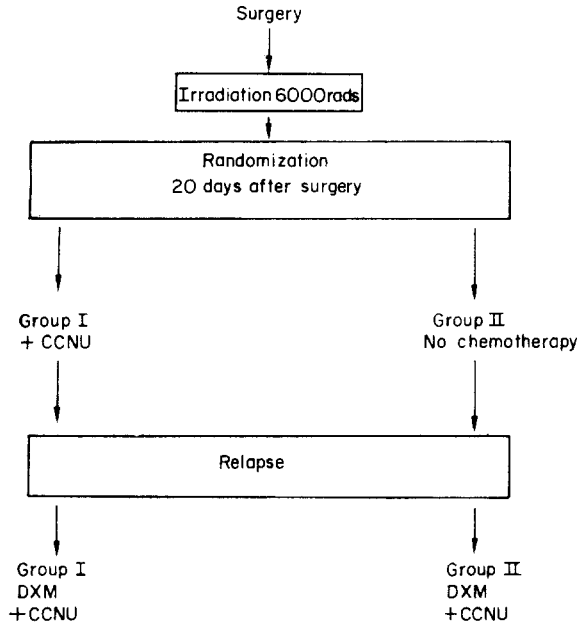
(*c*) length of free interval (from operation to recurrence).

1.1. *Trial* 01 (*Fig.* 1)

This trial is made up of 2 phases (A and B).

Phase A (from operation to relapse) aims to evaluate the effect of CCNU on the length of the free interval.

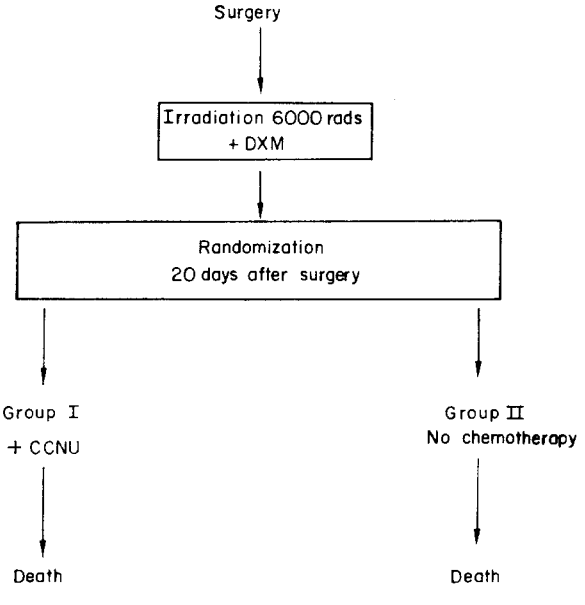
Phase B, starting after recurrence, will permit the determination of the remission rate. It will also try to establish whether CCNU should be given within 2 weeks after surgery or only after



recurrence, by comparing 2 groups of patients, the first receiving CCNU after surgery and the other after recurrence.

1.2. *Trial* 02 (*Fig.* 2)

It starts at surgery and ends at death and includes patients not fulfilling the special requests for trial 01. It takes only survival time into account.



2. SELECTION OF PATIENTS

2.1. *General conditions for admission in trials* 01 *and* 02

2.1.1. Diagnosis is based on the histology carried out within each institution.

Review of the tissue section

An unstained tissue section must be sent to:
Dr. Cl. Solheid,
Instituut Bunge,
Filip Williotstraat, 59,
2600, Berchem, Antwerpen, Belgium.

2.1.2. Operation will have taken place within 20 days before the start of chemotherapy. Maximum resection by standard procedure is requested for eligibility.

2.1.3. Patients must have an expected survival time of 8 weeks or more, and follow-up must be possible.

2.1.4. Patients must have normal hematopoetic, renal and hepatic function. The minimum acceptable standards being:
WBC $> 4{,}000/mm^3$, platelets $> 100{,}000/mm^3$, hematocrit $> 30\%$
BUN < 40 mg%, creatine < 1.5 mg%
bilirubin < 2 mg%

2.1.5. No anticancer agents can be given during this 2-week period after surgery. For nitrosourea the length of drug-free time is 4 weeks before the start of irradiation–chemotherapy.

2.1.6. Patients are excluded if they have other major medical illness.

2.2. *Additional criteria for admission in trial* 01

Patients eligible for that trial must fulfil the following conditions:

(*a*) Steroids therapy must be *stopped on the* 10*th day* after surgery. (Patients still requiring steroids will be treated in trial 02).

(*b*) The neurological examination should be either normal or show *incomplete* deficits which may be appreciated quantitatively and serve as criteria for further evaluation.

2.3. *Additional criteria for admission in trial* 02

All patients eligible for the study, but not for trial 01, will be treated in trial 02.

3. STUDY DESIGNS (Figs. 1 and 2)

Following surgery, the patients eligible for the study will be divided, according to the steroids dependence and neurological symptoms, into two categories. Each category will serve a separate trial (01 or 02).

3.1. *Trial* 01

3.1.1. Ten days after the complete discontinuation of the steroïds, the patients are randomized. Group I will receive CCNU, group II will serve as control. Patients from the two groups should be followed at the same frequency (once every two weeks) and quality of follow up.

3.1.2. When recurrence is established (see par. 7), phase A ends. Steroid administration is then permitted. In addition, patients from group II will receive CCNU. The survival *of all* patients will be reported.

3.2. *Trial* 02

3.2.1. Patients will be randomized 20 days after surgery. Group I will receive CCNU, group II will serve as control. Patients from the two groups should be followed at the same frequency and quality of follow up.

3.2.2. Survival will be measured in *all* patients.

3.3. *Randomization*

It will take place at the EORTC Coordinating Office in Brussels. For each patient entering the trial, call: 02/38.08.58 (8.30–11.30 hr and 13.00–17.15 hr—Monday to Friday).

4. CHEMOTHERAPY AND ADMINISTRATION OF STEROIDS

4.1. CCNU is given orally 130 mg/m^2 once every 6 weeks.

4.2. Dexamethazone (01 B and 02)
10 mg/m^2 per day: from day 1 to 28.
Decrease gradually to 0·0 mg from day 28.
If necessary, the administration of DXM may be resumed at 10 mg/m^2/day, the dosages of DXM will then be reported carefully.

5. IRRADIATION

Irradiation will start as soon as possible after surgery.

Radiation sources allowed: Betatron, Telecobalt or linear accelerator.

Dosage: 6000 rads in 6 weeks to the tumor (1000 rads/week).

Fields: 8×10 cm^2, may be reduced at the end of irradiation if necessary and if the tumor has really received the full dosage of 6000 rads.

6. TOXICITY OF THE DRUG

6.1. BWC, platelets, erythrocytes, B.U., creatinine and liver tests (LDH, GOT, GPT, Alc phosphatase, bilirubin, proteins) must be controlled before each administration of chemotherapy. Peripheral blood examination should be performed once a week in patients receiving CCNU.

6.2. CCNU will be discontinued if:
BWC $< 3.000/mm^3$ or platelets $< 60.000/mm^3$
BU > 60 mg% or creatinine > 2 mg%
Bilirubin > 2 mg%

7. EVALUATION OF THERAPY

7.1. *Criteria for recurrence*

The following will be considered as evidence for recurrence:

(*a*) Neurological deterioration of at least one symptom, chosen as a criteria for objective evaluation (2.2.b). This also includes appearance of *new* neurological signs, i.e., hemiparesis, hemisensory deficit, visual field deficit, or mental deterioration; the *return* of previously present signs or an *aggravation* of signs which never completely subsided.

(*b*) The appearance of intracranial pressure after having reached a stable condition post-operatively.

These must not have occurred solely as a postictal phenomena. Since the occurrence of neurological deterioration may be subtle at first and only confirmed by future events, there will be a distinction made between the first appearance of questionable signs (*suspected*) and when signs are more obvious (*confirmed*).

7.2. *Additional criteria for recurrence*

Although recurrence is defined as a clinical deterioration of neurological status, additional examinations should be performed when recurrence is suspected.

(*a*) EEG: Extension of a focal lesion over preoperative EEG (the first being *not earlier* than three months post-operatively) or an increase of focal lesion signs in two consecutive examinations done post-operatively will be considered as possible evidence of recurrence.

(*b*) Brain scan: an increase in tumor size over pre-operative brain scan seen on two consecutive brain scans one month apart (the first being not earlier than three months post-operatively), will be considered as possible evidence of recurrence. An increase in two consecutive brain scans will also be considered as possible evidence of recurrence.

(*c*) Angiography: an increase in tumor mass in an arteriogram compared to the most recent arteriogram (be it the pre-operative or a post-operative study) will be considered as possible evidence of recurrence.

(*d*) Shift of intracranial marker: a clear-cut shift in an intracranial marker placed at the time of resection compared with a *comparable base-line film* will be considered as possible evidence of recurrence. The shift must be in the direction indicative of tumor growth.

7.3. *Criteria for objective remission*

Objective remission may be observed only in trial 01 after relapse (Phase B).

The following will be considered as evidence of objective remission:

(*a*) Marked and durable (at least 4 weeks) improvement of at least neurological symptom without deterioration of any other neurological sign or apparition of new neurological deficits.

(*b*) Disappearance of intracranial pressure measured by marked and durable (more than 4 weeks) regression of pre-existing papillar oedema without deterioration of any other neurological sign or apparition of new neurological deficits.

Both (*a*) and (*b*) must persist at least 6 weeks after complete discontinuation of steroids.

8. SUPPORTIVE THERAPY

All patients may receive general neurosurgical supportive therapy including anti-epileptic drugs and physiotherapy. Blood component therapy is allowed and should be reported if given. Steroids, manitol, urea perfusion or chemotherapy other than what is scheduled cannot be used.

9. REPORTING

(All reports should be sent to the Coordinating Office).

9.1. All patients eligible for the study will be reported on Form I, which is to be sent *at the time of randomization* to the central office.

9.2. Form I will be sent to the central office* when the patients enter the study.

Forms II and III should be sent whenever patients quit the study whether by death or by dropping out.

10. SAMPLE SIZE

Trial 01: 28 patients in each group (mean duration of remission: 20 weeks; increase in duration 50%;

$$\alpha = 0{\cdot}05; \quad \beta = 0{\cdot}95; \quad \sigma_{\bar{x}} = 10 \text{ weeks}.$$

Trial 02: 49 patients in each group (mean survival time: 23 weeks; increase in survival time 50%;

$$\alpha = 0{\cdot}05; \quad \beta = 0{\cdot}95; \quad \sigma_{\bar{x}} = 15 \text{ weeks}).$$

Acknowledgement—The authors wish to thank M. D. Walker for long and fruitful duscussion of this protocol and S. Carter for providing CCNU for this study.

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Some *In Vivo* and *In Vitro* Antitumour Effects of the Deoxyfluoro-D-Glucopyranoses*

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Abstract—*The deoxyfluoro-D-glucopyranoses have been examined as antitumour agents against* (1) *a series of transplanted animal tumours (mainly the R-1 lymphoma) and against* (2) L5178Y *lymphoma cells in culture. 6-Deoxy-6-fluoro-D-glucose inhibited (up to* 90%) *the small solid tumour of the R-1 lymphoma but none of the other deoxyfluoro-D-glucopyranoses or deoxyhalogeno-D-hexopyranoses tested showed this effect. Several deoxyfluoro-D-hexopyranoses inhibited* L5178Y *cells in culture but all of these compounds were substituted at* C-2 (*e.g. 2-deoxy-2-fluoro-D-glucose*). *Only 6-deoxy-6-fluoro-D-glucose and 2-deoxy-2-fluoro-D-glucose of the deoxyfluoro-D-glucopyranoses produced toxic effects at the dose levels tested.*

INTRODUCTION

THE DEVELOPMENT of new synthetic routes [1] has made the complete series of deoxyfluoro-D-glucopyranoses readily accessible and thereby made possible evaluation of their biological activity. These compounds have been used [2] in enzyme specificity studies concerned with the design of selective inhibitors of hexokinase isoenzymes as potential antitumour agents. Only a few D-hexose derivatives have been previously shown to exhibit an antitumour effect and these have all been D-hexoses substituted at C-2[2-amino-2-deoxy-D-glucose (D-glucosamine) [3], 2-amino-2-deoxy-D-galactose (D-galactosamine) [4] and 2-amino-2-deoxy-D-mannose (D-mannosamine) [5]].

We now report the effects of deoxyfluoro-D-glucopyranoses on a range of transplanted animal tumours and L5178Y lymphoma cells in culture.

The following abbreviations are used throughout this paper: 2-deoxy-2-fluoro-D-glucose (2FG) [6], 3-deoxy-3-fluoro-D-glucose (3FG) [7], 4-deoxy-4-fluoro-D-glucose (4FG) [8], and 6-deoxy-6-fluoro-D-glucose (6FG) [9, 10].

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MATERIAL AND METHODS

The effects of deoxyfluoro-D-glucopyranoses on some transplanted animal tumours in vivo.

The R-1 lymphoma was induced in 1959, in CBA mice (at Mount Vernon Hospital, Northwood, Middlesex) by cobalt X-irradiation. The tumour has been passaged routinely by an intraperitoneal injection of ascites cells. A dose of 10^6 cells injected subcutaneously into the inguinal region of a mouse kills an untreated animal in approximately 10 days. Drug injections were all intraperitoneal and five daily doses were given from the third to the seventh day after transplantation. The effectiveness of drugs was measured by comparison of survival times in the treated and untreated groups of animals.

The procedure used for the murine lymphomas, L5178Y (strain, DBA^2 ♂) and TLX/5 (strain, CBA/LAC ♀), was the same as that employed for the R-1 lymphoma (strain, CBA/LAC ♀). The methods used for the other tumours, viz., ADJ/PC6A, Novikoff hepatoma and Walker carcinosarcoma 256, were the standard procedures used at the Chester Beatty Research Institute [11].

RESULTS

When 6FG was first tested against the R-1 lymphoma, in the dose range 30–1000 mg/kg, it produced no significant increase in survival time over that of the untreated controls. The tumours at the site of inoculation were, however, considerably smaller than those in the control, untreated group. A further series of animals was then grafted subcutaneously with R-1 lymphoma cells and treated with doses (100 and 150 mg/kg) of 6FG for the five days. These animals were killed and the tumours excised and weighed on the seventh day after transplantation. The tumours of treated animals were smaller than those of animals receiving no treatment.

If 6FG acts by competitively inhibiting the transport of glucose into the R-1 lymphoma cells, a lowering of the blood glucose concentration ought to increase the antitumour effect of 6FG. The blood glucose concentration was lowered by administering insulin B.P. ("Wellcome" brand). However, when well-tolerated doses of both 6FG (150 mg/kg) and insulin (2 units/animal) were given in combination, lethal toxicity resulted so that it was not possible to measure the antitumour effect of combination therapy. Toxic doses of 6FG produced high blood concentrations of 6FG. A single dose of 6FG of 1 g/kg produced, 30 min after injection, a blood concentration of 6FG three times the concentration of glucose (6FG and glucose concentrations in the blood were estimated by the method of Asatoor and King [12]).

If the antitumour effect of 6FG is due to inhibition of glucose transport into the cell, some reversal of the antitumour effect and the toxicity would be expected if the blood glucose level were raised. However, a dose of glucose (1 g/kg) 15 min prior to administration of 6FG (100, 200, 400, and 800 mg/kg, daily, days 3–7) did not reverse the antitumour effect.

In order to test whether the observed antitumour effect of 6FG on the R-1 lymphoma was specific to this compound, a series of 6-deoxy and 6-deoxy-6-halogeno derivatives of both D-glucose and D-galactose was synthesized by known procedures (see Table 1). This series of compounds was tested against the R-1 lymphoma in the same manner as that described above. The results, given in Table 1, show that the observed antitumour effect is specific to 6FG.

Table 1. The effect of 6FG *and some* D-*glucose and* D-*galactose derivatives on the solid tumour of the R-1 lymphoma*

Compound	Dose (mg/kg)	Average tumour weight (g)	Tumour weight as % of untreated control
6FG	125	0·12	50
	250	0·07	29·2
	500	0·025 3/5 deaths	10·4
6-deoxy-D-glucose [13, 14]	125	0·48	116
	250	0·48	116
	500	0·30	69·8
6-chloro-6-deoxy-D-glucose [15, 16]	250	0·40	93
	500	0·34	79·1
	1000	0·28	65·1
6-bromo-6-deoxy-D-glucose [9]	250	0·40	93
	500	0·34	79·1
	1000	0·38	88·4
6-deoxy-D-galactose (D-fucose)*	125	0·48	116
	250	0·48	116
	500	0·48	116
6-deoxy-6-fluoro-D-galactose [17]	125	0·20	83·3
	250	0·23	95·8
	500	0·22	91·7
6-chloro-6-deoxy-D-galactose [18, 15]	250	0·24	100
	500	0·19	79·2
	1000	0·23	95·8
6-bromo-6-deoxy-D-galactose [9]	250	0·25	104·2
	500	0·31	129·2
	1000	0·26	108·3

*Purchased from Koch-Light Ltd., Colnbrook, Bucks, U.K.

Methotrexate (3 mg/kg, daily, days 3–7) increases the survival time of R-1 lymphoma-bearing mice to 15 or 16 days (66% increase). Since 6FG has an effect on the solid tumour, the effect of the two compounds in combination was examined. No further increase in the survival time was observed when the two compounds were administered together; a slight increase (to 77%) was observed when 6FG (100 mg/kg daily, days 10–14) was given after the methotrexate treatment. 6FG showed slight activity when tested against the ADJ/PC6A plasma cell tumour; 70% inhibition being observed at 125 mg/kg. 6FG had no effect either on the TLX/5 lymphoma, a tumour similar to the R-1 lymphoma, or on the Lewis lung carcinoma*.

2FG, which was found to have a pronounced effect on L5178Y lymphoma cells *in vitro* (see following section), had only a slight effect (approx. 20% inhibition) on the solid tumour of the L5178Y lymphoma *in vivo* (daily doses of 2FG of up to 400 mg/kg, days 3–7). 2FG had no effect when tested against theR-1 lymphoma, the ADJ/PC6A plasma cell tumour, and the rat Novikoff hepatoma. Since 2FG is a substrate for hexokinase [2] and 2-deoxy-2-fluoro-D-glucose 6-phosphate is an inhibitor of both D-glucose 6-phosphate dehydrogenase [19] and D-glucose phosphate isomerase [20], some effect was expected against the Novikoff hepatoma. The Novikoff hepatoma is known to rely on hexokinase (rather than glucokinase) for the phosphorylation of D-glucose [21].

3FG, which was found to be relatively non-toxic, had no effect on the rat Walker 256 carcinosarcoma at dose levels up to 1·6 g/kg. 4FG was also found to be non-toxic up to 625 mg/kg but no antitumour tests were carried out on this compound because the large amounts of compound needed were not available. Only 6FG and 2FG of the deoxyfluoro-D-glucopyranoses produced a lethal effect in non-tumour-bearing animals at the dose levels tested. 6FG had an LD_{50} of approx. 200 mg/kg in both mice and rats when given in 5 or 6 daily doses. 2FG was less toxic and had an LD_{50} of approx. 600 mg/kg. 2FG, 3FG, and 6FG were also administered, at a single dose of 400 mg/kg by intraperitoneal injection into male Chester Beatty Wistar rats (approx. 200 g). Morphological changes were observed in the livers from these rats only in the case of 6FG. After 24 hr there was degeneration and some necrosis of centrilobular hepatocytes. This centrilobular necrosis was well-established by 48 hr, but after 6 days there was restitution of normal structure. Other tissues examined appeared normal. (R. L. Carter, personal communication).

The effects of deoxyfluoro-D-glucopyranoses on L5178Y mouse lymphoma cells in vitro.

Culture. The sensitive strain of L5178Y mouse lymphoma cells used for these experiments had been grown continuously in culture for several years. Normally, cultures were diluted when live cell numbers exceeded 2×10^5 cells/ml.

The L5178Y cells were grown in 14 ml McCartney bottles each containing 5 ml of Fischer's 1964 medium, with 10% added horse serum, which contains D-glucose (1 mg/ml). Duplicate cultures were set up for each compound, one pair of controls contained no extra glucose and another pair contained added glucose giving a total of 2 mg/ml. Each compound tested was dissolved in normal culture medium to give a concentration of 5 mg/ml. One millilitre of this solution was then added to 4 ml of a cell culture to give a final concentration of the compound of 1 mg/ml, this being equal to the concentration of glucose in the medium. In one further experiment with 2FG, the concentration of glucose in the medium was kept at 1 mg/ml and several different concentrations of the fluoro sugar were used.

Cell counts. The number of live cells was determined from counts using a haemocytometer slide viewed under phase contrast.

In plotting results the number of live cells was obtained by multiplying the number of cells/ml by any dilution made after the start of the experiment.

RESULTS

Figure 1 shows the effect of each of the deoxyfluoro-D-glucopyranoses (2FG, 3FG, 4FG, and 6FG) on the proliferation of the cells. 3FG, 4FG, and 6FG had no noticeable effect on the exponential growth rate of the cells seen in both the normal and in the control with added glucose. Thus, the initial number of 5×10^4 cells increased to a value of approx. 10^6 after 50 hr. 2FG, however, had a marked effect on the growth of the cells, so that after an initial rise in the number of live cells from 5×10^4 to approx. 10^5 in 24 hr, at 50 hr there had been a sharp decrease in the cell count to a value less than the initial count. Figures 2–4 show the effects of a series of sugars modified at C-2.

*This test was kindly carried out by Dr. K. Hellmann, Imperial Cancer Research Fund, Lincolns Inn Fields, London.

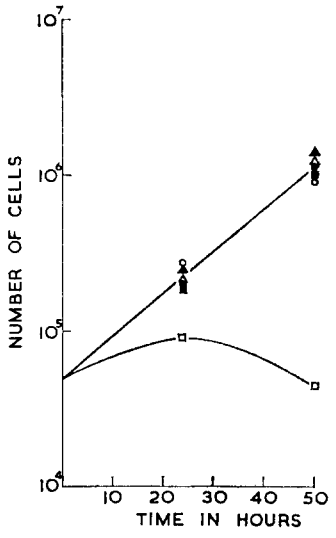

Fig. 1. *The growth of* L5178Y *cells in the presence of* D-*glucose* (△), 2FG(□), 3FG(●), 4FG(▲), *and* 6FG(■), *each at a concentration of* 1 *mg/ml* (5·5 *mM*). *The incubation medium contained glucose* (1 *mg/ml*) *and the growth of the cells in this medium was used as a control* (○).

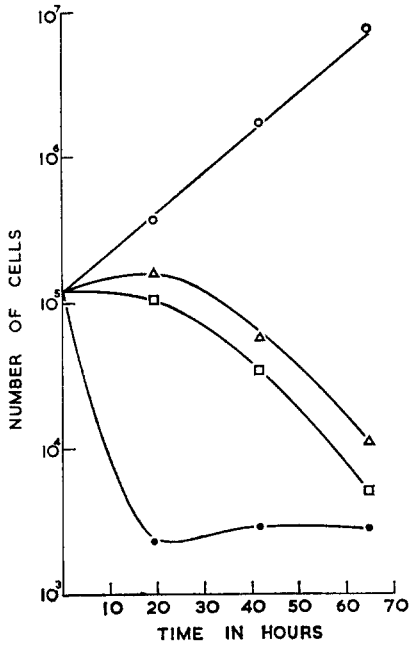

Fig. 2. *The growth of* L5178Y *cells in the presence of* 2-*deoxy*-2-*fluoro*-D-*hexopyranoses at concentrations of* 1 *mg/ml control* (○), 2FG(△), 2FM(□), 2FGal(●).

2-Deoxy-2-fluoro-D-mannose (2FM) [6] and 2-deoxy-2-fluoro-D-galactose (2FGal) [22] produced a similar effect (Fig. 2) to that of 2FG in that the live cell count after approx. 65 hr was considerably lower than the initial count. At 20 hr 2FGal caused a reduction in the number of live cells whereas 2FM closely resembled 2FG in allowing a small increase in cell numbers in the early stages of the incubation followed by considerable killing of cells in the later stages.

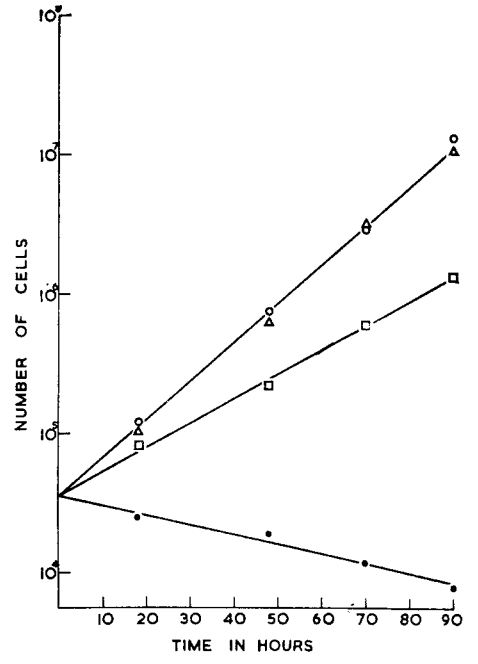

Fig. 3. *The growth of* L5178Y *cells in the presence of* 2,2diClG(△), 2ClG(□), *and* 2,2diFG(●) *at concentrations of* 1 *mg/ml;* (○) *control.*

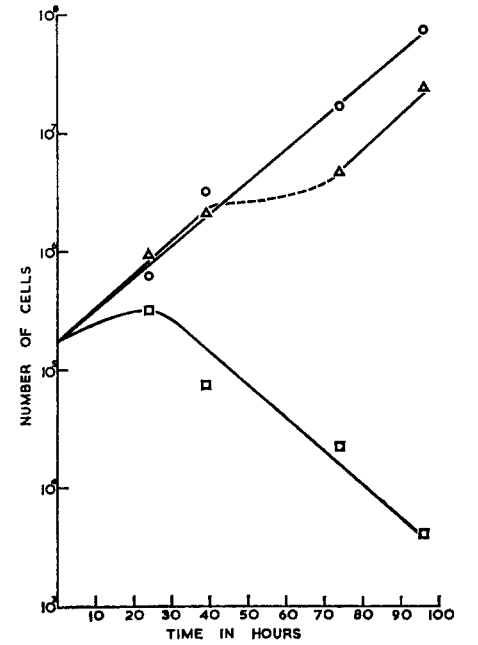

Fig. 4. *The growth of* L5178Y *cells in the presence of* 2MeG(△) *and* 2DG(□) *at concentrations of* 1 *mg/ml; control* (○). *The break in the line of* 2MeG *shows the time at which overcrowding of the cells made dilution necessary.*

No effect (Fig. 3) of 2,2-dichloro-2-deoxy-D-*arabino*-hexose (2,2diClG) [23] on the growth of the cells was observed whereas 2-chloro-2-deoxy-D-glucose (2ClG) [23] somewhat reduced the number of live cells. Thus, at approx. 90 hr the control cultures increased to 10^7 cells; in the presence of 2ClG the count was only a little above 10^6. 2-Deoxy-2,2-difluoro-D-

arabino-hexose (2,2diFG) [24] caused a continuous reduction in the number of live cells.

2-0-Methyl-D-glucose (2MeG) [25] had no apparent effect (Fig. 4) on the growth rate of the cells but 2-deoxy-D-*arabino*-hexose (2-deoxy-D-glucose, 2DG) by continuously reducing the number of live cells, exerted an effect similar to that of 2FG (cf. Fig. 2).

Finally, an experiment was carried out in which concentrations of 2FG less than that of the glucose in the medium (1 mg/ml) were used. The results are shown in Fig. 5. Whereas a concentration of 2FG at 0·03 mg/ml caused

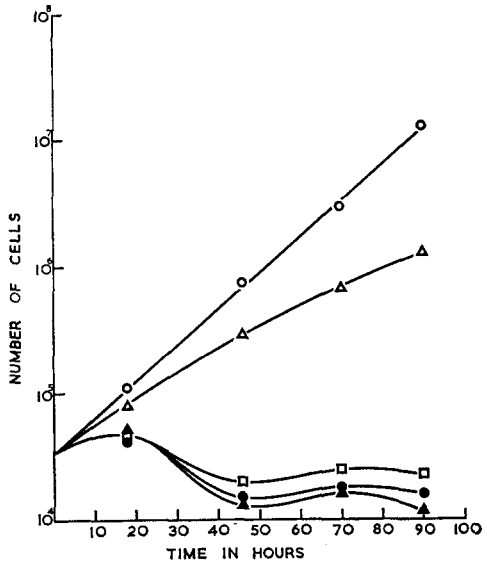

Fig. 5. *The growth of* L5178Y *cells in the presence of different concentrations of* 2FG(*mg/ml*): *control*(○), 0·031(△), 0·063(□), 0·125(●), 0·25(▲).

only a relatively small reduction in the number of live cells, concentrations of 0·063, 0·125, and 0·25 mg/ml each produced an almost constant cell count after the usual small rise in cell numbers in the early stages of the experiment.

DISCUSSION

The results show that two of the deoxyfluoro-D-glucopyranoses viz. 6FG and 2FG, can produce an antitumour effect. The antitumour effect of 6FG is specific in two respects.

(1) 6FG is effective only against one of the transplanted tumours,. i.e. the solid R-1 lymphoma. Also, 6FG was not cytotoxic to L5178Y lymphoma cells *in vitro*.

(2) 6FG was the only deoxyfluoro-D-glucopyranose to show this antitumour effect and, in addition, was the only active compound in a series of 6-deoxy-6-halogeno derivatives of D-glucose and D-galactose, including 6-deoxy-6-fluoro-D-galactose. Therefore, not only is the fluorine atom necessary, but it must be substituted at C-6 of a D-hexose in the *gluco*-configuration.

The antitumour effect of 2FG was specific in one respect, having a cytotoxic effect on L5178Y lymphoma cells *in vitro* but very little effect on the series of transplanted tumours. Indeed, it had little effect on the L5178Y lymphoma *in vivo*, but this could be due to phosphorylation of 2FG in the liver [2]. The effect of 2FG against L5178Y cells *in vitro* was, however, not specific since other C-2 substituted D-hexoses, namely, 2,2diFG, 2DG, 2FM, and 2FGal also had a cytotoxic effect.

At first it seemed that the cytotoxic effect of 6FG could be due to competitive inhibition of the active transport of glucose into the cell. Blakley and Boyer [26] have shown that although 6FG is a weak competitive inhibitor of yeast hexokinase (K_i 12mM) [19] and does not inhibit the fermentation of glucose or fructose by yeast extract; 6FG inhibits the fermentation of glucose and fructose by intact yeast. They suggested that 6FG competed with glucose for cellular uptake.

Wilson and Landau [27] showed that 6FG is well-transported across the intestinal wall of the hamster. Wick *et al.* [28] found that 6FG was transported across the muscle cell wall and that although insulin accelerated the rate of transport of glucose it did not accelerate the rate of transport of 6FG. These observations prompted the experiment when insulin and 6FG were given in combination. The plasma level of glucose was expected to decrease and that of 6FG to stay constant. This may well have happened since a non-toxic dose of 6FG and a non-toxic dose of insulin given at the same time produced a lethal effect.

Certain tumours have an increased rate of glucose transport. Hatanaka *et al.* [29, 30] have observed that normal mouse embryo cells transformed with murine sarcoma viruses show a significant increase in the rate of uptake of glucose. If 6FG acts by competitively inhibiting glucose transport, the antitumour effect and toxicity of 6FG should be reduced by increasing the concentration of blood glucose, but a dose of glucose (1 g/kg) given before 6FG did not alter either the antitumour effect or the toxicity. Also 6FGal, which has been shown [31] to be actively transported across the hamster intestinal wall as effectively as 6FG, did not exhibit this antitumour effect. Although transport studies on R-1 lymphoma cells using 6FG need to be done to clarify this situation, the antitumour effect of 6FG seems unlikely to be due solely to

a decrease in glucose uptake by the lymphoma cells. The antitumour effect and the liver toxicity caused by 6FG could be due to metabolism of 6FG. However, 6FG has, so far, only been shown to be a substrate for rat liver glucose dehydrogenase and the K_m is probably too high to be of physiological significance [32]. It is possible, but perhaps unlikely, that 6FG is converted into a highly toxic compound such as fluoroacetate by some, as yet unknown, metabolic pathway. More comment can be made about the cytotoxic effect of 2FG on L5178Y lymphoma cells *in vitro* since D-glucosamine, D-galactosamine, D-mannosamine, and D-mannose have been shown to inhibit protein, glycoprotein, RNA, and DNA synthesis in L5178Y lymphoma cells [33]. D-Glucosamine was shown by Quastel and Cantero [3] to be an inhibitor for neoplastic growth. Since D-glucosamine is known to compete with D-glucose for hexokinase and so inhibit glucose metabolism this was originally thought to be its mechanism of action. The inhibition, however, does not account significantly for the antitumour effect of D-glucosamine. Bosmann [33] has shown that D-glucosamine inhibits macromolecular synthesis in L5178Y cells and that its antitumour effect could be due to the resulting reduction in endogenous cellular pools of UMP, UDP, and UTP.

It seemed possible that the cytotoxic effect of these 2-deoxy-2-halogeno-D-hexoses could be due to competition with glucose for hexokinase since most of these compounds are good substrates for yeast hexokinase [2]. The order of K_m values for these sugars with yeast hexokinase are 2,2diFG hexose < 2FG < 2FM < 2ClG. The cytotoxicity of these compounds was approximately in the reverse order i.e. 2,2diFG was far more effective than 2ClG. However, 2FGal was the exception since it was as cytotoxic as 2,2diFG but is neither a substrate nor an inhibitor for yeast hexokinase [2]. Coe [34] has shown that 2FG inhibits hexokinase in Ehrlich ascites cells and also that the rate of formation of fructose 1,6-diphosphate is decreased. In addition, it is stated that the hexokinase inhibition is noncompetitive and that 1,6-anhydro-2-deoxy-2-fluoro-β-D-glucopyranose could be involved. This seems unlikely since 2FG competitively inhibits the rate of phosphorylation of glucose by yeast hexokinase [2, 19] and it has been shown by ^{19}F nuclear magnetic resonance that 2FG exists in aqueous solution at least 99% as 2-deoxy-2-fluoro-D-glucopyranose [6]. Only in very acid solution is the formation of the 1,6-anhydro sugar likely and then only to the extent of approx. 5%.

Although it seems that inhibition of hexokinase does not account for the cytotoxicity of the 2-deoxy-2-halogeno-D-hexoses, it is doubtful whether they could reduce the level of endogenous uridine nucleotides. The mechanism of action of these compounds, therefore, probably differs from that of the amino sugars. The time differences observed between the onset of cytotoxic effects of these compounds could even imply that each acts in a different manner.

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Some Approaches to Mapping the Redistribution of Parent-Cell H-2 Antigens on the Membrane of Cell Hybrids

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Abstract—*Cell hybrids were created by Sendai virus-induced fusion of A9 (H-2^k) cells and peritoneal exudate cells (presumably macrophages, H-2^d), and the distribution of parent-derived H-2 specificities was followed using two indirect methods. Competition within pairs of non-crossreacting H-2 specificities for the attachment of specific antibodies was measured by the blocking index. In cell hybrids, no blocking was detectable within the pairs H-2·4–H-2·11 and H-2·4–H-2·5 where the members of each pair were derived from different parents. When, however, both specificities of a pair (H-2·5, H-2·11) were from the same parent, their blocking relationship was similar as in the particular parent. In different experiments and with different types of cells, the presence of blocking between two H-2 specificities was found to be confined to the "double* cis" *configuration of their genetic determinants. No blocking was found in "single* trans", "*single* cis", *and "double* trans" *configurations. The difference in blocking could not be simply traced back to a different cell surface density of the antigens under test.*

Trans *specificities that do not compete (H-2·4, H-2·11) were shown to cooperate in a complement-dependent reaction with a specific antibody. The competition clearly indicates an intermixing of parent derived H-2 antigens on the cell hybrid membrane.*

The possible bearing of the data on the understanding of the control of the specificity of the cell-surface architecture is discussed.

INTRODUCTION

THE CHARACTERISTICS of the better-defined histocompatibility (H-) antigens, such as mouse H-2 antigens, fit fairly well the criteria for "integral" (in contrast to "peripheral") membrane proteins [1]. The term seems to imply that such proteins are essential to the structural integrity of the cell membrane. Surprisingly enough, H-2 antigens tend to enjoy a great freedom to disappear from and reappear on the cell surface; their expression may undergo variations, for instance, with the cell cycle [2], with the transformation into a tumour cell and appearance of a neoantigen [3] or in response to various experimental treatments [4]. The dynamics of such changes may be well-accommodated by the "fluid mosaic model" of the cell membrane structure [5]. At the same time, however, the cell-surface architecture seems to display certain patterns which clearly imply restrictions to an absolute freedom for the membrane components [6]. The rules of the interplay of various factors that favour conformation on the one hand and dynamics on the other are far from being understood. The question may also be asked whether there is, in mammalian cell membranes, any analogy to the great autonomy of the preformed cortical structures as demonstrated in *Paramecium*, where, for example, the structural specificity of a growing cortical graft is determined by its preformed pattern and not by the nucleus of the recipient cell [7].

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It was hoped that an analysis of the membrane distribution of parent-derived H-2 antigens on cell hybrids produced by virus-induced fusion of somatic cells might help to elucidate some of these questions.

MATERIAL AND METHODS

Cell hybrids

Cell hybrids used for the experiments were produced by Dr. J. Svoboda in 1970 from H-2^d (B10.D2) peritoneal exudate cells, presumably macrophages, and an H-2^k cell line (L-A9, kindly provided by Prof. B. Ephrussi). The fusion of the parental cells was induced by β-propiolactone-inactivated Sendai virus and the Littelfield's system [8] was employed for selecting the hybrids. After a number of passages, several clones were isolated and two of them used in the present study. The chromosome number ranged from 76 to 96 with a mean of 84 (after two years in the culture).

H-2 reagents

H-2 reagents were presumably monospecific antisera for specificities H-2·4, H-2·5 and H-2·11 prepared according to Snell's Catalog of mouse alloantisera [9]. They were titrated for cytotoxic activity using the ^{51}Cr-release assay with lymph node cells used as standard target cells [10, 11].

Blocking test

The blocking test was described in detail elsewhere [12]. Briefly, the absorption assay was performed in parallel with control cells and "blocked" cells (i.e., cells with an H-2 specificity —other than that tested for antibody-absorbing capacity—blocked with specific antibody).

The "blocking index" is the percentage difference in the numbers of the two cell types equivalent in their capacity to reduce on absorption the cytotoxic activity of the test reagent to 50%.

Lymph node cells served always as standard cells for the comparison with the cell hybrids.

RESULTS AND DISCUSSION

Competition between non-crossreacting antigens

The first approach to the problem was based on a well-demonstrated phenomenon that, under certain conditions, two non-crossreacting cell-surface antigens may compete with one another in that molecules of a specific antibody attached to one of them tend to interfere to some degree with antigen–antibody reaction at the other sites [12, 13, 14, 15]. The "blocking index" (see Material and Methods) is a well-reproducible parameter; it thus seems to reflect some topographical relationship between the two antigens under test—not necessarily a rigid spatial configuration, but rather a reproducible

Table 1. Blocking relationships within three pairs of H-2 specificities and the effect of chromosomal configuration of their genetic determinants

Chromosomal configuration	H-2 alleles	Strain origin	Blocking index (%)
+11 +4/+11 +4 double *cis*	a/a	B10.A, A	23, 25
+11 +4/−11 −4 single *cis*	a/b	(B10.A × B10)F_1 (A × B10)F_1	0
−11 +4/+11 −4 single *trans*	d/k	(B10.D2 × B10.BR)F_1	0
−11 +4/−11 +4/+11/−4/+11/−4 double *trans*	d/d/k/k	(B10.D2 × C3H)cell hybrid	0
+5 +4/+5 +4 double *cis*	a/a	B10.A, A	23, 24, 24, 25
+5 +4/−5 −4 single *cis*	a/f	(B10.A × B10.M)F_1	0
−5 +4/+5 −4 single *trans*	d/k	(B10.D2 × B10.BR)F_1	0
−5 +4/−5 +4/+5 −4/+5 −4 double *trans*	d/d/k/k	(B10.D2 × C3H)cell hybrid	0
+11 +5/+11 +5 double *cis* (diploid)	a/a k/k m/m	B10.A,A B10.BR B10.AKM	53, 56 61, 67, 59 65 } (mean = 60)
+11 +5/−11 −5 single *cis*	k/d	(B10.BR × B10.D2)F_1	0
+11 +5/+11/+5/−11 −5/−11−5 double *cis* (tetraploid)	k/k/d/d	(C3H × B10.D2)cell hybrid	38, 49, 75 (mean = 54)

surface pattern of a statistical nature. It was hoped that the possible variation in the blocking index due to the fusion of parental membranes in cell hybrids might throw some light on how the specificity of the cell-surface architecture is controlled. Three pairs of H-2 specificities were selected for this purpose and the blocking index was measured within individual pairs, but with cells differing in the chromosomal configuration of the genetic determinants of the tested specificities. Table 1 gives the results.

It can be seen that the blocking relationship between specificities H-2.4 and H-2.11 is reproducible even with cells of different strain origin (B10.A and A). With homozygous (double *cis*) cells +4 +11 the blocking index was 23–25%. No blocking was, however, found with heterozygous (single *cis* or single *trans*) cells +4 +11. This finding differs from that of Legrand and Dausset [16], who found the *cis* versus *trans* difference in blocking with cells heterozygous for HL-A specificities. The cause of this difference between the H-2 and HL-A systems is not clear; in H-2, the presence of blocking seems to require not only the *cis* configuration, but also a double gene dosage, which is probably reflected in a double density of the antigenic sites on the cell surface. If the latter factor was critical for blocking to occur, cell hybrids derived from parents homozygous for one or the other tested specificity, respectively, might meet the requirement in spite of being "double *trans*". The absence of blocking, repeatedly confirmed with the tested hybrid cell, clearly showed that a double gene dosage alone is not sufficient for this kind of competition between the two specificities. The same result was obtained with another pair of specificities, H-2·4 and H-2·5. When, however, the two chosen specificities were contributed by the same parent, as was the case with the H-2·5, H-2·11 pair, the presence of blocking in the cell hybrid resembled the relevant parent. It might thus be concluded that blocking requires a double gene dosage and the *cis* configuration. One tends to wonder, however, whether a double gene dosage in a practically tetraploid cell should not be equivalent rather to a single gene dosage in a diploid cell (single *cis*) where no blocking was observed. Similar blocking relationships would, of course, only be expected if the gene dosage/cell surface area ratio remained constant and no new variables occurred in the cell hybrid. In order to find out whether the surface density of the parent-contributed test antigens was changed, the hybrid and parent cells were compared as to their capacity to reduce on absorption the cytotoxic activity of two appropriate monospecific reagents to 50%. The results are seen in Fig. 1. The cell hybrid had clearly higher antibody-absorbing capacities than either parent although the increase was much more pronounced with regard to the macrophage (contributing specificity H-2·4) than to the A9 parent (H-2·5). A similar relative increase in the cell hybrid was, however, found in respect to standard (lymph node) cells homozygous

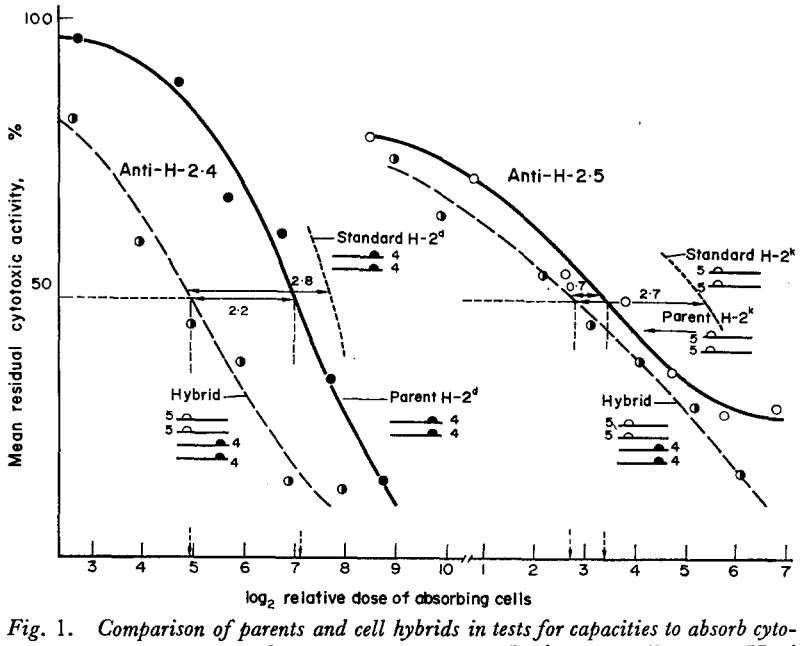

Fig. 1. *Comparison of parents and cell hybrids in tests for capacities to absorb cytotoxins against parent-specific H-2 antigens. Parent H-2^k = A9 cells, parent* H-2^d *= peritoneal exudate cells, standard = homozygous lymph node cells. The relative antigenicity is expressed in terms of log 2 differences in the numbers of the compared cells needed to reduce the cytotoxic activity of the monospecific test reagent to* 50 *per cent of its initial value.*

for one or the other test specificity. In both cases the cell hybrid had about sevenfold antibody absorbing capacities, which may be ascribed to its possessing accordingly larger absolute numbers of antigenic sites. Since the mean relative surface areas of the cloned cell hybrid population is not a sevenfold of that in the standard cells, the increase in the antigenicity per cell must be due—at least to some extent—to an increased surface density of antigenic sites in the cell hybrid. The fact, that the variation concerning the cell-surface area, antigenic density and the character of the cell may not essentially affect the value of the blocking index, might indicate one of two mechanisms.

(1) The blocking relationship between two antigenic sites is subject to an intrachromosomal regulation. The regulatory mechanism would have to control a non-random incorporation into the cell membrane of H-2 antigens that are not necessarily carried on the same molecule (such as specificities H-2·4 and H-2·11 controlled by two separate genes). It is hard to explain why the operation of such a mechanism would not be manifested in single *cis* diploid cells in contrast to double *cis* tetraploid and double *cis* diploid cells. The interpretation of the blocking index in terms of a configurational relationship of two antigens may be a crucial point here; the original view that the degree of blocking is inversely proportional to their spatial distance on the cell membrane can hardly accommodate the new facts.

(2) The ultrastructural surface pattern of the parent-derived membrane controls the differentiation of the newly forming membrane in the cell hybrid in a similar way as the cortical graft in *Paramecium* [7].

On the basis of the present data, the second mechanism appears to be more plausible.

Cooperation between non-crossreacting antigens

Another approach to the analysis of the antigenic structure of the cell hybrid membrane was based on the idea that while some antigens may compete, some might cooperate. A hypothetical basis for cooperation is illustrated in Fig. 2. Complement-dependent antigen–antibody reactions are believed to require the combination of C_{1q} with two different Fc sites of the antibody molecules. When the distribution on the cell membrane of antigens of two different specificities (black and white) was such as indicated, the chance for complement to bind might be smaller with a single monospecific reagent than with a 1:1 mixture of the two specific reagents even if the total number of antibody molecules were constant. Experimental evidence to support this hypothesis is available [17]. Without cooperation, mixing the two reagents should be equivalent to diluting one with another; the expected titration curve for the 1:1 mixture should then lie between those for individual reagents. Figure 3 illustrates the results of one of several experiments of this kind concerning specificities H-2·4 and H-2·11 in single *trans* (F_1 hybrid) and double *trans* (somatic hybrid) cells. It can be seen that with cells of both types cooperation is clearly indicated by the fact that the titration curves for the 1:1 mixture resemble those for the more

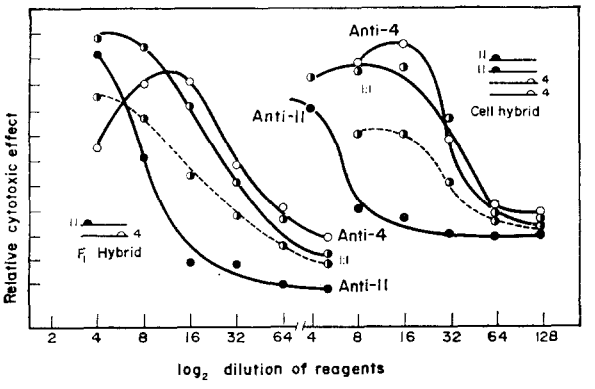

Fig. 3. Comparison of the distribution of specificities H-2·4 and H-21·1 on the membrane of F_1 and cell hybrids as judged by their capacity to cooperate in a complement-dependent reaction. Dotted lines: titration curves for 1:1 mixture expected on no-cooperation hypothesis.

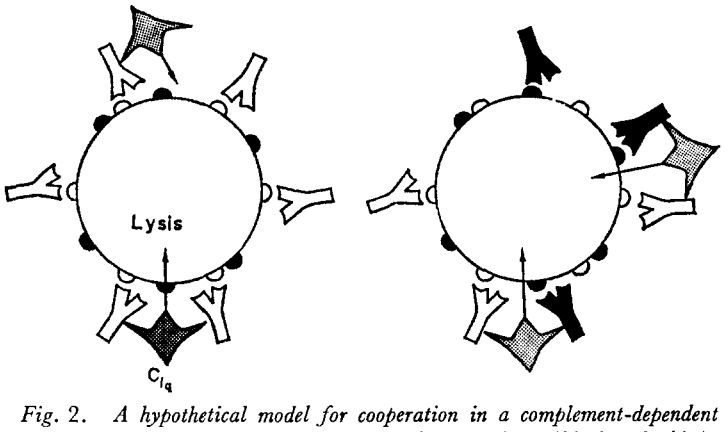

Fig. 2. A hypothetical model for cooperation in a complement-dependent reaction between two non-crossreacting membrane antigens (black and white).

potent reagent rather than the dotted curves constructed on no cooperation-hypothesis. No essential difference is seen between F_1 and somatic hybrid cells which suggests that even in the latter the antigens contributed by the two parents are close enough to one another for cooperation to be possible. Taking into account the fact that there was no blocking within the same pair of H-2 specificities (Table 1) one is inclined to regard the two approaches as a set of tools of different resolution power for an empirical estimation of the mutual positions of two membrane antigens. It seems only logical that blocking and cooperation are mutually exclusive; if a multimolecular complex has to be formed of complement and antibodies of different specificities occupying two adjacent antigenic sites, the configuration of the latter should not be conducive to blocking. Further data are needed, but the combination of the two methods seems to be promising. The observed cooperation between H-2 antigens which are contributed by different parents is in accord with intermixing of species-specific surface antigens in cell hybrids as demonstrated by direct methods [18, 19].

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4′-Demethyl-epipodophyllotoxin-β-D-thenylidene Glucoside (VM-26)—A Brief Review

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Abstract—*4′-Demethyl-epipodophyllotoxin-β-D-thenylidene-glucoside is as emisynthetic derivative of podophyllotoxin, a natural product from the May apple plant* (Podophyllum peltatum). *The drug is highly water insoluble and requires a lipid solvent for i.v. administration. The mode of action is at least two-fold, i.e., immediate metaphase arrest of dividing cells and subsequent prevention of cells from entering mitosis which is related to inhibition of DNA synthesis. In the L1210 system the drug is highly active, the best schedule being every three hours on days 1 and 9 or days 1, 5, and 9. VM-26 also has activity in intracerebrally inoculated L1210 mice. Phase I studies have arrived at a maximum tolerated dose (MTD), on a weekly schedule, of 67 mg/m². Other schedules employed include a twice weekly schedule of 1–3 mg/kg (40–120 mg/m²), a weekly regimen at 100–130 mg/m², and a schedule of 30 mg/m² daily × 5 repeated every 10–15 days. All schedules require drug administration by i.v. infusion over 30–60 min. Human toxicity includes leukopenia (dose related), hypotension on rapid administration, alopecia, occasional GI distress, and one episode of anaphylaxis. Early clinical studies indicate that VM-26 may have activity in Hodgkin's and non-Hodgkin's lymphomas, brain tumors and carcinoma of the bladder.*

INTRODUCTION

THE ANTITUMOR activity of podophyllotoxin derivatives has been studied since the late 1940s. Recently, two new congeners completed preclinical evaluation. 4′-Demethyl-epipodophyllotoxin-β-D-thenylidene glucoside (NSC 122819), known as VM-26, has undergone several Phase I and II clinical studies. The other compound, 4′-demethyl-epipodophyllotoxin-β-D-ethylidene glucoside (VP16-213; NSC 14154), is now in Phase I and early Phase II trials.

This review will summarize the present preclinical and clinical experience with VM-26 as a basis for conducting further investigations.

HISTORY

VM-26 is another member of a long line of medicinals that have been obtained in the past 250 years from the roots of *Podophyllum peltatum* (mandrake, May apple) [1]. Podophyllin, the resin, has found worldwide use as a cathartic, choleretic, and cholegogue, and was only dropped from the United States Pharmacopoeia in 1942. Interest in the drug was reawakened by Kaplan [2] who cured condyloma acuminatum with the topical use of podophyllin in oil. Further studies revealed that podophyllin and its crystalline derivative, podophyllotoxin, produced their therapeutic effect by acting as mitotic poisons [3]. Chemical analysis of podophyllin showed that the active tumor-damaging components included podophyllotoxin, α-peltatin, β-peltatin, and numerous other compounds [4, 5].

α-Peltatin was initially chosen for study in human neoplasia because of its greater activity compared to its congeners in several transplantable rodent tumors. Although the clinical

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trials revealed no clear-cut therapeutic efficacy, the drug did cause temporary regression of splenomegaly and lymphadenopathy in patients with assorted leukemias and lymphomas [6].

In 1963, Sandoz Laboratories synthesized two podophyllin derivatives that had minimal antitumor activity in clinical trials [7, 8] and were too toxic for general clinical use. These were SPI-77 (podophyllic acid, 2-ethyl hydrazide) and SPG-827 (podophyllotoxin, 4,6-0-benzylidene-β-D-glucoside), the latter obtained from the podophyllum plant native to India (*Podophyllum emodi*). SPI-77 caused severe chemical phlebitis and the SPG-827 produced intolerable gastrointestinal distress.

Sandoz Laboratories has recently synthesized the two newest derivatives, VM-26 and VP16-213 [9]. The structures of the podophyllin derivatives are shown in Fig. 1.

CHEMISTRY

VM-26 ($C_{32}H_{32}O_{13}S$) has a molecular weight of 656·7 and is poorly soluble in water. The clinical formulation contains several solvents (benzyl alcohol, dimethyl acetamide, polyethoxylated castor oil, and absolute alcohol) and maleic acid is added to bring the pH to 5·1. For intravenous administration, the drug is diluted with water and infused slowly in 5% glucose or normal saline over a period of 30–60 min.

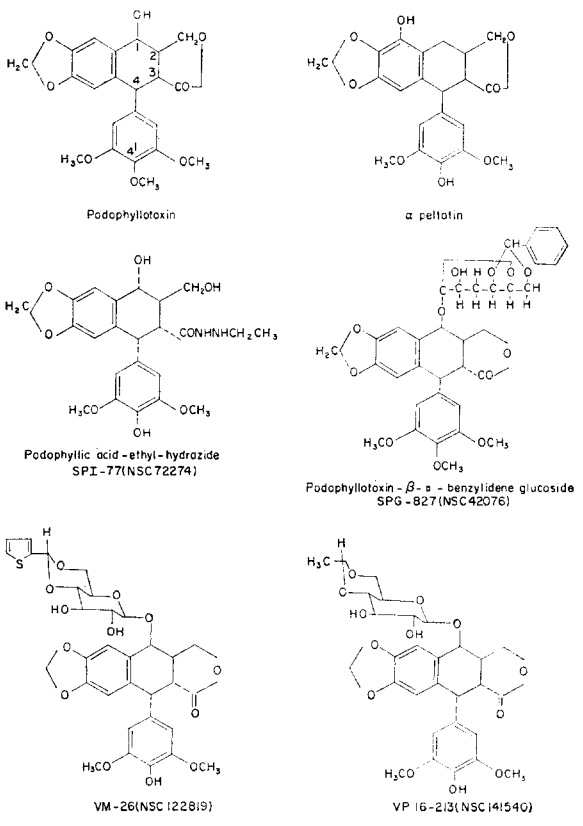

Fig. 1. Structures of podophyllin derivatives.

BIOCHEMISTRY

VM-26 has cytostatic activity *in vitro* against a variety of tumor-cell lines [10, 11]. The ED_{50}* of 0·005 mg/l against P-815 mastocytoma compares favorably with colchicine (0·004 mg/l), vinblastine (0·008 mg/l) and 0·02 mg/l for mechlorethamine [11].

The podophyllum derivatives are described as spindle poisons with their activity attributed to mitotic arrest [4]. This mode of action is only partially true for VM-26. Using fibroblasts in tissue culture, Stähelin [11] demonstrated that the initial effect of VM-26 is to arrest mitosis at metaphase in the classic manner of a spindle poison. However, 30–60 min later this effect is superseded by another, i.e. inhibition of the beginning of mitosis at drug levels between 0·1 and 1 mg/l *or* lysis of cells at concentrations ≧ 1 mg/l. Unlike colchicine and the podophyllum compounds of the pure spindle poison type, the effect of VM-26 in fibroblasts is virtually irreversible. It is clear that physiological concentrations of VM-26, unlike colchicine and, possibly, the vinca alkaloids, prevent cells from entering prophase and the action of the drug goes beyond that of a spindle poison.

Stähelin [11] also refers to studies indicating that VM-26 significantly inhibits H^3-thymidine uptake in P-815 cells, which would imply that the drug prevents DNA synthesis and could explain its preprophase mechanism of action.

ACTIVITY IN ANIMAL TUMOR SYSTEMS

VM-26 has been tested in several rodent tumor systems [11]. Only moderate activity was observed in Sarcoma 180, with a range of 41–72% reduction in tumor weight and no long-term survivors. Intraperitoneal administration of the drug in Ehrlich ascites tumor prevented development of ascites for 9 days but survival time was increased only 75% and there were no cures. The Walker carcinosarcoma was most responsive, especially on a day 1, 3, 6 schedule which produced 96% reduction in tumor weight.

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*ED_{50} = Concentration necessary to inhibit cell proliferation by 50%.

L1210 schedule dependency studies performed under contract for the National Cancer Institute, reported by Venditti [12], are summarized in Table 1. Conclusions on the material in suspension (saline + Tween-80) were unclear. However, the clinical formulation had excellent activity in L1210 with > 500% increase in life span (ILS) over controls and 60% 60-day survivors when given every 3 hr on either the day 1, 9 or 1, 5, 9 schedule.

The L1210 data of Stähelin [11], using a DMSO + Tween-80 vehicle, confirm the high order of activity of VM-26. The greatest activity (373% ILS) was obtained by twice-weekly administration of VM-26 at a dose of 6 mg/kg. Moderate activity (36–56% ILS) was also noted in mice inoculated intracerebrally with L1210.

Table 1. *Comparison of schedule dependency in L* 1210 *of VM-*26 *in suspension and solution* [12]

I.P. treatment schedule	Suspension* Optimal dose (mg/kg)	Suspension* Maximum I.L.S. (%)	Suspension* 60-day survivors (%)	Solution† Optimal dose (mg/kg)	Solution† Maximum I.L.S. (%)	Solution† 60-day survivors (%)
Once, Day 1	64‡	88	10	16	154	30
qd, Days 1, 9	64‡	90	10	8·0	246	40
qd, Days 1, 5, 9	32	43	0	8·0	151	30
qd, Days 1–9	8·0‡	40	0	1·0	131	10
q3h, Day 1	64/8	37	0	8·0/8	89	0
q3h, Days 1, 9	64/8‡	86	10	8·0/8	> 500	60
q3h, Days 1, 5, 9	64/8‡	37	0	8·0/8	> 500	60

*In saline + Tween-80.
†In epipodophyllotoxin vehicle solvent (benzyl alcohol, *N,N*-dimethyl acetamide, polyethoxylated castor oil, maleic acid, absolute alcohol, q.s. with isotonic sodium chloride).
‡Highest dose used.

CLINICAL STUDIES

Toxicity

Muggia *et al.* [13] performed a Phase I clinical trial in which VM-26 was given to 24 patients on a weekly schedule based on the animal tumor data showing greater efficacy for an intermittent schedule. Using dose increments reached by a modified Fibonacci scheme of escalation [14], the study concluded that 67 mg/m^2 per week was a safe dose producing consistent but reversible toxicity.

Leukopenia was the single most reproducible toxicity. Mild leukopenia appeared after 3, 4, and 6 weekly doses at 37·5 mg/m^2 and was reversible within one week. The range of leukopenia in 10 patients receiving starting doses of 67 mg/m^2 was 2100–4900/mm^3 after a median of 3 doses. The white count nadir generally occurred 1 week post-treatment but ranged from 3 to 14 days. Some patients developed a shift to the left in the granulocyte series but otherwise there was no preferential lowering of lymphocytes versus granulocytes, or vice versa. Thrombocytopenia due to the drug was negligible and was seen in only one patient.

The only other significant toxicity was hypotension after rapid IV push of VM-26. When administration of the drug was changed to a 30-min infusion, a mild episode of hypotension was noted in one patient at the start of a 50 mg/m^2 course. There was no delayed hypotension or cardiac toxicity.

Gastrointestinal toxicity was mild; nausea occurred in 2 patients during infusion of 67 mg and 90 mg/m^2, respectively, and diarrhea appeared on one occasion in 2 different patients. There was no renal or hepatic toxicity, and alopecia occurred in only 1 patient.

Trempe *et al.* [15] briefly reported a Phase I trial in 29 patients who received the drug by 20–40 min infusion because rapid administration produced cardiovascular side-effects (hypotension?). Toxicities noted were leukopenia, thrombocytopenia, and alopecia. Two regimens that produced moderate to severe toxicity were 1 mg/kg per day × 10 and 2 mg/kg per day × 3, followed by 2-day rest and, then, 2 mg/kg q2day × 3. A weekly schedule was not established but was estimated as 1–2 mg/kg per week, which is comparable to the schedule of Muggia *et al.* [13].

Dombernowsky *et al.* [16] gave the VM-26 intravenously twice weekly to 27 patients and

found, as in the other investigations, a predominance of hematologic toxicity. Leukopenia was most severe in lymphoma patients, who had prior extensive radiotherapy and/or chemotherapy, and was less common in patients with solid tumors. Toxicity was not noted until the cumulative dose reached 250–500 mg. At 1000 mg, hematologic toxicity was exhibited by all in the lymphoma group but by only one-half of the solid tumor patients. The clinically tolerable dose was felt to be in the range of 1–3 mg/kg twice weekly. Other toxicities included gastrointestinal symptoms such as nausea and vomiting a few hours after infusion, and 2 patients had diarrhea. Reversible alopecia was noted in two patients 4 weeks post-treatment. Another patient had a severe anaphylactic reaction on day 58 of therapy after reaching a dose level of 1 mg/kg. The patient recovered on cessation of treatment but suffered a second reaction to a small test dose several days later. Chemical phlebitis at the injection site was a constant problem in all patients and was thought due to the solvent system (dimethylacetamide, cremophor EL, and ethanol).

In a large scale study of the European Organization for Research on Treatment of Cancer (E.O.R.T.C.), VM-26 was given to 185 patients [17]. Thirty-seven (37) patients received 30 mg/m^2 per day (1-hr infusion) for 10–20 days but prolonged and severe myelosuppression in 27 (78%) required a dose change to 30 mg/m^2 per day × 5, repeated at 10–15 day intervals. Among 148 patients treated on this schedule, there were 3 fatalities due to pancytopenia but all of them had received extensive prior radiotherapy and chemotherapy. Nausea, vomiting, and diarrhea were the manifestations of GI toxicity in 17 (9%) of the 185 patients. Three patients also experienced stomatitis, and alopecia occurred in 16/185 (8·6%).

Because of unpublished preclinical data indicating the immunosuppressive effects of VM-26, the E.O.R.T.C. performed a battery of immunological tests. After VM-26 treatment, there was no change in delayed hypersensitivity measured by skin testing with BCG, candidin, and streptokinase. Similarly, PHA transformation of lymphocytes was unchanged by therapy. Immunoglobulin levels did not vary in pre- and post-drug administration measurements, suggesting that there is no change in immediate type hypersensitivity.

Sklansky *et al.* [18], in a preliminary communication, have reported the use of VM-26 in brain tumors at a dose of 100–130 mg/m^2 weekly for 6–8 courses. The principal toxicities were leukopenia and anemia, primarily in previously treated patients. Patients without prior treatment did not exhibit significant toxic effects.

Table 2 summarizes some of the dose schedules employed. The variation in doses used on weekly schedules probably reflects differences in patient characteristics in terms of prior therapy. Clearly, patients who have extensive prior radiotherapy and/or chemotherapy are more susceptible to the myelosuppressive effects of VM-26.

Table 2. VM-26 dose schedules

Investigator	Schedule
Muggia *et al.* [3]	67 mg/m^2 per week × 6
Dombernowsky *et al.* [16]	1–3 mg/kg ($\simeq$ 40–115 mg/m^2) biw
E.O.R.T.C. [17]	30 mg/m^2 per day × 5 q 21d
Sklansky *et al.* [18]	100–130 mg/m^2 per week × 6–8

Therapeutic results

The clinical efficacy of VM-26 in Phase I trials has been evaluable in only a small number of patients.

Muggia *et al.* [13] noted clinical and functional improvement in 2 of 7 patients who were evaluable on the basis of having received an adequate course of therapy. Both patients had brain tumors, which is interesting in view of the lipid solubility of VM-26 and its activity in IC-inoculated L1210 mice.

Dombernowsky *et al.* [16] reported 3 partial responses (PR), all in Hodgkin's disease, out of 6 patients treated longer than 3 weeks at doses leading to toxicity. The duration of remission ranged from 60 to 90 days. Significantly, these patients had previously received alkylating agents, vinca alkaloids, and procarbazine, which indicates that VM-26 does not exhibit clinical cross-resistance with these agents.

The Sloan–Kettering studies [15] produced 2 partial responses in 4 cases of Hodgkin's

disease and lowering of peripheral blood counts in 1 case of acute lymphocytic leukemia (ALL) and 1 case of chronic myelogenous leukemia (CML). There were only symptomatic or no responses in reticulum cell sarcoma and assorted tumors.

The large Phase II study of the E.O.R.T.C. emphasized lymphomas and other solid tumors [17]. In 22 patients with Hodgkin's disease, they found 1 CR (Stage IIIA disease) of brief duration (30 days) and 6 partial responses. VM-26 apparently was fairly active in reticulum cell sarcoma (13/25 or 52% PR) but 9 of the patients had no prior therapy and 2 others had only antecedent radiotherapy. There were 6 PR in 19 lymphosarcomas, with striking regression of pleural effusions in 3 patients.

The E.O.R.T.C. study of solid tumors included 119 patients but only 4 tumor types were represented by sufficient patients to assess drug activity. The responses in these tumors were 1 CR and 6 PR including cancer of the esophagus (0/12), head and neck (0/16), bladder (1 CR and 4 PR/24), and breast cancer (2 PR/22). On the basis of the E.O.R.T.C. investigation, VM-26 has antitumor activity against Hodgkin's disease and non-Hodgkin's lymphomas, especially reticulum cell sarcoma. There also is interesting activity in bladder carcinoma but only minimal effect in breast cancer and little activity in cancers of the esophagus and head and neck.

The use of VM-26 by Sklansky *et al.* [18] in primary brain tumors has shown objective neurologic improvement in 4 of 12 patients with symptomatic disease and only one progression in 7 asymptomatic patients. Responses occurred in 2 patients who had experienced progressive disease while under therapy with nitrosoureas.

Henderson [19] has also investigated VM-26 without observing response in 14 patients with ALL (12 children and 2 adults). He noted a reduction in circulating blasts in 3 patients and 1 PR in a child among 7 AML patients (5 adults, 2 children).

CONCLUSIONS

Clinical trials of VM-26 have shown definite antitumor activity in lymphomas and brain tumors. However, except for the minimal effect in breast cancer, there is not enough information to warrant further comment on the other signal solid tumors (colon, melanoma, pancreas, lung, and ovary).

There appears to be potential for the use of this agent in primary combination therapy of lymphomas, particularly in reticulum cell sarcoma. VM-26 is not unduly myelosuppressive and, otherwise, shows overlapping toxicity with the vinca alkaloids only in alopecia. At this time, none of the lymphomas appear to exhibit cross-resistance to VM-26 and the standard therapeutic agents used against them.

The sensitivity of brain tumors to VM-26 is very encouraging and combination of this agent with nitrosoureas seems a feasible area for further investigation. Another approach to combination chemotherapy with VM-26 could be developed considering cell kinetics. For example, a priming dose of VM-26 to arrest cells at mitosis, followed by a second drug having peak activity at this stage in the cell cycle (e.g., irradiation or bleomycin) [20, 21] would certainly be worth investigation.

In short, VM-26 is a new active antitumor agent that could be a valuable addition to our therapeutic armamentarium.

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Effect of Increasing Doses of Estrogen and Progesterone on Mammary Carcinogenesis in the Rat*

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Abstract—*Female Sprague–Dawley rats were fed a single dose of* 20 *mg DMBA at* 50 *days of age. Fifteen days later, groups of rats received injections in incremental or constant doses of estradiol benzoate or progesterone, alone or in combination, for the next* 21 *days. This hormone treatment was an attempt to mimic the effect of pregnancy on the appearance of DMBA-induced mammary tumors. The administration of incremental doses of progesterone plus a constant daily dose of estradiol benzoate, or the administration of incremental doses of progesterone alone showed a slight but insignificant stimulation of tumor appearance. Incremental doses of estradiol benzoate, either alone or in conjunction with progesterone, depressed tumor appearance.*

INTRODUCTION

It is well-established that hormone-responsive mammary tumors develop in female Sprague–Dawley rats following intragastric instillation of the polycyclic hydrocarbons, 7,12-dimethylbenz[α]anthracene (DMBA) or 3-methylcholanthrene (MCA). Growth of tumors induced by DMBA or MCA can be profoundly affected by modifying the hormonal environment of the host rat. For example, ovariectomy or hypophysectomy of the host rat results in regression of mammary tumors induced by MCA [1] or DMBA [2]. Moreover, pregnancy has been shown to stimulate both tumor appearance and tumor growth [5].

Huggins, Moon and Morii [3] have shown that the accelerating effect of pregnancy on the induction of mammary cancer with DMBA may be mimicked by the administration of 4 mg progesterone for 30 days beginning 15 days after the intragastric administration of the carcinogen. Furthermore, pharmacological doses of estradiol-17β (20 μg/day for 30 days) delayed tumor appearance, whereas the concommitant administration of the above doses of estradiol-17β and progesterone resulted in a 50% reduction in the incidence of mammary cancer. However, concomitant administration of estradiol-17β (0·1–1·0 μg) and progesterone (4 mg) did not abolish the cancer stimulating effect of progesterone.

Since it is generally accepted that both estrogen and progesterone are produced in increasing quantities during pregnancy [4], it seemed of interest to examine the effect of increasing doses of estrogen and progesterone on mammary tumor appearance in DMBA-fed, virgin rats. In this manner we hoped to gain an insight into the stimulating effect of pregnancy on the appearance of DMBA-induced mammary tumors.

MATERIAL AND METHODS

Virgin female Sprague–Dawley rats were obtained from the dealer at 43 days of age and

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were kept in a room artificially lighted during normal daylight hours and maintained at a temperature of 78 ± 2°F. Wayne Lab. Blox and tap water were given *ad libitum*.

At age 50 days, each rat received 20 mg DMBA in 1 ml sesame oil by stomach tube. Beginning 15 days after DMBA feeding, each rat received daily subcutaneous injections of estradiol benzoate and progesterone either alone or in combinations for 21 days. Four dosages of estradiol benzoate and 2 dosages of progesterone were used in these studies and were designated as follows:

Low incremental dose estradiol benzoate

0·01 μg, days 1–3; 0·05 μg, days 4–6; 0·1 μg, days 7–9; 0·2 μg, days 10–12; 0·4 μg, days 13–15; 0·8 μg, days 16–18 and 1·6 μg, days 19–21.

High incremental dose estradiol benzoate

1·0 μg, days 1–3; thereafter, dosage increased in 1·0 μg increments every third day, reaching a level of 7·0 μg at days 19–21.

Low constant dose estradiol benzoate

0·1 μg daily from days 1–21.

High constant dose estradiol benzoate

1·0 μg daily from days 1–21.

Incremental dose progesterone

1·0 mg, days 1–3; thereafter, dosage increased in 1·0 mg increments every third day, reaching a level of 7·0 mg at days 19–21.

Constant dose progesterone

3·0 mg daily from days 1–21.

The dosage schedule for each group was as follows: *Group I*—DMBA controls, 11 rats, no hormone treatment. *Group II*—Low incremental dose estradiol benzoate, 8 rats. *Group III*—High incremental dose estradiol benzoate, 8 rats. *Group IV*—Low incremental dose estradiol benzoate and constant dose progesterone, 6 rats. *Group V*—High incremental dose estradiol benzoate and constant dose progesterone, 13 rats. *Group VI*—Incremental dose progesterone, 10 rats. *Group VII*—Low constant dose estradiol benzoate and incremental dose progesterone, 7 rats. *Group VIII*—High constant dose estradiol benzoate and incremental dose progesterone, 10 rats. *Group IX*—Low incremental dose estradiol benzoate and incremental dose progesterone, 7 rats. *Group X*—High incremental dose estradiol benzoate and incremental dose progesterone, 13 rats.

Tumors were detected by daily palpation and all rats were observed for more than 5 months after DMBA feeding. No rats died during the treatment period, but several died during the intervening months due usually to tumor necrosis, abscess formation, and debility. The numbers of rats which did not survive to necropsy in each group were as follows: Group I–2, Group II–0, Group III–0, Group IV–1, Group V–0, Group VI–0, Group VII–1, Group VIII–1, Group IX–1, Group X–2. Such rats were necropsied when they died, and included in the final data. At necropsy, sections of all tumors were taken for histologic study, stained with hematoxylin and eosin, and classified as to tumor type.

RESULTS

The majority of the tumors which developed in all groups were classified as mammary adenocarcinomas, but a few benign fibroadenomas were also observed. A third tumor type was designated as "mixed". Essentially, the "mixed" tumor was composed of relatively equal portions of adenocarcinoma and an atypical fibrous stroma. A histologic description of these tumors has been reported [5].

The results pertaining to percent incidence, mean time of appearance of the first palpable tumor (MTA) and mean number of active centers (numbers of tumors in group/number of rats in group) are summarized in Table 1.

As noted in Table 1, all DMBA control rats developed mammary tumors. The mean number of active centers was 3·00 ± 0·37 and the mean time of appearance of first palpable tumor was 68·6 ± 5·1 days after DMBA feeding.

When low incremental doses (0·01–1·6 μg/day) estradiol benzoate were administered to rats in Group II, 7 of 8 rats developed tumors, and the MTA was significantly increased. Moreover, estradiol benzoate at the higher incremental dose (Group III) further depressed tumor formation, as only 5 of 8 rats developed tumors, and the mean number of active centers was reduced. Estradiol benzoate (low incremental dose) plus a constant dose of 3·0 mg progesterone/day (Group IV) had no significant effect on tumor formation. Finally, the combination of a high incremental dose estradiol benzoate plus a constant dose of 3 mg progesterone/day (Group V) depressed all parameters used to assess tumor appearance, as the incidence and mean number of active centers were decreased, while the MTA was increased.

When only incremental doses of progesterone (Group VI) were administered, there was no significant effect on the parameters we used to assess tumor formation. However, the first

Table 1. *Effect of increasing or constant doses of estradiol-benzoate (EB) and progesterone (P), alone or in combination on mammary tumor incidence and time of appearance in* DMBA-*fed female rats*

Group	Treatment	Tumor incidence (No. with tumors/ No. rats) (%)	Mean No. active centers[a] (± S.E.)	MTA[b] (± S.E.)	Range of first tumor appearance (days after DMBA)
I	None	11/11 (100%)	3·00 (±0·37)	68·6 (±5·1)	42–99
II	EB (LI)[c]	7/8 (87·5%)	2·13 (±0·68)	91·1[f] (±11·2)	39–117
III	EB (HI)[d]	5/8 (62·5%)	1·00[f] (±0·43)	78·0 (±26·6)	21–140
V	EB (HI)+ 3 mg P/day	8/13 (61·5%)	1·08[f] (±0·34)	113·5[f] (±7·4)	91–140
VIII	P (I)[e]+ 1.0 μg EB/day	6/10 (60·6%)	0·70[f] (±0·22)	97·0 (±23·3)	42–140
IX	EB (LI)+ P (I)	6/7 (85·7%)	1·14[f] (±0·28)	96·3[f] (±13·1)	38–117
X	EB (HI)+ P (I)	5/13[g] (38·5%)	0·69[f] (±0·30)	112·4[f] (±10·1)	88–140

[a]Mean number of active centers = Total tumors in group/number rats in group.
[b] MTA = Mean time of appearance of first palpable tumor, days after DMBA.
[c]EB (LI) = 0·01 μg, days 1–3; 0·05 μg, days 4–6; 0·1 μg, days 7–9; 0·2 μg, days 10–12; 0·4 μg, days 13–15; 0·8 μg, days 16–18 and 1·6 μg, days 19–21.
[d]EB (HI) = 1·0 μg, days 1–3; thereafter, dosage increased in 1·0 μg increments every third day reaching a level of 7·0 μg at days 19–21.
[e]P (I) = 1·0 mg, days 1–3; thereafter dosage increased in 1.0 mg increments every third day, reaching a level of 7·0 days 19–21.
[f]Significant from Group I, $P < 0·05$ or better.
[g]Significant from Group I, $P < 0·01$ or better.

palpable tumor appeared during the 21-day treatment period in 7 of these 9 rats. When increasing doses progesterone plus a low constant dose of 0·1 μg estradiol benzoate/day were given to rats in Group VII, all 7 rats developed tumors, with 4 rats having the first palpable tumor appear during the treatment period. No significant change in MTA or mean number of active centers was evident when compared to controls. On the other hand, the combination of incremental doses progesterone plus a high constant dose of 1·0 μg estradiol benzoate/day (Group VIII) decreased tumor incidence to 60%, and the mean number of active centers was decreased.

Rats receiving incremental doses of progesterone plus low incremental doses estradiol benzoate (Group IX) exhibited a reduced tumor incidence of 85·7%. In addition, the mean number of active centers was less than controls, while the MTA was increased. Incremental doses progesterone plus the high incremental doses estradiol benzoate administered to rats in Group X resulted in the most profound reduction in tumor appearance that was observed. Only 38·5% of rats in this group developed tumors, with the mean number of active centers decreased to 0·69 ± 0·30 and the MTA increased to 112·4 ± 10·1 days after DMBA feeding.

DISCUSSION

We have previously demonstrated that when female Sprague–Dawley rats are allowed to breed 15 days after DMBA feeding, all rats develop tumors during the ensuing pregnancy [5]. Pregnancy does not affect the mean number of tumors/rat, but the latent period of tumor development is considerably decreased when compared to DMBA-treated, virgin controls. These findings essentially agree with the conclusions of Dao *et al.* [6, 7] concerning MCA-induced mammary tumors, and with those of Huggins *et al.* [3] as regards DMBA-induced mammary tumors. On the other hand, the mechanisms by which gestation hastens tumor appearance is not readily apparent. That an endocrine mechanism is involved is suggested by the hormone-responsive nature of the tumors.

The ovaries are essential for the maintenance of pregnancy in the rat [8, 9]. Moreover, rats overiectomized early in pregnancy can be

maintained until parturition by the daily injection of suitable doses of progesterone [8, 9]. These findings point out the vital role of progesterone in maintaining gestation in the rat. That estrogen also may play a role in the maintenance of pregnancy in the rat is shown by the experiments of Lyons [8]. Rats overiectomized on day 6 or 8 of pregnancy show better fetal maintenance when treated with 2 μg estrone plus 4 mg progesterone/day than when only 4 mg progesterone/day is given.

It is generally accepted that both estrogen and progesterone are produced in increasing quantities during pregnancy [9]. Thus, in the present study estradiol benzoate and progesterone in various increasing doses were given to DMBA-fed, female rats for 21 days. These data suggested that the stimulatory effect of pregnancy on tumor appearance most likely does not result from increasing estrogen secretion. Tumor appearance was not hastened when 2 incremental dose levels of estradiol were given, and in fact, a suppression of tumor appearance was noted at the higher dose. When a constant daily dose of progesterone was added to the incremental estradiol treatment, a further inhibition of tumor appearance resulted. An even greater inhibition of tumor appearance occurred when both increasing increments of progesterone and estradiol were administered. The only treatments which stimulated tumor appearance in any manner similar to that seen during pregnancy was the administration of incremental doses of progesterone plus a constant dose of 0·1 μg estradiol benzoate/day, or the administration of incremental doses of progesterone alone (Groups VI and VII). Although neither the mean number of active centers/rat or the mean time of tumor appearance was significantly different from controls, the majority of rats developed tumors during the 21-day treatment period, as was seen in pregnant animals [1, 3, 5]. Perhaps larger doses of progesterone would have demonstrated a more dramatic stimulation of tumor formation. Thus, these data suggest that the increased secretion of progesterone during gestation in the rat stimulates the appearance of palpable mammary tumors.

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Triton WR 1339*: Effect on the Reticulo-endothelial System (RES) Activity in Tumor-Bearing Mice†

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Abstract—*The reticulo-endothelial system (RES) activity in normal and in intracerebral Sarcoma 180-bearing mice was investigated after acute or subchronic treatment with the non-ionic surface-active agent: Triton WR 1339 (TWR).*

While the single treatment is ineffective, the subchronic treatment markedly raises the phagocytic index not only in normal mice but also in tumor-bearing animals where the RES is depressed by the presence of disseminating cancer cells. A possible relation between the RES stimulatory capacity and the antimetastatic effect exerted by TWR is envisaged.

INTRODUCTION

SEVERAL years ago G. B. Mackaness [1] and J. E. Lovelock [2] suggested a possible activity of TWR‡ on the macrophage system to explain the antitubercular activity exerted by this compound.

Previous work from these laboratories has demonstrated that TWR shows a marked inhibitory effect on cancer dissemination and on metastases formation under various experimental conditions [3, 4, 5] without inhibiting the primary tumor growth or showing the cytotoxic symptoms common to most of the drugs utilized in cancer chemotherapy. It was, therefore, of interest to investigate the effect of TWR on the phagocytic activity of the reticulo-endothelial system (RES) in tumour-bearing mice. To this end the test of Sarcoma 180 implanted intracerebrally (IC) was selected because it induces depression of the RES phagocytic activity in mice [6] and because its dissemination in the lung is inhibited by TWR [4].

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*Triton WR 1339 = EORTC 863—formaldehyde polymer of polyoxiethylene ether of octylphenol.

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‡TWR = Triton W.R. 1339.

MATERIAL AND METHODS

1. *Animals and tumour*

Female CD_1 mice (Ha M/ICR Swiss) obtained from Charles River, Italy, with an average weight of 20 ± 2 g were used. The animals were kept in cages of Makrolon ($20 \times 26 \times 13$ cm), 10 per cage, at a constant temperature of 22°C, a relative humidity of 60%, and free access to water and food (Charles River 4RF 21).

Sarcoma 180 (S180) was maintained in ascites form in this strain of mice for more than 300 passages. Only ascitic fluids obtained from mice innoculated 7–10 days before were used in order to keep cell clumps and dead cells to a minimum. Intracerebral (IC) tumors were obtained by transplanting 1×10^5 S180 cells in a volume of 0·01 ml in the thalamic area on the median line, according to a technique previously described [7].

2. *Experimental design*

Triton WR 1339 (kindly supplied by Winthrop Co., U.S.A.) was administered at two different schedules of treatment, namely, single injections of 50, 200 or 500 mg/kg intraperitoneally (i.p.) in CD_1 normal mice and repeated injections for 6 successive days at the dose of 50 or 500 mg/kg i.p. in both normal and S180 IC-bearing CD_1 mice. Treatment was given from day 1 to day 6 after the tumor implantation. Phagocytic acitivity of the RES was evaluated 24, 48 and 72 hr after the last TWR injection. The liver, spleen and lungs of each animal were collected 1 hr after the intravenous (IV) injection of colloidal carbon and the weight relative to 20 g of mouse b ody weight was recorded.

3. *Evaluation of the colloidal carbon clearance from the blood*

The carbon suspension $C_{11}/_{1431}$a Pelikan (John Hemchel and Co., New York City) was diluted with saline up to a concentration of 16 mg of carbon/ml and injected in the tail vein at a dose of 160 mg/kg.

Samples of blood (0·02 ml each) were collected from the retro-orbital venous plexus at 3, 6, 9, 12 and 15min. Each blood sample was diluted with 5 ml of haemoglobin diluent (1 g $NaHCO_3$, 50 mg KCN and 200 mg $K_3Fe(CN)_6$ in 1 l. of water). Carbon concentration was determined by its absorption at 540 nm in a Beckman D.U. spectrophotometer. The phagocytic index K, determined with the method of Biozzi *et al.* [8], is the slope of the line relating the logarithm of the carbon concentration in the blood with the time (3, 6, 9, 12 and 15 min) after the carbon administration.

4. *Statistical analyses*

The average value of the phagocytic index (K) for each group was calculated as a weighted mean, the weights being indirectly proportional to the error of the regression line [9].

Acute treatment with TWR. The variances of the K values were shown to be homogeneous and the volumes of distribution (*Vd*) did not differ significantly since all the confidence intervals overlap. Therefore, an analysis of variance was carried out by treating the experimental results

Table 1. *Acute treatment with Triton WR* 1339 (*TWR*) *and activity of the reticulo-endothelial system* (*RES*) *in* CD_1 *female mice*

		Controls	TWR 50 × 1	TWR 200 × 1	TWR 500 × 1
24 hours	K·100	4·3 ± 0·2	4·2 ± 0·2	5·1 ± 0·2	4·3 ± 0·2
	Vd	0·7 (0·6–0·8)	0·8 (0·7–0·9)	0·9 (0·8–1·0)	0·8 (0·7–1·0)
	Liver wt.	1·1 ± 0·1	1·3 ± 0·04	1·3 ± 0·02	1·6 ± 0·1
	Spleen wt.	0·14 ± 0·007	0·13 ± 0·01	0·19 ± 0·001	0·15 ± 0·01
	Lung wt.	0·19 ± 0·01	0·19 ± 0·01	0·19 ± 0·001	0·21 ± 0·02
48 hours	K·100	4·7 ± 0·3	4·5 ± 0·3	5·2 ± 0·2	4·8 ± 0·3
	Vd	0·8 (0·7–0·9)	0·8 (0·7–0·9)	0·8 (0·7–0·9)	0·7 (0·6–0·9)
	Liver wt.	1·3 ± 0·02	1·3 ± 0·03	1·4 ± 0·02	1·4 ± 0·07
	Spleen wt.	0·14 ± 0·01	0·15 ± 0·007	0·18 ± 0·01	0·18 ± 0·01
	Lung wt.	0·22 ± 0·008	0·26 ± 0·01	0·23 ± 0·01	0·24 ± 0·009
72 hours	K·100	3·8 ± 0·3	3·4 ± 0·2	4·5 ± 0·3	3·6 ± 0·2
	Vd	0·9 (0·8–1·0)	0·9 (0·8–1·0)	0.8 (0·7–0·9)	0·8 (0·7–0·9)
	Liver wt.	1·3 ± 0·05	1·4 ± 0·03	1·5 ± 0·07	1·4 ± 0·07
	Spleen wt.	0·13 ± 0·007	0·15 ± 0·007	0·17 ± 0·01	0·17 ± 0·01
	Lung wt.	0·20 ± 0·009	0·20 ± 0·01	0·24 ± 0·01	0·25 ± 0·02

CD_1 female mice were injected with TWR at the doses of 50, 200, and 500 mg/kg i.p. The clearance of colloidal carbon in the blood was evaluated 24, 48 and 72 hrs after injection. K (phagocytic index) is the slope of the line relating the logarithm of the carbon concentration in the blood with the time (3, 6, 9 and 15 min) after intravenous injection of a dose of 160 mg/kg of carbon, and it is always a negative number (± S.E.).

Vd is the volume of distribution of the carbon and it is equal to the ratio between the dose (mg/kg) administered and the estimated concentration of carbon in the blood at time 0. The values in brackets are the upper and the lower 95% confidence limites for *Vd*.

Each value is based on data from 6 animals, divided into 2 groups of 3.

Details concerning the factorial analysis are reported under the Statistical analyses section. No difference is statistically significant. The weights of liver, spleen and lungs are expressed in g (± S.E.)/20 g body weight. The Student's test was used for establishing the statistical significance between each group and its controls. No difference is significant.

as a 4 × 3 factorial design (4 doses of TWR: 0, 50, 200 and 500 mg/kg and 3 times: 24, 48 and 72 hr after TWR treatment).

Repeated treatment with TWR. These data were considered as a 3 × 2 × 3 factorial design (3 different times: 24, 48, and 72 hr after TWR treatment; 2 different types of animals: normal and tumor-bearing mice; and 3 different doses of TWR: 0, 50, and 500 mg/kg). Since the variances of the *K* values were not homogeneous, the analysis was carried out by calculating the linear combinations of the data relating to the factorial effects and by comparing the resulting values with the appropriate combined variance using an approximation to Student's test.

RESULTS AND CONCLUSIONS

Results obtained on the RES phagocytic activity after a single injection of TWR at the dose of 50, 200, and 500 mg/kg are summarized in Table 1. There is practically no effect exerted by a single dose of TWR on the volume of the distribution and on the clearance of colloidal carbon in blood in respect to controls at all the time interval considered, i.e., 24, 48, and 72 hr after TWR administration.

Table 2 shows instead that a repeated treatment with TWR (500 mg/kg × 6 days) in normal animals does not affect the *Vd* of colloidal carbon but it does induce a marked and prolonged increase of the phagocytic index which was still significantly higher in respect to control value even 72 hr after TWR administration. This effect was accompanied by a significant increase in liver and spleen weight. The lower dosage (50 mg/kg × 6), on the other hand, was ineffective at this schedule of treatment. The presence of a disseminating tumor, S180 IC, significantly decreased the phagocytic index, thus confirming previous findings [6]. However, TWR at the dose of 500 mg/kg markedly raised the phagocytic index together with a significant increase in liver, spleen, and, at given times, lung weight.

It is important to note that this effect was obtained by treatment with TWR at doses (500 mg/kg × 6) able to significantly inhibit tumor-cell dissemination in the system used [2], whereas non-antimetastatic schedules (50 mg/

Table 2. Subchronic treatment with Triton WR 1339 (TWR) and activity of the reticulo-endothelial system (RES) in normal and tumor-bearing CD_1 female mice

		Normal			Sarcoma 180▲		
		Controls	TWR 50 × 6	TWR 500 × 6	Controls	TWR 50 × 6	TWR 500 × 6
24 hours	*K*·100	3·8 ± 0·4	5·1 ± 0·5	16·0 ± 1·8	2·9 ± 0·1	2·9 ± 0·2	8·7 ± 0·7
	Vd	0·9 (0·7–1·1)	0·9 (0·7–1·2)	0·6 (0·2–1·6)	0·7 (0·7–0·8)	1·0 (0·9–1·2)	0·9 (0·6–1·3)
	Liver wt.	1·1 ± 0·02	1·1 ± 0·02	1·5 ± 0·04*	1·2 ± 0·03	1·1 ± 0·03	1·4 ± 0·04†
	Spleen wt.	0·08 ± 0·004	0·11 ± 0·006*	0·19 ± 0·008*	0·11 ± 0·008	0·11 ± 0·004	0·14 ± 0·009†
	Lung wt.	0·15 ± 0·07	0·16 ± 0·006	0·17 ± 0·008	0·17 ± 0·004	0·17 ± 0·002	0·20 ± 0·01†
48 hours	*K*·100	4·0 ± 0·2	3·9 ± 0·2	9·1 ± 0·9	2·8 ± 0·1	2·6 ± 0·3	4·9 ± 0·8
	Vd	0·9 (0·8–1·0)	1·2 (1·0–1·4)	0·8 (0·5–1·4)	0·8 (0·8–0·9)	0·9 (0·8–1·1)	1·0 (0·6–1·6)
	Liver wt.	1·1 ± 0·03	1·2 ± 0·03	1·5 ± 0·06*	1·1 ± 0·02	1·1 ± 0·02	1·3 ± 0·06†
	Spleen wt.	0·10 ± 0·005	0·14 ± 0·01*	0·14 ± 0·01†	0·12 ± 0·01	0·12 ± 0·05	0·14 ± 0·01
	Lung wt.	0·17 ± 0·01	0·18 ± 0·01	0·18 ± 0·01	0·19 ± 0·003	0·18 ± 0·007	0·18 ± 0·006
72 hours	*K*·100	4·0 ± 0·2	4·8 ± 0·2	6·5 ± 1·2	2·3 ± 0·03	3·1 ± 0·4	4·4 ± 0·4
	Vd	1·0 (0·9–1·1)	1·0 (0·9–1·1)	0·9 (0·5–1·9)	0·7 (0·7–0·8)	1·0 (0·8–1·3)	0·9 (0·7–1·2)
	Liver wt.	1·1 ± 0·05	1·1 ± 0·02	1·4 ± 0·05*	1·0 ± 0·03	1·0 ± 0·03	1·4 ± 0·02*
	Spleen wt.	0·11 ± 0·007	0·12 ± 0·007	0·11 ± 0·07	0·09 ± 0·01	0·10 ± 0·005	0·10 ± 0·006
	Lung wt.	0·15 ± 0·008	0·17 ± 0·008	0·17 ± 0·01	0·17 ± 0·007	0·19 ± 0·01	0·18 ± 0·009

▲CD_1 mice were injected on day 0 with 10^5 Sarcoma 180 cells intracerebrally. TWR was given from day 1 to day 6 at the doses of 50 and 500 mg/kg i.p. The clearance of colloidal carbon (160 mg/kg i.v.) was evaluated 24, 48, and 72 hr after the last injection of TWR. *K* and *Vd* are defined and calculated as for Table 1. Each value is based on data from 6 animals divided into 2 groups of 3.

Details concerning the factorial analysis are reported under the Statistical analysis section. The *Vd* were not statistically different among the various groups. For the *K* values, the overall effect of TWR is significant ($P < 0{\cdot}001$). There are also significant interactions between TWR and type of animals ($P < 0{\cdot}01$) and between TWR and time ($P < 0{\cdot}01$). The weights of liver, spleen and lungs are expressed in g (± S.E.)/20 g body-weight. The Student's *t*-test was used to establish the statistical significance between each group and its controls.

*$P < 0{\cdot}01$; †$P < 0{\cdot}05$ in respect to control value.

kg × 6) also remained ineffective on the RES activity. It is difficult to establish if the increase of the RES activity induced by TWR both in normal and in tumor-bearing animals is explained only by the increase of liver and spleen weight or if other mechanisms are operating as well.

In conclusion, TWR is able to increase the RES activity not only in normal mice, but also in animals with an impairment of this activity induced by the presence of a disseminating tumor. This effect is long-lasting although it is more marked in normal than in tumor-bearing mice. Whether this increase of phagocytic activity is responsible for the effect exerted by TWR on cancer dissemination remains to be established.

Finally, it may be recalled that the activity of TWR resembles that of other agents, such as BCG or Caryobacterium Parvum, which are known to be stimulators of the RES and, at the same time, inhibitors of tumor growth [10, 11].

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The Prolonged Binding of Ethyl Carbamate-(^{14}C) to DNA in Regenerating and Intact Mouse Liver*

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Abstract—*The binding of ethyl carbamate-(1-^{14}C), or a metabolite, to mouse liver DNA persisted longer if it was given to partially-hepatectomised mice compared with intact mice. Chromatographic examination of acid hydrolysates of the liver DNA from the partially hepatectomized mice showed that the radioactivity associated with the DNA was not present as a result of incorporation into the bases during DNA biosynthesis. Ethyl carbamate produced a greater inhibition of DNA turnover in the liver when given to partially-hepatectomized mice compared with intact mice.*

INTRODUCTION

ETHYL CARBAMATE (urethane), or a metabolite, binds covalently with liver DNA, RNA and protein in mice and rats [1, 2, 3]. A quantitative difference between the level of binding to liver DNA of intact and partially hepatectomized mice has been shown [4]. The present experiments were initiated to determine if (a) the binding of ethyl carbamate to liver DNA was maintained for a longer period than had previously been studied; (b) the quantitative difference in binding referred to above [4] was also maintained over a prolonged time; and (c) there was a correlation between the inhibition of liver DNA synthesis produced by ethyl carbamate and the maintenance of binding of ethyl carbamate to liver DNA [4, 5].

MATERIAL AND METHODS

Animals

Male Crackenbush mice, 7 weeks old at the start of each experiment, were used.

Radiochemicals

Ethanol-(1-^{14}C) (59·0 mCi/m mole), thymidine-(6-^{3}H) (5·0 Ci/m mole) and thymidine-(2-^{14}C) (56 mCi/m mole) were obtained from the Radiochemical Centre, Amersham, U.K. Ethyl carbamate-(1-^{14}C) (26·7 μCi/m mole) was prepared from ethanol-(1-^{14}C) [6].

Animal experiments

Two-thirds partial hepatectomy was performed under light anaesthesia. Only animals which survived the operation without complications, which made a rapid recovery and were not visibly jaundiced were subsequently used.

All chemicals and radiochemicals were given by intraperitoneal injection in sterile saline, thymidine-(^{3}H) (0·25 μCi/g) one hour before death and thymidine-(^{14}C) (0·01 μCi/g) daily for the five days prior to partial hepatectomy and/or the administration of unlabelled ethyl carbamate. Ethyl carbamate-(^{14}C) (6 μCi; 20 mg) was given as indicated in the text.

Extraction of nucleic acids

For the measurement of the binding of ethyl carbamate-(^{14}C), liver DNA was extracted by the phenol technique [7]. For the measurement of the incorporation of thymidine-(^{3}H) and thymidine-(^{14}C), liver DNA was extracted by a modification of the Schmidt–Thannhauser technique [8]. DNA was estimated colorimetrically by the diphenylamine method [9].

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Chromatographic examination of DNA

Liver DNA was hydrolysed with aq. 72% (w/v) $HClO_4$ (15 μl/mg DNA). After 1 hr at 100°C, the hydrolysate was diluted threefold with water and clarified by centrifugation. The hydrolysate was applied to a column (20 × 1·2 cm) of Dowex 50 (H^+ form) and eluted with a linear gradient of 0·5–4·0 M–HCl (650 ml) in 6 ml fractions at 0·5 ml/min. The E_{254} of each fraction was monitored and the radioactivity in each fraction was measured after 1 ml aliquots of each fraction had been evaporated to a small volume (0·1–0·2 ml) in the scintillation vials.

Each fraction, which corresponded with either an increase of ultraviolet absorption or radioactivity above the normal levels was examined by descending paper chromatography on Whatman No. 1 paper using as solvents, (i) butan-1-ol: acetic acid: water (12 : 3 : 5; by volume), (ii) butan-1-ol: water (43 : 7, by volume) and (iii) butan-1-ol: NH_4OH: water (43 : 2 : 5). Spots were localized with an ultraviolet light at 254 nm wavelength.

Radioactivity measurements

Radioactivity was measured with a liquid scintillation spectrometer. The scintillation fluid comprised: naphthalene (100 g), 2,5-diphenyloxazole (3 g), xylene (385 ml), 1,4-dioxane (385 ml) and ethanol (230 ml).

Statistics

Statistical significance was established using Student's *t*-test.

RESULTS

Persistence of binding of ethyl carbamate-(1-^{14}C)

Ethyl carbamate-(1-^{14}C) (6 μCi; 20 mg) was given to intact mice and to partially-hepatectomized mice 18 hr post-operatively. Groups of eight mice were killed 10 hr and 1, 2, 5, 8 and 12 weeks later. Extraction of the DNA with ether for 10 hr, acid hydrolysis of the DNA and chromatographic examination of the hydrolysate verified results obtained with tritiated ethyl carbamate [3] and the radioactivity associated with the DNA was considered to represent covalently bound material. The amount of material bound to DNA is shown in Table 1.

These values have been obtained by dividing the radioactivity found to be associated with the DNA by the specific activity of the ethyl carbamate-(^{14}C) used. It is not intended to imply that the whole ethyl carbamate molecule is bound, indeed it is known that only the ethyl group with or without the oxygen atom linking carbon-1 to the carbonyl carbon, binds [6]. As the radioactivity is in the ethyl group, the specific activity of the ethyl group is the same as that of the ethyl carbamate molecule.

Table 1. *Binding to liver DNA of intact and partially-hepatectomized male Crackenbush mice after a single dose of ethyl carbamate-(1-^{14}C)* (6 μCi; 20 mg)

Time after dosing (weeks)	Specific activity of DNA* (μ moles bound material/g DNA)†	
	Intact	Partially hepatectomised
0†	3·29 ± 0·45	2·99 ± 0·64
1	1·38 ± 0·19	2·62 ± 0·15
2	0.67 ± 0·11	1·87 ± 0·34
5	0.22 ± 0·03	0·94 ± 0·10
8	0·05 ± 0·02	0·89 ± 0·15
12	none found	0.86 ± 0·08

*Mean ± standard deviation.

†These values were obtained by dividing the specific activity of the extracted liver DNA, measured in nCi/g by the specific activity of the ethyl carbamate-(1-^{14}C).

‡These values were obtained from the mice killed 10 hr after dosing.

In intact mice, the binding declined rapidly during the first two weeks, then less rapidly until at 8 weeks it was 0·05 ± 0·02 μ moles/g DNA. Radioactivity was not detected in the liver DNA of intact mice after 12 weeks. In the hepatectomized mice, the maximum binding was less than that observed in intact mice but its decline was much slower and by 5 weeks appeared to have reached a constant level. The binding at 5 weeks was 0·94 ± 0·1 μ moles/g. DNA, at 8 weeks, 0·89 ± 0·15 μ moles/g DNA and at 12 weeks 0·86 ± 0·08 μ moles/g DNA.

Chromatographic examination of the acid hydrolysates of liver DNA from the hepatectomised mice killed 5, 8 and 12 weeks after dosing indicated an identical pattern of hydrolysis products. On paper chromatograms only one radioactive spot was found in the hydrolysates and this did not correspond to any of the hydrolysis products of liver DNA from intact untreated mice. The nature of the material in this spot remains to be determined.

Effect of ethyl carbamate on thymidine-(^{3}H) incorporation

The effect of a single dose of ethyl carbamate (20 mg) on liver DNA synthesis was determined by measuring the incorporation of thymidine-(^{3}H) into liver DNA in four groups of mice. The groups were: A: partially hepatectomized; B: partially hepatectomized and given ethyl carbamate (20 mg) 18 hr later; C: intact and D: intact and given ethyl carbamate (20 mg).

The mice were killed at 0 (10 hr), 1, 2, 5, 8 and 12 weeks. One hour before death they were given thymidine-^{3}H (0·25 μCi/g). The specific activity of the liver DNA is shown in Table 2.

In group A (partially hepatectomized) the rate of DNA synthesis declined to a constant level between 2 and 5 weeks. DNA synthesis in group B (partially hepatectomized plus ethyl carbamate at 18 hr) was lower (82·2 ± 29·4 d.p.m./μg DNA) at zero time (10 hr) than that in group A (343·3 ± 67·9 d.p.m./μg DNA) but rose to a maximum at 2 weeks. By 5 weeks it had reached the control level (45·4 ± 12·2 d.p.m./μg DNA). In group C (intact) DNA synthesis remained at approximately the same level throughout the experiment. The specific activities of liver DNA at 0 and 12 weeks were 56·5 ± 19·1 and 49·9 ± 21·5 d.p.m./μg deoxyribose, respectively. DNA synthesis was inhibited in the intact mice treated with ethyl carbamate (group D), but had returned to normal by 5 weeks. In an earlier study, ethyl carbamate was shown to inhibit DNA synthesis in the livers of intact and partially-hepatectomized mice by 63 and 50% respectively within 8 hr of dosing [4].

Table 2. Specific activity of liver DNA from intact and partially-hepatectomized male Crackenbush mice with and without ethyl carbamate (20 mg)

Time after partial hepatectomy and/or dosing with ethyl carbamate (weeks)	Specific activity (d.p.m./μg DNA; mean ± S.D.) (A) Partially hepatectomized	(B) Partially hepatectomized ethyl carbamate* (20 mg)	(C) Intact	(D) Intact ethyl carbamate* (20 mg)
0	348·3 ± 67·8	82·2 ± 29·4	56·5 ± 19·1	25·7 ± 4·8
1	209·6 ± 86·7	167·9 ± 80·6		16·5 ± 4·0
2	116·7 ± 41·6	231·8 ± 47·2		42·4 ± 10·9
5	45·4 ± 12·2	39·0 ± 4·5	51·2 ± 3·2	53·1 ± 10·3
8	45·4 ± 4·0	45·6 ± 7·4		61·3 2·7
12	41·4 ± 7·7	44·8 ± 3·7	49·9 ± 21·5	52·3 ± 7·4

*Ethyl carbamate was given 18 hr after partial hepatectomy.

Effect of ethyl carbamate on DNA turnover

The effect of ethyl carbamate on DNA turnover was determined in mice whose liver DNA had been labelled with thymidine-(2-^{14}C) (0·01 μCi/g; i.p.), given on each of the five days prior to partial hepatectomy or the administration of ethyl carbamate. On the sixth day, two groups of sixteen mice were randomly selected and partially hepatectomised. The pieces of liver that were removed at partial hepatectomy were immediately frozen in liquid nitrogen and were used to determine the specific activity at zero time.

Sixteen of the partially-hepatectomized mice were given ethyl carbamate (20 mg; i.p.) 18 hr after partial hepatectomy. Groups of eight mice were killed 8 and 12 weeks later. The remaining sixteen partially-hepatectomized mice were given saline (0·2 ml; i.p.) 18 hr after partial hepatectomy and were used as controls. Groups of eight mice were killed 8 and 12 weeks later. The specific activity of the liver DNA was determined as is shown in Table 3.

There was no significant difference in the specific activities of liver DNA from the two intact groups killed at 8 weeks and only a "probably significant" difference between the specific activities at 12 weeks (6·82 ± 0·53 and 8·01 ± 0·64 d.p.m./μg DNA for, respectively, the intact mice with and without ethyl carbamate; $0·05 < P < 0·01$). The specific activity of the liver DNA of the partially-hepatectomized mice that had been given ethyl carbamate was higher than that of the partially-hepatectomized mice, at both 8 and 12 weeks. In both instances the differences were highly significant ($0·001 < P$).

DISCUSSION

After the administration of a single dose of ethyl carbamate-(^{3}H) to male Crackenbush mice, a metabolite was found to bind to liver DNA and protein but not to RNA [3]. Because of the low level of binding to and the rapid removal of bound material from protein only the binding to DNA was examined in subsequent studies [4, 5, 6]. Using ethyl carbamate-(1, 2-^{14}C)

Table 3. Specific activity of liver DNA after two-thirds partial hepatectomy and/or the administration of a single dose of ethyl carbamate (20 mg)*

Time after partial hepatectomy and/or dosing with ethyl carbamate (weeks)	Specific activity (d.p.m./μg DNA; mean ± S.D.)			
	(E) Intact	(F) Intact ethyl carbamate* (20 mg)	(G) Partially hepatectomized	(H) Partially† hepatectomized ethyl carbamate* (20 mg)
0‡	50·4 ± 6·1	50·4 ± 6·1	50·4 ± 6·1	50·4 ± 6·1
8	5·4 ± 3·8	8·1 ± 1·0	11·3 ± 4·1	17·4 ± 1·1
12	6·8 ± 0·5	8·0 ± 0·6	10·2 ± 1.5	15·5 ± 1·6

*The mice were given thymidine-(^{14}C) (5 × 0·01 μCi/g) before partial hepatectomy or administration of ethyl carbamate.
†Ethyl carbamate was given 18 hr after partial hepatectomy.
‡The specific activity for 0 weeks was determined from the pieces of liver removed at partial hepatectomy.

and ethyl carbamate-(carbonyl-^{14}C) the bound molecule was shown to be either an ethyl or an ethoxy group [6].

In the same study using ethyl, methyl, propyl and butyl carbamates labelled with carbon-14, only binding of ethyl carbamate to liver DNA could be detected.

If the binding to DNA is involved in carcinogenesis, the mechanism by which a permanent change could be produced in subsequently formed DNA falls into one of two categories. It has been suggested that material bound to DNA is removed along with the base to which it was bound and that an aberration could be produced in the newly-synthesized DNA [10, 11].

An alternative category involves mechanisms in which the carcinogen, or a metabolite, remains bound at least until cell division has occurred, as only then could mutations or chromosome aberrations be produced [12]. Many chemical carcinogens, or their metabolites, remain bound to liver DNA for at least two to three months [12, 13, 14, 15]. In a study of the binding of ethyl carbamate-(^{3}H) to mouse liver DNA in intact and regenerating liver, the binding appeared to persist for longer in the regenerating liver [4]. The present study confirms this observation for while binding could not be detected in the livers of intact mice after 8 weeks, binding was detected in the livers of partially hepatectomized mice 12 weeks after dosing. In the partially-hepatectomized mice, the binding at 5, 8 and 12 weeks after dosing appeared to have reached a constant level. It is not known if the nature of the binding, i.e., the site of binding and the nature of the bound molecule are the same in intact and partially-hepatectomized mice.

The extent of binding to liver DNA in the partially-hepatectomized mice, 5, 8, and 12 hours after dosing was about 0·9 μ moles/g DNA or a molecule per $3·6 \times 10^3$ nucleotides. This compares favourably with the extent of ethylation obtained in other circumstances. A single injection of ethionine (500 mg/kg; 1 mCi) labelled DNA to the extent of 2·7 nmoles/g or 1 guanine residue in 230,000 [16].

Other workers have shown that the binding of a carcinogen to liver DNA was higher in regenerating liver than in intact liver [17, 18] although the comparative durations of binding in intact and partially-hepatectomized animals were not determined.

In earlier studies there appeared to be a correlation between the maintenance of binding to liver DNA and the inhibition of liver DNA synthesis produced by the dose of ethyl carbamate [4] and 7,12-dimethylbenz[a]anthracene [17]. In the present study four groups of mice were used: intact, intact and given ethyl carbamate, partially hepatectomized and partially hepatectomized and given ethyl carbamate 18 hr post-operatively. Using the incorporation of tritiated thymidine into liver DNA as a measure of DNA synthesis no significant difference could be detected in the rate of DNA synthesis in any of the four groups at 5, 8 and 12 weeks after treatment. The failure to detect any difference could have been due to the insensitivity of the techniques employed. When the

liver DNA was labelled with thymidine-(^{14}C) before any further treatment (partial hepatectomy and/or administration of ethyl carbamate), a significant difference was observed in the level of labelling of liver DNA in the four groups at times after the treatment. By 8 weeks, the level of labelling in all four groups had fallen to a low level. There was no significant difference between the levels of labelling in the two intact groups (E and F) or between the labelling in the intact group (E) and the partially-hepatectomized group that were not subsequently treated with ethyl carbamate (G) (Table 3).

In the two partially-hepatectomized groups (G and H) the levels of labelling were significantly different at 8 and 12 weeks ($P < 0{\cdot}001$). The levels of labelling in the two groups that were given ethyl carbamate (F, intact and G, partially hepatectomized) were also significantly different ($P < 0{\cdot}001$). These results suggest that when ethyl carbamate was given to partially-hepatectomized mice during a certain phase of liver regeneration (i.e., 18 hr postoperatively), the DNA in some of the liver cells is prevented from replicating or from being involved in semi-conservative repair replication. If this were not the case, the specific activity of the liver DNA which had been labelled before partial hepatectomy and the administration of ethyl carbamate would have fallen to the same levels as those from either intact mice or partially-hepatectomized mice that had not been treated with ethyl carbamate. It is not known if the cells that are involved in this inhibition of DNA synthesis are those in which ethyl carbamate, or a metabolite, remains bound.

SUMMARY

The binding of ethyl carbamate-(1-^{14}C), or a metabolite, to liver DNA was examined in intact and partially-hepatectomized mice up to 12 weeks after dosing. In the intact mice, binding could not be detected after 8 weeks, whereas in the hepatectomized mice, binding was still detectable at 12 weeks. In the hepatectomized mice the levels of binding at 5, 8 and 12 weeks were not significantly different.

The effects of ethyl carbamate on DNA synthesis and DNA turnover in the liver were examined in intact and partially-hepatectomized mice. DNA synthesis, measured by thymidine-^{3}H incorporation, in intact and partially-hepatectomized mice with and without ethyl carbamate treatment, followed patterns that were predictable in the light of existing knowledge. DNA turnover was measured by measuring the carbon-14 content of liver DNA of mice that had been given thymidine-(^{14}C) before partial hepatectomy and/or the administration of ethyl carbamate. The carbon-14 content was measured at 0 (immediately before treatment), 8 and 12 weeks. In intact mice there was very little difference in the degree of labelling in mice treated with ethyl carbamate compared with untreated mice. However, in the partially-hepatectomized mice the degree of labelling in the mice also given ethyl carbamate was significantly higher than in mice which had not received ethyl carbamate.

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Immune Response to Adenovirus 12-Induced Tumour Antigens, as Measured *in vitro* by the Macrophage Migration Inhibition Test*

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Abstract—*Cellular immunity to an adenovirus* 12*-induced CBA mouse tumour was demonstrated* in vitro *by the macrophage migration inhibition test. The specific production of MIF was demonstrated for sensitized spleen cells incubated for* 3 *days at* 37°C *in the presence of cell-free tumour extract, and was detected by the ability of supernatant from spleen cell cultures to inhibit the migration of guinea pig peritoneal macrophages. In addition, cellular immunity was demonstrated in mice bearing large transplantable tumours,* 28 *days following tumour transplant. Spleen cells from tumour-bearing mice taken* 14 *and* 24 *days after tumour transplant did not produce MIF in the presence of tumour extract. Serum from mice bearing large tumours was shown to inhibit the production of MIF from sensitized spleen cells incubated with tumour extract. The nature of such factors and their mechanism of action is not yet known, but it is likely that they are either antibody or antigen–antibody complexes.*

INTRODUCTION

PREVIOUS studies have shown that immunity to transplanted adenovirus 12-induced tumour cells can be demonstrated for animals previously immunized with the homologous virus or inactivated, sub-lethal doses of tumour cells [1, 2]. In addition, immunization with cell-free extracts from an adenovirus 12-induced CBA mouse tumour was found to produce immunity to challenge with homologous tumour cells [3, 4]. The immunity produced with cell-free tumour extracts was shown to be cell-mediated, since adoptive transfer of spleen cells from immunized mice transferred transplantation immunity, whilst mice inoculated with immune sera did not reject transplanted tumour cells [5].

The above studies were all carried out using CBA mice; attempts to demonstrate the immune response using *in vitro* colony inhibition tests [6] were not successful with a cell line derived from our *in vivo* adenovirus 12-induced tumour line [5]. In the present study, the immune response of CBA mice bearing adenovirus 12-induced tumours and tumour-immune mice was measured by the macrophage migration inhibition test [7]. The production of macrophage migration inhibition factor by "sensitized" CBA mouse spleen cells incubated in the presence of cell-free tumour extracts was measured, together with the added effect of serum from tumour-bearing mice. The relationship between these findings and the *in vivo* transplantation immunity was compared.

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MATERIAL AND METHODS

Adenovirus 12 *tumour cells*

Adenovirus 12-induced CBA mouse tumours, maintained by subcutaneous transplantation in CBA mice at 2–3 week intervals, were used in all experiments. Tumour cell suspensions were

prepared as described previously [3]. Excised tumours of 10–15 mm diameter were free of necrotic tissue, chopped finely, and treated with 0·25% trypsin to obtain a single cell suspension. Cells were washed twice with Eagle's Minimal Essential Medium containing 2% inactivated calf serum, resuspended in the same medium to a concentration of 5×10^6 viable cells/ml, and used immediately.

Tumour extract

Transplanted adenovirus 12-induced tumours, diameter 15–30 mm, were excised from groups of 5–10 mice, finely chopped, washed in Hanks' saline and the tissue centrifuged at 600 ***g*** for 10 min. The tumour fragments were then suspended in Hanks' saline at a concentration of 2% (v/v), homogenized in a Waring blender, and frozen at −80°C. After thawing at room temperature, the extracts were centrifuged at 100,000 ***g*** for 1 hr, and the supernatant fluid filtered through a Millipore filter of 0·45 μ pore size. The cell-free tumour extracts were kept frozen at −80°C until used.

Spleen cell suspension

Spleen cell suspensions were prepared from normal CBA mice, mice immunized with tumour extract and tumour-bearing mice in the same manner. Spleens were removed, chopped finely, and suspended in medium "199". The fragments were further disrupted by extrusion through a syringe without a needle, and the resulting suspension was filtered through sterile cotton gauze to remove undisrupted tissue. The spleen cells were centrifuged at 100 ***g*** for 10 min, and distilled water was added to the deposited cells for 30 seconds (3 ml of water per 0·1 ml sedimented spleen cells) to lyse the red cells, and the medium was restored to isotonic strength by the addition of an equal volume of double strength saline (0·3 M NaCl). The cell suspension was then washed three times in medium "199" and suspended in the same medium to a concentration of $1{\cdot}0 \times 10^7$ spleen cells/ml.

Mouse sera

Mice were killed, and serum was collected from mice by allowing the blood to drain into the thoracic cavity after the heart and lungs had been removed. Pools were prepared from both normal CBA mice and from mice bearing transplanted adenovirus 12-induced tumours of 20–30 mm diameter. The blood was kept overnight at 4°C, centrifuged at 2000 ***g*** for 10 min, and the serum stored at −20°C.

Guinea pig macrophages

Guinea pigs, weighing 250–300 g were inoculated intraperitoneally with 20 ml of sterile paraffin oil. Seven to ten days later the animals were killed and the peritoneal cavity washed with 200–300 ml of Hanks' saline. The peritoneal washings were placed in a separating funnel to allow the paraffin oil to separate, and the lower layer of macrophages was collected and centrifuged at 500 ***g*** for 5 min. The deposited macrophages were washed three times in medium "199" and resuspended in medium "199" to a 5% (v/v) concentration.

Macrophage migration inhibition (MMI) tests

The capillary tube method for detecting inhibition of macrophage migration was used, as described by George and Vaughan [7] and modified by David *et al.* [8]. Spleen cells were suspended at a concentration of 1×10^7 cells/ml in medium "199", and 2–4 ml quantities were placed in each of two 45 mm dia. petri dishes (Sterilin). To one petri dish was added tumour cell extract, to a final concentration of 10% (v/v), while the second culture served as a control. The cultures were then incubated for 3 days at 37°C in an atmosphere of 5% CO_2 in air. After incubation, tumour cell extract was added to the control culture to 10% of the total volume, and both cultures were centrifuged at 2000 ***g*** for 10 min to sediment the spleen cells. The supernatant fluids were removed and dialysed for 48 hr at 4°C against medium "199". Following dialysis, heat-inactivated foetal calf serum (Flow Laboratories, Irvine, Scotland) was added to the supernatant culture fluids to a 10% (v/v) final concentration, the pH was adjusted to 7·6 by the addition of 7·5% sodium bicarbonate solution, and the fluids were then tested for macrophage-inhibiting factor (MIF).

Haematocrit tubes (100 μl volume) were filled with guinea pig macrophage suspension, sealed at the one end with Cristoseal (Hawksley & Sons Ltd., Lancing, Sussex), and centrifuged at 500 ***g*** for 5 min. The tubes were then cut at the sedimented cell-liquid interface, and the packed macrophages were attached to the bottom of a plastic migration chamber (Sterilin) with silicone grease; one capillary tube was placed in each well. The wells were filled with fluids from the spleen cell cultures using approximately 0·4 ml of supernatant per well. Each well was sealed with a glass cover-slip, fixed with silicone grease, and incubated at 37°C. The area of the migration of the macrophage was measured after 24 and 48 hr incubation by planimetry. Two to four capillary tubes

were used to test each supernatant. The per cent migration was calculated according to the formula:

$$\% \text{ inhibition} = 100 - \left[\frac{\text{Average migration of guinea pig macrophages in presence of test supernatant}}{\text{Average migration of guinea pig macrophages in presence of control supernatant}} \times 100 \right]$$

The statistical significance of differences was calculated using Student's *t*-test.

RESULTS

MIF production by spleen cells from tumour-immune mice

Groups of 3–4 week old CBA mice were immunized subcutaneously with three doses, each of 0·1 ml volume, of adenovirus 12-induced tumour cell extract; these mice, termed tumour-immune mice, have been shown previously to be relatively immune to challenge with transplanted tumour cells [3, 5]. Four–ten days after the third inoculation, the spleens were removed and cell suspensions prepared; cell suspensions were also prepared from normal mice spleens. Both cell suspensions were incubated with tumour cell extracts, and the fluids later removed and tested for MIF. The results are shown in Table 1.

Spleen cells from tumour-immune mice produced MIF when incubated with tumour cell extract since fluids from these cultures inhibited guinea pig macrophage migration. This result was found in two separate experiments shown in Table 1, and in five further experiments. In the two experiments shown, fluids from spleen cell cultures from tumour-immune mice incubated with tumour cell antigen inhibited macrophage migration by 37 and 45% after 24 hr incubation. This inhibition, which was statistically significant ($P = < 0{\cdot}05$), was maintained after 48 hr incubation, and in some experiments, the inhibition was greater at this time than after 24 hr incubation. Spleen cell cultures from normal mice did not produce MIF in the presence of tumour cell extract, since fluids from these cultures did not cause significant inhibition of migration of guinea pig macrophages, compared with control supernatants (Table 1).

MIF production by spleen cells from tumour-bearing mice

Groups of CBA mice aged 3–4 weeks were inoculated subcutaneously with 5×10^5 viable adenovirus 12-induced tumour cells; this dose of cells was known to induce tumours in 90–100% of mice in the period 4–5 weeks after inoculation [3, 4]. At various times after inoculation, the mice were killed, the spleens removed and spleen cell suspensions prepared. These cells were incubated in the presence of tumour cell extract, and the fluids then tested for MIF. In each case, spleen cells from normal mice were incubated with tumour cell extract to provide control preparations. The results are shown in Table 2.

Spleen cell cultures from CBA mice bearing tumours of mean diameter 0·62 and 0·65 cm at 14 days after tumour cell inoculation did not produce detectable MIF when incubated with tumour cell extract (Table 2). In further tests, not shown in Table 2, spleen cells from animals bearing tumours of mean diameter 1·36 and 1·70 cm at 24 days after inoculation also failed to produce MIF; in both cases fluids from these spleen cultures enhanced the migration of

Table 1. *Production of MIF by spleen cells from tumour-immune or normal CBA mice in the presence of tumour cell extract*

Exp. No.	Spleen cells from	Tumour cell extract	Macrophage migration area (± Standard deviation)		Inhibition (%)
1	Tumour-immune mice	+	3·37	(±0·71)	37*
		−	5·47	(±0·80)	
	Normal mice	+	5·79	(±0·63)	7·5
		−	6·25	(±0·55)	
2	Tumour-immune mice	+	4·76	(±1·61)	45*
		−	8·64	(±0·11)	
	Normal mice	+	9·32	(±0·83)	−2
		−	9·09	(±1·92)	

*Significant inhibition of macrophage migration ($P < 0.05$).

guinea pig macrophages. In contrast, spleen cells from mice bearing transplanted tumours 28 days after inoculation produced MIF in the presence of tumour cell extract; the mean diameter of tumours was 1·50 and 2·26 cm. In both cases, the inhibition of macrophage migration was significant compared to that of controls ($P = < 0{\cdot}05$), and this difference was demonstrable after both 24 and 48 hr incubation. Spleen cells from normal CBA mice did not produce MIF when incubated with tumour extract. Thus, the appearance of sensitized lymphocytes in mice bearing transplantable adenovirus 12-induced tumours was specific and was related to the time after inoculation of tumour cells.

Effect of serum from tumour-bearing mice on MIF production

Pooled sera from CBA mice bearing transplanted adenovirus 12-induced tumours was inactivated at 56°C for 30 min and mixed with an equal volume of tumour cell extract to provide a control preparation. Each serum-tumour cell extract preparation was incubated at room temperature for one hour and then added to spleen cell cultures prepared from tumour-immune mice and from normal mice; the final concentration of serum and of tumour cell extract in each case was 10%. After three days incubation at 37°C, the culture fluids were harvested, dialysed against medium "199" for 48 hr, and tested for MIF against guinea pig macrophages. The results of two replicate experiments are shown in Table 3.

In initial experiments the effect of normal mouse serum on MIF production from sensitized spleen cells was investigated. The results are shown in Table 3. The inhibition of macrophage migration by supernatant fluids from cultures of sensitized spleens and tumour extract was 18%. In contrast, supernatants from cultures of sensitized cells incubated with tumour extract and normal mouse serum inhibited macrophage migration by 27%; this result was significant ($P = < 0{\cdot}05$). Neither tumour extract nor tumour extract and normal sera stimulated MIF production from normal spleen cells. Thus, normal mouse sera did not interfere with MIF production from sensitized spleen cells.

The effect of sera from tumour-bearing mice on MIF production from sensitized spleen cells is shown in Table 4. Since normal sera did not inhibit MIF production by sensitized spleen cells in the presence of tumour extract, these cultures were used as a control to estimate the effects of sera from mice with tumours. Supernatants from cultures of sensitized spleen cells and tumour extract in the presence of sera from tumoured mice did not produce significant inhibition of macrophage migration. This result was shown in two separate experiments (Table 4).

These results suggest that MIF production by "sensitized" lymphocytes is not affected by the presence of normal mouse serum, but that factors present in serum from tumour bearing mice inhibit the production of MIF from tumour-immune spleen cells.

Table 2. Production of MIF by spleen cells from tumour-bearing mice in the presence of tumour cell extract

Days following inoculation	Mean tumour size	Spleen cells from:	Tumour cell extract	Macrophage migration area (± Standard deviation)		Inhibition (%)
	0·62 cm	Tumour bearing mice	+	3·76	(±0·18)	−19
			−	3·11	(±0·57)	
		Normal mice	+	2·9	(±0·39)	−11
			−	2·62	(±0·25)	
14						
	0·65 cm	Tumour bearing mice	+	3·7	(±0·30)	−16
			−	3·25	(±0·47)	
		Normal mice	+	4·04	(±0·49)	−7
			−	3·79	(±0·15)	
	1·5 cm	Tumour bearing mice	+	2·66	(±0·17)	37*
			−	4·17	(±0·15)	
		Normal mice	+	4·51	(±1·46)	4
			−	4·72	(±0·73)	
28						
	2·26 cm	Tumour bearing mice	+	1·92	(±0·12)	50*
			−	3·6	(±0·72)	
		Normal mice	+	3·81	(±0·35)	−36
			−	2·81	(±0·28)	

*Significant inhibition of macrophage migration ($P < 0.05$).

Table 3. Effect of normal CBA mouse sera on MIF production by sensitized spleen cells

Spleen cells from:	Spleen cells incubated with: N. serum	Tumour extract	Macrophage migration area (± Standard deviation)		Inhibition (%)
Immunized mice	−	+	2·52	(±0·32)	18
	+	+	2·25	(±0·07)	27*
	−	−	3·07	(±0·11)	
Normal mice	−	+	2·15	(±0·35)	6
	+	+	2·25	(±0·78)	4
	−	−	2·35	(±0·07)	

*Significant inhibition of macrophage migration ($P = <0·05$).

Table 4. Effect of serum from tumour-bearing mice on MIF production by spleen cells from mice immunized with tumour extract

Exp. No.	Spleen cells from:	Spleen cells incubated at 37°C/3 days: N. serum	Tumour serum	Tumour extract	Macrophage migration area (± Standard deviation)		Difference (%)
1	Immunized mice	+	−	+	2·03	(±0·98)	28
		−	+	+	2·83	(±0·08)	
	Normal mice	+	−	+	2·06	(±0·27)	−28
		−	+	+	1·66	(±0·32)	
2	Immunized mice	+	−	+	2·1	(±1·27)	31*
		−	+	+	3·0	(±0·35)	
	Normal mice	+	−	+	3·1	(±0·14)	8
		−	+	+	3·37	(±0·53)	

* Significant difference ($P<0.05$) between effect of normal mouse sera and sera from tumour-bearing mice on MIF production.

DISCUSSION

The macrophage migration inhibition test (MMI) has been used extensively as a reliable *in vitro* test for the detection of delayed hypersensitivity, and has also been used to measure cellular immune reactions of animals to chemical- or virus-induced tumours [9, 10, 11, 12]. The results of the present study using the MMI test show that antigens present in cell-free extracts of adenovirus 12-induced tumours elicit a delayed hypersensitivity reaction from spleen cells from mice immunized with cell-free tumour extracts, and from mice bearing large transplanted tumours. We have occasionally observed in these studies the production of MIF by normal mouse spleen cells in the presence of tumour extract. This was a feature of some preparations of tumour extract, and the reasons for this reactivity were not known. Tumour cell extract preparations were therefore screened for their ability to induce MIF from normal spleen cells, and batches of extract having this property were discarded.

Immunization of mice with cell-free extracts of transplanted adenovirus 12-induced tumour cell induced transplantation immunity [3, 4], thus showing the extracts to be antigenically active. In addition, adoptive transfer of spleen cells from tumour-immune and tumour-bearing mice was shown to confer immunity to transplanted homologous tumour cells, but adoptive transfer of sera from tumour-bearing mice enhanced subsequent tumour development [5]. In the present study, spleen cells from tumour-bearing mice or tumour-immune mice both produced MIF in the presence of homologous tumour cell extracts. This MIF production was inhibited by sera from tumour-bearing mice. The results of *in vivo* studies using cell and serum

transfer techniques and the present findings were in agreement.

Although definitive information on the immune reactions to tumour formation should be carried out in animal experiments, *in vitro* techniques greatly facilitate these studies. Since the results of the MMI tests parallel results obtained from animal studies, this technique probably measures the immune reaction to tumour growth and of tumour immunity. Similar results have been obtained with other animal tumours using colony inhibition tests [6, 13]; however, we have been unable to adapt these techniques to the present tumour system.

The finding that sera from tumour-bearing mice was capable of preventing the production of MIF by "sensitized" spleen cells demonstrated the existence of factors capable of "blocking" this immune reaction. The nature of such factors is not clearly understood, but evidence indicates that the blocking is due to either antibody or antibody–antigen complexes [13, 14]. In the present system, these factors may act by direct blocking of tumour specific transplantation antigens present in tumour extract, thus preventing them from stimulating lymphocytes to produce MIF. Alternatively, they may affect the immune cells rendering them incapable of reacting to the transplantation antigens. Both these possibilities are at present under investigation.

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The Karyotype Evolution in Chronic Granulocytic Leukaemia—I. The Chromosomes Gained and Lost During Initiation of the Evolution*

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Abstract—*In a material of* 3633 Ph[1]*-positive metaphases from* 48 *patients with chronic myelogenous leukaemia, the distribution among the various chromosome groups of supernumerary and missing chromosomes was analysed in karyotype variants showing gain and/or loss of one chromosome. The frequencies of missing as well as supernumerary chromosomes were found to be inversely related to the average chromosomal size in the various groups, a relation very similar to that of missing chromosomes observed in studies on normal cells. Further, each morphological chromosome group contributed much the same to chromosome gain as well as chromosome loss. Together, these observations suggest that the mitotic aberrations responsible for simple abnormal leukaemic karyotype variants are the same as those operating in normal cells, and, in addition, in leukaemic cells the same mitotic aberrations appear to cause both chromosome loss and gain. Further, the observations may suggest that karyotype evolution is caused by defective elimination rather than excessive production of abnormal variants.*

INTRODUCTION

CYTOGENETICALLY, the acute transformation of chronic granulocytic leukaemia (CGL) is characterized by an evolutionary process leading to the establishment of a number of abnormal, mostly hyperdiploid Ph[1]-positive haemopoietic clones. It is beyond doubt that the distribution among the various chromosome groups of the supernumerary chromosomes in these clones is non-random; this applies also to the chromosomes sporadically missing in the hyperdiploid karyotypes [1–5].

It is obscure, however, whether mitotic errors primarily lead to preferential gain and loss of specific types of chromosomes or, alternatively, gain and loss are random but selection permits predominant survival of variants with specific karyotypes. Whereas aneuploid haemopoietic clones do not occur, normal individuals do show sporadic hypo- and hyperdiploidy [6]. It is consequently possible that the karyotype polymorphism of advanced CGL may be due to defective elimination rather than to increased development of aneuploid karyotype variants.

The present paper focusses on the types of chromosomes gained and lost during the initial steps of the CGL evolution by analysing the karyotypes showing one supernumerary and/or one missing chromosome. The object is to investigate whether the results differ from those obtained from studies on normal cells.

MATERIAL AND METHODS

Karyotype studies were performed in 48 (17 female and 31 male) patients with clinically and haematologically typical *Ph*[1]-positive CGL. Between 1 and 7 (average 2) studies were carried out in each patient at different stages of the disease. When first investigated, 10 patients were newly-diagnosed and untreated. The

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remainder were investigated in the chronic phase and/or after active transformation.

Thirty-six bone marrow specimens and 72 short-term leucocyte cultures were studied. The marrow was prepared without preceding *in vitro* incubation according to a slight modification of the method described by Tjio and Whang [7]. The blood samples were cultured for 48 hours at 37°C and prepared for karyotype analysis following a slightly modified version of the method of Moorhead *et al.* [8]. The metaphases were spread by gentle evaporation of the fixative over a flame and then Giemsa stained. All karyotype analyses were performed under direct observation in the microscope and carried out by the author. From each blood culture and bone marrow sample between 3 and 73 (average 33·6) metaphases were analysed. The material comprises a total of 3633 metaphases, all of them Ph^1-positive.

The results of the present investigation are comparable with observations on normal cells only provided the influence of aneuploid clones occurring in the material is eliminated. Metaphases showing the same abnormal karyotype and obtained from the same patient may or may not be members of the same abnormal clone. It was chosen to consider such metaphases clonal members although it was recognized that it might lead to some underestimation of the actual prevalence of aneuploid karyotype variants. The alternative decision, to count all cytogenetically identical aneuploid metaphases as independent variants would, however, invariably lead to a substantial overestimate of the prevalence. In some samples such cells comprised more than 50% of the karyotyped cells and were probably clonal members. Consequently, for each patient, each abnormal karyotype was registered and included the material once only, without considering the number of metaphases showing the karyotype in the sample(s) obtained from that patient.

The study concerns karyotype abnormalities due to gain and/or loss of one chromosome, i.e., variants with 47 chromosomes showing one extra and no missing chromosome, 45-chromosome variants missing one but without any additional chromosome and, finally, 46-chromosome variants showing gain as well as loss of one chromosome. To simplify, these three categories are termed 47(1+/0−), 45(0+/1−), and 46(1+/1−) variants, respectively.

For statistical analysis *t*-tests were used. If the numbers were too small to allow this test, Fisher's exact test was applied.

RESULTS

Table 1 shows the observed profiles of supernumerary chromosomes in 47(1+/0−) and 46(1+/1−) variants together with the profiles calculated on the assumption that each chromosome pair has the same probability of being involved in numerical aberrations. In the 47(1+/0−) group 47,G(Ph^1)+variants were significantly more frequent and 47,B+ significantly less frequent than expected. In the 46(1+/1−) group supernumerary Ph^1 and morphologically normal G members were more frequent and chromosome gain in the C, B and A groups less frequent than expected. In no chromosome group did the frequency of supernumerary members differ significantly between the two categories of abnormal variants.

Table 1. *Distribution of supernumerary chromosomes in* 47(1+/0−) *and* 46(1+/1−) *variants*

Chromosome groups	47(1+/0−) Observed	47(1+/0−) Expected	*P*	46(1+/1−) Observed	46(1+/1−) Expected	*P*
Ph^1	9	0·87	<0·001	20	2·96	<0·001
G	6	3·22	>0·05	24	10·94	<0·001
F	4	3·48	>0·05	17	11·83	>0·05
E(17–18)	4	3·48	>0·05	18	11·83	>0·05
E(16)	1	1·74	>0·05	10	5·91	>0·05
D	5	5·22	>0·05	16	17·74	>0·05
C	10	13·30	>0·05	31	45·23	<0·05
B	—	3·48	0·03	—	11·83	<0·001
A(3)	—	1·74	>0·05	—	5·91	<0·02
A(2)	—	1·74	>0·05	—	5·91	<0·02
A(1)	1	1·74	>0·05	—	5·91	<0·02
Total	40	40·01		136	136·00	
Number of variants	40			136		

Table 2. Distribution of missing chromosomes in 45(0+/1−) *and* 46(1+/1−) *variants*

Chromosome groups	45(0+/1−) Observed	Expected	*P*	46(1+/1−) Observed	Expected	*P*
G	20	11·18	<0·01	27	11·20	<0.001
F	19	12·09	<0·05	19	12·09	<0·05
E(17–18)	21	12·09	<0·01	23	12·09	<0·005
E(16)	14	6·04	<0·005	8	6·05	>0·05
D	19	18·13	>0·05	28	18·13	<0·02
C	37	46·24	>0·05	28	46·24	<0·005
B	1	12·09	<0·005	1	12·09	<0·005
A(3)	1	6·04	<0·05	2	6·04	>0·05
A(2)	2	6·04	>0·05	—	6·04	<0·02
A(1)	2	6·04	>0·05	—	6·04	<0·02
Total	136	135·98		136	136·01	
Number of variants	136			136		

Table 2 shows the observed and calculated profiles of missing chromosomes in 45(0+/1−) and 46(1+/1−) variants. In the 45(0+/1−) group G, F, E(17–18) and E(16) chromosomes were missing in significantly more and B chromosomes in significantly less variants than expected. In the 46(1+/1−) group a similar picture emerged. The observed number of variants missing E(16) members differed, however, insignificantly from the expected one; and loss of D members occurred more frequently than expected. However, in no chromosome group did the frequency of chromosome loss differ significantly between the 45(0+/1−) and the 46(1+/1−) variants.

It appears from Tables 1 and 2 that with increasing chromosome size gain as well as loss of chromosomes becomes relatively rarer. In order to analyse further the relation between these two variables, the following calculation was carried out in each individual chromosome group:

$$\frac{O-E}{E} \times 100,$$

where O is the observed and E the expected number of numerical aberrations in each chromosome group as listed in Tables 1 and 2. The figures calculated in this way were then related to the average chromosomal size in each individual chromosome group [9] after correction for the chromosomal sex of the material. The Ph[1] size was arbitrarily chosen to be half the average size of the G(21–22) members and listed separately. As significant differences were not observed between the composition of the supernumerary chromosome population in the 47(1+/0−) and the 46(1+/1−) groups these were pooled. For the same reason, the various categories of missing chromosomes were added in the 45(0+/1−) and the 46(1+/1−) groups.

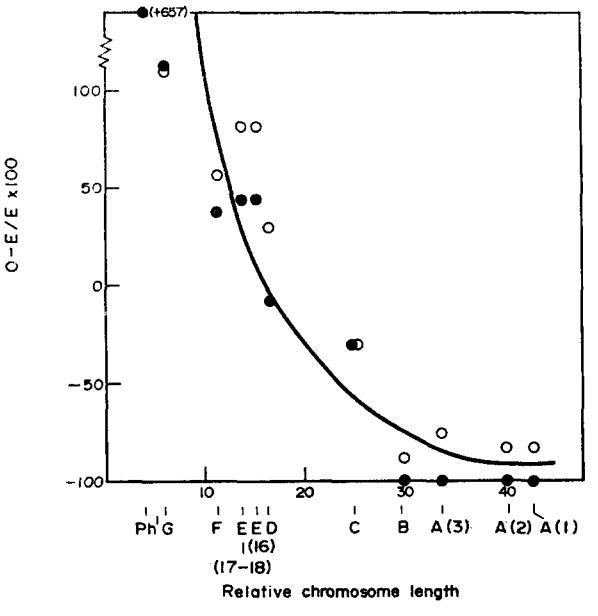

Fig. 1. Relation between observed (O) and expected (E) frequencies of supernumerary (●) and missing (○) chromosomes in karyotype variants with one supernumerary and/or missing chromosome plotted against average chromosome size in each chromosome group. Expected frequencies calculated on assumption of equal probability of chromosome gain/loss in each chromosome pair. Ordinate values calculated from the formula: $[(O-E)/E] \times 100$.

As appears from Fig. 1 the rates of gain and loss are both inversely related to chromosomal size, although E(17–18) and E(16) appear to be involved in both types of aberration somewhat more frequently than the F members.

In Fig. 2 the percentage distribution in the various chromosome groups of supernumerary chromosomes in the pooled 47(1+/0−) and 46(1+/1−) variants are related to the distribution of missing members in the total population of 45(0+/1−) and 46(1+/1−) variants. Figure 2 shows that each chromosome group covers almost the same proportion of the supernumerary and of the missing chromosomes.

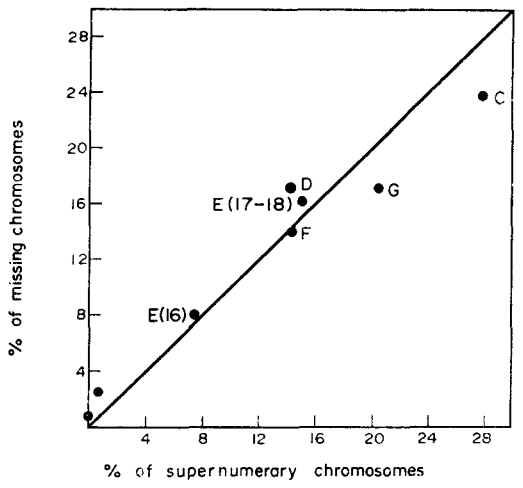

Fig. 2. Relation between frequencies of chromosome gain (abscissa) and loss (ordinate) in the various chromosome groups. Coefficient of regression: +1·00. *Coefficient of correlation:* +0.98. $P < 0{\cdot}001$.

DISCUSSION

The distributions of supernumerary and lost chromosomes in Ph^1-positive CGL metaphases were investigated by Pegoraro *et al.* [5] who found an inverse relationship to chromosomal size when missing and supernumerary chromosomes were pooled. However, the highest frequency of loss was observed in the E(16–18) group, and the supernumerary chromosome population was dominated by G, F, and C members. The discrepancy between these and the present results is probably due to the fact that Pegoraro *et al.* included patients showing advanced karyotype evolution, whereas the present material covers only the very first steps of the karyotype evolution. Advanced aneuploid Ph^1-positive karyotypes are primarily characterized by gain of G, F, and C and loss of E(17–18) members [1–4, 10].

Earlier studies on chromosomes lost from 45-chromosome metaphases of normal individuals have demonstrated distributions essentially similar to those presented here [10, 11]. Although in other studies relatively low frequencies of lost G members were observed, small chromosomes were predominantly lost [6, 12]. Further, in accordance with the present observations, loss of relatively more E(16–18) than F chromosomes (Fig. 1) was observed in 3 of the studies [6, 11, 13].

This similarity between normal and leukaemic metaphases suggests that the same cellular mechanisms may be responsible for chromosome loss in both. Further, the demonstration in the present material of almost identical distributions of lost and gained chromosomes suggests that the same mechanism (such as e.g. non-disjunction) is responsible for chromosome loss and gain.

This conclusion agrees well with observations by Kato and Yosida [14]. Using a Colcemid-reversal technique they produced artificial non-disjunction in Chinese hamster cells. They found that gain as well as loss took place predominantly among the small chromosomes. Further, the fact that the X chromosome was never missing but frequently occurred as a supernumerary member indicated that the observed pattern of numerical chromosome aberrations was due to a selection rather than to a primary gain and loss in specific chromosome categories.

The data obtained in the present study do not show whether the observed distribution of gained and lost chromosomes was due to preferential involvement of small chromosomes or resulted from a selection among karyotypes produced by random gain and loss.

The karyotype polymorphism of the material on which the study was based makes it difficult to compare the frequencies of $45(0+/1-)$ and $47(1+/0-)$ variants with those observed in normal cells. However, if the number of $45(0+/1-)$ variants are related to the number of cells without numerical chromosome aberrations a frequency of 9·1% is found, which is very similar to those observed in normal cells [6, 12, 13]. This agreement indicates that mitotic aberrations may occur with much the same frequency in normal and CGL cells. The fact that when calculated in the same way the $47(1+/0-)$ variants occur somewhat more frequently in the present material (2·7%) than among normal cells (1% or less) [6, 12] may suggest that some hyperdiploid cells are selectively favoured in the leukaemic *in vivo* environment. This idea agrees well with the fact that hyperdiploid clones are allowed to develop in the leukaemic but not in the normal organism. The CGL cytogenetic evolution may consequently be due to defective elimination rather than increased production of aneuploid karyotype variants.

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The Karyotype Evolution in Chronic Granulocytic Leukaemia—II. The Chromosome and Karyotype Pattern of Advanced Evolution*

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Abstract—*The trends of karyotype evolution were investigated in a material consisting of* 3633 *karyotyped* Ph^1*-positive metaphases obtained from* 48 *patients with chronic granulocytic leukaemia. In agreement with observations by others, the majority of the chromosomes gained during progressive evolution were observed to be* Ph^1, F *and, in particular,* C *members and most of those lost to be* E(17–18) *chromosomes. The vast majority of the hyperdiploid karyotypes fell in two distinct groups: one showing excess numbers of C chromosomes with or without other supernumerary chromosomes* (C *variants) and another presenting the same cytogenetic characters except for presence of a second* Ph^1 (Ph^1C *variants). With progressive evolution the* Ph^1C *variants covered increasing proportions of the variant population whereas the* C *variants decreased. Apparently, during evolutionary progress, gain of a second* Ph^1 *confers selective advantage.*

INTRODUCTION

IT IS STILL far from clear whether the wide variety of cytogenetically abnormal, haemopoietic clones occurring in the terminal acute transformation of chronic granulocytic leukaemia (CGL) are causally related to the accelerated course of the disease. Probably, cells with different karyotypes have different phenotypes. Consequently, the polymorphic cytogenetic picture of the abnormal karyotypes is supposedly associated with a corresponding polymorphism as far as cellular phenotypes are concerned. It is, however, difficult to accept that a wide variety of different cellular phenotypes are all compatible with or even well-adapted to the leukaemic microenvironment. On the other hand, a number of studies agree that in this cytogenetically polymorphic picture common features do occur. Among the supernumerary chromosomes, members of the G(21–22, Ph^1), F(19–20) and C(6–X–12) groups predominate whereas loss of E(17–18) members is more frequent than loss of any other chromosome category [1–5]. And, as far as the relation between karyotype and cellular phenotype is concerned, presence of excess numbers of C chromosomes has been found to be associated with immature blood pictures [6] and abnormal myeloblast differentiation [7].

As described elsewhere [8] the initial steps of karyotype evolution involve gain and loss of members of a variety of chromosome groups. This contrasts to the predominant gain and loss of specific chromosome categories at later evolutionary stages (see above) and raises the question whether the initial polymorphic cytogenetic picture is gradually narrowed down to a relative oligomorphism. If so, a study of advanced, abnormal karyotypes may allow characterization of a few cytogenetic features which are conditions *sine qua non* for prolonged clonal survival in the leukaemic haemopoietic tissues. If such features do exist, they may be useful in further studies on the relation between karyo-

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type aberrations and the morphological and biochemical characteristics of leukaemic cells.

Abnormal Ph[1]-positive karyotypes observed in 48 CGL patients have been analyzed at different stages of disease evolution in order to investigate whether or not the surviving clones have specific cytogenetic features in common. As a primary feature of karyotype evolution is acquisition of increasing numbers of supernumerary chromosomes [2, 4], these numbers were chosen as a parameter of evolutionary progression.

MATERIAL AND METHODS

The patient and metaphase material as well as the techniques applied for leucocyte culture and chromosome spreads are described elsewhere [8].

As the present study concerns the cytogenetic character of abnormal karyotype variants at different levels of evolution the number of metaphases showing the same karyotype in each patient is irrelevant. Consequently, from each patient each abnormal karyotype variant observed was included into the material only once, regardless of the number of metaphases showing it and, in patients investigated repeatedly, regardless of the number of samples in which it was observed.

The variants were grouped according to the number of supernumerary chromosomes occurring in them. These groups are referred to in this study by means of formulae which state the chromosome number, the number of excess and the number of missing chromosomes. For example, the formula 49(3+/0−) refers to a 49-chromosome variant having three supernumerary and no missing chromosomes.

RESULTS

Table 1 shows the supernumerary chromosomes occurring at different stages of evolution. The presence of additional C chromosomes becomes increasingly frequent during evolution. In the late stages karyotype variants with excess numbers of A(3) members increase in frequency whereas the frequencies of additional F members remain constant and supernumerary Ph[1] chromosomes show slightly decreasing frequencies. However, when related to the numbers of karyotype variants in each of the 5 groups of Table 1,

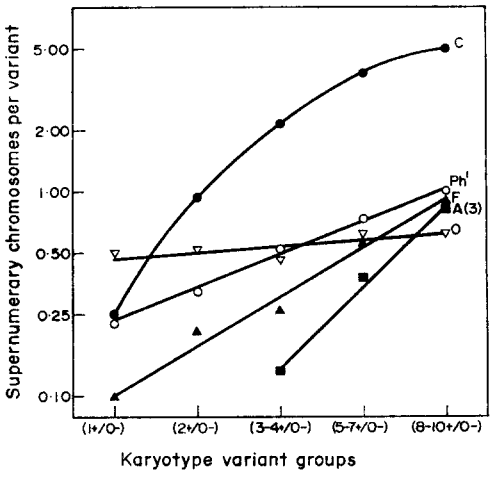

Fig. 1. Number per variant of supernumerary C, Ph[1], F, A(3) and total other chromosomes (O) in karyotype variants with increasing numbers of excess chromosomes. Semi-logarithmic plot. Curve and lines fitted by hand.

Table 1. Distribution of supernumerary chromosomes in karyotype variants with different chromosome numbers

	47 (1+/0−)	(%)	48 (2+/0−)	(%)	49–50 (3−4+/0−)	(%)	51–53 (5−7+/0−)	(%)	54–56 (8−10+/0−)	(%)
Ph[1]	9	22·5	6	15·8	8	14·8	12	12·3	11	12·0
G	6	15·0	6	15·8	3	5·5	4	4·1	4	4·4
F	4	10·0	4	10·5	4	7·4	9	9·2	10	10·9
E(17–18)	4	10·0	1	2·6	1	1·9	1	1·0	—	—
E(16)	1	2·5	—	—	—	—	—	—	—	—
D	5	12·5	3	7·9	3	5·5	2	2·0	2	2·2
C	10	25·0	18	47·4	33	61·1	61	62·3	55	59·8
B	—	—	—	—	—	—	—	—	—	—
A(3)	—	—	—	—	2	3·7	6	6·1	9	9·8
A(2)	—	—	—	—	—	—	—	—	—	—
A(1)	1	2·5	—	—	—	—	3	3·1	1	1·1
Total	40	100·0	38	100·0	54	99·9	98	100·1	92	100·2
Number of variants	40		19		15		16		11	

Table 2. Distribution of missing chromosomes in karyotype variants with different chromosome numbers

	45 (0+/1−)		46 (1+/1−)		47 (2+/1−)		48–50 (3−5+/1−)		51–58 (6−13+/1−)	
		(%)		(%)		(%)		(%)		(%)
G	20	14·7	27	19·9	4	9·3	6	20·7	1	3·6
F	19	14·0	19	14·0	11	25·6	2	6·9	3	10·7
E(17–18)	21	15·4	23	16·9	11	25·6	13	44·8	18	64·3
E(16)	14	10·3	8	5·9	3	7·0	—	—	1	3·6
D	19	14·0	28	20·6	4	9·3	6	20·7	4	14.3
C	37	27·2	28	20·6	6	14.0	—	—	—	—
B	1	0·7	1	0.7	—	—	1	3·4	1	3·6
A(3)	1	0·7	2	1·5	2	4·7	—	—	—	—
A(2)	2	1·5	—	—	1	2·3	1	3·4	—	—
A(1)	2	1·5	—	—	1	2·3	—	—	—	—
Total	136	100·0	136	100·1	43	100·1	29	99·9	28	100·1
Number of variants	136		136		43		29		28	

the content per variant of supernumerary Ph[1], F, C and A(3) chromosomes increases steadily during progressive evolution.

This is illustrated further in Fig. 1, which shows that the C group is primarily responsible for the chromosome gain. However, in the late stages practically all the variants contain two Ph[1] chromosomes (Ph[1]-disomic variants) and an extra F as well as an extra A(3) chromosome. Together, the remaining chromosome types comprise on the average a total of about half a chromosome per variant at all stages of the evolution. Whereas this is equivalent to about 50% of the variants in the 47(1+/0−) group, in the 54–56(8–10+/0−) group it means that the total contribution of the E(17–18), E(16), D, B, A(2) and A(1) groups is on the average only half a chromosome out of 8–10 supernumerary chromosomes. Almost all the gain is consequently covered by the Ph[1], F, C and A(3) chromosomes. All the A(3)+ and, in the groups with 6–10 additional chromosomes, most of the F+ variants originated from a single patient. A(3) gain may consequently be of less general importance than gain in the other chromosome groups. Figure 1 illustrates that the relatively polymorphism of the supernumerary chromosome population observed in the early stages [8] gradually narrows down to an oligomorphic picture as a consequence of advancing evolution.

Table 2 shows the missing chromosomes at various levels. Loss of an E(17–18) member is observed more frequently whereas the remaining chromosomes show either relatively constant or decreasing frequencies of missing members with progressive evolution. As all the variants included in Table 2 have lost only one chromosome, the percentage values equal the average chromosome loss per variant. In the 51–58-chromosome group about two thirds of the

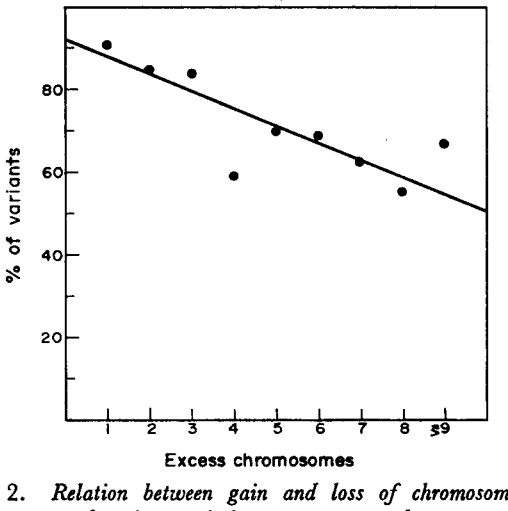

Fig. 2. Relation between gain and loss of chromosomes: percentages of variants missing one or more chromosomes in groups of cells with increasing numbers of supernumerary chromosomes. Regression coefficient: −3·70. *Correlation coefficient:* −0·81. $P < 0{\cdot}01$.

(6–13+/1−) variants lack an E(17–18) member.

Figure 2 shows the frequencies of variants missing one or more chromosomes in groups of cells with increasing numbers of excess chromosomes. It appears that the proportion of such variants gradually fall.

Analysis of the chromosomes predominantly gained and lost during karyotype evolution suggests that primarily poly-C+ and Ph[1]-disomic karyotypes become more and more frequent. As appears from Fig. 3, Ph[1]-disomic variants containing at least one supernumerary C member with or without additional members of other chromosome groups (Ph[1]C variants) represent increasing proportions of the variant population. On the other hand, variants differing from these only by having one instead of two Ph[1] chromosomes (C variants) show continuously decreasing frequencies.

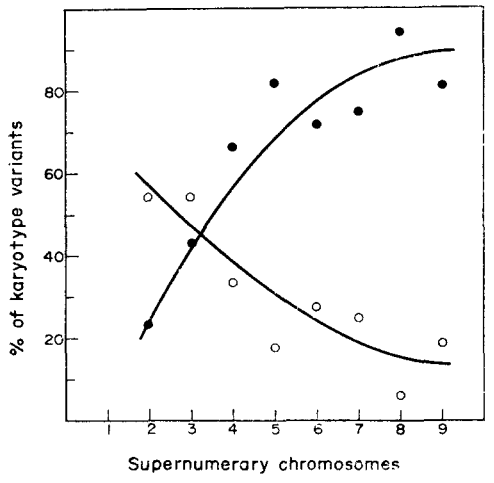

Fig. 3. Percentages of karyotype variants showing Ph¹C (●) and C (○) karyotypes (for definitions see text) related to progressive gain of supernumerary chromosomes. Curves fitted by hand.

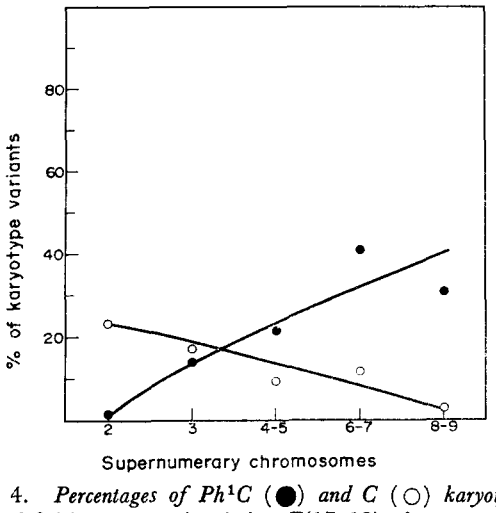

Fig. 4. Percentages of Ph¹C (●) and C (○) karyotypes (for definitions see text) missing E(17–18) chromosomes in relation to progressive gain of supernumerary chromosomes. Curves fitted by hand.

Figure 4 shows that Ph¹C and C variants each missing one or two E(17–18) members behave similarly during progressive evolution. The proportions of the total Ph¹C and C variant populations showing E(17–18)− karyotypes do not change significantly ($P > 0{\cdot}05$) during the evolution.

DISCUSSION

The present observations that supernumerary chromosomes are recruited mainly from the G, F, and C groups and that chromosome loss occurs particularly among the E(17–18) members are in accordance with the results of earlier studies [2, 4, 5, 9].

The karyotypes characteristic of advanced evolution fall into two distinct groups: Ph¹C and C karyotypes. Apart from the number of Ph¹ chromosomes there were no cytogenetic differences between the two groups. The predominant event during the evolutionary progress was, in both groups, the addition of C chromosomes. In spite of these similarities, the Ph¹C variants were apparently selectively favoured whereas the C variant prevalences gradually decreased. Apparently, the different fates of the two karyotype categories were due solely to the presence or absence of a second Ph¹. This observation awaits confirmation from other studies.

In other studies the presence of supernumerary C members has been shown to be associated with subnormal differentiation of the CGL granulocyte precursors [6] as well as with premature development of periodic acid-Schiff positive substances in myeloblasts, which is possibly due to asynchronous nuclear-cytoplasmic differentiation in these cells [7]. Clones of granulopoietic cells showing delayed differentiation may undergo more cell divisions before they mature into the non-proliferating cell compartments than do normally differentiating cells and may thus have a substantial selective advantage over the latter. However, in both studies, defective differentiation was apparently unrelated to the occurrence of supernumerary G members (including additional Ph¹ chromosomes).

The present study suggests, however, that the advantage associated with excess numbers of C chromosomes depends entirely on the inclusion of a second Ph¹. But the consequences of Ph¹-disomy for the cellular phenotype remain obscure. However, Spiers and Baikie [10] suggest that leukaemic cell proliferation may be inhibited by the genetic information present in the distal part of the long arm of the G chromosome pair to which the Ph¹ belongs and promoted by genes present elsewhere in the pair.

The almost identical behaviour of C and Ph¹C variants with and without E(17–18) loss suggests that elimination of these chromosomes is not associated with demonstrable advantages. The increasing predominance of E(17–18) loss during progressive evolution (Table 2) is only relative. This may indicate that, in contrast to loss of other chromosomes, E(17–18) loss is no major disadvantage. However, in some variants from CGL patients metacentric C marker chromosomes have been observed which are believed to be isochromosomes for the long arm of chromosome No. 17 (2). Thus, apparently E(17) monosomic cells may, in fact, be trisomic for the long E(17) arms. If such isochromosomes occur more than sporadically in CGL, it follows that possible selective advantages may be associated with excess rather than loss of

E(17–18) substance. This would be in accordance with the hypothesis proposed by Spiers and Baikie [11] that the long arms of one of these chromosomes contain genetic information promoting cell proliferation.

Considerations such as these may serve to indicate how little is in fact known concerning the role of the karyotype abnormalities occurring in leukaemic cells. In most cases these karyotypes can be characterized only in terms of numerical aberrations in the various chromosome groups, because up till now the quinacrine and Giemsa banding techniques for recognition of individual chromosome pairs have apparently not been usefully applicable to metaphases of leukaemic cells.

Thus, the demonstration in Fig. 2 that karyotypes missing one or more chromosomes become more infrequent with progressive evolution may be explainable on the assumption that trisomies and monosomies occurring in the same chromosome group may "neutralize" each other. If this is true, it follows that many supernumerary chromosomes actually occurring in the present material have not been recognized and that the profile of karyotype evolution drawn in the present paper may be far from a true portrait.

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Inhibition of Granulopoiesis by Endogenous Granulocyte Chalone Studied with the Diffusion Chamber Technique*

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Abstract—*This study showed that growth of granulocytes, both normal and leukaemic, is inhibited in a specific manner in diffusion chamber cultures carried by chloroma-bearing host rats (→ elevated granulocyte chalone content in the body). This inhibition could be demonstrated directly by a reduced formation of granulocytes and by measuring the ^{3}H-thymidine uptake in the cells. Non-granulocytic cell lines (mastocytoma and HeLa cells) were not detectably inhibited when grown in identical conditions. The inhibition of granulocytic cells did not depend on a selective immunological reaction developed against granulocytic cells in the chloroma-bearing host rats.*

INTRODUCTION

GRANULOCYTE chalone is a tissue-specific, species non-specific regulator substance which inhibits cell proliferation in normal and leukaemic granulocytes [1]. Chemically this substance seems to be a hydrophilic polypeptide with an apparent chain length of 20–30 amino-acids [2].

The biological properties of granulocyte chalone have so far been studied in a number of assay systems; in all of these a crude or partially purified chalone preparation has been used, either by adding it to the assay cultures *in vitro* or by injecting it into test animals *in vivo*. This general experimental approach has certain disadvantages; among them is the difficulty of performing long-term *in vivo* studies because large quantities of sufficiently well purified material and frequent injections are needed.

The present study was specifically designed to avoid the difficulties encountered in the use of exogenous chalone. Two developments have made a new approach possible. First, it has been shown that chloroleukaemia cells produce granulocyte chalone—and also respond to it—and that this substance is present in excess in rats suffering either from a local chloroma tumour or from a generalized leukaemia [3–7]. Secondly, it has been shown that the closed *in vivo* culture system utilising diffusion chambers is a convenient tool for the quantitative study of normal and leukaemic granulopoiesis [8, 9], and that the rate of granulopoiesis in the chambers can be inhibited by injecting granulocyte chalone into the host animals [10]. It thus follows that granulopoiesis in diffusion chambers implanted into the peritoneal cavity of chloroma-bearing rats with an elevated endogenous chalone content in the body should be inhibited compared with the situation in control hosts.

In this study we specifically wanted to show (i) that inhibition by the granulocyte chalone is tissue specific, (ii) that it does not depend on a selective immunological reaction or cytotoxity against granulocytic cells, and (iii) that the

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inhibition is detected without the need of measuring cell proliferation by ^{3}H-thymidine labelling. The last point is important because of some recent criticism of chalone experimentation [11].

MATERIAL AND METHODS

General experimental plan and host animals

A series of 12 experiments was made to study the effect of a high endogenous granulocyte chalone content on five cell lineages grown in diffusion chambers *in vivo*. In these experiments a total of 126 male and female rats of an inbred Wistar strain, 3–6 weeks old and weighing 50–140 g, were used as chamber recipients.

Half of the animals suffered from a local subcutaneous chloroma tumour or from a generalized leukaemia. The other half, serving as controls, were either healthy normal rats or animals from which a chloroma tumour had regressed spontaneously or was surgically removed at the time of chamber implantation. In two of the experiments the chamber recipients received 700 rads of whole body irradiation one day before chamber implantation, with the tumours shielded in the experimental group. The purpose of using all these different types of controls was to confirm that the results are not explicable in terms of a selective immunological reaction against granulocytic cells.

Cell suspensions for leukaemia transplantation were obtained from chloroma tumours as described earlier [4, 7]. Local tumours were induced by injecting $1–2 \times 10^6$ cells into the subcutaneous tissue in the back of the rats 2–3 weeks before the start of an experiment; generalized leukaemia usually showed ascitic growth of chloroma cells at the time of chamber implantation; rats with a local tumour had no ascites.

In three additional experiments with 26 pairs of rats, we studied the effect of a decreased endogenous chalone content on chloroma cells grown in diffusion chambers. To this end, half of the chamber recipients received 700 rads of whole body irradiation one day before chamber implantation; the other half was not irradiated and served as controls.

Irradiation technique

For the irradiation the animals were transported in a warm car to the Institute of Radiation Physics, Helsinki. They were exposed to 700 rads of whole body irradiation in groups of 3–4, each rat in its own ventilated cardboard box measuring $5 \times 5 \times 20$ cm. The dose was controlled with film badge dosemeters. The irradiation was produced by a 190 kV X-ray apparatus; the physical conditions were: current 10 mA, 2·0 mm Al filter, target distance 55 cm, and dose rate 65 rad/min. Local chloroma tumours of the experimental rats were shielded by a lead cup with a wall thickness of 3 mm.

Cell lineages cultured

Diffusion chamber cultures were initiated with five lineages of cells: normal rat and mouse bone marrow cells yielding relatively pure granulocyte cultures [12, 13], chloroma cells, mastocytoma cells, and HeLa cells.

Rat bone marrow cells were collected from adult male and female animals of the inbred Wistar strain by flushing the femoral cavity with Hanks' solution containing antibiotics (penicillin and streptomycin, 100 μg/ml of each). Mouse bone marrow cells were obtained from adult CBA/H-T6T6 mice by carefully crushing the femora in Hanks' solution with antibiotics and collecting the supernatant; more stem cells are recovered by this technique than by flushing the femoral cavity (Bøyum, personal communication). Chloroma cells were obtained from local tumours as described before [9, 14, 15]. Mastocytoma cells, subline P–815-X2, were obtained from ascitic tumours grown in female DBA/2Jax mice; before use for diffusion chamber cultures the cells were cultured *in vitro* for 1·5–2 days in Orion spinner medium.* HeLa cells were obtained from ordinary *in vitro* cultures by trypsinisation.*

Rigorous aseptic techniques were applied in all steps of cell collection and preparation of the diffusion chamber cultures.

Diffusion chamber technique

Diffusion chambers with a capacity of ~130 μl were made of acryl rings and Millipore filters as described earlier [9]. Two or three chambers glued together in a row (Tensol Cement) were implanted into the abdominal cavity of each recipient rat.

The chambers were filled with 100 μl of cell suspension usually containing 10×10^5 of bone marrow cells (rat or mouse), 5×10^5 of chloroma cells, 5×10^5 of mastocytoma cells, or 5×10^5 of HeLa cells. In the experiments where mastocytoma and HeLa cells were used, each of the three chambers was inoculated with a different lineage of cells; in the rest of the experiments all the chambers were filled with the same cell suspension.

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After culture periods of 1–10 days the chambers were recovered and shaken unopened for 70 min in a 0·5% Pronase (Pronase, B grade, 45,000 p.u.k./g, Calbiochem., Los Angeles, California) solution at room temperature. The cells were then collected by a Pasteur pipette, rinsing the inside of the chamber several times. The volume of the cell suspension was measured and the number of intact cells was determined haemocytometrically after crystal violet staining.

Labelling of cultures

In several experiments the diffusion chamber cultures were labelled with ^{3}H-thymidine (methyl-T; sp.ac. > 10 Ci/mmole; The Radiochemical Centre, Amersham, England). One hour before the termination of the cultures 10 μCi of ^{3}H-TdR in 1 ml of Hanks' solution was injected into the abdominal cavity of the host rats. Radioactivity incorporated in the cultured cells was determined radiochemically and, in a few cases, also autoradiographically.

Total radioactivity incorporated in the cultured cells was determined from a small aliquote of the cell suspension prepared for cell count analysis. A measured volume was pipetted into a centrifuge tube, the cells were spun down, washed several times with Hanks' solution, dried overnight at 110°C, and dissolved in Soluene (Packard Co.) at 55°C. After adding an ordinary toluene-based scintillation liquid to the samples they were counted in a Packard Tri-Carb liquid scintillation counter for 10 min. Owing to the small sample sizes and some quenching, the count rates were occasionally very low, especially in strongly inhibited cultures. Usually, however, the relative counting error was less than $\pm 5\%$.

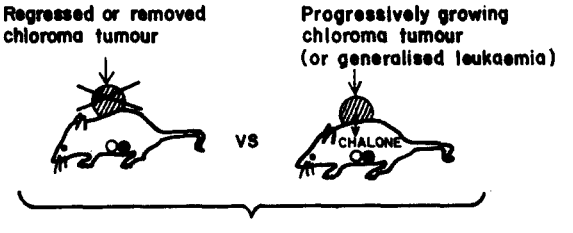
Autoradiograms were made of cytocentrifuge (Shandon) preparations as described before [9, 15]. They were covered with Kodak AR–10 stripping film, exposed for two weeks, processed, and stained with Mayer's haematoxylin. The average background was less than one grain per 100 μm^2; cells with $\geqq 10$ grains were scored as labelled. To obtain the labelling index about 1000 intact-looking cells were scored per preparation; the mean grain count was determined from 10 randomly selected labelled cells per preparation. Two persons evaluated the autoradiograms independently.

Statistical treatment

The statistical significances of the differences between the experimental and control groups were tested by Wilcoxon's non-parametric methods. In these tests comparisons were made between animals in the different assay groups rather than between individual diffusion chamber cultures. To compare the pooled results from different experimental series it was necessary to scale the measurements to a common arbitrary level in order to eliminate systematic differences between experiments and different culture times. To this end, the mean obtained for each pair of control and experimental rats was given the value 100; hence all scaled individual values are between 0 and 200 with an overall mean of 100.

RESULTS

It has been shown [3–5] that chloroma cells produce granulocyte chalone and that this substance is present in excess in chloroma-bearing animals. In these experiments the chalone was prepared either from chloroma tumours or from serum of tumour-bearing animals and tested in a short-term *in vitro* culture using normal and leukaemic bone marrow cells as the target tissue. Here we used a completely different assay system as illustrated below:



Granulocytic (●) and non-granulocytic (○) cells were cultured for 1–10 days in diffusion chambers and analysed for cell yields and incorporation of ^{3}H-TdR.

In a series of 12 experiments with 63 pairs of rats carrying 346 diffusion chamber cultures, we measured the effect of an elevated endogenous chalone content on the growth of five different cell lineages. The results obtained for cell yields are shown in Table 1 and in Figs. 1 and 2.

The growth characteristics of the five cell lineages used as target tissue differed considerably from one another. Cultures initiated with rat bone marrow cells and HeLa cells seemed to attain a steady-state condition with no apparent net growth during the culture periods studied. Mouse bone marrow cells (Fig. 1), chloroma cells (Fig. 2; for details of the growth characteristics, see: 9, 15) and mastocytoma cells optimally grew at a relatively fast rate for about five days, though the variation from one experiment to another was large (for causes of this variation, see: 15). The shortest initial doubling time obtained (~ 12 hr) was for mastocytoma cells. The pulse labelling index for the whole

Table 1. *Inhibition of growth of normal and leukaemic granulocytes in* 1–10 *day-old diffusion chamber cultures carried by chloroma-bearing host rats. The results of the different experiments have been scaled to a common arbitrary level by denoting the mean for each pair of control and experimental animals as* 100. N *is the number of animal pairs studied; each host rat carried* 2 *or* 3 *diffusion chambers inoculated with the same or different lineages of target cells*

Experiment	Target cells	Chamber hosts	*N*	Number of cells harvested (arb. units) Exp.	Control	S.E.†
I	Prolif. granulocytes (rat)	Local chloroma vs normal	10	84	116	8
II	Prolif. granulocytes (rat)	Gen. leukaemia vs normal	6	85	115	19
III	Prolif. granulocytes (rat)	Gen. leukaemia vs normal	5	65	135	13
IV	Prolif. granulocytes (mouse)	Local chloroma vs normal	4	68	132	11
V	Prolif. granulocytes (mouse)	Local chloroma vs normal	4	81	119	33
VI	Prolif. granulocytes (mouse)	Local chloroma vs tumour regressed	4	66	134	9
VII	Prolif. granulocytes (mouse)	Local chloroma vs tumour regressed	5	85	115	20
VIII	Chloroleukaemia cells	Local chloroma vs normal	7	94	106	18
IX	Chloroleukaemia cells	Local chloroma vs normal	8	84	116	9
VII	Chloroleukaemia cells	Local chloroma vs tumour regressed	5	66	134	17
X	Chloroleukaemia cells	Local chloroma vs normal*	3	81	119	11
XI	Chloroleukaemia cells	Local chloroma vs tumour removed*	3	86	114	18
XII	Chloroleukaemia cells	Gen. leukaemia vs normal	4	83	117	21
			Weighted mean	80·2	119·8	
			Inhibition	33%		
V	Mastocytoma cells	Local chloroma vs normal	4	94	106	19
VII	Mastocytoma cells	Local chloroma vs tumour regressed	5	104	96	18
XII	Mastocytoma cells	Gen. leukaemia vs normal	4	108	92	6
V	HeLa cells	Local chloroma vs normal	4	80	120	16
XII	HeLa cells	Gen. leukaemia vs normal	4	140	80	15
			Weighted mean	105·1	94.9	
			Inhibition	—		

*All host rats received 700 rads of whole body irradiation one day before chamber implantation. The chloroma tumour of the experimental hosts was shielded during irradiation.

†Owing to the method of scaling of the cell count values, the standard error of both means is the same.

population was about 12% in 2–7 day control cultures of rat bone marrow cells, about 20% in control cultures of mouse bone marrow cells, and about 30% in control cultures of chloroma cells.

In spite of the different growth characteristics of the target cells, and of the large variation between different experiments, the results given in Table 1 are unequivocal. They show that in every experiment the number of normal and leukaemic granulocytes recovered from the diffusion chambers was lower in the chloroma-bearing rats than in the controls. The overall mean of the scaled values was 80·2 for the experimental rats and 119·8 for the control rats (S.E.=4·2 for both groups) and, hence, the average inhibition was 33%. The inhibition is statistically significant at $P < 0{\cdot}001$ (Wilcoxon test for paired values).

The pattern of granulocyte growth in chloroma-bearing and control rats is seen in Fig. 1, where the raw data obtained for mouse bone marrow cell cultures are plotted against time. As already pointed out, the variation in the cell yields of individual experiments was large; however, the regression line obtained for the control cultures suggests an exponential growth of five days with a population doubling time estimated at 25 hr. The scatter of the experimental group values was even larger than that of the control group, presumably owing to the additional heterogeneity of the material (e.g. differences in tumour mass between the host animals), and numerical estimation of the population doubling time is therefore not meaningful. In spite of this, it is clear from Fig. 1 that the population growth was significantly slower in the chloroma-bearing host rats than in the controls. This conclusion is confirmed by a similar analysis performed on chloroma cell cultures; regression equations through the raw data indicate that the average population doubling time was 52 hr for control rats and 88 hr for chloroma-bearing rats (Fig. 2).

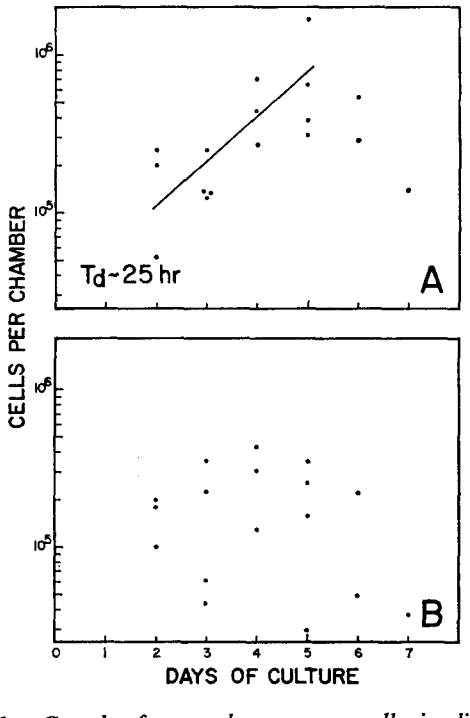


Fig. 1. Growth of mouse bone marrow cells in diffusion chambers in control (A) and chloroma-bearing (B) host rats. Each dot is the cell yield obtained for a single host rat carrying 1–3 diffusion chambers; raw data of four different experiments (IV–VII, see Table 1).

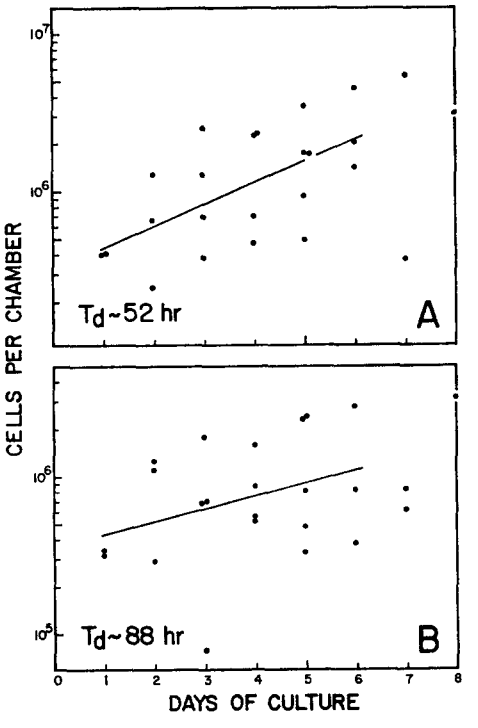


Fig. 2. Growth of chloroma cells in diffusion chambers in control (A) and chloroma-bearing (B) host rats. The dots are the cell yields obtained for the host rats, each carrying 1–3 diffusion chambers; raw data of six different experiments (VII–XII, see Table 1). Note that the number of dots is less than the number of animal pairs studied (see Table 1), because in some experiments all the rats were killed on the same day and, to avoid placing undue weight on these days, only the mean values were plotted.

In contrast to the growth inhibition obtained for normal and leukaemic granulocytic cells, diffusion chamber cultures of mastocytoma cells and HeLa cells were not detectably inhibited in chloroma-bearing host rats (Table 1). The overall mean of the scaled cell count values was 105·1 for chloroma-bearing rats and 94·9 for the controls (S.E. = 7·7; number of animal pairs studied = 21). The variation between the

experiments was again large; however, the average cell yield in the chloroma-bearing rats happened to be even higher than in the control rats, and hence a biologically significant inhibition of mastocytoma and HeLa cell cultures by host chloroma can be excluded with confidence. In agreement with this, the difference between granulocytic and non-granulocytic cultures is statistically significant even when the analysis is limited to rats each carrying both types of cultures ($P < 0{\cdot}01$; Wilcoxon test for paired values). It thus follows that the inhibitory effect of the host chloroma on the population growth of normal and leukaemic granulocyte cultures is tissue specific in nature.

The results given in Table 1 demonstrate further that the tissue-specific inhibition is not based on a selective immunological reaction developed in the chloroma-bearing animals against chloroma cells and cross reacting with corresponding normal cells. This possibility is excluded by the significant inhibition (43%; $P < 0{\cdot}01$) detected in those experiments where the control group consisted of rats with spontaneously regressed chloroma tumours, because an immunological reaction against chloroma cells is evidently as strong in the control rats showing spontaneous regression of the tumour as in the experimental rats showing progressive growth of the tumour. Furthermore, whole body irradiation of the host rats (chloroma tumour shielded) did not obviate the inhibition (Table 1, experiments X and XI). It may also be worth noting that cell-mediated immune reaction is excluded by the very nature of the assay system, because the cultured cells are effectively separated from the host cells by the thick Millipore filters (cf. 16).

In addition to the measurement of total cellularities in the diffusion chamber cultures, cell proliferation was determined in several experiments by injecting ^{3}H-TdR into the chamber-carrying host rats one hour before killing. Total radioactivities incorporated into the cells were analysed as described under "Material and Methods" and the counts were scaled to a common level.

The results are given in Table 2. They show that incorporation of ^{3}H-TdR was strongly inhibited in the granulocyte cultures carried by chloroma-bearing host rats compared with the controls. The average inhibition for all granulocyte cultures was 48% ($P < 0{\cdot}01$), and this appears to be even stronger than the average inhibition obtained for cell yields (33%).

In agreement with the results obtained for cell yields, incorporation of ^{3}H-TdR was not detectably inhibited in mastocytoma and HeLa cell cultures carried by chloroma-bearing host rats (Table 2).

In order to study the mechanism of growth inhibition further, an autoradiographic analysis was performed on the cultures of experiment VI (see Table 1). There were several reasons for choosing this particular experiment for the analysis: (i) inhibitions detected for population size and for total incorporation of ^{3}H-TdR were especially strong; thus, distinct changes should be observable autoradiographically; (ii) the control group consisted of rats with spontaneously regressed chloroma tumours, which excludes a selective immunological reaction as a possible cause of group differences (see above); (iii) the source of the inhibitor (a subcutaneous tumour in the back of the host rat) was not in close contact with the target cultures; hence a reduced uptake of ^{3}H-TdR in the cultured cells could not be explained in terms of label utilization by the chloroma cells growing in the ascitic fluid.

The results are given in Table 3. They show that the population size, the pulse labelling index and the mean grain count were all inhibited by 50–60% in the cultures carried by the chloroma-bearing rats. The product of these three parameters should be proportional to the total incorporation of ^{3}H-TdR in the cultures and, as can be verified from the data given in Table 3, this appears to be the case. Hence the autoradiographic findings indicate that the reduced uptake of ^{3}H-TdR per labelled cell, which contributes much to the total incorporation of ^{3}H-TdR, was an important factor in the extremely strong inhibition observed in this experiment ($86 \pm 4\%$).

Irradiation of the host rat prior to chamber inoculation decreases the number of granulocytes in the body; consequently, a decrease in the chalone content should also occur. The situation is essentially reciprocal to that seen in chloroma-bearing host rats, and granulocyte growth may thus be expected to be stimulated in diffusion chamber cultures carried by pre-irradiated rats. Enhanced growth of granulocyte cultures has in fact been demonstrated in irradiated host mice [17, 18] and hence the published observations are in agreement with the expectation, even if the enhanced growth may also depend on humoral stimulators such as granulocytic antichalone [19, 20].

We made a series of three experiments with the purpose of confirming that pre-irradiation stimulates granulocyte growth also in rats. Chloroma cells were used as the target population because stimulation by humoral factors such as granulopoietin, believed to induce the

Table 2. Inhibition of ^{3}H-TdR incorporation in proliferating normal and leukaemic granulocytes grown in diffusion chambers in chloroma-bearing host rats. Radioactivities measured for each pair of control and experimental rats have been scaled to a common arbitrary level (cf. Table 1). The experimental group consists of host rats suffering from chloroleukaemia (local tumour or generalized leukaemia); the control group consists of both normal rats and rats in which a local chloroma tumour had regressed. N is the number of animal pairs studied; each animal carried 2 or 3 diffusion chamber cultures

Target cells	*N*	Incorporation of ^{3}H-TdR (arb. units) Exp.	Control	S.E.*	Inhibition (%)
Proliferating granulocytes (rat and mouse)	35	67	133	8	50
Chloroleukaemia cells	10	75	125	14	40
Non-granulocytic cells (mastocytoma and HeLa cells)	21	99	101	13	2

*S.E. is the same for both means (cf. Table 1).

Table 3. Inhibition of granulocyte proliferation in diffusion chambers carried by chloroma-bearing host rats (experiment VI). The experimental group consists of host rats suffering from a large subcutaneous tumour; the control group consists of rats of the same litter in which the tumour had spontaneously regressed. Each rat carried three diffusion chambers; the values given are the means of these three cultures

Duration of culture days	Number of cells harvested ($\times 10^{-3}$) Control	Exp.	Total incorporation of ^{3}H-TdR (counts/min) Control	Exp.	Pulse labelling index (%) Control	Exp.	Mean grain count of labelled cells Control	Exp.
2	248	102	192	37	16·5	7·2	92	57
3	135	44	222	28	25·5	14·7	148	67
4	271	130	495	20	21·0	5·0	157	13
5	311	263	275	57	18·5	9·5	84	38
Average inhibition (%; mean ± S.E.)	48 ± 11		86 ± 4		56 ± 7		60 ± 11	

conversion of a secondary stem cell into the first recognizable member of the granulocyte series [1], may not affect leukaemic cells which have already "differentiated" beyond this stem cell level. The results are given in Fig. 3. They show that the population doubling time was shortened from 64 hr in the control cultures to 44 hr in the experimental cultures, as estimated from the regression equations through the cell count values on days 1–6. The overall average of the cell yields in the irradiated rats was about 20% higher than in the controls (comparison between animal pairs). The difference is significant at $0{\cdot}1 > P > 0{\cdot}05$ if all the values on days 1–8 are taken into consideration. However, it has been shown in mice that enhancement of granulocyte growth is detectable from cell count values only after 3–4 days of culture [18] and thus the comparison of cell yields may be restricted to a time period excluding the values on days 1 and 2. If this is done, the difference between the irradiated and control rats is significant at the level $P < 0{\cdot}05$ (Wilcoxon test for paired values).

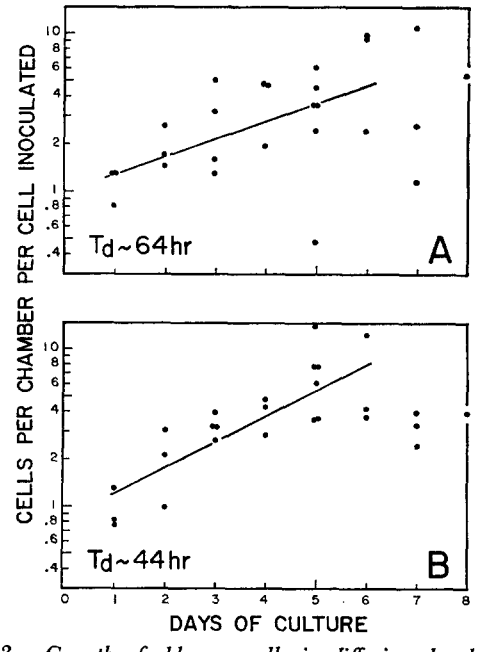


Fig. 3. Growth of chloroma cells in diffusion chambers in normal (A) and pre-irradiated (B) host rats. Each dot is the relative cell yield obtained for a single host rat carrying 2–3 diffusion chambers; raw data of three experiments with different initial cell counts (1·0 – 5·2 × 10^5 chloroma cells).

DISCUSSION

The results show that growth of granulocytes, both normal and leukaemic, is inhibited in diffusion chamber cultures carried by chloroma-bearing host rats compared with different types of controls. The average decrease in cell yields was 33% (range in 12 different experiments 11–52%), and for total uptake of ^{3}H-TdR 48%; both these effects were statistically significant. Growth inhibition did not occur in cultures of non-granulocytic cell lines grown in identical conditions, and it did not depend on immunological mechanisms developed against granulocytic cells.

The tissue specificity of the effect was confirmed with mastocytoma and HeLa cell cultures; these cells were not detectably inhibited in chloroma-bearing host rats compared with the controls. Thus, the inhibition of granulocyte growth is not explicable in terms of essentially non-specific factors, such as deficient nutrient supply, accumulation of toxic metabolites, oxygen deficiency, or changes in pH. Other authors [21–24] have also shown that trivial environmental factors do not usually play an important role in the regulation of cell division even in assay conditions which appear to be more drastic for the cells than the conditions used in many of our experiments (such as those involving chamber hosts with a relatively small local tumour—less than half of the lethal size—in the back). In particular, Bichel [22, 23] demonstrated that when two different ascites tumours, both reaching a plateau before the death of the host animal, were inoculated into the same mouse, both tumours developed independently. Bichel actually showed that the decrease in the growth rate of an ascites tumour is based on specific feedback inhibition, i.e. on a chalone-like mechanism (see below).

Besides trivial environmental factors, the inhibition of granulocyte growth observed in the present study could also have been due to a selective immunological reaction developed against granulocytic cells in the chloroma-bearing host rats. This possibility was excluded in many ways. The best evidence is provided by the experiments showing a strong and statistically significant inhibition of granulocyte growth (average decrease in cell yields 43%) in the cultures carried by chloroma-bearing rats—which had thus failed to reject the tumour—compared with otherwise identical host rats that had rejected the chloroma tumour. It may also be worth noting that cell-mediated reaction is excluded by the very nature of the assay system, because the target cultures were effectively separated from the host cells by thick Millipore membranes.

Because the inhibition of granulocyte growth in chloroma-bearing animals does not depend either on trivial environmental factors or on immunological reaction, the results obtained indicate that the effect must be based on a specific feedback mechanism originating from the chloroma cells of the chamber-carrying host rats. Besides the work of Bichel [22, 23] on ascites tumours, essentially similar findings and conclusions have been reported by Ferris [25] in a preliminary paper on chloroleukaemia cells. He observed that the growth rate of these cells, but not of Ehrlich's ascites carcinoma cells, was inhibited when grown in leukaemic host rats. On the other hand, when chloroleukaemia cells were grown in diffusion chambers in mice with Ehrlich's ascites carcinoma, growth inhibition was not detected compared with normal host mice.

Apart from these findings, the existence of a specific feedback inhibitor originating from chloroma cells was known from earlier studies based on short-term *in vitro* assays (3–5). It had been shown that chloroma cells produce granulocyte chalone, the tissue-specific inhibitor of granulocyte proliferation [1], and that this substance is present in excess in rats suffering either from a local chloroma tumour or from a generalized leukaemia. Furthermore, injections of exogenous granulocyte chalone into mice carrying diffusion chamber cultures had been shown to inhibit ^{3}H-TdR uptake in proliferating granulocytes, but not in immunoblasts and macrophages [10]. Consequently, the purpose of the present study was not only to re-confirm the existence of this specific feedback inhibitor with the aid of a new assay system, but to show that the effect of the granulocyte chalone can be directly demonstrated by the reduced formation of granulocytes. This was not possible in the earlier short-term experiments.

Demonstration of reduced target tissue "mass" (cell numbers) by chalone treatment *in vivo* is difficult and sometimes not even possible. There are several reasons for this, such as counteraction by other regulatory links and possible prolongation by chalone of the life span of postmitotic cells [26–28]. Even a complete and permanent regression of a tumour induced by chalone treatment [6, 7, 29] is not an unequivocal experimental demonstration of reduced cell formation, because chalone-independent cell death is an essential component of the regression [1, 15]. Furthermore, any role of chalone in tumour regression may appear in doubt now that Mohr *et al.* [30] have reported

that their results on melanoma regression [29] after all did not depend on melanocyte chalone, but rather on bacterial spores contaminating the preparations used.

Because of these findings, and the recent criticism of chalone experimentation stemming from the use of ^{3}H-TdR as the basic indicator of cell proliferation [11], we wanted to show that it nevertheless is possible to demonstrate the chalone effect both by reduced formation of normal and leukaemic granulocytes and by measuring cell proliferation with ^{3}H-TdR. As already indicated, both these parameters of growth rate were significantly inhibited (33% and 48%, respectively) in closed *in vivo* cultures in the presence of a high endogenous chalone content.

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Letter to the Editor

In Vivo Effect of Adenosine 3′,5′–Monophosphate on Ehrlich Ascites Tumour Cells*

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THE ACTIVITY of adenylcyclase [1] and the concentration of cyclic AMP [2] become enhanced in untransformed cells as the cell population density increases, but are decreased in transformed cells [3, 4]. Moreover, the addition of cyclic AMP or dibutyryl cyclic AMP to the culture medium of transformed or malignant cell cultures can inhibit cell division [5, 6, 7, 8] and restore morphological characteristics [9] of untransformed cells. These observations *in vitro* suggest that density-dependant cessation of growth is controlled by intracellular cyclic AMP levels and that in some malignant cells this mechanism is defective. However, little work [10] has been done to test whether cyclic AMP is effective as a carcinostatic agent *in vivo*. We report the effect of administration of cyclic AMP to mice inoculated with Ehrlich tumour cells.

Young adult female A strain mice were injected with ten million Ehrlich ascites tumour cells, either subcutaneously between the scapulae to produce a solid tumour, or intraperitoneally. Three days later drugs were given intraperitoneally and continued twice daily for 4-5 days. One group of animals received cyclic AMP, 10 mg/kg body weight, immediately preceded by aminophylline, 50 mg/kg each in 0·1 ml of saline (test group). A second series of mice received the same dose of aminophylline and 0·1 ml of saline (aminophylline group), while other mice received 0·1 ml of saline only (control group).

After 8 days growth, the subcutaneous tumours were solid, white structures without cysts but with a well-established blood supply. The mean tumour weight in 15 controls was 0·12 g (S.E., 0·017) which was double that in 14 test mice (mean 0·06 g, S.E., 0·007) (P = 0·01–0·001). The aminophylline group had a mean tumour weight of 0·09 g (S.E., 0·009). This was significantly different when compared with the test animals (P = 0·02–0·01) but not when compared with the controls (P = 0·2–0·1).

The mean total number of ascites tumour cells in the peritoneum 8 days after inoculation was nearly twice as high in 15 control mice (1,076 million cells, S.E. 136 million) as in 15 test mice (548 million, S.E. 83 million) (P = < 0·001). In 15 other animals which received aminophylline the number of cells (mean 605 million, S.E. 115 million) was also significantly lower than in the control group (P = 0·05–0·02).

The mean total packed cell volume, a measure of the cellular material present [11], in the test group was 86, (S.E. 11·8) which was significantly lower than that of the controls (mean 153, S.E. 18·0) (P = 0·01–0·001). The volume for the aminophylline series (mean 113, S.E. 16·0) was intermediate between the test and control values, but the differences were not significant at the 5% level (P = 0·2–0·1).

Half the test mice had no ascitic fluid, and in all of them three muscle layers of the abdominal wall appeared normal histologically. In contrast all the control mice had abundant ascitic fluid and the muscle layers were distorted, contained numerous capillaries and were infiltrated with lymphocytes and larger cells which resembled Ehrlich cells.

Under phase contrast microscopy the living tumour cells from the control mice (Fig. 1)

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had the same appearance as those from untreated mice. Each cell was round with a tuft of hair-like structures at one pole, but few hair-like projections around the rest of the cell periphery. The nucleus was irregular and usually orientated at the opposite pole to the tuft.

Most of the tumour cells derived from test mice (Fig. 2) were round and larger than the control cells. Almost all of them lacked localised tufts but bore many hair-like projections, around the whole cell periphery. The nucleus was larger and regularly shaped, either round or oval. Tumour cells from the aminophylline-treated mice resembled those from untreated animals, but were more regular in shape.

Administration of aminophylline alone, also impeded tumour growth. This suggests that inhibition of phosphodiesterase allowed enough native cyclic AMP to accumulate and limit cell growth.

In summary, the administration of cyclic AMP together with aminophylline to mice, curbed the growth of Ehrlich tumour cells both in the solid and ascitic forms by approximately 50%, when compared with saline treated controls. Drug treatment suppressed formation of ascitic fluid, altered the morphology of the tumour cells, and abolished the inflammatory reaction in the abdominal wall, but at the concentrations used, appeared to have no toxic properties.

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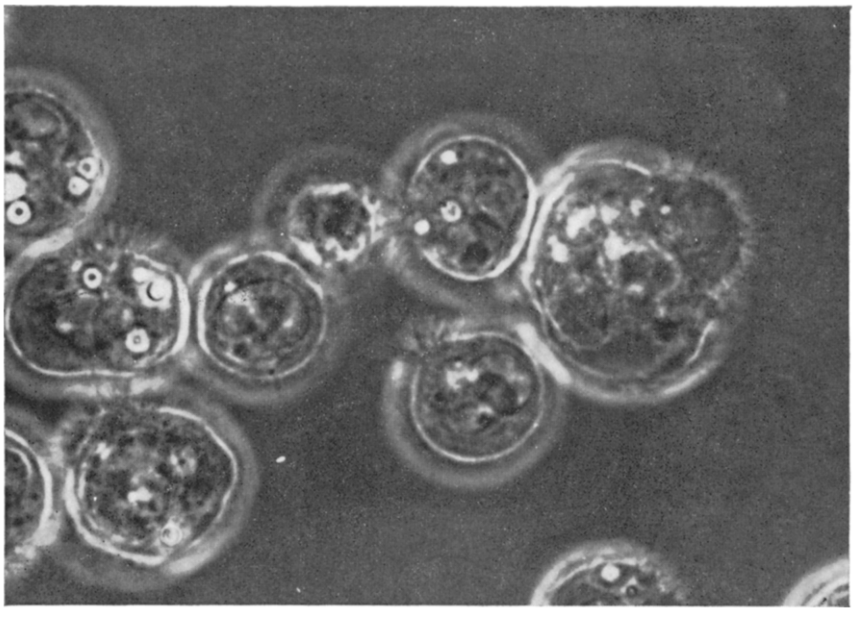

Fig. 1. *Morphology of living Ehrlich ascites tumour cells* 8 *days after inoculation into peritoneal cavity. Phase contrast microscopy from a saline treated mouse.*
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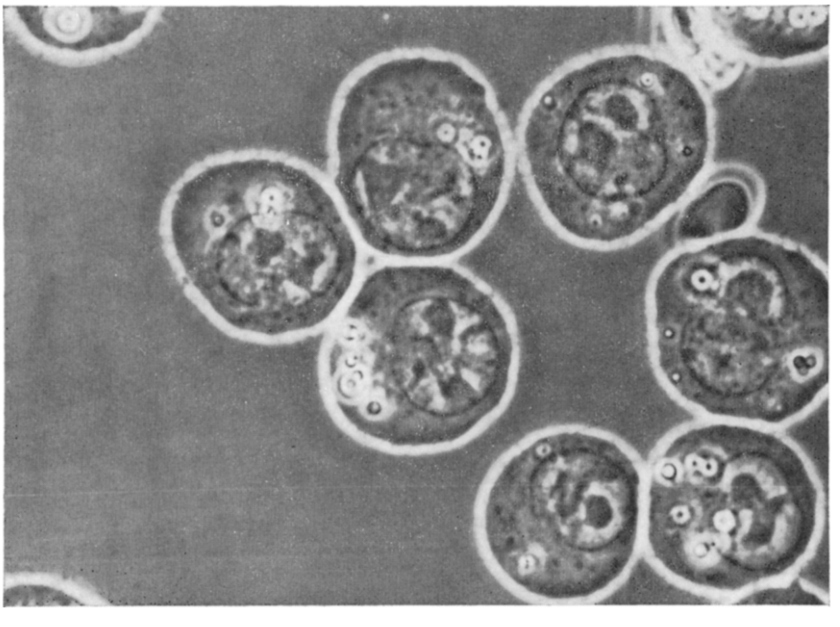

Fig. 2. *Morphology of living Ehrlich ascites tumour cells* 8 *days after inoculation into peritoneal cavity. Phase contrast microscopy from a cyclic AMP and aminophylline treated mouse.*
× 2000

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Letter to the Editor

Excretion of 5-Hydroxyindole Acetic Acid and N^1-Methylnicotinamide in Advanced Cancer Patients

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IN RECENT years there has been considerable interest in the metabolism of tryptophan in cancer patients. The kynurenine pathway in patients with bladder cancer has been found to be incomplete [1, 2]. These patients have an abnormally high urinary excretion of some metabolites along the pathway of tryptophan metabolism. Malignant carcinoid (argentaffinoma) has been considered to be an abnormality in tryptophan metabolism in which a much greater proportion of tryptophan than normal is metabolised by way of the hydroxyindole pathway [3]. The urinary concentrations of 5-hydroxytryptamine and 5-hydroxyindole acetic acid are greatly increased in these patients. Boyland and his co-workers [4] reported that the urinary concentrations of 5-hydroxyindole acetic acid and 5-hydroxytryptamine in the patients with cancer of the larynx and bronchus were lower than that in the patients with argentaffinomas but higher than in urine from cancer-free patients.

We have studied the urinary excretory level of 5-hydroxyindole acetic acid (5-HIAA), the end product of the hydroxyindole pathway and the excretory level of N^1-methylnicotinamide, the end product of the kynurenine pathway in patients with advanced malignant tumours at various sites.

A total of 58 patients with advanced cancer have been studied. These included patients with cancer of the breast, brain, respiratory tract, mouth, cervix and caecum. All had been treated by surgery or radiotherapy. A fresh overnight specimen of urine was collected from each patient. Similarly, urine specimens were collected from 15 healthy subjects and 18 hospital patients without cancer but with debilitating or sub-acute diseases, such as tuberculosis, chronic bronchitis, coronary thrombosis and hypertension.

The 5-HIAA in urine was estimated using the method of Udenfriend and his associates [5]. The amount of N^1-methylnicotinamide excreted in urine was measured by the fluorimetric method described by Johnson *et al.* [6]. In order to ensure that the N^1-methylnicotinamide excreted in urine was derived from tryptophan, the excretory level of the vitamin was measured only in those subjects who did not have any record of preceding intake of the vitamin supplements.

As it was not possible to collect a 24 hr-urine specimen from each subject, all results were expressed per g of creatinine, which is normally accepted as being extremely constant in its excretion.

The results of the estimations of 5-HIAA and N^1-methylnicotinamide in the urine of cancer patients and control subjects are shown in the Table. The 5-HIAA levels in the patients with malignant tumours of various sites averaged 13·9 mg with a range of 8·6–24·0 mg/g of creatinine. Control estimations of 5-HIAA on 15 healthy subjects averaged 2·2 mg with a range of 1·2–3·6 and on 18 hospital patients without cancer but with debilitating diseases averaged

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Urinary levels of 5-hydroxyindole acetic acid and N^1-methylnicotinamide in cancer patients

Subjects	No. of subjects	Age (yr)	5-hydroxyindole acetic acid (mg/g creatinine)	N^1-methyl-nicotinamide (mg/g creatinine)
Cancer patients	58	62 (53–71)	13·9 (8·6–24·0)	2·7 (0·4–3·0)
Control (healthy subjects)	15	52 (45–65)	2·2 (1·2–3·6)	9·0 (3·8–11·2)
Control (patients with non-malignant diseases)	18	59 (55–69)	2·7 (1·4–3·6)	8·1 (2·1–18·4)

Each figure is the mean value for the number of subjects shown in the table with a range in parenthesis.

2·7 mg with a range of 1·4–3·6 mg/g creatinine. Thus, the HIAA excretory levels in cancer patients were above both controls. Conversely, urinary excretory levels of N^1-methylnicotinamide in cancer patients were markedly decreased compared with those of the controls.

It should be noted in the present data that, unlike in control groups, the urinary concentrations of the 5-HIAA in cancer patients fall within the wider limits. No variation in the concentration of 5-HIAA with the various site of tumours studied was however observed. It seems therefore probable that the wider range of the 5-HIAA could be either due to reflection of the greater number of specimens or perhaps the state of the disease condition.

Although the changes in urinary excretion of 5-HIAA in general cancer patients are quantitatively not as pronounced as in the patients with argentaffinomas [3], the increased level of 5-HIAA and decreased level of N^1-methylnicotinamide in urine of cancer patients studied suggest that, to a certain extent, the greater proportion of tryptophan is metabolized in these patients by way of the hydroxyindole pathway. These results are sufficiently interesting to merit further investigation in early cancer patients, since this could provide the method for early diagnosis of the disease. This work, however, is currently being investigated in our laboratories.

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Letter to the Editor

Inhibition of the Growth of Ehrlich Ascites Tumors by Treatment with the Respiratory Inhibitor Rotenone*

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IT WAS suggested, in a previous paper [1], that the anti-cancer agent VM–26 could exert its antimitotic action through the inhibition of cellular respiration with NADH-linked substrates.

In order to investigate whether the inhibition of NADH-linked respiration could represent a new approach to experimental anti-cancer chemotherapy, we have studied the effects of the known mitochondrial inhibitor rotenone [2] on the growth, survival, and mitotic fraction of Ehrlich ascites tumors.

Thirty-six mice (Swiss strain) weighing 25 g each were injected intraperitoneally with a massive inoculation of 10×10^6 washed Ehrlich ascites cells. Beginning with the 24th hr after inoculation, 15 mice were injected daily with 3 mg of rotenone (K & K laboratories) per kg of animal weight dissolved in 0·1 ml of sunflower oil. Fifteen controls were injected with 0·1 ml of the solvent alone. The treatment was continued until the animals died, and the weight and survival times of the animals in both groups were recorded. The 6 remaining mice were allowed to develop a tumor during 6 days and 19 hr, after which 3 mice were injected with a single dose of 80 μg of rotenone dissolved in 0·1 ml of 25% ethanol. The other 3 mice were injected with the solvent alone. Samples of ascites liquid were collected from these 6 mice at 1, 2, 4 and 6 hr after the injection of rotenone, and cell smears were stained with May Grunwald-Giemsa for evaluation of the mitotic fraction and percentage of mitotic phases.

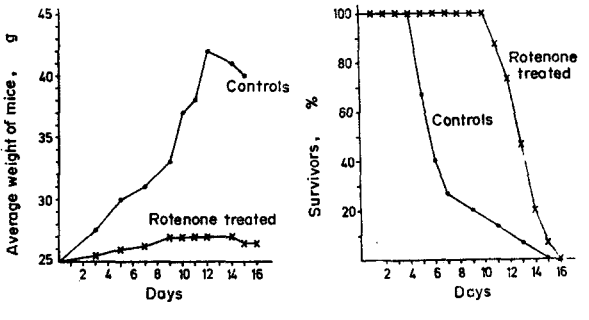

Fig. 1. (Left): Average weight of two groups of fifteen mice during 16 days after inoculation with 10×10^6 Ehrlich ascites cells. Controls: group injected intraperitoneally with the solvent daily. Rotenone treated: groups injected intraperitoneally with 80 μg of rotenone per animal daily. The standard deviation (not shown) of each value of average weight ranged between 0·5 and 1 g, depending on the number of surviving animals. Total packed cell volume reached 3·5 ml in the controls on the 12th day while it was undetectable at necropsy in the treated group.

(Right): Percentage of surviving animals in the same experiment as that shown by the figure on the left.

Figure 1 (left) shows the average weight of both groups of 15 mice during 16 days following the transplant of the tumor. It can be observed that the control group gained weight progressively until the 12th day due to the growth of the tumor in each animal. In comparison, the average weight of the 15 mice treated with rotenone was maintained at steady

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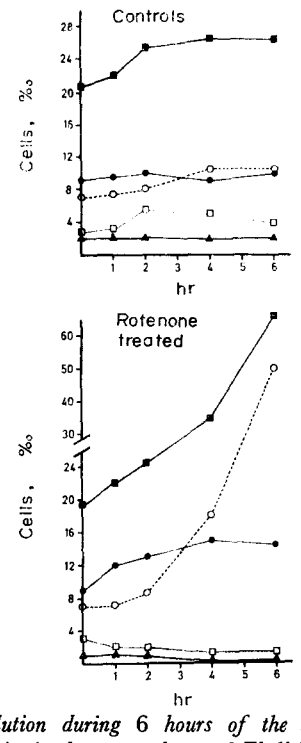

Fig. 2. Evolution during 6 hours of the average mitotic fraction and mitotic phases per thousand Ehrlich ascites cells in two groups of 3 mice. Controls: group treated with a single injection of the solvent at zero time. Rotenone treated: group treated with a single injection of 80 μg of rotenone per animal at zero time.
Closed circles: prophases; open circles: metaphases; open squares: telophases; closed triangles: anaphases; closed squares: mitotic fraction.

values due to the absence of tumor growth in this series. Ascitic fluid and cells were undetectable in these animals at necropsy. Rotenone does not cause a weight loss in normal mice at the dose used (3 mg/kg), which is $\frac{1}{3}$ of the LD_{50}.

Figure 1 (right) shows the percentage of surviving animals. It can be observed that the control group shows a high mortality rate from the 6th to the 7th day, and on the 15th day the last animal died. The rotenone-treated group shows 100% survival up to the 10th day, which compares favorably with the 13% survival in the control group at the same time. After the 10th day, a high mortality rate in the rotenone treated group can be seen due to drug toxicity, and on the 16th day the last animal died. The toxicity of rotenone seems to be due to severe hepatic injury (fat degeneration). The mean life span of the control group was 6 days and of the treated group, 12·7 days, Thus, there was a 110% increase of mean life span in the treated group.

Figure 2 shows the average mitotic fraction and mitotic phases per thousand cells in the two groups of 3 animals treated with a single injection the 7th day after transplant (3000 cells in each sample were counted for each animal). It can be observed that in the control group the mitotic fraction and the percentage of mitotic phases remain almost steady during the 6 hr of observation. The rotenone treated group shows a marked increase in the percentage of cells in metaphase at the 6th hr after the injection, and this increase in the main cause of the increase in the mitotic fraction at this moment. A discrete increase in prophases and a discrete decrease in telophases and anaphases are also observed in the samples of the rotenone-treated group.

Figures 1 and 2 represent the typical results of experiments realized under optimal conditions. Doses of rotenone less than 3 mg per kg of weight, subcutaneous injections of the drug, and shorter treatments show essentially similar results. Table 1 shows a dose-response relationship for the effects of rotenone against the growth of Ehrlich ascites tumors.

From our results it can be concluded that rotenone inhibits the growth of Ehrlich ascites tumors and increases temporarily the survival

Table 1. Dose–response relationship of the effect of rotenone on Ehrlich ascites tumors

Inoculum	Host	Treatment		Dose (mg/kg)	% Anti-tumor activity		Mean increase life span	No. of exp.
		Start day	Injection		By tumor weight	By packed cell volume	(%)	
10×10^6 cells i.p.	Swiss	2nd	daily, 8 days	2	66	67	83	2
10×10^6 cells i.p.	Swiss	2nd	daily, 8 days	3	74·8	83·3	110	3
10×10^6 cells i.p.	Swiss	2nd	daily, 8 days	4	83·9	68	100	2
3×10^6 cells i.p.	Swiss	2nd	daily, 16 days	3·5	100	100	100	2

Each experiment included 30 treated animals and 30 controls. Fifteen animals of each group were used for evaluation by tumor weight and survival time and the other fifteen for evaluation by total packed cell volume. Tumor weight was calculated by difference between inoculated controls and non inoculated rotenone treated animals (10 normal animals for each dose level). Rotenone did not cause weight loss at the used doses. The figures of antitumor activity and increase of mean life span are the average of 2 or 3 experiments as indicated.

time of the animals. The inhibition of growth seems to be due to the arrest of mitosis in metaphase.

Rotenone has been shown to be a very potent agent which blocks electron transport in the mitochondrial respiratory chain at site I of oxidative phosphorylation [2]. Therefore, its main action is the inhibition of respiration with NADH-linked substrates, as we reported for the anti-cancer agent VM–26 [1]. One of the effects of low doses of VM–26 is a transient arrest of mitosis in metaphase [3].

Meisner and Sorensen [4] showed *in vitro* that rotenone blocks mitosis in metaphase. Due to the lack of release of the mitotic inhibition with the partial release of respiration adding succinate, these authors estimated that the action of rotenone on mitosis did not appear to be related to its action on mitochondrial respiration. However, Tobey *et al.* [5], more recently, showed that several mitochondrial inhibitors such as rotenone, antimycin, oligomycin, dinitrophenol, prevent the completion of mitosis and specified that a respiration-linked process is required during mitosis for progress through metaphase. The antimitotic action of rotenone could, consequently, be explained by the inhibition of the production of the energy necessary for the metaphase–anaphase transition [5].

The high toxicity of rotenone and its low therapeutic index make it a poor candidate for further studies as a chemotherapeutic agent. However, the action of rotenone reported here indicates that an anti-cancer chemotherapy based on the inhibitors of NADH-linked respiration may be worth investigating.

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Proliferative Properties of Malignant Cell Populations Cultured in Intraperitoneal Diffusion Chambers

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Abstract—*The proliferative characteristics of four different types of malignant cells cultured in intraperitoneal diffusion chamber (ID-chamber) were investigated, and compared with the corresponding data obtained under conditions of cell culture* in vitro *and growth* in vivo. *The cell types were: BICR/M1R$_K$ cells, a permanent malignant cell culture line originating from a transplantable mammary tumour of the Marshall rat; Fortner A Mel* 1 *cells, a transplantable malignant melanoma of the golden hamster in the ascitic form; a transplantable ethylnitrosourea-induced leukemia (L5222) of the BD IX rat; and finally leukemic cells from two human patients with untreated acute myeloblastic leukemia.*

After an initial decline in the cell number, all cell types investigated proliferated in ID-chambers implanted into BD IX rats, for periods of 8–13 *days. The tumour cell populations grew exponentially for* 2–5 *days, with an actual doubling time corresponding to that obtained during growth* in vivo. *The human leukemic cells behaved as a "steady-state" system, where the rate of proliferation was apparently compensated by a corresponding rate of cell death. The rate of proliferation in ID-chambers was slower than under cell culture conditions* in vitro.

Ten min after intravenous injection of hydroxyurea (HU) into the host animals, the HU concentration in the ID-chambers had equilibrated with the blood concentration of HU, when measured on the third day after implantation of the chambers. On the 7th day, the rate of diffusion of the compound between the blood and the chambers was slower, probably due to an inflammatory reaction on the external side of the chambers.

The mode of proliferation of the investigated cell populations during diffusion chamber culture may be more representative of the normal growth conditions in vivo *than of those in cell culture* in vitro.

INTRODUCTION

THE PROLIFERATIVE properties of malignant cell systems and their dependence on host control mechanisms have been the object of extensive investigation under both *in vivo* and cell culture conditions. Although cell populations in culture *in vitro* are generally preferred for technical reasons, they evidently lack many characteristics of the highly controlled cell systems in the intact organism. Part of this disadvantage may be circumvented when cells are cultured in diffusion chambers implanted into the peritoneal cavity of laboratory animals (ID-chambers). These chambers are impermeable for cells, but their porous walls permit the exchange of diffusible substances between host and chamber via the peritoneal fluid. Various types of mammalian cells have been reported to proliferate in ID-chambers [1–12]. Since the cells tend to grow in clots formed within the chambers, precise cell counts have been difficult, until it was shown that proteolytic

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enzymes, such as pronase, could be used to dissolve the clots [8]. The ID-chambers now offer the advantage of a closed *in vivo* cell culture system where the cell number per chamber can be determined at any time.

The present report is concerned with the proliferative properties of different malignant cell populations in ID-chambers. The results are compared with the corresponding data obtained for the same cell populations growth both in the form of tumour transplants *in vivo* and in monolayer cell cultures. In addition, the water soluble agent hydroxyurea [13] was used as a model substance for measuring the diffusion kinetics between the blood of the host animal and the ID-chambers.

MATERIAL AND METHODS

Cell types

(a) *BICR/M1R transplantable rat tumour.* This

millilitres of blood from each patient were allowed to sediment for 30 min. The cells in the plasma-layer were then diluted to the desired cell concentration with culture medium.

Medium

All cell types were suspended in a modified Eagle's medium [22] supplemented with 10% calf serum.

ID-Chamber technique

A modification of the technique described by Nettesheim *et al.* [8] and Benestad [2] was used. The chambers were made of perspex rings (3 mm thick, internal diameter 10 mm) covered with Millipore filters (pore size 0·22 μm). A slightly conical hole in the perspex ring was used for pipetting the cell suspensions into the chambers; it was then sealed with a plastic stopper and a drop of Tensol Cement No. 6 (I.C.I., Welwyn Garden City, Herts., England). Before use, all chambers were tested [illegible]

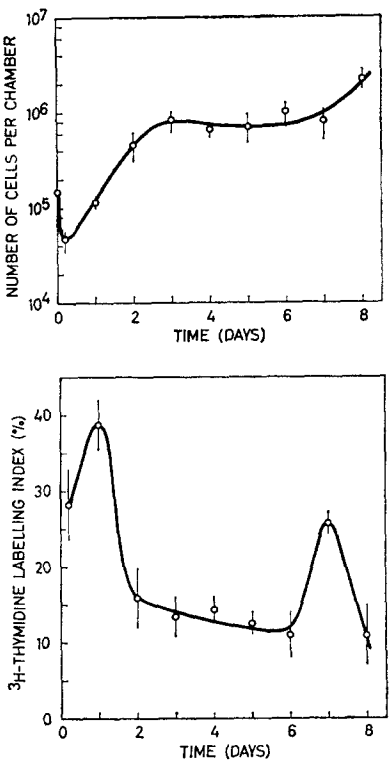


Fig. 2. (a) cell number/chamber, and (b) L.I. of hamster melanoma cells during ID-chamber culture in rats. Each point is the mean of 4–8 chambers (± S.E.).

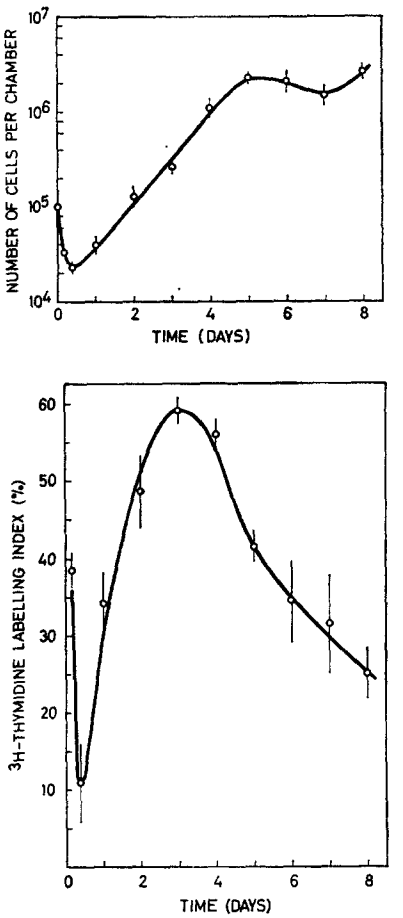


Fig. 3. (a) cell number/chamber, and (b) L.I. of rat leukemia cells from peripheral blood during culture in ID-chambers. Each point is the mean of 4–12 chambers (± S.E.).

made after suspending the cells in serum. They were air dried and fixed in methanol.

Autoradiography

Thirty minutes prior to sacrifice, the host animals were injected i.p. with 0·5 μCi of ^{3}H-methyl- thymidine (^{3}H-Tdr; specific activity 5 Ci/m mole; Radiochemical Centre, Amersham, England; diluted to 100 μCi/ml with 0·9% NaCl-solution) per g of body weight.

Autoradiograms of the cell smears were prepared by the "dipping" procedure, using Ilford K5 nuclear emulsion (Ilford Ltd., Ilford, Essex, England). The exposure time was 2 weeks. After developing, the autoradiograms were stained through the emulsion with Giemsa at pH 5·1. Based on background determinations, nuclei with ≧6 silver grains were scored as labelled. ^{3}H-Tdr labelling indices (L.I.; number of ^{3}H-labelled cells/total cell number) were determined on the basis of 300 counted cells.

Hydroxyurea measurements

Hydroxyurea (HU; Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.), dissolved in sterile 0·9% NaCl-solution, was injected i.v. into male host animals at a dose of 100 μg per g of body weight. At different time (10 min–3 hr) after injection, the animals were sacrificed. The chambers (5 from each animal) and 1 ml of heparinized blood from the caval vein were immediately placed on ice, and the concentrations of HU were determined as described by Fabricius and Rajewsky [23]. The chamber content was taken out without previous pronase treatment and measurements were performed on the pooled contents from 5 chambers.

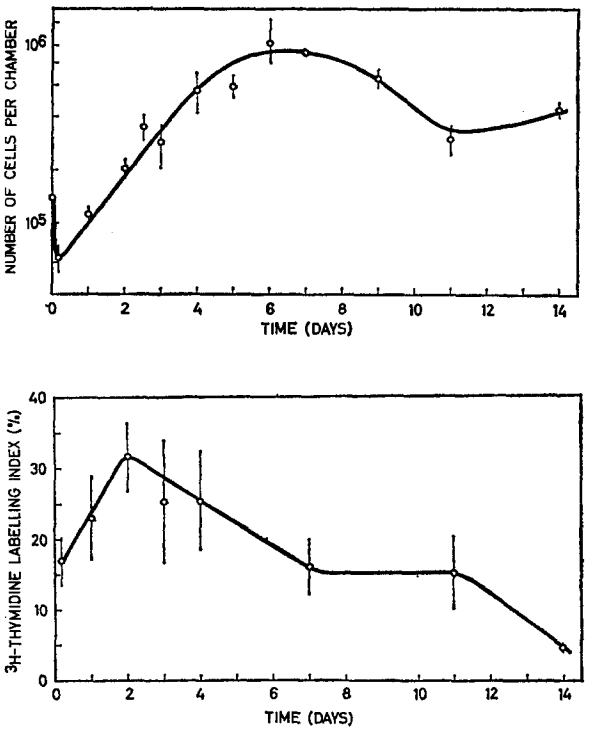


Fig. 1. *(a) cell number/chamber, and (b) L.I. of BICR/M1R$_K$ cells during culture in diffusion chambers. The first 7 points of the curve are means of* 8–23 *chambers, the others of* 4–5 *chambers (± S.E. indicated as vertical bars).*

Table 1. *Comparison between the proliferative characteristics of different cell lines in diffusion chambers,* in vitro *and* in vivo. t_D *refers to the actual doubling times, except for the BICR/M1R$_K$ tumour* in vivo, *where also the potential doubling time* ($t_{D_{pot}}$) *is given*

Cell type	Chamber t_D (hr)	Chamber L.I. (%)	*In vitro* t_D (hr)	*In vitro* L.I. (%)	*In vivo* t_D (hr)	*In vivo* L.I. (%)
BICR/M1R$_K$ tumour	29·5 (26·4–34·8)	32	13·2 (7·8–16·3)	39	48 $t_{D_{pot}}$ 29	36 [14]
Fortner A Mel 1 hamster melanoma	14·1 (8·7–17·6)	38			7–18	
L 5222 BD IX rat leukemia	16·0 (13·1–18·5)	58			14	42 [17]
Human blast leukemia						
Patient H	—	23			t_C 30–150	3·4–14·5 [28]
Patient D	—	5			P.F. 0·11–0·28	
BD IX rat NE-1 neurinoma	28·8 (23·5–33·7)	29	17·3 (15·2–19·3)	(27)		

The 95% confidence intervals for the t_D values are given in brackets. The t_D for the hamster melanoma cells is calculated from a curve [16].

The ^{3}H-Tdr labelling indices (L.I.) are maximum values observed.

References are given in brackets.

The ranges for cell cycle time (t_C) and Proliferative Fraction (P.F.), and L.I., observed for acute myelogenic leukemia *in vivo* are also shown in the right column.

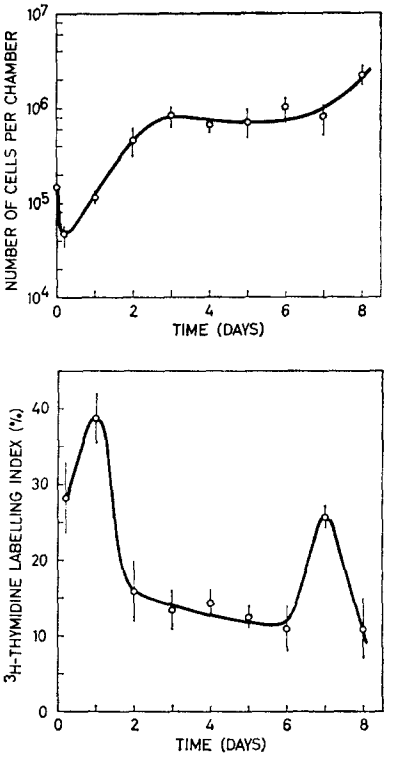


Fig. 2. (a) cell number/chamber, and (b) L.I. of hamster melanoma cells during ID-chamber culture in rats. Each point is the mean of 4–8 chambers (± S.E.).

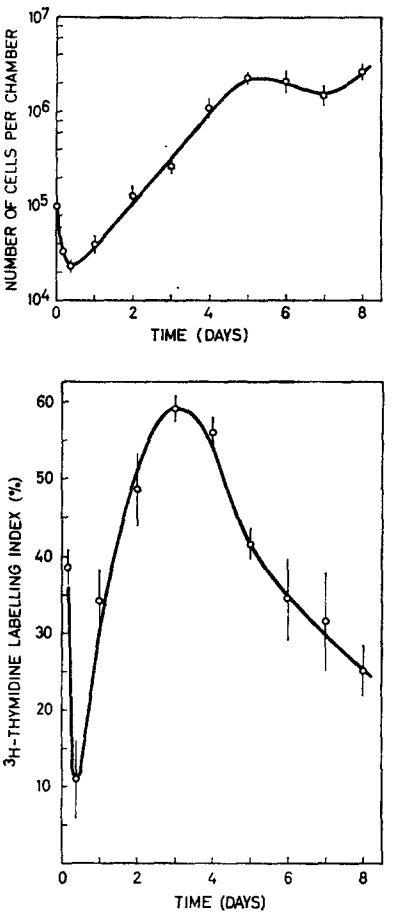


Fig. 3. (a) cell number/chamber, and (b) L.I. of rat leukemia cells from peripheral blood during culture in ID-chambers. Each point is the mean of 4–12 chambers (± S.E.).

Table 2. Saturation density of different tumour lines cultured in ID-chambers, in vitro, *or grown* in vivo

Cell type	Chamber/mm³	Number of cells *In vitro*/mm²	*In vivo*/mm³
BICR/M1R$_K$ tumour	$3{\cdot}9 \times 10^3$	$1{\cdot}4 \times 10^3$	$\sim 1 \times 10^5$
Fortner A Mel 1 hamster melanoma	$4{\cdot}3 \times 10^3$	*	$\sim 5 \times 10^5$
L 5222 BD IX rat leukemia	$11{\cdot}1 \times 10^3$	*	$\sim 5 \times 10^5$
BD IX rat NE–1 neurinoma	$8{\cdot}7 \times 10^3$	$1{\cdot}9 \times 10^3$ [27]	Not measured

The *in vivo* values refer to solid tumour tissue in the case of the BICR/M1R tumour, to the ascitic form in the case of Fortner A Mel a melanoma cells, and to the peripheral blood for L 5222 BD IX rat leukemia cells [14, 15]. The *in vitro* values refer to number of cells per mm² in Falcon plastic dishes of total size 21 cm².

*No data available.

RESULTS

The proliferative behaviour of the different cell types cultured in ID-chambers is shown in Figs. 1–5. Under appropriate experimental conditions, no significant differences were found in the cell yield per chamber from different animals at the same experimental point.

For BICR/M1R$_K$ rat cells, hamster melanoma and L 5222 leukemia cells, a similar pattern of proliferation was found: during the first 4–8 hr the number of cells per chamber was rapidly reduced to $\frac{1}{2}$ or $\frac{1}{3}$ of the original implant. The cells then proliferated approximately exponentially from the first until the 2nd or 5th day, depending on the cell type. At the same time, a high L.I. was observed. For L 5222 leukemia cells the maximum L.I. was nearly 60% (Figs. 3b and 7). The estimated doubling times (t_D) of the different cell types during exponential growth are shown in Table 1 together with the maximum L.I. values observed.

Cell proliferation then gradually leveled off, and different maximum values were reached for the cell number per chamber again depending on the cell type (Figs. 1–3). The saturation density of the different cell types in the ID-chambers is shown in Table 2.

When the maximum cell number was reached, all three cell types showed a declining, but still rather high L.I. This indicates that the rate of cell production is now balanced by a corresponding rate of cell death. After 8 days, the number of melanoma cells per chamber showed a further increase, preceded by an increased L.I. (Fig. 2). On the other hand, the number of BICR/M1R$_K$ cells per chamber gradually decreased and approximately 50% of the maximum value was observed on day 14.

After a few days of culture of melanoma cells an almost pure population of these large tumour cells was found, although the original cell suspension taken from the hamster had been contaminated with peritoneal cells and erythrocytes.

The number of inoculated human leukemic cells also dropped initially (Fig. 4). The cells from patient H then increased from the first to the second day, and thereafter remained at a constant value for 2 weeks. In the other case (D), the number of cells per chamber remained at a similar, but lower level during the same period. The H-cells had a maximum L.I. of 23% on the first day, while the D-cells never exceeded a value of 5% (see Fig. 8).

Cell passages between ID-chambers

L 5222 leukemia cells which had been grown in ID-chambers for 1 week were taken out, diluted with medium and inoculated into new chambers which were then implanted into new hosts. This procedure was repeated at weekly intervals. The L 5222 cells were thus easily kept for 6 passages without any apparent impairment of the cell yield. As many cells remained in suspension in the chambers, the

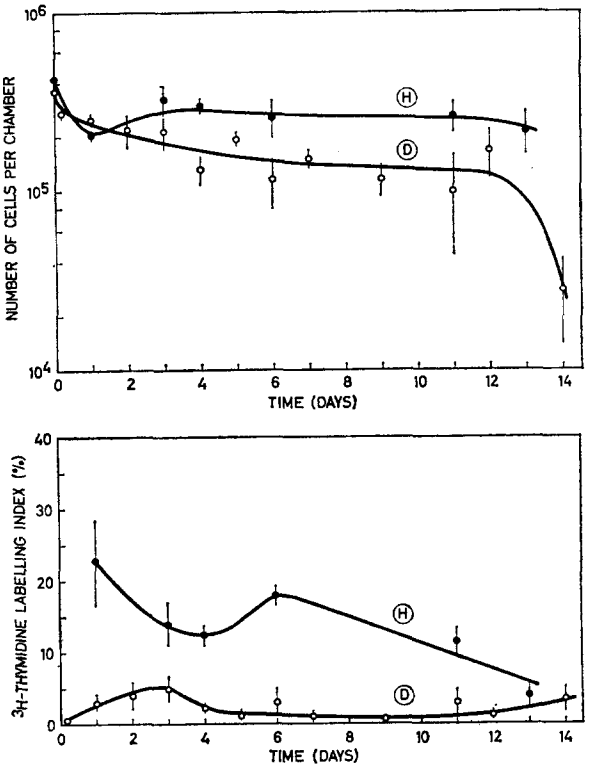


Fig. 4. (a) cell number/chamber, and (b) L.I. of leukemic cells from two patients with acute myeloblastic leukemia (H and D) during culture in ID-chambers. Each point is the mean of 4 chambers (± S.E.).

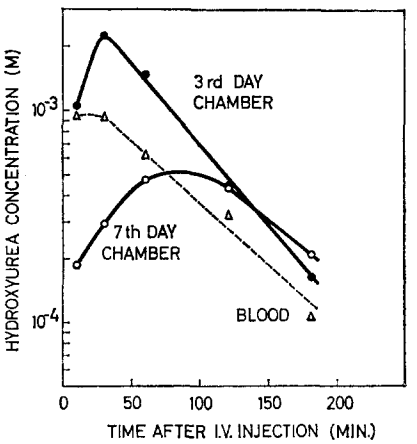


Fig. 5. The concentration of hydroxyurea (HU) in chambers and blood after a single intravenous injection (100 mg of HU/kg body weight) into the host animals at 3 and 7 days after implantation of L 5222 rat leukemia cells. Each point is the mean of 2–4 measurements, each representing a pooled sample from 5 chambers.

pronase treatment could be omitted when exact cell counts were not required.

Hydroxyurea determinations

Hydroxyurea (HU) was injected i.v. into male host rats on days 3 or 7 after the implantation of chambers containing L 5222 leukemia cells. On day 3 almost identical concentrations of HU were found in the blood and in the chambers, 10 min after the injection (Fig 5). For about 30 min, the concentration further increased in the chambers, but not in the blood. After 1 hour, the HU concentration in the blood and in the chambers decreased in a similar way. Between 0·5 and 3 hr after the injection of HU, the half-life ($t_{\frac{1}{2}}$) of the HU-concentration in the blood of male BD IX rats was about 50 min.

On day 7 only 20% of the blood concentration was found in the chambers at 10 min after injection. After 1 hr the chamber concentration reached the same level as in the blood. The subsequent decline of the HU concentration in these chambers was slower than in 3-day cultures.

DISCUSSION

In the present experiments, three different types of malignant cells have been shown to proliferate in ID-chambers. In all cases, a common proliferative pattern was observed, with an initial decline in the number of cells per chamber, followed by a phase of temporary exponential proliferation.

The rate of proliferation, as reflected by the L.I. and the population doubling time ("actual Doubling Time") was different for each cell type. When this is compared to the *in vitro* culture conditions, it is seen that the BICR/M1R$_K$ tumour cells grow considerably faster *in vitro* than in the ID-chambers, with an actual doubling time of about one half of the corresponding value in the ID-chambers (see Table 1). On the other hand, the actual doubling time of these cells in chambers was in very good agreement with the "potential" doubling time derived for the same cells when proliferating *in vivo* in the form of solid tumours (Table 1). This was measured by the labelled mitosis technique [24] in primary s.c. re-implants of BICR/M1R$_K$-cells into Marshall rats [14]. The actual doubling time of these tumours, however, was longer ($\sim$48 hr), due to a considerable degree of cell loss (death). This difference regarding the actual doubling time between the same tumour cells growing "accessibly" as tumours and "protected" in ID-chambers may in part be due to the lack of host immune reactions in the latter case. However, the larger size of a solid tumour as well as its dependence on vascularization make a direct comparison to a small chamber clot with free diffusion conditions difficult.

The actual doubling time of the hamster melanoma cells in the ID-chamber was of the same magnitude as that reported for the ascitic form [16]. However, a precise doubling time for these cells in ascitic form is difficult to measure exactly, as cells stick to the abdominal wall, and the release of cells into the ascitic fluid may not be constant.

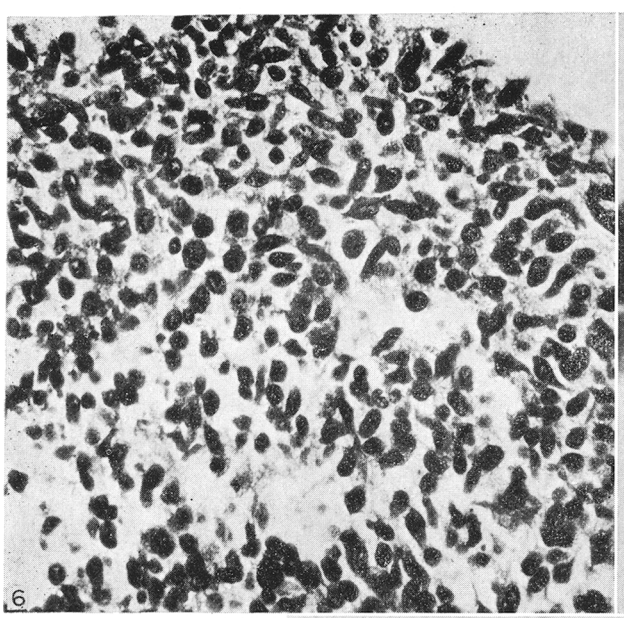
The L 5222 leukemia cells showed about the same population doubling time in the ID-chambers as in the peripheral blood. The lower L.I. observed in the latter case may be due to a contamination with lymphocytes which are difficult to distinguish from the undifferentiated leukemic cells. It is also a possibility that the proliferative fraction may be larger in leukemic bone marrow than in the blood. In a recent publication, Harriss and Hoelzer [20] reported a median cell cycle time of 12·6 hr for the L 5222 leukemia cells *in vivo*, which is compatible with the actual doubling time of 14 hr observed in our laboratory [17].

Similarly, murine granulocytic cells have a population doubling time of the same magnitude in ID-chambers as during exponential regenerative growth *in vivo* [25, 26]. On the other hand, in analogy to the BICR/M1R$_K$ tumour cells discussed above, cells from the transplantable BD IX rat neurinoma NE-1 have a longer doubling time in ID-chambers than in the form of a permanent cell line in culture [27] (Table 1). Unfortunately, normal and malignant cells derived from the bone marrow are dependent on the agar culture technique *in vitro*, from which kinetic data of our cells are not available.

From the available experimental data, it appears that the rates of cell proliferation in ID-chambers may be more comparable to the values observed under *in vivo* conditions than to the rates of proliferation found in the corresponding permanent cell lines *in vitro*.

Human leukemic blasts seem to behave differently from the other cell types cultured in rat hosts as they almost immediately exhibit the properties of a "steady-state" system (Fig 4). The L.I. in the case of patient H suggests a rather high rate of cellular proliferation which, in turn, must then be compensated by a rate of cell loss of similar magnitude.

On the other hand, a high L.I. does not



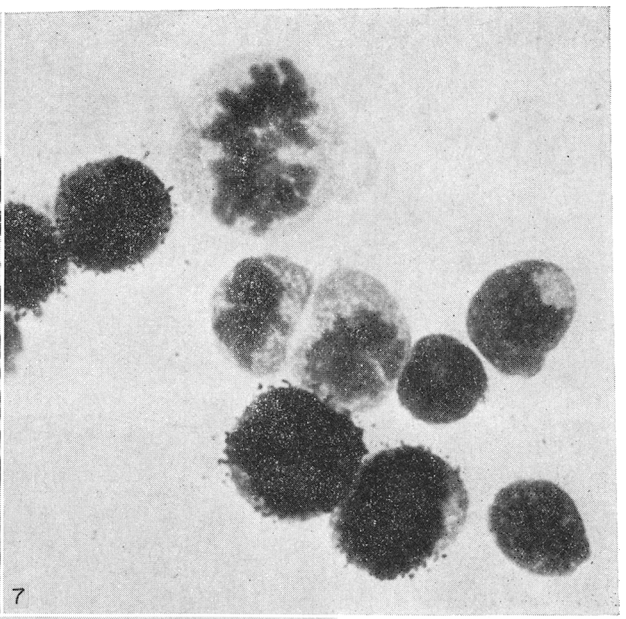


Fig. 6. *Histological section of BICR/M1R$_K$ tumour cells after* 5 *days of culture in ID-chambers. The cells grow in a loose, fibrinous clot; however, solid tumour clumps have also been observed. Haematoxylin-eosin stain, magnification* × 289.

Fig. 7. *Autoradiogram of a smear of L* 5222 *rat leukemia cells after* 3 *days of culture in ID-chambers, during the exponential growth phase. Giemsa stain. Oil immersion, magnification* × 733.

Fig. 8. *Autoradiogram of a smear of human leukemia cells after* 24 *hr of culture in ID-chambers. At this stage, the L.I. was* 23%. *Giemsa stain. Oil immersion, magnification* × 733.

(to face p. 538)

necessarily reflect an equally high rate of proliferation, since a long S-period relative to the cell cycle time will also increase this index. However, the interpretation of these results is complicated by the fact that leukemic cells may exhibit great variations in the rate of cell proliferation as well as in the durations of the different periods of the cell cycle [28, 29] (Table 1).

In the case of very long cell cycle times, even a slight increase in the rate of cell loss might be sufficient to outweigh the rate of cell proliferation. For some cell types in ID-chamber culture this may be overcome by various procedures to stimulate cell proliferation. For example, normal as well as leukemic lymphoblasts proliferate rapidly in chambers following PHA stimulation [30], while the culture of human bone marrow cells in chambers apparently requires the use of irradiated host mice [31].

Our experiments show that the concentration of a small molecule such as HU, which is freely diffusible in the body, rapidly equilibrates between the blood and the chambers (Fig. 5). Thereafter, for unknown reason, a temporary accumulation of the substance was seen in the chambers. The following elimination of HU occurred with the same rapidity as in the blood, but came after some time lag. This time lag could be due to the fact that since HU is mainly catabolized by the liver [23], the substance first has to diffuse back to the blood before being catabolized.

However, while on the third day after implantation, equilibration between blood and chamber was observed at 10 min after injection, the diffusion of HU in both directions was retarded on the 7th day. This is probably due to the inflammatory reaction on the external side of the chambers, which is always seen after 5–6 days of culture.

On the other hand, it appears that ID-chambers never become entirely impermeable for diffusible peritoneal substances, since cells may remain viable for very long periods in the chambers [4, 5, 9]. This was particularly evident in the case of the human leukemic cells investigated, which could be recovered from the chamber for up to 33 days after implantation (not shown in Fig. 4).

Similar to our experiments, Johnson *et al.* [6] observed a high L.I. of rat lymphocytes 10 min after i.p. injection of ^{3}H-Tdr on the 2nd day after implantation of the ID-chambers. Furthermore, Amos and Wakefield [32] refer to experiments by Urbach, indicating that when host animals breathe pure oxygen, the oxygen tension in the peritoneal cavity rises almost immediately, and 5 min later within the chambers. Approximately one hour is required for the concentration of large molecules such as antibodies to equilibrate with the interior of ID-chambers after i.p. injection [32].

From these data it is apparent that diffusion across the chamber membranes is very rapid during the first days after implantation, a period which coincides with the time of exponential growth of the cells (Figs. 1–3). This underlines the possibility of studying the effects of exogenous agents on cells in ID-chambers via i.v. administration to the host, provided that the dependence of the rate of diffusion on the age of the chambers is taken into account.

Although at 3–5 days the rate of diffusion is still high, the cell number per chamber reached a maximum at this time. However, the cessation of proliferation was reversible at least for 7 days, as the L 5222 leukemia cells could easily be subcultured into new chambers without any decrease of cell production.

The maximum cell density was different for the individual cell types investigated (Table 2), and this may depend on the size of the cells as well as their different nutritional requirements. The fact that all the cell types exhibited a lower density in ID-chambers than under conditions of "free" growth *in vivo* also suggests that the conditions of chamber culture as such are a limiting factor.

On the other hand, it is also possible that the cessation of proliferation in the chambers may in part be caused by specific inhibitors ("chalone"-like factors). Although such substances have mostly been studied in normal cell populations, their existence has been reported for ascites tumour cells [33] as well as for leukemic cells [34]. Possible factors and mechanisms involved in the control of cell proliferation in cell cultures and under *in vivo* conditions have recently been reviewed [35, 36].

We conclude that the malignant cell lines used in these experiments are able to grow exponentially in diffusion chambers and that their proliferative parameters during this type of *in vivo* culture are more representative of the normal *in vivo* conditions that of those observed in cell cultures *in vitro*.

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Experimental Studies on the Uptake and Retention of Labelled Proteins in a Rat Tumour*

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Abstract—*The initial uptake and later retention of labelled albumin and fibrinogen was studied in a transplantable rat tumour and compared to various normal tissues of the rat. A high uptake and retention of both proteins was found in tumour as compared to normal tissues. This mechanism is probably based on a high capillary permeability of the tumour capillary wall also for large protein molecules.*

INTRODUCTION

THE UPTAKE of intravenously administered fibrinogen in tumours has, until recently, been suggested as due to a local conversion in tumours of fibrinogen to fibrin by a high thromboplastic activity of tumour tissue [1]. This mechanism has also been discussed and proposed to be applied to the diagnosis and therapy of malignant tumours and metastases [2, 3].

However, the results obtained in a recent series of experimental studies on the initial uptake in transplantable rat tumours of fibrinogen and other proteins with varying molecular size suggested a passive leakage of proteins into the extravascular space of tumours [4, 5, 6]. This mechanism of a passive equilibration of labelled proteins in tumour tissue seemed to be based on a high permeability of the tumour capillary wall, also for large protein molecules. Furthermore, a large extracellular space was found in the experimental rat tumours compared to various normal organs of the rat.

The experimental observations of a high permeability of the tumour capillary wall and of a large extracellular space in tumour tissue might be of importance for the accumulation of different agents to tumour tissue in the diagnosis and therapy of malignant tumours. However, from this aspect, the accumulation of active agents would not only depend upon the initial uptake but also upon the retention in the tumour.

Thus, the aim of the present study was to follow the initial uptake and the later retention of two labelled proteins with different molecular size (albumin and fibrinogen) in a transplantable rat tumour as compared to different normal tissues of the rat.

MATERIAL AND METHODS

Animals and tumours

Inbred rats from a hooded strain, 3–4 months old and with a mean weight of 200 g were studied. A 20-methylcholanthrene induced sarcoma in its 43rd and 45th transfer generation, propagated and transplanted intramuscularly into one hindleg of the animals, was studied about 2 weeks after transplantation and with a mean tumour diameter of about 10 mm.

Labelled proteins

Each animal was injected intravenously in a tail vein with the radioactive tracer in saline.

Fibrinogen. Homologous rat fibrinogen (M.W.: 340·000) was prepared and labelled with ^{125}I

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as described previously [1]. Each animal was given 0·6 ml of the fibrinogen solution corresponding to 2 mg of fibrinogen and 10 μCi ^{125}I. The clottable activity of the labelled fibrinogen was 85% and the $t_{\frac{1}{2}}$ about 20 hr.

Albumin. For practical reason *human* albumin (M.W. : 69·000) labelled with ^{125}I (^{125}I-RISA) was used. Each animal was given 0·5 ml of the labelled albumin solution containing 10 μCi ^{125}I. The $t_{\frac{1}{2}}$ for the human albumin was about 15 hr.

Radioactivity measurements

Measurements of isotope in blood or tissue specimens were made in a well-scintillation counter with a one channel pulse analyzer. At least 10^4 counts were registered. Due correction was made for background activity.

Registration of results

The activities in counts/min per ml blood and counts/min per g tissue were calculated. Hematocrit was used to calculate the activity per ml plasma assuming zero activity in red blood corpuscles. Since in each experiment all animals were given the same dose of isotope and since animal and tumour weights were slightly varying, the data obtained are first presented as ratios between the activity per g of tissue and the activity per ml of plasma. This gives the retention of activity in tissue in relation to plasma at different sampling times. To get an estimation of the uptake in relation to the injected amount of labelled protein, the activity per ml of plasma at 1 hr after injection was set to 100%. The activities per ml of plasma or per g of tissue at later sampling times were then related to that arbitrary value.

EXPERIMENTS

Procedure

Thirty animals, transplanted with the 43rd transfer generation, were injected with ^{125}I-RISA, and thirty animals, transplanted with the 45th tumour transfer generation, were injected with ^{125}I-labelled rat fibrinogen. In each experimental group, 5 animals respectively were sacrificed by bleeding under ether anaesthesia 1, 8, 20, 48, 72 and 96 hr after isotope injection. One millilitre of blood and biopsies from macroscopically vital tumour tissue, muscle, liver, spleen, stomach, upper small intestine, large intestine and lower thoracic vertebrae ("bone-marrow") were taken. The activity in blood and tissues was registered and calculated as counts/min per ml of plasma and counts/min per g of tissue. In registration of bone marrow activity it was not possible to make any correction for solid bone tissue weight.

Results

The ratios between the activity/g of tissue and the activity/ml of plasma are given in Fig. 1. Mean values ± 1 S.D. (N = 5) are presented. The extracellular volume (ECV–ml/g tissue) in tumour and normal tissues, as determined in a previous study [5] by ^{51}Cr-EDTA, and the plasma volume (PV–ml/g tissue), as determined in the same study by early tissue/plasma ratios of albumin, are included in the diagrams.

The increase of the ratios during the experiments indicate an equilibration of labelled proteins with the extravascular space. In tumours a rapid equilibration was found during the first 20 hr. A further increase of the ratio was found for fibrinogen after 72 hr with values approaching the ECV ratio.

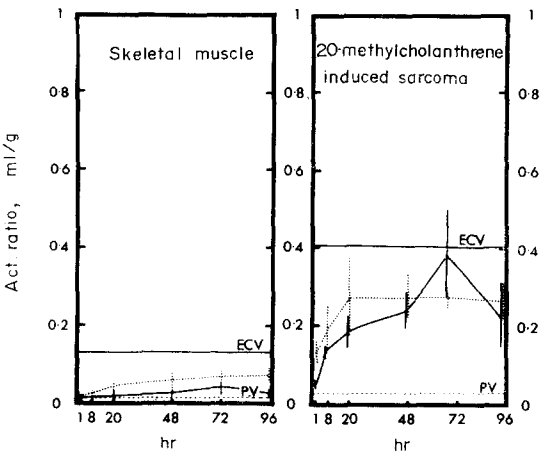

Fig. 1A.

Compared to tumour the equilibration of both proteins with the extravascular space of muscle was slow. In specimens from the gastrointestinal tract, high ratios were found especially in the stomach. In this organ, the albumin ratios exceeded and the fibrinogen ratios nearly reached the ECV ratio. Comparatively, high ratios were also found in liver and spleen while the equilibration in the bone marrow was low. (The ECV ratio in liver is not possible to estimate by ^{51}Cr-EDTA, since this isotope seems to be excreted to some extent in the bile [5].)

The plasma activity/ml at 1 hr is arbitrarily set to 100% in Fig. 2. Based on this plasma activity, the remaining activity per g of tissue in the tumour, small intestine, and muscle was calculated. As can be seen from the figure, more than 5% of the initial plasma activity remained in

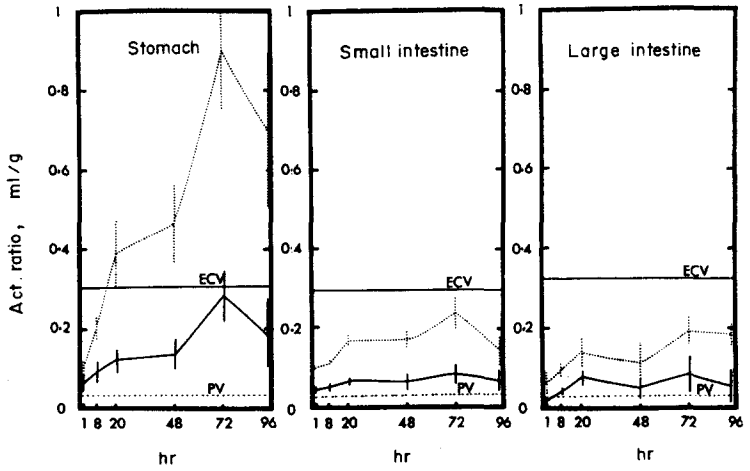

Fig. 1B.

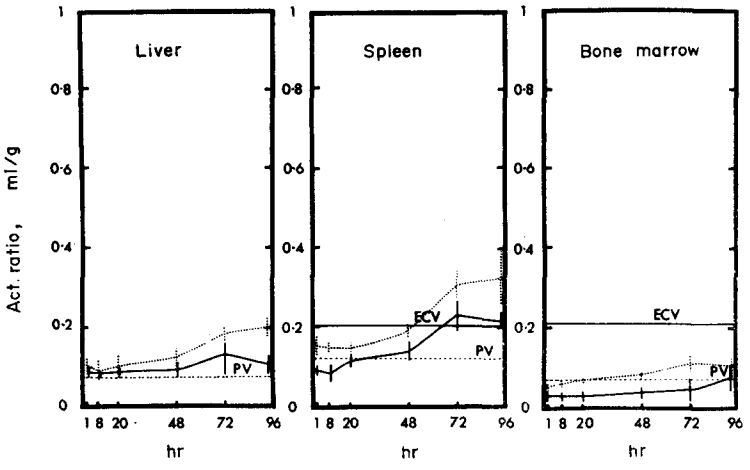

Fig. 1C.

Fig. 1. *Tissue/plasma ratios in tumour and normal tissues* 1, 8, 20, 48, 72 *and* 96 *hr after intravenous injection of labelled albumin (dotted lines) and labelled fibrinogen (unbroken lines). The ECV and the PV, as determined in a previous study* [2], *are included. All data are based on mean values from* 5 *animals.*

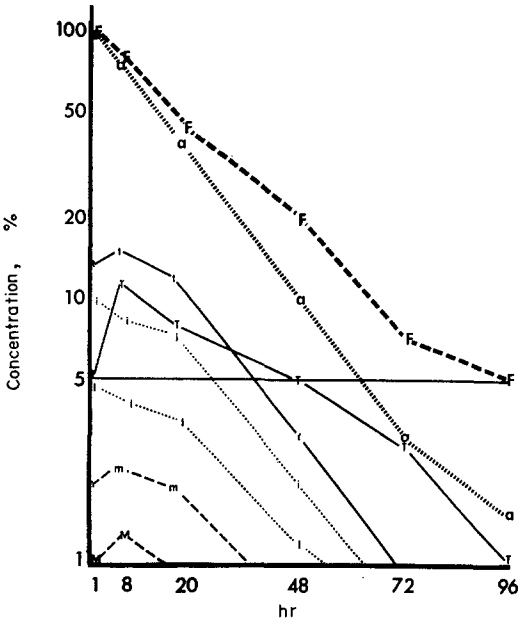

Fig. 2. *Labelled albumin (a) and fibrinogen (F) activity per ml of plasma* 1, 8, 20, 48, 72 *and* 96 *hr after intravenous injection with the activity registered after* 1 *hr given as* 100%. *Tissue concentration of albumin (small letters) and fibrinogen (capital letters) in tumour (t,T), proximal small intestine (i,I) and muscle (m,M) in per cent of the* 1 *hr plasma activity.*

the tumour for about 48 hr, while the activity in the normal tissues was comparatively low and decreased rapidly. Thus, after an initial equilibration period, the activity of labelled proteins in all tissues decreased in proportion to the plasma activity at the same registration time.

DISCUSSION

A fast permeation of labelled albumin and fibrinogen was previously found in experimental tumours. A similar initial increase of ratios for fibrinogen and albumin in the tumour supported the hypothesis of a passive leakage of both proteins into the extravascular space of the tumour [4, 5, 6]. In the present study, the activity ratios for proteins in the tumour remained unchanged from 48 to 96 hr, which further supports a passive equilibration between tumour and plasma. When related to the initial plasma concentration, the activity of labelled protein per g of tumour was comparatively high up to 48 hr after isotope injection. A cellular absorption and even intracellular uptake in tumours of labelled albumin, reported in some experimental studies, [7, 8] has not been verified for the tumour used in this study [5].

The activity in normal organs increased comparatively slowly and did not reach the same levels as in the tumour. However, extremely high ratios for albumin was found in the stomach. This is most probably explained by albumin being metabolized in the stomach and proximal intestine [9] and, thus, by iodine activity in split products in specimens from this organ. The metabolism of albumin might also explain the high ratios in liver and spleen, which could be based on an uptake of split products in the reticulo-endothelial system of these organs. Such a mechanism might also explain the high fibrinogen ratio after 72 hr in the spleen.

Thus, the present study supports the suggestion of a passive equilibration of large protein molecules with the extravascular space of experimental tumours due to a rapid equilibration with the ECV and to a large ECV. The tissue activity of labelled proteins was comparatively high up to about 48 hr after injection. This activity concentration was then passively following the plasma activity. The biological $t_{\frac{1}{2}}$ for both proteins was short, which together with a rapid tumour–plasma equilibration should counteract a sustained high extravascular concentration.

The possibility of maintaining a high tissue concentration of large molecules by the utilization of the unspecific mechanism of passive exchange between plasma and the extravascular space of tumour during a prolonged period, without interfering with normal tissues, might be used in diagnosis and therapy of malignant tumours. This is, however, not only correlated to a high capillary permeability and a large extravascular space in tumours, but is also highly dependant upon the possibility of maintaining a high plasma concentration during a prolonged period.

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Acid Phosphatase Activity in the Liver of Mice with Transplanted Leukaemia

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Abstract—*The activity of acid phosphatase, used as a lysosomal marker enzyme, was studied in the livers of mice 2, 4, 6, 8 and 10 days after inoculation of a strain-specific lymphatic leukaemia. Total and unsedimentable activities of the enzyme were assayed in whole homogenates of the liver, whereas a lysosome-rich preparation obtained by gradient centrifugation was used for measurements of total and free activities.*

Unsedimentable and free activities increased 4 days after inoculation of leukaemic cells. Thereafter, the unsedimentable activity remained high until death 10 days post inoculation, while the free activity decreased to the control values on day 6 and again increased on days 8 and 10. On the other hand, total activity of the enzyme, both in homogenates and in the lysosome-rich preparations, decreased slightly but progressively until death.

These alterations were interpreted as indicating an in vivo *release of acid phosphatase in the liver as a response of liver reticuloendothelium against leukaemic cells, and progressive infiltration of this organ with lysosome-poor leukaemic cells.*

INTRODUCTION

LONG-TRANSPLANTED leukaemias probably meet feeble resistance by syngeneic hosts, since selection pressure during repeated transfers has reduced their antigenicity [1]. It is possible, however, that the tissues and organs invaded by leukaemic cells resist this infiltration by phagocytosis and activation of their lysosomes, rather than by specific immune mechanisms. Lysosomes of the leukaemic cells, on the other hand, do not appear to assist their infiltration and spread [2].

The present work was intended to provide information on the role of lysosomes in defence of the organism against malignant cells of low antigenicity. To this end, acid phosphatase, as a marker enzyme for the lysosomes, was studied in livers of mice at various stages of infiltration with cells of a long-transplanted lymphatic leukaemia.

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*Some of the data presented in this paper were included in V. Šverko's M.Sc. thesis.

MATERIAL AND METHODS

Animals

A lymphatic leukaemia, specific for A-strain mice, described earlier by Boranić and Tonković [3] was used in these experiments. Inoculation of 10^6 leukaemic spleen cells into the tail vein results in death with massively enlarged spleen and liver 9–11 days later. Infiltrates of leukaemic cells in the liver can be seen by microscopy 4 days post inoculation, although by the bioassay, i.e. by transfer of the tissues of inoculated mice to secondary recipients, the leukaemic cells can be detected in the liver as soon as 2 days post-inoculation.

Adult female mice of A strain weighing 18–20 g were inoculated with normal or leukaemic spleen cells suspended in physiological saline, and 8 from each group were sacrificed 2, 4, 6, 8 or 10 days later. The livers were removed as rapidly as possible, weighed, and washed thoroughly but carefully by flushing with an ice-cold solution containing 0·25 M sucrose and 1 mM EDTA, pH 7·0, until visibly free of blood. Livers of non-inoculated control mice receiving no cells were collected in the

same manner. During the experimental period the animals were supplied with food (commercial pellets) and water *ad libitum*.

Liver homogenates

All experiments were carried out on homogenates of 8 livers pooled as to provide enough material for fractionation and enzyme assays. Washed livers were cut into small pieces with scissors and were homogenized in 0·25 M sucrose containing 1 mM EDTA, pH 7·0, with 1000 rev/min strokes in a Potter–Elvehjem homogenizer.

Total and unsedimentable activities in homogenates of whole liver

The total activity was assayed under conditions designed to ensure complete release of enzymes from lysosomal structures. This was accomlished by performing the assay in the presence of 0·1% Triton X-100 according to Wattiaux and de Duve [4]. The reliability of this procedure was verified in specimens treated with repeated freezing and thawing instead of Triton X-100. On the other hand, this concentration of Triton X-100 did not inhibit enzyme activity under the conditions described.

The unsedimentable activity was determined in the supernatant after the homogenate had been subjected to high centrifugation force ($3{\cdot}6 \times 10^6$ *g*/min) so as to spin down all particulate matter. The enzyme assay medium contained the same concentrations of Triton X-100, substrate and buffer as for the determination of the total activity.

The ratio of unsedimentable to total activity served as a measure for *in vivo* release of acid phosphatase from lysosomes into the cytoplasm.

Lysosome-rich preparation

The whole homogenate of the liver (10% w/v) was fractionated by differential centrifugation according to the method of de Duve *et al.* [5]. Nuclei and cell debris were removed by centrifugation at 7,000 *g* (average field, g_{max} 1,000). The supernatant was centrifuged at 33,000 *g* (average field, g_{max} 3,300) to separate mitohondria, and further at 220,000 *g* (average field g_{max} 22,000) to obtain finally the lysosome-rich pellet.

Total and free activities in the lysosome-rich preparation

The lysosome-rich pellet was washed twice with ice-cold 0·25 M sucrose, resuspended in that medium, and assayed for total and free acid phosphatase activities as follows.

To determine the total activity, the lysosome-rich preparation was assayed in the presence of 0·1% Triton X-100. This concentration of the detergent was found beforehand to be sufficient for causing complete release of acid phosphatase from the lysosomes in lysosome-rich preparation.

Free activity was measured under conditions which permit quantitative measurements of the free enzyme and at the same time prevent the enzyme within intact particles from reacting with the substrate added. These requirements were met provided that the assay was performed in the presence of 0·25 M sucrose, with pH no lower than 5·0. The mixture was incubated at 37°C for 10 min.

The ratio of free to total activity (expressed as per cent) proved to be a reproducible parameter, and was used as an index of *in vitro* release of the acid phosphatase from the lysosomes.

Enzyme assay

Acid phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2) was assayed by the method of Berthet *et al.* [6]. Units of the enzyme activity are expressed as μmoles of inorganic phosphate liberated within 1 min per total wet weight of the liver.

RESULTS

After intravenous inoculation of 10^6 normal or leukaemic cells into mice, 8 animals from each group were sacrificed every second day for a period of 10 days. Animals dying spontaneously were discarded. Figure 1 summarizes changes in the wet weight of the liver, in total and unsedimentable activities of acid phosphatase in the liver homogenates, and in total and free activities of that enzyme in the lysosome-rich preparation. Data obtained in non-inoculated mice are indicated at zero time.

The first changes of the acid phosphatase activity, either in the whole homogenate or in the lysosome-rich preparation, were recorded 4 days after transplantation of leukaemic cells. The total activity in the whole homogenate and in the lysosome-rich preparation decreased for about 21 and 19%, respectively, below control values. After day 4 both curves declined slightly but steadily until the last day of observation.

On the other hand, the unsedimentable activity and the free activity of the enzyme began to increase as soon as 2 days post-inoculation, and reached their maxima on

day 4. Whereas the unsedimentable activity remained high until death of the mice, the free activity decreased to the starting level on day 6, and then rose rapidly again before death, i.e. 8 and 10 days post inoculation.

In mice inoculated with normal cells from the syngeneic spleen, only minor shifts were observed in all the parameters followed.

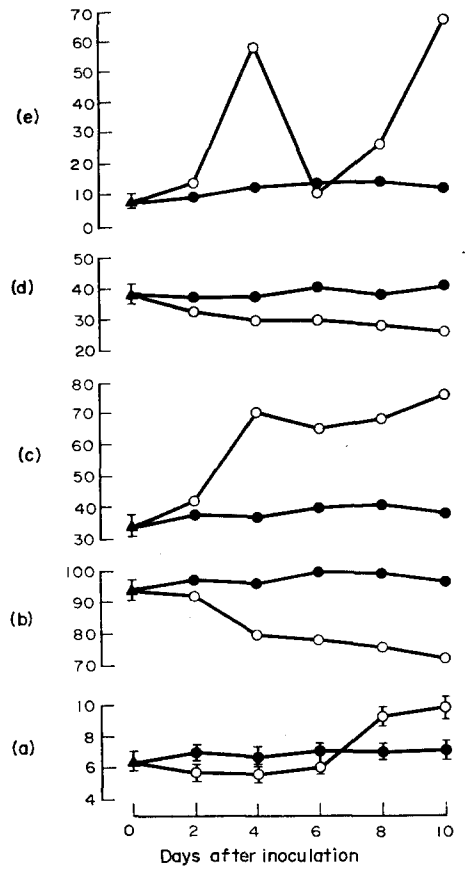

Fig. 1. *A-strain mice before* (▲) *and after inoculation of* 10^6 *normal* (●) *or leukaemic* (○) *spleen cells. a—liver wet weight* (% *of body weight); b—total acid phosphatase activity of the whole liver homogenate* (*units per total wet weight of liver); c—unsedimentable activity* (% *of total activity in the whole liver homogenate); d—total acid phosphatase activity of the lysosome-rich fraction* (*units per total wet weight of liver); e—free acid phosphatase activity* (% *of total activity in the lysosome-rich fraction). Each point represents the measurement of* 8 *pooled livers. Except values for relative liver weight which are given as means* ± *standard errors of the means for* 8 *animals, and acid phosphatase activities of inoculated mice* (*zero time*) *which are expressed as means* ± *standard errors of the means for* 3 *groups of* 8 *mice each.*

DISCUSSION

Responses of hepatic lysosomes to inoculation of normal and leukaemic cells were followed by measuring the activity of acid phosphatase, a lysosome marker enzyme, in liver homogenates and in lysosome-rich preparations from the liver. The method employed did not discriminate between various cells of the leukaemic liver, i.e. the enzyme as well the lysosomes studied may have originated from the parenchymal cells, Kupffer's cells, or the infiltrated leukaemic cells. The following discussion is based on the assumption that initial changes of lysosomal activity we observed refer to alterations in reticuloendothelial compartment of the liver, ie. Kupffer cells.

Marked increase observed in the unsedimentable and free activities 4 days after inoculation of leukaemic cells coincided with a steep rise of the blood leukocyte count, which occurs when leukaemic cells from the extravascular compartment enter the circulation (Tonković and Boranić, unpublished). This may indicate *in vivo* labilization of the lysosomal membrane and release of acid phosphatase into the cytoplasm of Kupffer cells, as a response of their lysosomes against circulating leukaemic cells. A similar process has been proposed by de Duve [7] to explain the degradation and removal of thymic lymphocytes after X-irradiation, and the alteration of cartilage ground substance after treatment with an excess of vitamin A [8].

The fall in free acid phosphatase activity found 6 days after inoculation of leukaemic cells could be attributed to regeneration of lysosomes. This supposition finds support in data of Paris *et al.* [9] who observed a reactive increase in the number of primary lysosomes and autophagic vacuoles in cells injured by X-rays. However, the rate at which the newly-formed lysosomal structures become engaged in lytic processes triggered by leukaemic cells seems to outweigh the rate of their generation. The persistent elevation of the unsedimentable activity from day 4 post-inoculation until death on day 10, and the terminal increase in the free activity on days 8 and 10, support this view.

Total acid phosphatase activity in the whole liver homogenate and in its lysosome-rich preparation decreased slowly after the inoculation of leukaemic cells. Maximum decrease was reached 4 days post inoculation and persisted until death. A possible interpretation of this finding might be that lysosome-rich cells of liver reticuloendothelium or even parenchyma were gradually depleted and replaced by lysosome-poor leukaemic cells. This idea agrees with data by Brandes *et al.* [9] who established by electron microscopy that leukaemic cells showed no reaction with stains specific for acid phosphatase. It also accords with data of Paris *et al.* [10] that the activities of lysosomal enzymes were lower in rapidly-growing adeno-

carcinomas than in the original glandular tissues, as well as with results of Allison *et al.* [11] showing that lysosomes tend to disappear from dividing cells. Our attempts to directly measure the activity of lysosomal enzymes in the homogenates of leukaemic cells have so far been unsuccessful due to technical difficulties.

In conclusion, the phenomena found for the acid phosphatase, as a reference enzyme for the lysosomes, suggest that further studies of other representative lysosomal enzymes may cast light on the role of lysosomes in defence against long-transplanted leukaemias and tumours of low antigenicity.

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Plasma Oestradiol and Progesterone in Benign Breast Disease

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Abstract—*The concentrations of plasma oestradiol and progesterone have been measured in women with benign breast disease and in a control group of ostensibly normal women.*

There were no significant differences in the steroid levels of the two groups in the follicular, ovulatory and luteal phases of the menstrual cycle. The hormone levels were not related to any of the histological diagnoses made on tissue obtained at biopsy from the women with benign breast disease.

INTRODUCTION

THE MAJORITY of diseases of the breast classed as "benign" can be divided into two main categories, those in which the lesion is confined to the lobules such as fibroadenoma and cystic disease, and those in which the ductal system is affected such as duct ectasia and epitheliosis. In many cases both types of lesion occur and it has been generally assumed that the proliferative nature of epitheliosis is due to hypersecretion of one or more of the hormones known to be involved in the development of the mammary gland.

Measurement of the endocrine environment associated with particular benign breast diseases is of interest since the results might give a further insight into the aetiology of the benign conditions, and also because there is considerable evidence that women with benign breast disease have a greater risk of subsequent breast cancer [1–7]. Studies on benign disease might therefore provide information about the role of the endocrine system in the genesis of breast cancer.

There is little published work on the hormonal status of women with benign breast disease [8–12], and these studies are confined to assays of urinary metabolites. Recently, methods have been devised for the measurement of oestrogens and progesterone in blood and since these estimations may reflect more closely the hormonal stimuli to the tissues, a study of the plasma concentrations of these compounds in normal pre-menopausal women and in patients with benign breast disease was undertaken.

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MATERIAL AND METHODS

The patients in this study had been admitted to the Guy's Hospital Breast Unit for the biopsy of lumps considered to be benign. The lesions seen in the histological study of this group included epitheliosis, adenosis, fibroadenoma, cystic disease, duct abscess and simple cysts. Blood samples were collected by venepuncture on the day prior to operation and the plasma stored at −20°C. The control group consisted of women living in Guernsey, none of whom had any history of menstrual disorders or breast disease. Both groups were within the age range 20–44 years, and the women in the control group were matched for age and the day of the menstrual cycle with the hospital patients; blood was collected and the plasma stored at −20°C.

Tissue specimens obtained at biopsy were fixed in formalin and embedded in paraffin wax. Sections (5 μm thick) were cut and stained with haematoxylin and eosin.

Plasma oestradiol was measured by a modification of the method of Corker [13]. ^{3}H-Oestradiol (2000 counts/min) was added to plasma (4 ml) which was extracted twice with dichloromethane (10 ml). The dried extract

was chromatographed on columns of Sephadex LH20 with benzene: methanol (85 : 15) as elution mixture. The oestradiol fraction was evaporated to dryness, dissolved in tris-hydrochloric acid buffer (0·01 M, pH 7·4) an aliquot taken for estimation of procedural losses and duplicate aliquots for oestradiol assay in the system described by Corker.

Plasma progesterone was estimated by a competitive protein binding assay which used diluted guinea-pig serum as the specific binding agent [14].

RESULTS

The plasma samples were collected at various times in the menstrual cycle. This makes exact comparisons between patients and controls difficult since there is considerable variability between individuals in cycle length and time of ovulation. The cycle has therefore been divided into three phases: the follicular phase (for plasma specimens collected between day 6 and day 12); the ovulatory period (day 13–day 17) and the luteal phase (day 18–day 26).

The plasma oestradiol concentrations of the controls and patients with benign breast disease in these three phases are shown in Fig 1. The plasma oestradiol concentrations of women with benign breast disease who were in the ovulatory and luteal phases are marginally lower than those in the control groups but the differences are not significant. The mean values are shown in Table 1.

The plasma progesterone concentrations in the two groups of women are shown in Fig. 2.

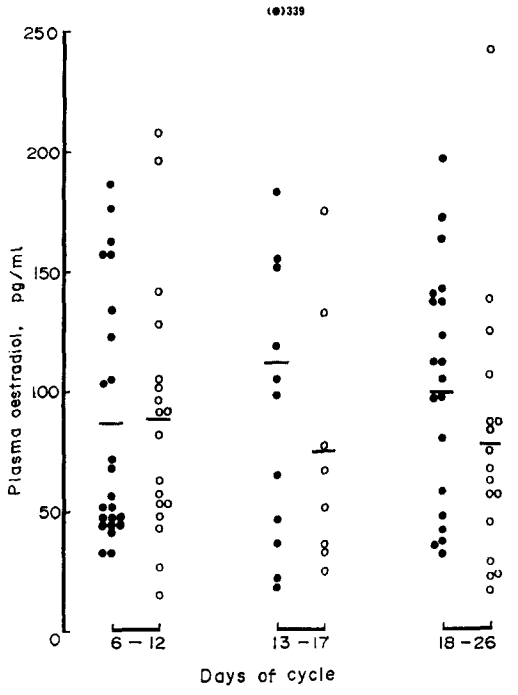

Fig. 1. *Plasma oestradiol concentrations during the follicular, ovulatory and luteal phases of the menstrual cycle.*
● *represents control group.*
○ *represents patients with benign breast disease.*
The horizontal lines indicate the mean values for each group.

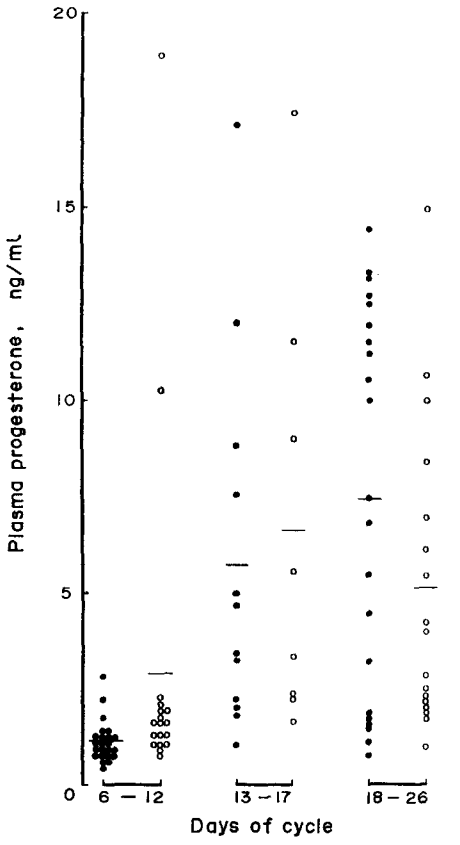

Fig. 2. *Plasma progesterone concentrations during the follicular, ovulatory and luteal phases of the menstrual cycle.*
● *represents control group.*
○ *represents patients with benign breast disease.*
The horizontal lines indicate the mean values for each group.

Table 1. *Plasma oestradiol concentrations in normal pre-menopausal women and in patients with benign breast disease*

Days of cycle	6–12		13–17		18–26	
	Control	Benign	Control	Benign	Control	Benign
No. of subjects	23	18	12	8	21	17
Mean concentration of plasma oestradiol (pg/ml) ± S.E.M.	87 ± 11	89 ± 13	112 ± 26	75 ± 19	100 ± 12	78 ± 13

Once again there are no significant differences between the plasma concentrations in the normal women and those with benign breast disease, although the latter have marginally higher progesterone concentrations in the follicular and ovulatory phases and lower concentrations in the luteal phase. The mean values are shown in Table 2.

A study of the histology of the benign lesions is in progress. Current results show that there were 15 women with marked epithelial proliferation, the lesion considered to carry the greatest risk of subsequent malignancy [3, 6]. The oestradiol and progesterone concentrations in these women were distributed evenly throughout the normal range of values. While further work is required it seems highly unlikely that this condition is associated with abnormalities in the plasma concentrations of the female sex hormones.

Table 2. Plasma progesterone concentrations in normal pre-menopausal women and in patients with benign breast disease

Days of cycle	6–12		13–17		18–26	
	Control	Benign	Control	Benign	Control	Benign
No. of subjects	23	18	12	8	21	17
Mean concentration of plasma progesterone (ng/ml) ± S.E.M.	1·17 ± 0·11	2·93 ± 1·06	5·76 ± 1·40	6·64 ± 1·98	7·48 ± 1·07	5·14 ± 0·95

Assays of plasma oestradiol and progesterone in a single plasma sample taken in the luteal phase of the cycle do not provide conclusive evidence whether or not ovulation has taken place, and it is obvious that if the frequency of anovulatory cycles differed between the normal controls and the patients with benign breast disease, a comparison of mean steroid values in the two groups would lead to bias.

For the purpose of this paper, anovulatory cycles have been defined as those in which the luteal phase oestradiol concentration was less than 60 pg/ml coupled with a progesterone concentration of less than 3 ng/ml. By this definition 4 normal women and 5 patients with benign breast disease would be classed as having an anovulatory cycle.

When the results for such patients and controls are excluded the mean oestradiol and progesterone concentrations in the luteal phase of the normal women were 114 pg/ml and 8·93 ng/ml, respectively, compared with 91 pg/ml and 6·46 ng/ml in the patients with benign breast disease. The differences are not significant.

DISCUSSION

There are obvious limitations in a study in which single plasma specimens are taken in the menstrual cycle. In ideal conditions, serial samples would be much more informative. Nevertheless, the present results indicate that there are no discernible differences between the plasma oestradiol and progesterone concentrations in patients with benign breast disease and normal women.

In a study in which blood samples were collected from a large number of ostensibly normal women, 5 women have subsequently developed breast cancer. Plasma oestradiol was measured in the samples from these women and in controls with no evidence of breast disease, matched for age and day of the menstrual cycle. Preliminary results show no apparent abnormalities in plasma oestradiol concentrations in the pre-clinical phase of the disease [15].

It is generally believed that oestradiol is an important hormone in the genesis of human breast cancer [16]. However, in women with benign breast disease and in women who subsequently developed breast cancer, normal concentrations of plasma oestradiol and progesterone were found.

One explanation for these findings is that raised levels of plasma oestradiol are not a necessary factor in the aetiology of either benign or malignant breast disease. Alternatively, an effective imbalance in oestrogenic and progestational stimuli could be mediated by abnormalities in target cell response to normal peripheral concentrations of the relevant hormones.

The only convincing endocrine abnormality in patients with breast disease is a subnormal plasma level of androgen sulphates and of urinary androgen metabolites [17]. It would be tempting to suppose that oestrogenic and progestational stimuli to the breast are modified by androgen secretion and that deficiencies in androgen production lead to an abnormal

stimulus to breast tissue by plasma levels of oestradiol and progesterone that are within the normal range.

Whatever the final explanation of our results may be, the original concept of oestradiol as a putative breast carcinogen, which stems from the clear demonstrations of its role as such in rodents, appears less tenable in man.

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Composition of Leukemic Cell Populations in AKR Leukemia and Effects of Chemotherapy

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Abstract—*With the use of a cell sedimentation technique at* 1**g**, *the cytokinetic behavior of leukemic cells from the thymuses of mice with spontaneous AKR leukemia was determined. It was demonstrated that small sized cells were derived from the rapidly-proliferating larger sized cells and appeared to be in a prolonged* G_1 *or resting phase. However, from serial observations of* ^{3}*H-TdR specific activities and radioautography, these small cells appeared to be constantly renewed, and a significant fraction of these cells were subsequently observed as labeled non-viable cells. This continual transition of cells between compartments may represent one of the major modes of cell death, i.e. age related within the tumor cell population. The relatively high resistance of small cells to various cytotoxic agents was demonstrated.*

INTRODUCTION

AN UNDERSTANDING of tumor cell kinetics is essential if complete tumor eradication is to be achieved with cytotoxic agents currently available. As a tumor grows, the tumor cell population becomes heterogenous secondary to either an increased variability in cell cycle time and/or to an increased proportion of non-proliferative cells (decreased growth fraction) [1–5]. Both the number of tumor cells and this heterogeneity seem to be directly related to the difficulty with which large tumors can be successfully treated with anti-tumor drugs since the effects of such agents depend to a large extent on the proliferative state of the target cells. [6–9].

A cytokinetic analysis based on cell size in the leukemic cell population of spontaneous AKR leukemia has recently been reported [10]. It was shown in this experimental system that differences in cell size appeared to be closely related to proliferative status as previously reported in human acute leukemia [11, 12]. Since this animal model demonstrates many similarities to acute leukemia, especially with respect to the response to chemotherapy [13], it appeared important to gain further insight into the characteristics of the AKR tumor cell population and, particularly, into the effects of anti-tumor drugs in relation to the various cell sizes.

Accepted 14 May 1973.

MATERIAL AND METHODS

Animals and chemotherapeutic agents

AKR mice, 6–12 months of age with advanced spontaneous leukemia, were obtained through the Mammalian Genetics and Animal Production Section, National Cancer Institute. Body weight varied from 21 to 35 g (mean, 26 g). The size of thymus, spleen, and lymph nodes and their statistical variations in a large number of animals have been described elsewhere [14] For *in vivo* labelling studies with tritiated thymidine (^{3}H-TdR), only the larger thymuses were used for further study in order to provide more uniform and comparable leukemic cell populations. The thymuses thus selected varied from 380 to 1080 mg with a mean of 570 mg. Chemotherapeutic agents listed below were made available through the

courtesy of Dr. Harry B. Wood, Jr., Drug Development Branch, NCI.

^{3}H-TdR labeling studies and cell separation

Animals were given a single intraperitoneal injection of 3μCi/g of ^{3}H-TdR (specific activity 6·7 or 9·1 Ci/mM, New England Nuclear Corp.) at time 0, and then kept under standard laboratory conditions for up to 7 days until sacrifice. Single cell suspensions were prepared from the thymuses and incorporation of ^{3}H-TdR was determined by liquid scintillation counting, as described previously [10]. Radioactivity was expressed as counts/min per 10^6 cells. Approximately 1×10^8 cells were separated according to cell size by a sedimentation technique at 1*g* [15, 16] as described in detail elsewhere [10, 17]. Cells separated into 30 fractions were concentrated by centrifugation and slides were prepared for radioautography. A known number of cells was processed for liquid scintillation counting with the addition of 5×10^6 unlabeled cells as carriers. Radioautographs were exposed for 3 months in order to maximize detection of lightly labeled cells.

Chemotherapy experiments

Animals were examined prior to drug administration for degree of splenomegaly and lymph node enlargement, and clinical response was assessed by the reduction in size of these organs on palpation. Drugs were administered intraperitoneally in approximately 0·5 ml. Doses used for the various drugs were: vincristine (NSC-67,574) 1 mg/kg, cyclophosphamide (NSC-26,271) 75 mg/kg, prednisolone (NSC-9151) 30 mg/kg, 1,3-*bis* (2 chloroethyl)-1-nitrosourea (BCNU, NSC-409,962) 40 mg/kg, Palm O-ara-C (NSC-135,962) 150 mg/kg, cytosine arabinoside (Ara-C, NSC-63,878) 30 mg/kg. Animals were sacrificed 2 days after the single injection unless otherwise indicated. For the combination regimen of vincristine, prednisolone, and cyclophosphamide, the following schedule was employed: vincristine and prednisolone on day 0; cyclophosphamide and prednisolone on day 1; and cyclophosphamide on day 2. These animals were sacrificed on day 3, 5, or 11. In studies of Ara-C by repeated injection, the drug was given at intervals of 4–6 hr for 27 hr and the animals sacrificed 2 days after initiation of the treatment.

Since the number of cells surviving the treatment were, in most cases, too few for adequate separation and, in order to visualize clearly changes in cell size distribution, ^{51}Cr labeling was utilized. The surviving cells were labeled and separated after being mixed with untreated, unlabeled leukemic thymus cells. Labeled cells composed a relatively small proportion of the total cells separated so that the contribution of the labeled cells to the profile of the separated cells should be small (Table 1). Approximately 100 μCi of $Na_2^{51}CrO_4$ (Rachromate-51, Abbott Laboratories) were added to a 10 ml suspension of the cells from the treated

Table 1. *Relevant data on chemotherapy experiments*

Drug	Dose (mg/kg)	Time of sacrifice after initiation of treatment	Clinical response*	Thymus† (mg)	Surviving cells in thymus ($\times 10^7$)	*In vitro* ^{3}H-TdR labeling index (%)‡	% of ^{51}Cr labeled cells for separation§
Palm O-ara-C	150 × 1	23 hr	+	100	4·0	52·7	27·0
Ara-C	30 × 6 q6h	2 days	+ + +	40	0·7	5·4	6·8
Vincristine	1·0 × 1	2 days	+ +	210	2·0	16·3	13·3
Cyclophosphamide	75 × 1	5 days	+ + +	40	0·7	3·6	6·0
BCNU	40 × 1	2 days	+ + +	145	2·5	52·2	18·0
Vincristine and Prednisolone	1 × 1 30 × 2	40 hr	+ +	160	3·5	32·0	26·0
Vincristine Prednisolone Cyclophosphamide	1 × 1 30 × 2 75 × 2	3 days	+ + +	80	1·1	2·0	8·8
Ditto	Ditto	5 days	+ + +	50	0·13	1·7	2·0
Ditto	Ditto	11 days	+ + +	100	0·42	19·3	5·8

*Response to the treatment was classified primarily according to the degree of reduction in size of spleen and lymph nodes.

†The mean thymus weight of 73 untreated animals was 526 mg which yielded approximately $3·5 \times 10^8$ cells.

‡Labeling index of unseparated cells.

§[No. of ^{51}Cr labeled cells/No. of ^{51}Cr labeled cells + No. of untreated "carrier" cells] × 100.

animals and incubated for 25 min at room temperature according to the method described by Eyre *et al.* (18). Radioactivity in 3 ml aliquots of each fraction was determined using an auto gamma counter, Model 1085 (Nuclear Chicago). Radioautography was performed on slides prepared with the Cytocentrifuge (Shandon Scientific Co.) from each fraction according to the method described by Ronai [19], exposed for 2 weeks, developed and stained with Giemsa stain.

RESULTS

^{3}H-TdR labeling study

^{3}H-TdR uptake over a period of 7 days after a single injection of ^{3}H-TdR is shown in Fig. 1. Differences in specific radioactivity of

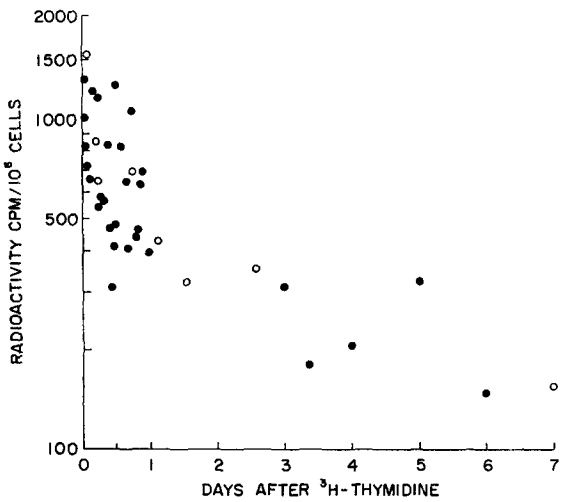

Fig. 1. Radioactivity per 10^6 cells after single i.p. injection of 3 μCi/g of ^{3}H-TdR, S.A.: 6·7 Ci/mM or 9·1 Ci/mM.

the radioisotope did not appear to affect the pattern of incorporation. However, variation in radioactivity among individual animals was of considerable magnitude in spite of the fixed experimental conditions. Radioactivity per cell appeared to follow an early exponential reduction up to 1 day; a more gradual decrease was noted during the rest of the period.

The successive profiles of specific radioactivity at various intervals after a single injection of ^{3}H-TdR are shown in Figs. 2 and 3. At 1·5 days, which corresponds roughly to 2 cell cycle times of rapidly proliferating cells, significant amounts of radioactivity remained in the small or medium sized cell fractions, although the highest activity appeared in the larger cell fractions. As the interval after labelling was prolonged, the radioactivity in the larger cell fractions decreased gradually and relatively high radioactivity in the small

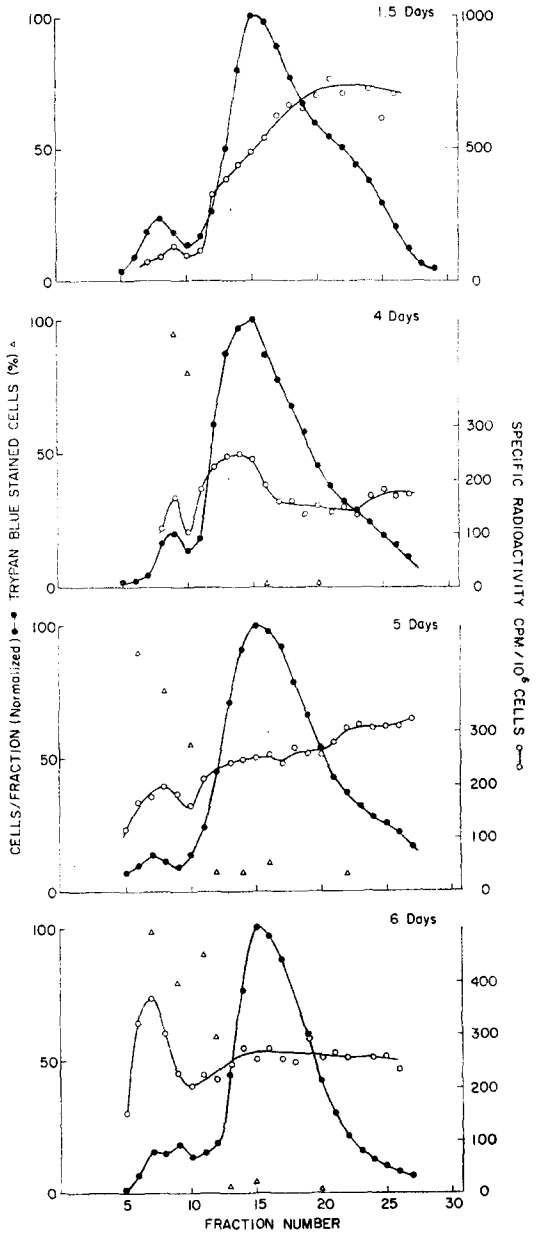

Fig. 2. Distribution of radioactivity at varying times after ^{3}H-TdR injection. Cell size correlates with increasing fraction number.

cell fractions became evident. In addition, the radioactivity in the earlier fractions (fraction 6–9, the minor cell count peak) appeared to increase gradually and consistently, and the highest relative activity was observed after 6 or 7 days. These fractions contained mostly dead cells and large cell fragments as indicated by trypan blue exclusion.

Radioautographic analysis revealed that relatively heavily labeled cells existed most frequently in the smaller cell fractions after 3 1/2 cell cycle times (2·6 days) when significant desynchronization of the cohort initially labeled had presumably occurred. The lowest frequency of heavily labeled cells occurred in the

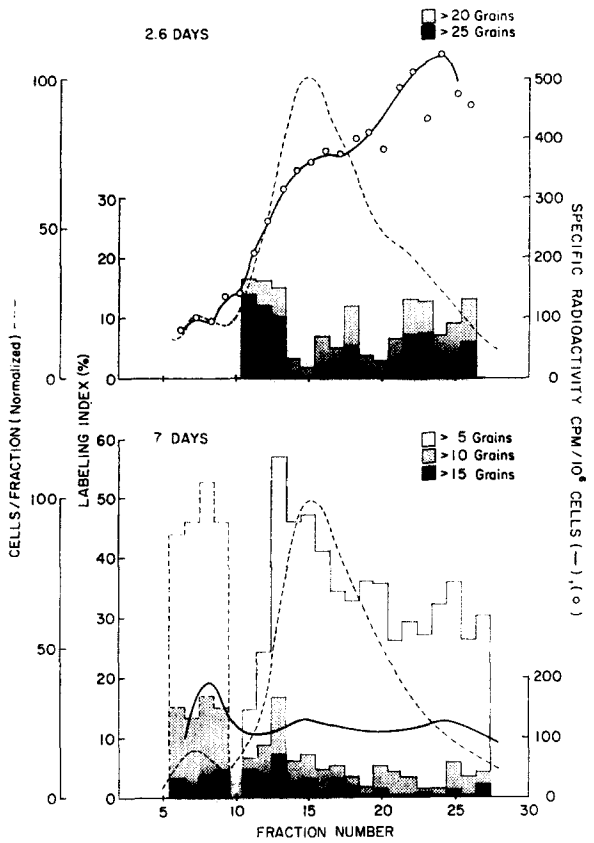

Fig. 3. ^{3}H-TdR labelling indices and radioactivity profiles at 2·6 days (A) and 7 days (B) after ^{3}H-TdR injection. Labelling indices of fractions 6–9 were performed on the "smudged" cells.

middle sized cell fractions (fractions 14–15) (Fig. 3A). This is consistent with a relatively high level of reutilization of isotope in the leukemic thymus and/or significant G_2 arrest.

A gradual shift of radioactivity became more evident after day 7 (Fig. 3B). The highest labeling index, as well as the highest frequency of heavily labeled cells, was attained in the small cell fractions. Cells in fractions 6–9, which were mostly dead cells and were probably the "smudged cells" on the slides, also showed a relatively high concentration of heavily labeled cells.

Chemotherapy experiments

Figure 4 shows the cell separation pattern and ^{51}Cr radioactivity distribution for the untreated leukemic cells. In this preliminary experiment, an aliquot of the cell suspension was labeled and then mixed with another aliquot (unlabeled) from the same suspension. The result indicates that the labeling procedures with ^{51}Cr do not affect cell size significantly and that the larger sized cells take up greater amounts of ^{51}Cr. In accordance with this, grain counts per cell were directly related to cell size; e.g. representative small, medium, and large cells showed mean grain counts of 7·7, 11·5, and 17·7, respectively. It is therefore concluded that the ^{51}Cr radioactivity

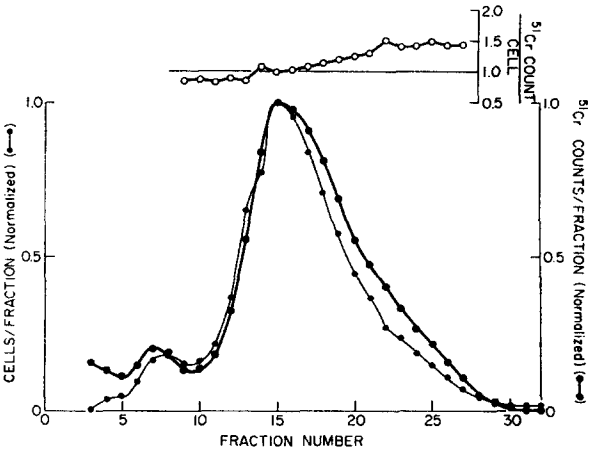

Fig. 4. Relationship between cells with varying sizes and ^{51}Cr labeling intensity. A portion of leukemic cells was labeled with ^{51}Cr before being separated along with the unlabeled cells from the same animal.

profile could be interpreted as an approximate representative of cell numbers present in the fractions with adequate corrections as indicated above.

In Table 1 are shown some relevant data on the chemotherapy experiments; the cell separation profiles for each study are shown in the figures (Figs. 5–7). Drugs with different modes of action were used singly or in combination. The dose schedules used are all known to be effective against AKR leukemia in terms of increased survival as reported by Skipper *et al.* [20]. The most tumoricidal regimens were adopted for combination therapy even though these might also be the most toxic in order to obtain the maximum drug effects on the leukemic cell population.

The cell size distributions of the surviving cell populations in these experiments are shown for the single (Figs. 5 and 6) and the combination treatments (Fig. 7) as represented by ^{51}Cr radioactivity profiles. The absolute ^{51}Cr counts on the ordinate are not of significance since the relative number of ^{51}Cr tagged cells to the total cells applied varied greatly from one experiment to the other depending on the availability of cells. Instead, the relative configuration of the profile reflects the perturbation of the population as a result of therapy.

All of these clinically effective treatments produced marked alterations of the profiles but with certain distinct characteristics depending

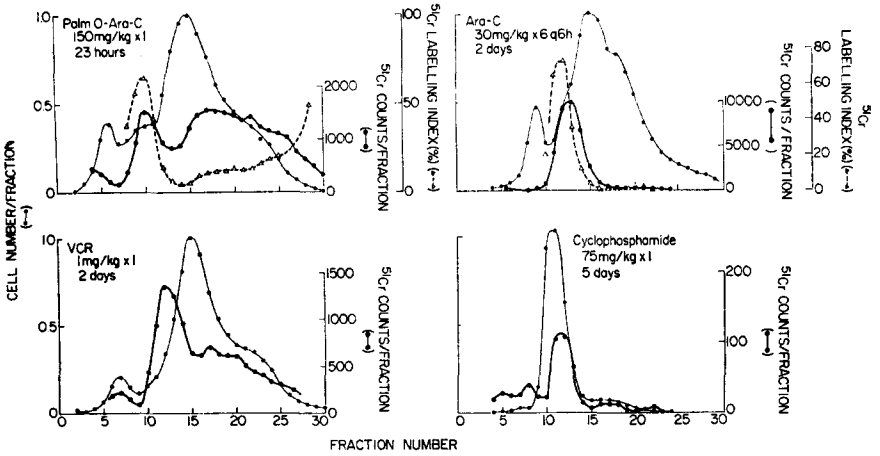

Fig. 5. Separation patterns of surviving cells after a single treatment with various chemotherapeutic agents. The ^{51}Cr radioactivity profile represents the cell size distribution of the surviving population in the treated animals. The lighter line is the cell distribution profile of carrier leukemic cells except for the cyclophosphamide chart where the profile of normal thymus cells is shown for comparison.

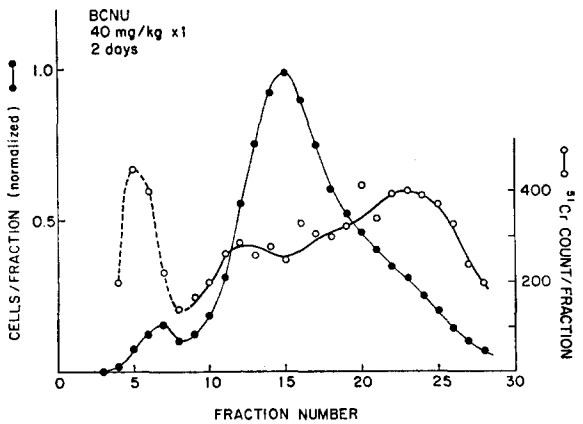

Fig. 6. Separation pattern of surviving cells 2 days after a single dose of BCNU. The cell distribution of leukemic carrier cells is illustrated by the lighter line.

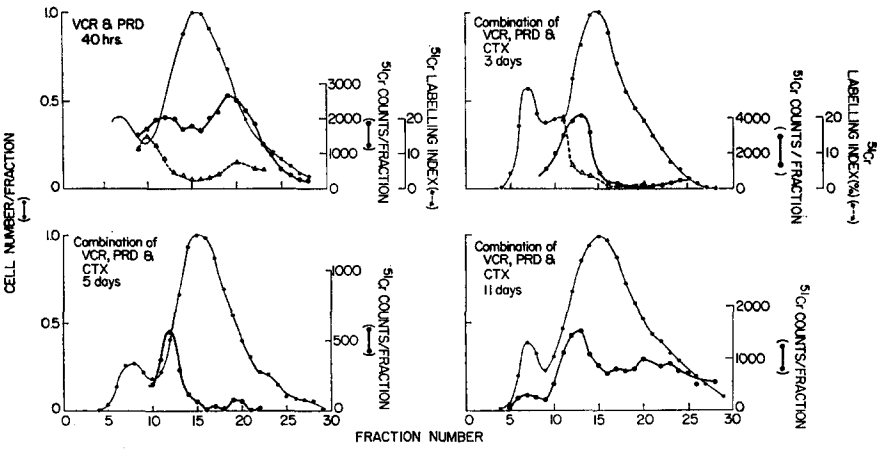

Fig. 7. Separation pattern of surviving cells after combination chemotherapy of vincristine, prednisolone, and cyclophosphamide. The lighter unbroken line represents the distribution profile of the leukemic carrier cells.

upon the type of drug employed. Palm O-ara-C resulted in marked reduction of the medium sized cells as reflected in the formation of a shallow trough between the large and small cells. The high labeling index was due to the presence of relatively abundant numbers of large cells with obvious morphological alterations but still capable of incorporation of ^{3}H-TdR *in vitro*. (Table 1). As reported in detail elsewhere [21], removal of the lethally damaged large cells leaves a prominent peak in the small cell fractions. The effect of multiple injections of Ara-C after a longer period of time (2 days) might be interpreted in the same way. Small cells along with some medium sized cells constituted the majority of the surviving population, and these demonstrated a marked decrease in ^{3}H-TdR labeling index. The results with vincristine were similar to those obtained with Ara-C and Palm O-ara-C in that all three agents demonstrated relative sparing of the small cell population. When administered along with prednisolone, vincristine resulted in accumulation of mitotically arrested cells which were seen microscopically to comprise the broad peak in the larger cell fractions (Fig. 7). It should be noted that although the cell size distribution observed is a reflection of the overall effect *in vivo* of drug treatment, it is also evident that this represents only a fixed segment of constantly changing, dynamic processes occurring within the leukemic cell population.

Cyclophosphamide, one of the most potent single agents against this animal model [19], resulted in the eradication of the vast majority of cells and left only a group of small cells slightly larger than the normal small thymus cells 5 days after a single injection. BCNU resulted in the relatively selective elimination of medium sized cells 2 days after treatment (Fig. 6).

A regimen of vincristine, prednisone and cytoxan, the most potent combination in AKR, capable of "cell cures" in a significant fraction of animals [20], killed most of the proliferating as well as non-proliferating cells at the time of maximum drug effect (Fig. 7, 3 and 5 days), although the relative resistance of the small cells was again observed. However, with recurrence of the disease after a maximum response, recognized clinically by the increasing size of the palpable organs, the restoration of the leukemic cell population was reflected by an alteration in cell size distribution (Fig. 7, 11 days). The larger cells had started to reappear and the ^{3}H-TdR labeling index increased. The reason for the persistence of peaks in the small cell fractions has not been clarified by the present investigation.

DISCUSSION

Based on the findings described here, together with the earlier observations [10, 17], a hypothetical cytokinetic profile of leukemic thymus cells in AKR mice is schematically illustrated in Fig. 8. This diagram depicts (1) the correlation between phases of the proliferative cycle and cell size achieved by the cell separation technique utilized, and (2) cytokinetic relationships between the proliferative and non-proliferative compartments as indicated by arrows. The observations that the small cells became labeled by multiple injections of ^{3}H-TdR after a prolonged period [10] and that radioactivity continuously shifted after pulse labeling towards the small cell fractions and eventually to the non-viable fractions suggest that the non-proliferating compartment is constantly renewed. The slow decrease of ^{3}H-TdR specific activity of the unseparated cells after the first day of rapid decrease is also compatible with the notion that transition of label to the non-proliferative compartment has occurred as the labeled cells transform into the smaller cells after division. This was also observed by radioautography. At the same time, the results of radioautography suggest that significant reutilization of label by the proliferating cells occurs within the leukemic thymus.

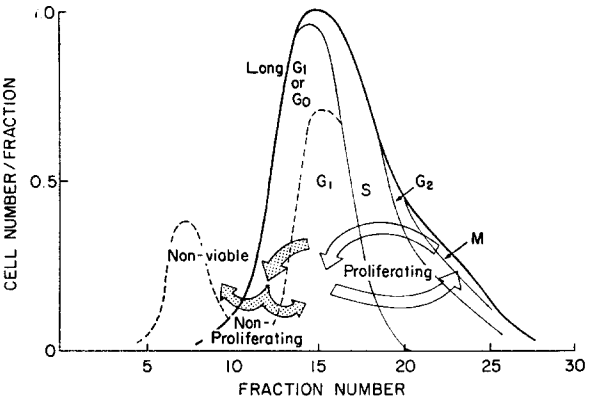

Fig. 8. Schematic illustration of the composition of the leukemic cell population in AKR leukemia. Arrows indicate movement of the cells within the population.

The significant role of cell loss in the growth of tumor cell populations has been demonstrated in human solid tumors [22–24] as well as in experimental tumors [1, 3, 4, 25]. Data are available from other studies both in this

laboratory and elsewhere to permit calculation of cell loss in spontaneous AKR leukemia. The mean pulse labeling index *in vitro* of 30 untreated leukemic animals was 34% [21] and the duration of DNA synthesis and cell cycle time has been estimated as 8 and 18 hours respectively [10]. The growth fraction, calculated as the ratio of the observed pulse labeling index to the fraction of the proliferating population in DNA synthesis [2], turns out to be approximately 75%. Schabel *et al.* estimated the tumor doubling time as approximately 5 days based upon observations of a large number of animals with advanced disease [26]. Using these estimates the cell loss factor as defined by Steel [22] was calculated to be 0·80. The cell loss might be ascribable largely to cell death within the population since cell loss due to other factors such as migration or metastasis is presumably much less significant [27]. Although the possibility of random death at any stage of the cycle has not fully been examined in this study, the results seem to be consistent with age dependent cell death through the continual transition of cells from proliferative to non-proliferative, and then to non-viable compartments. The major role of age related elimination as a mode of cell death has recently been described by Lala in Ehrlich ascites tumor systems [28]. Cell loss of this nature has also been incorporated into computer modeling of human leukemic cell populations [29], and the theoretical analysis of various types of cell loss has been reported by Steel and Hanes [30].

Alteration of cell size distribution due to chemotherapeutic agents seems to support the cytokinetic hypothesis proposed for AKR leukemia. Palm O-ara-C, Ara-C and vincristine have been classified as cell cycle specific agents, and cyclophosphamide and BCNU as cell cycle non-specific [31]. The cell cycle specific agents resulted in a marked reduction of proliferating cells with relative sparing of the non-proliferating cells in the small cell fractions. The relative resistance of small cells to treatment with cycle non-specific agents, or to the combination of both types was also demonstrated. It has been suggested by other investigators that these small cells might be less sensitive to various agents [7, 9, 32] including cycle-non-specific agents [7, 8, 33].

The proliferative capacity of the surviving cells has not been determined in these studies. A more detailed study with Palm O-ara-C suggested that cells with proliferative capacity which originally existed in the small cell fractions could be reduced markedly in number by intensive treatment for 3 days [21]. However, since the cells in the non-proliferative compartment constitute a major portion of the surviving cells after less intensive treatment and a significant fraction of these cells may still retain the proliferative capability, it is possible that these could, at least in part, be responsible for relapse. Our preliminary observations on human acute leukemic cell populations by a similar technique suggests the existence of analogous relationships among cells of varying sizes (G. P. Sarna, M. Omine and S. Perry unpublished observation) although there is a relative increase in the size of non-proliferative compartment. The importance of this type of cytokinetic characterization in the treatment of human disease remains to be determined.

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Antigenic Structure of Murine Mammary Tumour Viruses

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Abstract—*Rabbit antisera against purified standard murine mammary tumour virus (MTV-S) precipitate the virions of four different virus strains in double immunodiffusion: MTV-S, -L, -P and -O. The sera detect eight soluble antigens of MTV-S and -O, but only five of -P and three of -L. The effect of trypsin, pepsin, HCl RNAase, phosphilipase C and acridine orange on the appearance of precipitation lines of MTV-S antigens was investigated.*

On the basis of these results, it could be concluded that two soluble trypsin-sensitive antigens are located inside the nucleoid. They seem to contain some RNA fragments which do not influence antigenicity. They are group specific. Three other soluble antigens are membrane-constituents. Phospholipase C seems to increase their reactivity in immunodiffusion. They all contain a protein moiety which is essential for antigenicity. Two of the membrane antigens are involved in immunoprecipitation of the intact virion; one of them being group-specific.

INTRODUCTION

THE ANTIGENICITY of murine mammary tumour viruses (MTV) to natural and heterogeneous hosts has been firmly established by the induction of antibodies which can neutralize oncogenic activity or precipitate virus-specific antigens [see Blair's reviews 1, 2].

Up to now, five different soluble antigens of the standard virus-strain (MTV-S) have been described by Daams [3] and Nowinski *et al.* [4]. Their localization inside the virion was not completely clear. We have previously studied the structure of the MTV-S virion by treating the particles with various enzymes [5]. In an attempt to obtain some insight into the antigenic structure of MTV, it seemed worthwhile to test the sensitivity of MTV antigens to these enzymes.

MATERIAL AND METHODS

MTV Strains

The standard virus strain (MTV-S), with which most experiments have been performed, was isolated from the C3H mouse strain. The low oncogenic variant (MTV-L) was purified from C3Hf mouse strain materials, while the plaque-inducing virus (MTV-P) was derived from GR mice. MTV-O is a latent virus in BALB/c mice [6] which has been passaged in a virulent form in the same mouse strain [7].

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Virus isolation

Virions (B-particles) were isolated from mammary tumours according to Calafat and Hageman [8] utilizing ultracentrifugation of homogenates on a Ficoll-D2O discontinuous gradient followed by rate zonal centrifugation on continuous urografin or sucrose gradients. The quantity of purified virus was estimated by measuring the amount of scattered light by use of the fluorimetric attachment of a Zeiss PMQ II spectrophotometer at 365 nm. The purified virus contained 1 mg of virus protein per ml buffer (0·01 M Tris pH 7·0; 0·14 M NaCl).

Virus nucleoids were prepared by shaking a suspension of purified virus with 1 mg/ml Tween 80 for 30 min. An equal amount of ether was added and the mixture was shaken for 60 min. The emulsion was broken up by centrifuging for 10 min (1000 *g*). The water phase was washed with ether and the ether phase with buffer. The combined water phases were layered on top of a continuous sucrose or urografin gradient. The disrupted preparation was centri-

fuged for 14–16 hr on a continuous urografin or sucrose gradient in Spinco rotor SW 50 at 50,000 rev./min. The band at density 1·24–1·25 contains nucleoids, as seen with the electron microscope. The sedimentation constant of nucleoids in a 5–20% sucrose gradient is approx. 180 S. In some experiments, the disrupted virus preparation was incubated with phospholipase C (1 mg/ml in tris buffer) for 30 min in order to remove any remaining membrane fragments from the nucleoids before placing it on the top of the gradient.

Membrane fractions were obtained from the urografin or sucrose gradients by collecting the fraction having a buoyant density of 1·16.

Preparation of antisera—New Zealand rabbits were injected intracutaneously into the four footpads with 0·5 mg purified virus in 0·5 ml phosphate (0·05 M)-citrate (0·01 M) buffer emulsified in 0·1 ml incomplete Freund's adjuvant. A week later, a series of four weekly intramuscular injections was begun with the same amount of virus but this was emulsified in 0·1 ml complete Freund's adjuvant. A week after the last injection, the animals were bled from the ears and serum was collected.

Absorption of antisera—20 mg/ml of lyophilized, defatted BALB/c mouse milk and 50 mg/ml of lyophilized, perfused, O20 mouse liver was added to the antiserum and incubated for 1 hr at 37°C. Thereafter the serum was centrifuged for 15 min at 2000 rev./min. in a clinical centrifuge and then in a Spinco ultracentrifuge for 30 min at 200,000 × *g* producing an entirely clear fluid. Criteria for good absorption were the absence of reaction in double diffusion tests when tested against milk, liver, spleen, and mammary gland homogenates from 4 month old C57BL mice. In addition, antisera were tested in the indirect immunofluorescence test [3] and regarded as well-absorbed if no reaction occurred with acetone-fixed frozen sections of mammary glands, liver, and spleen from young C57BL mice.

Immunodiffusion tests—Two different Ouchterlony techniques were used. First, the micro-immunodiffusion set of LKB employing 0·6% agarose in phosphate buffered saline (PBS) (4 ml) plus 0·001% merthiolate per microscope slide. The wells contained 15 µl of reagent, but were sometimes refilled. The second test was the micro-immunodiffusion test of Wadsworth [9] and Crowle [10] as adapted for MTV by Fink *et al.* [11]. Plastic templates with 4 mm wells were superimposed on microscope slides covered with a 0·175 mm layer of 0·75% agarose in phosphate buffered saline containing 0·001% merthiolate. The wells were filled with 20 or 40 µl of reagents.

The slides were incubated for 48 hr in a partly humidified box at room temperature or, when testing complete particles, at 4°C. Thereafter, the templates were removed and the slides were incubated for 1 hr in cadmium–barbital buffer (250 mg cadmium acetate in 1 l. barbital buffer, pH 8·4, from LKB, Stockholm, Cat. 3276–VB). The slides were photographed with a Polaroid Cu 5 camera at a 3 × enlargement on Polaroid 3000 Black and White or on Polacolor film. The slides were then washed in distilled water for 6 hours and air dried.

Acridine orange staining—A 0·01% solution of acridine orange in McIlvaine's buffer (pH 3·8) was used to stain the slides which were treated after drying with Carnoy's fixative for 5 min. After 5 min staining, they were washed two times for 5 min in McIlvaine's buffer and thereafter incubated for 15 min in 0·15 M sodium phosphate. Following this, they were examined under u.v. light.

Electron microscopy—For observation of precipitation lines, LKB immunodiffusion tests were fixed overnight in 3·5% glutaraldehyde (Phosphate buffer, pH 7·8). Where lines were present, parts of the gel were cut out and then fixed in 1% osmium tetroxide in 0·1 M phosphate buffer, pH 7·2. During dehydration in alcohol, the preparations were stained with 2% uranyl acetate. Thereafter, they were embedded in a mixture of Epon and Araldyte. Thin sections were then studied with a Philips EM 200 electron microscope.

Virus disruption was achieved in various ways: (*a*) shaking 2 mg of virus suspended in 2 ml buffer with 40 ml ether for 30 min at room temperature with continuous aspiration of the evaporating ether through a Pasteur pipette connected to a waterpump; (*b*) rapid freezing (in a −30°C freezer) and thawing in a 37°C incubator three times; (*c*) sonication (20 kHz) for 10 sec; (*d*) tenfold dilution in PBS for 30 min at 37°C.

Enzyme incubations of virions, membranes, or nucleoids were performed for 60 min at 37°C as previously described [5].

The following enzymes were used:

Phospholipase C (Type I from Clostridium welchii, Sigma), 4·8 mg/ml in 0·01 M Tris, pH 7·4 + 0·01 M $CaCl_2$ + 0·1 M KCl,

Pepsin (NBC 3 × crystallized) 0·5% in 0·1 M HCl.

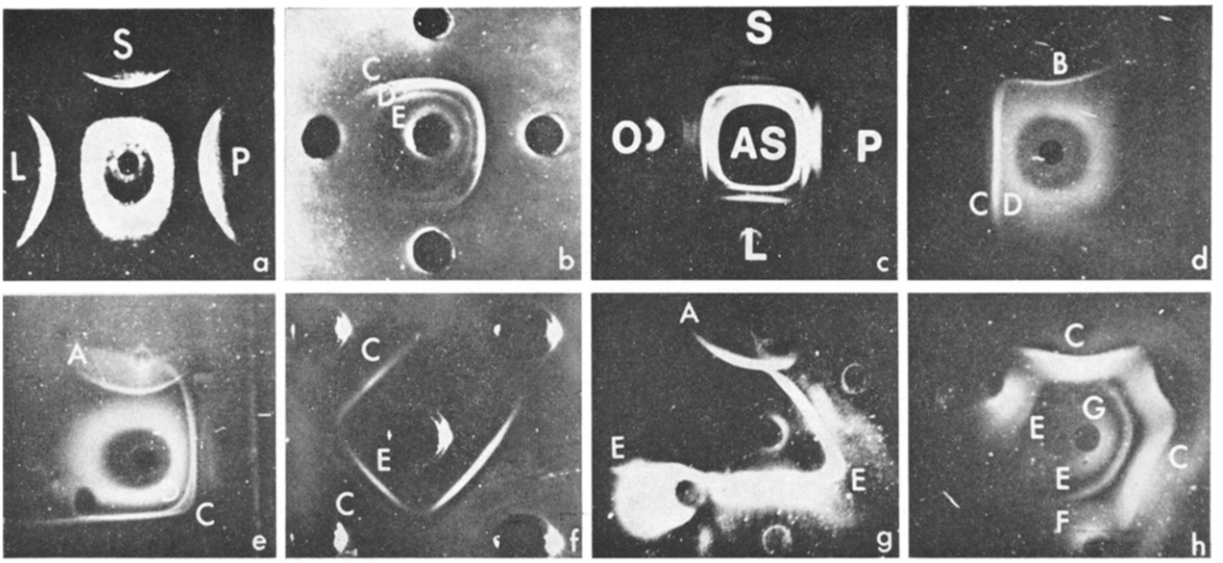

Fig. 1. *Immunodiffusion plates of MTV tested against rabbit anti MTV-S serum (always in centre well).*

(a) *Production of the precipitation line A with intact virions of MTV-S, -P and -L (micro-Oucht.).*

(b) *Production of precipitation lines C, D, and E with ether-disrupted MTV-S particles in clockwise decreasing concentrations in the outer wells (LKB-Oucht.).*

(c) *Production of several precipitation lines with ether-disrupted virions of MTV-S, -L, -O, and -P virions (micro-Oucht.).*

(d) *Production of precipitation line B with purified nucleoids of MTV-S (upper well) and C+D with ether-disrupted nucleoids (left well). Note fusion of C and D with B (micro-Oucht.).*

(e) *Crossing of lines C+D from disrupted MTV-S virions (left well) with line A produced by intact virions (upper well) (micro-Oucht.).*

(f) *Change in intensity of lines C(+D) and E. Left upper well: isolated MTV-S nucleoids producing a very strong C line and a line E too weak to be discerned on this photograph. Left lower well: membrane fractions producing a weak but discernible line C and a strong line E. Right lower well: disrupted virions producing lines C and E (LKB-Oucht.).*

(g) *Partial fusion of line E, produced by the MTV-S membrane fraction (right and lower well) with line A produced by intact virions (upper well) (LKB-Oucht.)*

(h) *Production of lines E, F, and G by the MTV-S membrane fraction. Lower well: membrane fraction. Two right wells: disrupted virions. Upper and left wells: nucleoids (micro-Oucht.).*

(to face p. 568)

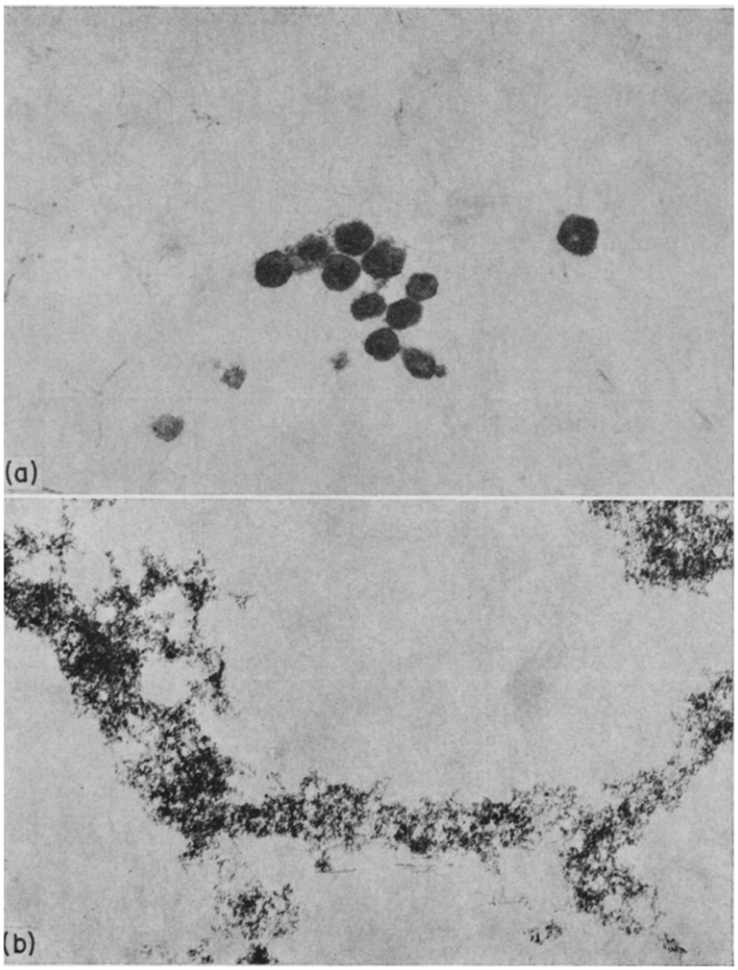

Fig. 3. Electron micrograph of precipitation lines: (a) *of line A,* (b) *of line C.*

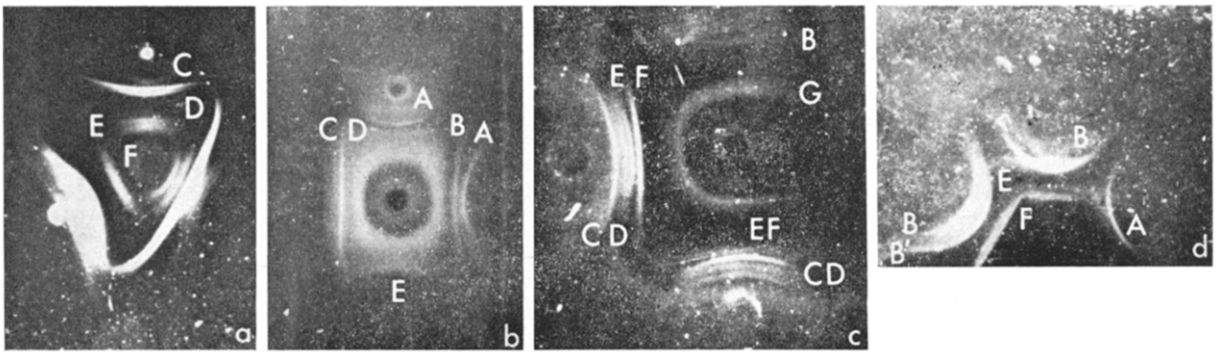

Fig. 4. Influence of pretreatments of MTV-S on the appearance of precipitation lines (All micro-Ouchterlony).

(a) *Upper well: virions incubated with acridine orange producing thin C and D lines. Right well: ether-disrupted virions. Left well: virions disrupted by repeated freezing and thawing.*

(b) *Upper well: intact virions producing line A. Right well: virions incubated with trypsin. Lower well: disrupted virions incubated with trypsin. Left well: ether-disrupted virions.*

(c) *Upper well: HCl treated virions producing lines B and G. Right well: pepsin-treated virions producing no lines. Lower well: phospholipase C-treated virions producing a thickened E and F line. Left well: disrupted virions.*

(d) *Upper well: nucleoids. Right well: RNAase-treated virions. Left well: RNAase-treated nucleoids producing an additional line* B^1.

Trypsin (Sigma, Type I) 0·25% in Dulbecco's phosphate buffered saline (PBS)
Ribonuclease (Sigma, Type Ia, 5 × crystallized) 0·1% in 0·01 M Tris (pH 7·0), 0·14 M NaCl.

To terminate the reactions, the samples were placed at 4°C. In addition to this, pepsin activity was halted by the addition of a neutralizing amount of 0·1 M NaOH, and the trypsin incubation was ended by adding 1% trypsin inhibitor (Sigma, Type I-S from soybean) in PBS at 1/4 of the volume of the trypsin used.

Acridine orange—1%: 0·01 ml was added to 0·1 ml of the MTV stock suspension. After 1 hr incubation at 37°C, the mixture was dialyzed against PBS at 4°C for 24 hr.

RESULTS

Depending on the experimental conditions, different precipitation lines can be observed in immunodiffusion tests using MTV preparations and anti-MTV antisera (Fig. 1). The ten separate lines which were observed in the various plates are represented in Fig. 2. The first line, A, which occurs near the antigen well and is strongly curved towards this (Fig. 1a) is formed only when freshly isolated virions and thin agar (0·75% or less) are used. Its appearance is facilitated by the addition of 0·04% bovine serum albumin or 0·25 M sucrose (final concentrations) to the agar. The electron microscopic picture of a thin section made from this line (Fig. 3a) corresponds with particles of the approximate size of 100 nm surrounded by a fringe of gamma globulins. Thin sections of other precipitation lines show only granular amorphous structures (Fig. 3b). All four MTV-strains produce line A with an antiserum against MTV-S. In Fig. 1(a), only three of these have been presented.

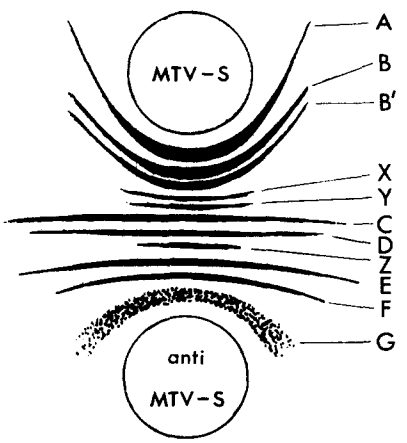

Fig. 2. Representation of the different precipitation lines of MTV-S with rabbit anti-MTV-S as observed in the various immunodiffusion plates.

After disruption of the virus particles, as confirmed by electron microscopic observation, line A does not appear, but other lines are produced at more distant sites. After centrifugation for 1 hr at 200,000 × *g*, the supernatant produces all these new lines, indicating that the corresponding antigens are soluble. In the LKB-Ouchterlony plates, three lines become visible (Fig. 1b); in the micro-Ouchterlony, eight appear (Fig. 1c) with the S and O strains of MTV. It has been consistently found that the P strain produces only five lines and the L strain three, with an antiserum to MTV-S, although the amount of virus was the same in all four tests.

Purified nucleoids of MTV-S produce line B which shows a curvature towards the antigen well and is developed at a greater distance than is A (Fig. 1d). This line is not produced when agar of more than 1% is used. Incubation of the nucleoid preparation for 1 hr at 37°C results in the appearance of lines C and D instead of B. They are produced approximately halfway between the wells. Lines C and D are often indistinguishable, but when the antiserum is added 16 hr before the antigen, they develop at separate sites nearer to the antiserum well. Line D is weaker than C; the relative thickness of the line varying with the different antisera. Both lines seem to fuse with B which is produced by the intact nucleoid, but cross line A (Fig. 1e). All four MTV strains produce lines C and D.

The membrane fractions produce a C+D line (Fig. 1f), although it is very weak as compared to the line produced by the nucleoid fraction. The membrane fraction is responsible for the appearance of another strong line, E, in the LKB–Ouchterlony test. This line is barely visible at the side of the well containing nucleoids. In Fig. 1(g), line E forms a spur with line A. The asymmetrical appearance of the latter is remarkable in this test.

In micro–Ouchterlony plates, the membrane fraction and disrupted virus particles produce two additional lines near the antiserum well. Line F is closely situated to line E, as can be discerned in Fig. 1(h). The broad translucent line G, which is strongly curved towards the antiserum well is very difficult to photograph. It has a red colour when illuminated longitudinally at a 45° angle. Line G is never formed when line A appears. In contrast to the E and F lines, G is produced by all four MTV strains. Line E is not produced with MTV-L and line F is not seen with MTV-L and -P.

Three more lines, X, Y, Z, which are rather faint, are occasionally produced by MTV-S and -O if large amounts of virus particles are tested with an antiserum directed against MTV-S. These lines have never been observed with the nucleoid and membrane fractions.

Acridine orange stains lines A and B bright orange–red. Sometimes C and D are weakly coloured, while the other precipitates always remain unstained. Incubation of disrupted MTV with this compound leads to a significant weakening of C and D, whereas the other lines are not affected (Fig. 4a).

The effect of pretreatment of MTV-S particles with enzymes on the various precipitation lines is summarized in Table 1. Lines X, Y, and Z are not mentioned in this table.

(Fig. 4d). Unfortunately, in this case, the nucleoid preparation is contaminated with membrane fragments which give rise to lines E and F.

DISCUSSION

The production of eight different precipitation lines after disruption of the MTV-S or -O virions might raise doubt about the specificity of the antiserum. Some lines may consist of precipitated mouse materials. However, in the immunofluorescence test, absorbed sera do not stain mammary glands or spleens from C57BL mice which normally do not show the presence of an MTV [3]. Specificity of the antiserum can also be deduced from the fact that an antiserum

Table 1. Effect of enzymatic treatment of MTV-S in precipitation lines of virus specific antigens with rabbit antiserum to MTV-S

Precipitation line	Trypsin	Pepsin	HCl	RNAase	Phospholipase C
A	0	–	–	0	–
B	–	–	0	+*	0
C	–	–	0	0	0
D	–	–	0	0	0
E	0	–	–	0	+
F	0	–	–	0	+
G	0	–	0	0	+

– = disappearance of the line; 0 = no effect; + = intensification of the line; * = appearance of another line.

Incubation of the intact particles with trypsin has no effect whatsoever, even when the virions are disrupted with ether after the enzymatic treatment. However, incubation of dissociated virus particles (Fig. 4b), of nucleoids, or membrane fractions inhibits the development of lines C and D.

Pepsin treatment seems to destroy all antigens, since no line is formed (Fig. 4c). The control experiment with HCl alone leads to the formation of a faint line B and line G (Fig. 4c). Incubation of disrupted particles with HCl affects the appearance of lines E and F so that these become quite thin.

The effect of phospholipase C is comparable to that of shaking with ether: Line A is not formed, but lines C to G inclusive appear (Fig. 4c). Lines E, F, and G are considerably thickened, regardless of whether intact or disrupted virions have been exposed to this enzyme.

When nucleoids are incubated with RNAase, a new line, B^1, appears in the vicinity of B. It has not been observed in any other experiment

to MTV-S, harboured in the C3H mouse strain, reacts with only three soluble antigens of MTV-L which is present in the C3Hf mice. The two mouse strains are genetically identical and, therefore, the five additional antigens in MTV-S can certainly not be host materials. The antiserum reacts with MTV-S isolated from mammary tumours, originating in infected BALB/c mice. It does not react, however, with Rauscher leukaemia virus isolated from leukaemic spleens of infected BALB/c mice.

The remarkable curvature of line A towards the antigen well indicates that the precipitated antigen must be of a large size [12]. This can also be deduced from the fact that agar of less than 1% must be used to produce this line. According to Wieme [13] the pore size in that case is larger than 100 nm. As disruption of the virion prevents the development of line A, it seems very likely that this line represents the precipitation line of intact virions as described by Blair [14, 15]. Electron microscopic examination of this line supports this idea.

Line B, which is produced by the nucleoid

fraction, is somewhat less strongly curved towards the antigen well than is line A. It must, nevertheless, represent a large particle. This can also be concluded from the necessity of soft agar for its formation.

Treatment with HCl separates the viral membrane from the nucleoid [5] and induces the appearance of lines B and G instead of A, strongly suggesting that line B consists of precipitated nucleoids.

The trypsin-sensitivity of lines C and D and their fusing with line B strongly indicates that C and D are nucleoid antigens.

The bright staining of A and B by acridine orange indicates the presence of RNA in the corresponding antigen. The occasional staining of C and D, or their weakening following preincubation with RNAase, may be explained by the adherence of some RNA fragments to the protein antigens. However, the RNAase insensitivity indicates that the RNA fragments are not essential for antigenicity. We assume that C and D, together with the RNA backbone, constitute the threadlike structure which forms the nucleoid.

The appearance of the B^1 line after treatment of nucleoids with RNAase is puzzling. An explanation may be that RNAase destroys RNA at certain unprotected sites. Thereafter, other antigenic determinants may then be exposed which give rise to a different precipitation line.

Nowinski *et al.* [4] described two protein antigens, S1 and S2, which they claim to be nucleoid antigens. Antisera prepared against purified nucleoids from MTV-S particles detect only these antigens. These antisera which were kindly provided by Dr. R. C. Nowinski, produce the C and D lines under our experimental procedures. Since the C and D antigens are present in all four MTV-strains, they may be regarded as group specific. According to Nowinski [4], the electrophoretic mobility of S1 (our C antigen) is comparable to that of the gs-1 antigen of the murine leukaemia viruses.

The insensitivity of lines E, F and G produced by the membrane fractions to trypsin accords with the resistance of the viral membrane to digestion by this enzyme. It can be stated that the corresponding antigen of G contains a protein moiety, in view of its sensitivity to pepsin. HCl seems to denature the E and F antigens to such an extent that they are no longer antigenic.

Phospholipase C exerts some effect on the structural integrity of the virus membrane. It is, therefore, understandable why, after incubation with this enzyme, all soluble antigen lines appear. This enzyme seems to increase the reactivity of E, F, and G in the immunodiffusion test. An explantation is that E, F, and G contain phospholipids which somewhat mask their antigenicity. Line E becomes visible in both the LKB- and the micro-Ouchterlony tests and can, therefore, be regarded as the major antigenic component of the membrane of MTV-S. Its partial interference with the development of the virion line A, and the spur formation between E and A, indicates that E is an antigen of the outer coat of MTV-S and is involved in the immunoprecipitation of the virion.

This may also hold true for G in view of the mutual exclusivity of A and G. The red colour of line G suggests a regular structure of the antigen which produces an optical interference phenomenon. Line G is found in all four MTV-strains. It seems to be the group-specific membrane antigen. Most likely G is the antigen which can be held responsible for the group-specific precipitation of the MTV virion as observed by Blair [16, 17], and in this study. After cross-absorption, she found type-specific virion precipitation [16.] Antigen E may be such a type-specific component which contributes to the immuno-precipitation of the intact virus particles. There are several other such type-specific antigenic determinants [16], which we, obviously, cannot detect as soluble antigens in immunodiffusion.

From the report of Nowinski *et al.* [4] one may deduce that the S4 and S5 proteins of MTV-S, as separated with polyacrylamide gel electrophoresis represent type-specific antigens. We assume that S4 and S5 correspond to our E and F antigens. Nowinski's S3 protein seems to be a group-specific membrane antigen. It is most likely our G antigen.

The X, Y, and Z lines, which only become visible when large amounts of MTV-S are deposited in the wells, corresponds with either very weak antigens or antigens which are present in small amounts.

The smaller number of precipitation lines observed with MTV-P and -L are probably not due to a smaller number of proteins. It seems that the corresponding entities of these virus strains are so different from those of MTV-S that the antiserum directed against the standard strain will not recognize enough antigenic determinants to precipitate the homologous antigen of the other MTV strains.

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Metabolic Controls in Precancerous Liver—V. Loss of Control of Cholesterol Synthesis During Feeding of the Hepatocarcinogen 3′-Methyl-4-Dimethylaminoazobenzene

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Abstract—*We have examined dietary control of cholesterol synthesis in rat liver during the feeding of 3′-methyl-4-dimethyl-aminoazobenzene (3′MeDAB), using an assay which permits repeated tests on the same animal. Synthesis was measured as plasma levels of 7-dehydrocholesterol following a single dose of AY-9944 (trans1,4-bis (2-chlorobenzylaminomethyl) cyclohexane dihydrochloride), a drug which inhibits the conversion of 7-dehydrocholesterol to cholesterol.*

During 3′MeDAB feeding there was a significant loss of inhibition of sterol synthesis in response to cholesterol feeding, but control rapidly returned to normal as soon as the carcinogen treatment was discontinued. The loss of control observed here was similar to that previously reported for 3 other hepatocarcinogens.

INTRODUCTION

Loss of dietary "feedback" control of cholesterol synthesis is a consistent feature both of primary and transplantable hepatomas [1, 2] and also of the livers of animals treated with the hepatocarcinogens aflatoxin, N-2-fluorenylacetamide and ethionine [3–8]. If this particular defect is important in the carcinogenic process in the liver, as has been suggested [3, 6], then it must be at least a common feature of treatment with all hepatocarcinogens. Hence we examined the regulation of cholesterol synthesis by dietary cholesterol during the course of feeding another commonly-used carcinogen, 3-methyl-4-dimethylaminoazobenzene (3′MeDAB).

Arcos, Griffith and Cunningham have reported [9] that feeding 3′MeDAB for less than 4 weeks results in a very low incidence of hepatomas, while feeding it for more than 6 weeks gives an incidence of nearly 100%. Thus, in an attempt to learn whether the control of cholesterol synthesis undergoes any marked change during this transition, we fed the carcinogen for 2, 4, 6 and 8 weeks.

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MATERIALS AND METHODS

Animals and diets

Fifty male Hooded-Wistar rats were used, aged 2–3 months at the beginning of the experiment and maintained throughout under a 12 hr day : 12 hr night light regime (lights on at 6.00 a.m., off at 6.00 p.m.). They were fed a commercial laboratory ration *ad libitum* (powdered Charlicks M164 mouse cubes), with a cholesterol content of 0·07 ± 0.01 % (measured as digitonin-precipitable sterols). They were divided into 5 groups, which received 0·06% 3′ MeDAB (K & K Laboratories, Inc., Plainview, N.Y., U.S.A.) for 0, 2, 4, 6 and 8 weeks respectively.

Each week half the animals in each group (the alternate half each week) received for two days a high-cholesterol diet, i.e. 5% cholesterol (Pierce Chemical Co., Rockford, Ill., U.S.A) mixed with the powdered feed.

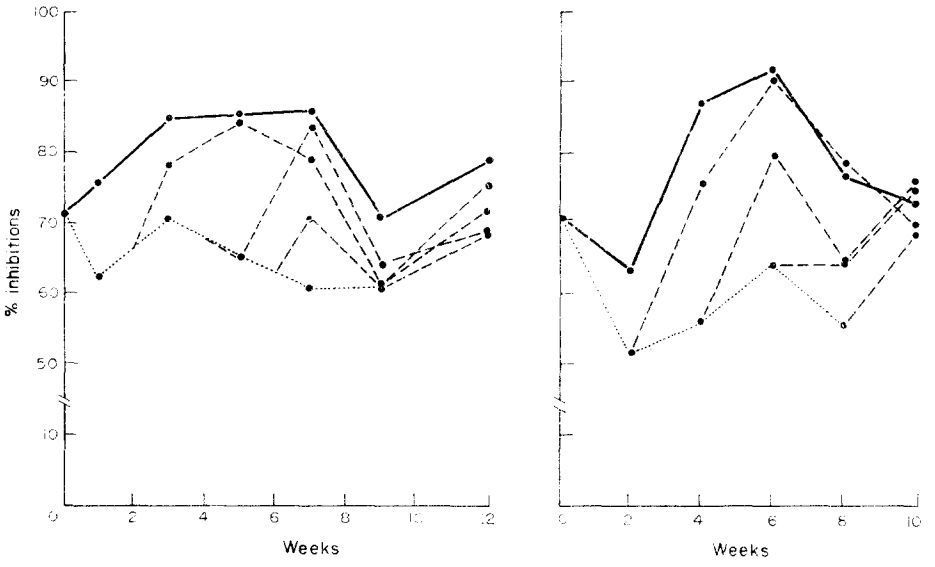

Fig. 1. Percentage of inhibition of cholesterol synthesis by dietary cholesterol during 3'MeDAB feeding (0·06%). The upper solid lines represent the control groups (not fed 3'MeDAB), while the lower dotted lines indicate the mean values of all the rats fed 3'MeDAB up to the times indicated. The broken lines indicate the mean values of the groups removed from the 3'MeDAB diet at 2, 4, 6 or 8 weeks respectively. The inhibition of cholesterol synthesis in the rats fed 3'MeDAB was significantly impaired (P < 0·05) after 1, 3, 4, 5, 6 and 8 weeks of feeding, but not at any later time. All groups contained 5 rats, and each rat was fed a high-cholesterol diet (5%) for 2 days every alternate week.

Vival assay for liver cholesterogenesis*

Every week each animal was tested for hepatic cholesterol synthesis *in vivo*. This assay has been described in detail previously [11]. Briefly, the rats were injected intraperitoneally with 10 μmoles/kg AY-9944† (the gift of Dr. Morris Givner, of Ayerst Research Laboratories, Montreal, Canada). Blood was collected from the tail vein 24 hr later, saponified, and extracted with light petroleum (b.p. 40–60°C). After evaporation of the petroleum the 7-dehydrocholesterol level was measured, as "fast-acting sterols", by colour development with Liebermann–Burchard reagent [12].

Every alternate week each rat received the high-cholesterol diet for two days, i.e. one day prior to the injection of AY-9944 and during the one-day test period. Thus, for each animal, there was a measure of cholesterol synthesis on both high- and low-cholesterol diets, and percentage of feedback inhibition was calculated from the level of synthesis while on the high cholesterol diet compared with that on the normal diet in the previous and following assays. Experience had shown that approximately 10% of young Hooded-Wistar rats normally appear to have poor feedback control of cholesterol synthesis [6], but these were eliminated before the 3'MeDAB feeding began.

*Green has suggested [10] that the terms "vival" and "vitral" should be used in place of "*in vivo*" and "*in vitro*" when these are required as adjectives.

†AY-9944: Trans-1,4-*bis*(2-chlorobenzylaminomethyl) cyclohexane dihydrochloride.

RESULTS

The levels of percentage of inhibition of cholesterol synthesis by cholesterol feeding are shown in Fig. 1, up to 12 weeks from the beginning of 3'MeDAB feeding.

The carcinogen caused a small, but generally statistically-significant, loss of dietary feedback control of hepatic cholesterol synthesis. This was evident after only one week on the carcinogenic diet. In almost all cases, however, including those animals fed 3'MeDAB for 8 weeks, when the carcinogen was removed from the diet, inhibition immediately returned towards normal, and in fact subsequently did not differ from control values at any time up to and including 48 weeks.

In these studies with 3'MeDAB, as in those reported previously with other hepatocarcinogens [5–8], we observed both that cholesterol synthesis measured in animals on a low-cholesterol diet was generally increased by the carcinogen treatment, and also that the extent of the loss of feedback control varied widely between different animals.

Twenty months after beginning 3'MeDAB feeding the 20 surviving rats were killed and examined for the presence of liver tumours. Of those animals that died before this time tumours were not found in any on which autopsies could be carried out. The results (Table 1) are in agreement with those of Arcos, Griffith and Cunningham [9] who reported that the period of 3'MeDAB feeding from 2 to 6 weeks is critical for hepatoma induction.

Table 1. *Tumour incidence in rats* 20 *months after beginning* 3'*MeDAB feeding*

Weeks on 3'MeDAB diet	Number of rats with liver tumours	Number of rats with other tumours*
0	0/5	4/5
2	1/5	4/5
4	1/3	3/3
6	2/4	3/4
8	3/3	2/3

*Most of these tumours were in the lungs or testes. There was no apparent relationship between their incidence and the length of 3'MeDAB feeding.

Our earlier work [6] had indicated that the ultimate development of liver cancer in animals treated with a hepatocarcinogen, in that case N-2-fluorenylacetamide, may have been proportional to the severity of early loss of feedback control of cholesterol synthesis. But, in these present studies with 3'MeDAB, we could not draw this conclusion.

DISCUSSION

These studies indicate that 3'MeDAB causes a loss of dietary feedback control of cholesterol synthesis in a manner similar to that previously observed with three other hepatocarcinogens: aflatoxin [6] N-2-fluorenylacetamide [5, 6, 7] and ethionine [8]. This is further evidence in favour of the hypothesis that loss of feedback control of cholesterol synthesis is an important element in carcinogenesis in the liver.

We interpret the observed variable degree of loss of control as indicating that at any time during carcinogen treatment only a proportion of the liver cells are defective in their control of cholesterol synthesis [2, 6]. Our results, reported here with 3'MeDAB and earlier with other carcinogens [5–8], show that on ceasing carcinogen treatment most if not all of the affected cells can recover. Yet all fully developed hepatomas display complete loss of feedback control of cholesterogenesis [1, 2]. Hence further studies are needed to determine whether following carcinogen treatment a few cells may fail to ever regain control, and whether these are the source cells of the tumour.

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Cell Proliferation in Rectal Carcinoma and Rectal Mucosa. A Stathmokinetic Study

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Abstract—*A stathmokinetic technique was used* in vivo *to estimate the rate of cell proliferation in a group of* 19 *carcinomas of the large bowel. We compare this rate of proliferation with that found using the same technique on normal rectal mucosa from seven tumour-bearing patients. The mean apparent cell cycle time* ($t_{c(a)}$) of the *tumours was estimated to be* 192 *hr, and that of the normal rectal mucosa to be* 82 *hr. The duration of mitosis* (t_m) *was* 2·3 *hr in tumour cells and* 1·2 *hr in normal rectal mucosal cells. The relevance of these findings to cancer therapy is briefly discussed. Speculative estimates of the rate of cell loss and of growth fraction in the tumours are made by combining our results with those obtained by other workers using different kinetic techniques.*

INTRODUCTION

THE STIMULUS for much of the kinetic study of human tumours has been the hope that radiation therapy and chemotherapy may thereby be put on a more rational basis. Success, particularly with chemotherapy, has so far been limited. One of the major problems is the lack of specificity shown by most anticancer agents in that they are toxic to normal proliferating cells as well as to malignant cells. It is well known that the cell kinetic profile of tumours is important in determining their response to both chemotherapy and radiation [1, 2]. For reasons such as these the study of cell sociology is clearly relevant to the problems raised by human cancers.

In this paper we report the results of a kinetic study of rectal carcinomas using a stathmokinetic technique. This involves the injection into the patient of an agent, such as colchicine, colcemid or one of the vinca alkaloids, that block cells in metaphase. Kinetic information can be obtained from the rate at which blocked metaphases accumulate. Refsum and Berdal [3] measured the rate of cell proliferation in 61 human tumours, mainly squamous epithelial carcinomas, using colcemid. One of us [4] used a similar method to study cell proliferation in neuroblastoma and nephroblastoma. We then decided to apply the technique to the more common and more accessible tumours of the large bowel. This also enabled us to compare cell proliferation in "morphologically normal" rectal mucosa with that in carcinomas.

Accepted 25 May 1973.

MATERIAL AND METHODS

Each of the patients in this study was given a single intravenous injection of vincristine sulphate (Lilly) at a dose of 0·045 mg/kg. This was the maximum dose recommended by the manufacturers and appeared to give an effective block as judged by the complete absence of telophases in normal mucosa and only very rare and usually abnormal telophases in the tumour. In almost all of the cases the injection was given several hours prior to excision of the tumour. A biopsy was obtained from each tumour before injection of vincristine and one or two biopsies were obtained at times up to four hours after the injection of vincristine. The time from

injection of vincristine to immersion of the biopsy in fixative was carefully noted. A final tumour sample was usually taken from the excised specimen.

Biopsies of carcinoma were obtained from 19 patients, 14 male and 5 female, and biopsies of normal mucosa before and after vincristine injection from 7 patients, 5 male and 2 female. Mucosal specimens were always taken from sites distant from the tumour. The ages of the patients ranged from 52 years to 81 years with a mean age of 67 years. All patients included in the series had invasive carcinomas.

The biopsies, or small pieces of excised tumour, were placed in Carnoy's fixative immediately after removal from the body. Prior to fixation specimens of mucosa were carefully attached to coverslips, muscularis downwards. Paraffin embedded blocks were processed in the usual way, and 3 μ sections were stained with haematoxylin and eosin. Using an eyepiece graticule 6,000 tumour cells were counted and the number of metaphases and other stages of mitosis were noted. In the biopsies of normal mucosa whole crypt counting was employed and the total number of cells and mitoses in 100 crypt columns per biopsy were counted.

Although all stages of mitosis were scored where present, metaphase counts rather than mitotic counts were used for the purposes of calculation. Metaphase counts were considered to be more reliable because prophases in particular proved quite difficult to recognize and may have been underestimated.

Only results obtained from biopsies taken prior to the start of the excision operations were used in the calculation of kinetic parameters because the samples from the excised tumours were found to be unreliable. This is presumably due to such factors as the period of ischaemia and physical trauma the tumours are subjected to during operation. Other important factors may be the degeneration or the escape of blocked metaphases. Degeneration, for example, has been shown to have a marked effect on the number of metaphases seen quite soon after mitotic-blocking has begun [5]. To minimize such effects vincristine-treated biopsies were taken only up to four hours after injection.

RESULTS

The mathematical interpretation of results in this type of study depends very much on the age structure of the tumour cell population. If the growth fraction is near unity and the rate of cell loss is small, the exponential model is often a moderately good approximation. In this study the growth fraction was small and the rate of cell loss was large; it follows that the bias towards young cells in the age distribution of the cell population was negligible. Following Lala [6] we therefore felt justified in using a very simple steady state model as the optimal approximation to our tumour.

Tables 1 and 2 show the results of metaphase and mitotic counts made on tumour and normal mucosa respectively. The results from the 19 tumour cases were pooled and metaphase index was plotted against time from injection of vincristine. The graph shows the results from pre-excision biopsies. The regression equation for y, calculated by the method of least squares, is

$$y = 0{\cdot}52x + 1{\cdot}07$$

The slope m of the regression line gives the mean rate of entry into metaphase. By inspection, therefore, the rate is 0·52% of the cell population per hour. The reciprocal of this rate gives the parameter usually called the potential doubling time (t_p) though in our steady state model the term "apparent cell cycle time" ($t_{c(a)}$) seems more apt.

$$\bar{t}_{c(a)} = 1/0{\cdot}0052 = 192 \text{ hr}$$

The standard error of the slope is 0·0569 and the 95% confidence interval in therefore

$$0{\cdot}52 - 2(0{\cdot}0569) < m < 0{\cdot}52 + 2(0{\cdot}0569)$$

It follows that the apparent cell cycle time, as we have computed it, would lie between 159 hr and 244 hr in 19 out of every 20 repetitions of the study. The relative standard error of $\bar{t}_{c(a)}$ is 0·1218.

The duration of metaphase, and of mitosis as a whole, can be computed from the equations

$$t_{met} = I_{met}/r_{met};\ t_m = I_m/r_m;$$

where t_{met} = duration of metaphase, and t_m that of mitosis;

I_{met} = untreated metaphase index, and I_m the natural mitotic index;

r_{met} = rate of entry into metaphase, and r_m into mitosis.

The values obtained are 2·0 hr for metaphase and 2·3 hr for mitosis.

The rate of entry into metaphase, as determined from the excised tumour specimen, is 0·27% of the cell population per hour, which is only about half the rate given by the biopsy samples. If we restrict ourselves to those excision specimens which were available within 4 hr of the vincristine injection the rate of

Table 1. *Metaphase and mitotic indices in tumours before and after treatment with vincristine*

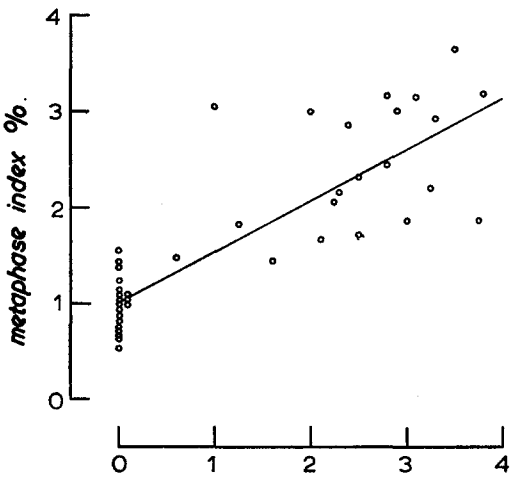
Patient	Sex	Age	Duke* Stage	Pre-vincristine biopsies: Metaphase index %	Pre-vincristine biopsies: Mitotic index %	Post-vincristine biopsies: Duration of arrest (hr)	Post-vincristine biopsies: Metaphase index %	Post-vincristine biopsies: Mitotic index %	Excision samples: Duration of arrest (hr)	Excision samples: Metaphase index %	Excision samples: Mitotic index %
JC	M	77	B	1·3	1·7	3·8	3·2	3·4	5·3	0·7	0·8
JM	F	69	C	1·0	1·3	3·1	3·2	3·4	4·1	3·3	3·4
KW	F	77	C	1·4	1·5	3·0	1·9	1·9	3·5	2·1	2·2
JH	M	71	C	1·0	1·0	3·8	1·9	2·0	5·1	2·6	2·7
LS	F	58	A	0·9	1·0	2·8	2·5	2·6	3·7	2·9	2·9
AY	M	52	B	0·5	0·6	2·1	1·7	1·8	3·1	2·6	2·7
TJ	M	73	C	1·5	1·7	2·5	2·3	2·6	6·0	1·2	1·3
TA	M	73	B	0·8	1·0	3·3	2·9	3·2	4·3	3·3	3·4
ER	F	61	C	1·1	1·4	(i) 0·6 (ii) 1·6	1·5 1·5	1·7 1·7	—	—	—
HW	M	81	B	0·7	0·8	2·5	1·7	1·8	3·6	1·9	2·0
AA	M	64	B	1·1	1·3	2·0	3·0	3·3	2·9	2·4	2·5
CW	M	61	B	1·1	1·3	2·3	2·2	2·2	2·6	2·0	2·1
LC	F	65	B	1·6	1·9	2·4	2·9	3·1	3·4	2·4	2·5
GJ	M	62	B	1·0	1·1	3·5	3·7	3·9	4·2	2·1	2·4
JMc	M	72	B	0·7	0·8	2·9	3·0	3·3	3·7	1·8	1·8
JW	M	67	C	0·9	0·9	2·8	3·2	3·4	4·0	1·7	2·0
PC	M	53	B	0·7	0·9	3·3	2·2	2·3	4·6	2·3	2·5
JMc	M	68	C	0·8	0·9	1·3	1·8	1·9	2·3	1·7	1·9
GP	M	61	A	1·0	1·1	(i) 1·0 (ii) 2·3	3·1 2·1	3·3 2·4	3·3	3·4	3·6

*Key

A—the tumour has not penetrated the muscularis mucosae.

B—the muscle coat is penetrated by tumour but there are no lymph node metastases.

C—the tumour has penetrated bowel wall and lymph node metastases are present.



entry into mitosis is estimated as 0·37 % of the population per hour.

The results obtained for the apparent cell cycle time ($\bar{t}_{c(a)}$) of the normal rectal mucosa are shown in Table 2. The mean value is $\bar{t}_{c(a)}$ = 82 hr, with a standard error of 13·96 hr, and therefore a relative standard error of 0·1702.

DISCUSSION

An advantage of the stathmokinetic technique when applied to human subjects is that it does not entail any serious or long term hazard to their welfare. The only adverse symptom produced in our study was an occasional, partial and transient loss of hair. We did, however, find it very difficult to obtain more than two or three biopsies per patient without causing undue distress. For this reason we were unable to obtain reliable estimates of kinetic parameters for *individual* patients and could only obtain mean values for the *group of tumours* studied. However, we became increasingly aware, as we progressed with this work, that there are large variations in mitotic index between different areas of the same tumour. Lieb and Lisco [7] found similar large variations in labelling index, also in large bowel carcinomas. For this reason even if we had been able to obtain multiple biopsies it is still doubtful whether we would have been able to characterize individual tumours reliably.

Included in Table 1 is the Duke stage of each tumour. No correlation could be found between staging and rate of cell proliferation as judged from the vincristine study. Fulker *et al.* [8] using a stathmokinetic technique to study cell proliferation in bladder carcinomas found a negative correlation between the degree of differentiation and the rate of cell proliferation. Again

Table 2. Metaphase and mitotic indices in mucosa before and after vincristine treatment

			Pre-vincristine biopsies			Post-vincristine biopsies				
Patient	Sex	Age	Mean crypt length (Number of cells)	Metaphase index %	Mitotic index %	Mean crypt length (Number of cells)	Duration of arrest (hr)	Metaphase index %	Mitotic index %	Apparent cell cycle time (hr)
AY	M	52	66	1·5	2·1	81	2·1	5·6	6·1	50·0
TA	M	73	75	0·7	1·3	79	3·3	3·5	4·3	122·0
ER	F	61	83	0·7	1·1	(i) 87	0·6	1·6	1·9	93·0
					(ii)	(ii) 90	1·6	2·4	2·9	
HW	M	81	71	0·9	1·4	99	2·5	7·5	7·9	38·0
AA	M	64	89	0·7	1·5	81	2·0	2·3	3·2	125·0
CW	M	61	73	0·9	1·3	84	2·3	3·2	3·6	102·0
LC	F	65	72	1·2	1·7	65	2·4	6·4	6·9	46·0

no such correlation could be demonstrated in this group of rectal carcinomas. The failure to find a correlation between proliferation and either Duke stage or degree of differentiation could well be due to the very high degree of variation in mitotic activity, discussed above, between different areas of the same tumour found in rectal carcinomas.

We are studying the problem of heterogeneity in these tumours and preliminary impressions suggest that variation within a single tumour may be of the same order as between different tumours.

If we compare our results with those obtained by other workers using different techniques then we can obtain interesting, if speculative, estimates of further kinetic parameters of large bowel carcinomas. Lipkin *et al.* [9] using an *in vivo* pulse labelling technique obtained fraction-labelled-mitosis (FLM) curves from which they estimated the cell cycle time (t_c) of human large bowel carcinomas to be between 24 and 48 hr. Welin and co-workers [10] investigated the rate of increase in external size of 20 large bowel carcinomas and found the mean tumour volume doubling time (t_d) to be 620 days. If we accept these results as representative of this type of tumour we can calculate values for the growth fraction and cell loss, as follows.

Combining Lipkin's range of values for t_c and our findings for $t_{c(a)}$ the growth fraction (I_p, or f_G) is found from the formula $f_G = t_c/t_{c(a)}$ to lie in the range 0·13 to 0·25. Cell loss may be represented by Steel's ϕ [11], i.e. the ratio of cell loss rate to cell birth rate:

$$\phi = 1-(t_{c(a)}/t_D) = 1-(190/14880) = 0{\cdot}987.$$

These values, though speculative, do fit with the relatively slow growth of these tumours and with their expected high rate of cell loss.

It may be worth noting that our calculated value for the duration of mitosis (t_m) is quite long for mammalian cells and may in part explain the apparent anomaly of high mitotic indices in slowly growing tumours. A second point of some interest is that the excised tumour may be an unreliable object for kinetic studies; if possible biopsy material should be used when it is surgically justifiable to obtain it.

The results obtained from the normal mucosa are in accord with those of Hoffman and Post [12], who concluded from *in vivo* labelling studies that proliferation is faster in normal large bowel mucosa than in the tumour. Normal mucosa is, of course, in a kinetic steady state, where cell birth balances cell loss. In the tumour this balance is lost; there is a small but significant *decrease* in cell loss and this, rather than an increase in proliferation, appears to be responsible for their progressive growth. As Hoffman *et al.* [12] remarked, this type of information is relevant to the question of the chemotherapy of human cancer. Most anti-cancer drugs act only on proliferating cells and success will be limited unless normal tissues can be protected from the action of these compounds.

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Survival of Chronic Myeloid Leukaemia Patients Treated by Dibromomannitol

*Report of the Dibromomannitol Cooperative Study Group**

Abstract—*The median survival of* 122 *patients with chronic myeloid leukaemia treated by dibromomannitol was* 43 *months. The survival function, and the causes of death of patients treated by dibromomannitol were similar to those previously found in patients treated by busulfan, indicating that dibromomannitol does not affect negatively the course of chronic myeloid leukaemia, and suggesting that new strategies should be developed in order to postpone the onset of blastic crisis.*

THE CONTROL of chronic myeloid leukaemia (CML) can be easily achieved with several drugs and with radiation therapy up to the development of an acute leukaemia syndrome commonly called Blastic Crisis [1, 2, 3, 4]. It is very hard at present to assess how much anti-leukaemic treatment has prolonged the survival of CML patients [3]. A comparative randomized trial of radiotherapy and busulfan has indicated the superiority of busulfan [5], so that any other drug for CML should be compared at least with the latter as to survival duration, efficacy, and toxicity.

In the last ten years several other drugs have been shown to be effective for CML and are currently employed in the control of the disease [3]. One of these compounds is 1,6-dibromo-1, 6-dideoxy-D-mannitol or dibromomannitol (DBM), a sugar derivative with alkylating activity first prepared and essayed in Hungary [6, 7]. Several reports have provided detailed information on its efficacy and scheduling, and have shown that the drug has a low haematologic toxicity, is well tolerated, and produces no side-effects clinically recognizable [8, 9, 10, 11, 12, 13, 14]. A comparative trial of busulfan and DBM would be likely to require many years and many patients, and most probably, whatever the answer, it would be overcome by new therapeutic trends [4, 15, 16, 17]. Therefore a retrospective study on DBM treated CML patients has been planned in order to collect available information, and to evaluate if DBM is likely to modify the duration of CML as compared with other treatments, with special reference to busulfan.

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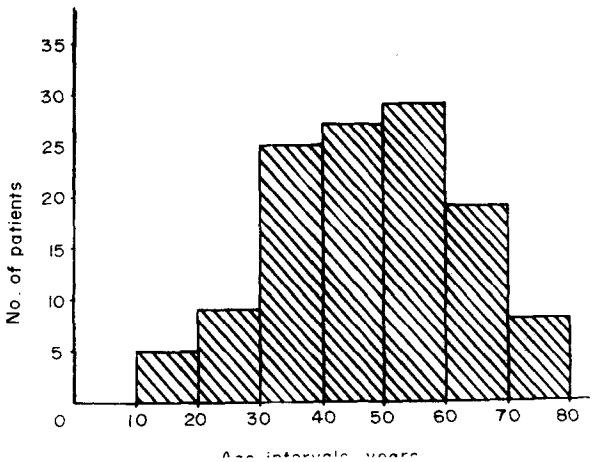
MATERIAL AND METHODS

Ten different Institutions accepted to cooperate to the study, and filled in special forms with the data pertaining to all CML patients treated only by DBM through the chronic phase of the disease, or for at least half of its duration. The diagnosis of CML was based on clinical findings, leukocyte count, and peripheral blood and bone marrow picture [1]. At March 31, 1972, data were collected on 166 patients



the sole treatment for more than half the duration of the disease, having been previously treated also by busulfan, radiotherapy, or one or more of the drugs listed in Table 1. These patients will be identified as Group B.

The duration of the disease was computed from diagnosis, i.e. from the date of the first blood examination consistent with CML. Survival has been defined through a method substantially similar to the death-table method for generations, while considering that the present series cannot be managed as a close generation, as a substantial number of patients are still alive, and 4 patients died from causes

Table 2

Induction treatment	No. of patients Group A	Group B	Group A + B
None	8	4	12
6 mg/kg of b.w./day × 15–17 days (range 3–14 mg/kg of b.w. per day × 45–4 days) Total dose 63–96 mg/kg of b.w. per treatment	49	45	94

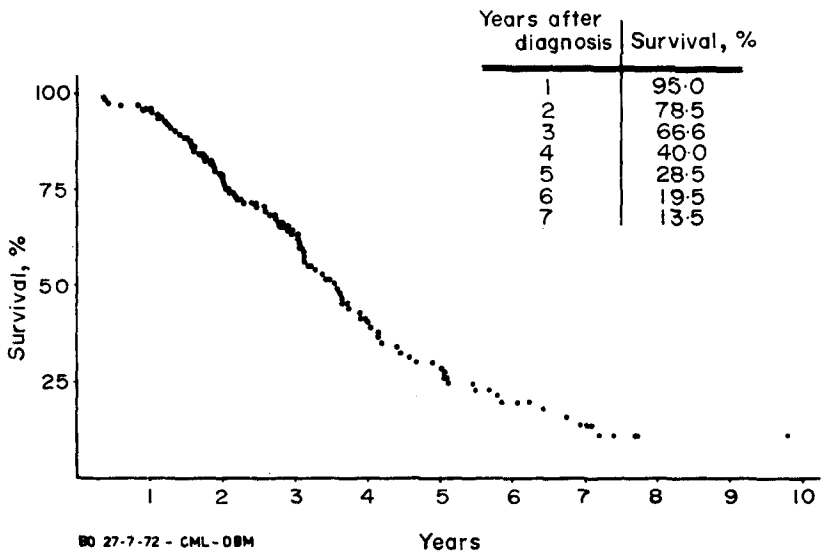


Fig. 2. Survival curve of the whole series of 122 patients. Half-life period is equal to 43·3 months, or 3·6 years.

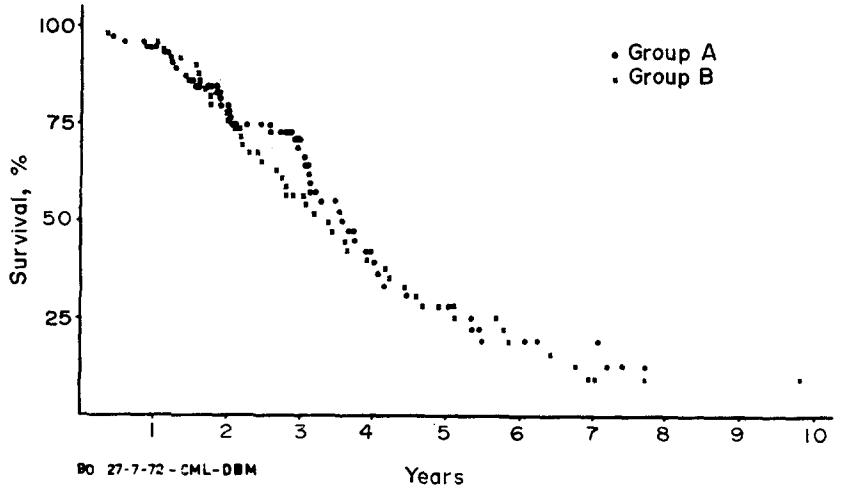


Fig. 3. Survival curves for separated Group-A and Group-B patients.

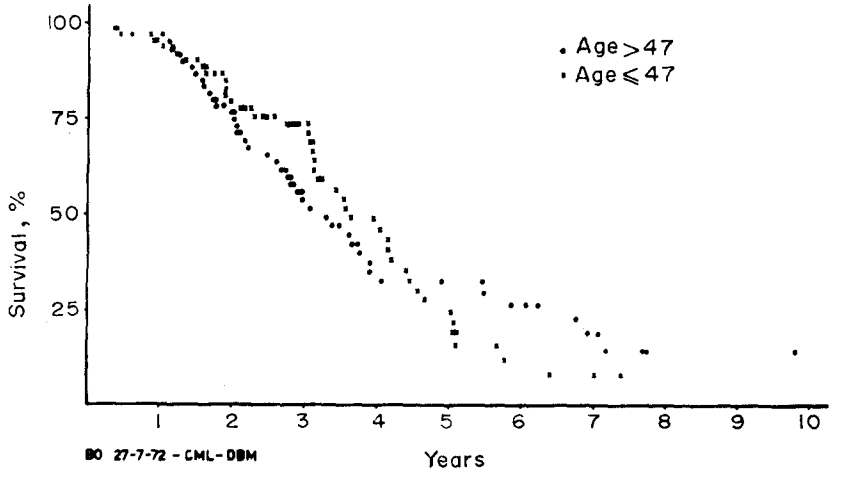


Fig. 4. Separated survival curves for patients aged under and above 47 years.

Table 2

Induction treatment	No. of patients Group A	Group B	Group A + B
None	8	4	12
6 mg/kg of b.w./day × 15–17 days (range 3–14 mg/kg of b.w. per day × 45–4 days) Total dose 63–96 mg/kg of b.w. per treatment	49	45	94
5 mg/kg of b.w. per day × 28–42 days Total dose 140–210 mg/kg b.w. per treatment	16	—	16
Total	73	49	122
Maintenance treatment			
None	14	12	26
10 mg/kg of b.w. per week in three doses (range 5–21 mg/kg of b.w. per week in 1–6 doses)	59	37	96

curves do not differ significantly at any interval.

As death-tables of these patients have been calculated without considering the age of the patients, an attempt has been made in order to evaluate the possible age's influence on survival. Considering the age distribution, the whole series has been divided in two groups, one group for patients less than 47 years old, and one group for the others. Pertinent survival curves are shown in Fig. 4. Here again they are similar, and statistical analysis (χ^2) failed to show any difference.

The doses and schedules of DBM for both induction and maintenance treatments are reported in Table 2. Twelve patients did not receive induction treatment (as defined by a minimum of 3 mg/kg of body weight for at least ten consecutive days). Ninety-four patients (or 77%) received induction treatment with a total dose ranging in between 63, and 96 mg/kg of body weight, with a mean and median daily dose of 6 mg/kg. The last 16 patients had more prolonged induction treatments, with a total dose ranging from 140 to 210 mg/kg b.w. Twenty-six patients (or 21·3%) did not receive any maintenance treatment. All the others had continuous, or partially continuous maintenance treatment, with a mean and median dose of 10 mg/kg b.w. per week given in three separate doses.

At the time of the collection of the data, 83 patients were dead. Nineteen of them had received DBM until death. In the remaining 64 cases, DBM was discontinued before death because of blastic crisis (BC) (37 cases), inefficacy (7 cases), marrow failure (10 cases), and other causes (10 cases). The mean interval between DBM discontinuation and death was 83 days in the case of BC, 129 days in the case of inefficacy, and 56 days in the case of marrow failure.

The causes of death are listed in Table 3. Fifty-seven patients developed BC, five others died with clinical and haematological findings not typical of, but consistent with BC. Six patients died while being cytopenic, with aplastic marrow, and one because of thrombocytopenia. Respective survival of these patients was of 4·7, 23·2, 40·0, 43·3, 44·0, 49·4, and 62·3 months (mean 38·1 months). Ten patients died because of causes possibly related with CML; these causes included twice each uraemia,

Table 3

Causes of death	No. of cases
Blastic crisis	57 (68·7%)
Probable blastic crisis	5 (6·1%)
Marrow failure	6 (7·2%)
Isolated thrombocytopenia	1 (1·2%)
Causes possibly related with CML	10 (12·0%)
Causes probably unrelated with CML (myocardial infarction)	4 (4·8%)
Total	83

pneumonia, and hepatitis, and once each tuberculosis, septicaemia, vireamia, and pericarditis. Myocardial infarction developed in four patients, and it was considered to be probably unrelated with CML.

DISCUSSION

The interpretation of present data is subjected to the restrictions common to any retrospective study. The way of patients' collection is open at special criticism, as two undeterminated mechanisms could have played a role, the first being possibly operating at the moment of the allocation of patients to DBM therapy, and the second at the moment of the collection of the data for the retrospective analysis. It should be remembered that most of the above general considerations also apply to all previous reports on CML duration [18, 19, 20], but the prospective randomized trial of busulfan and radiotherapy [5]. However, present data come from ten different Institutions, in which patients have been treated and followed up independently, so that some compensation of sampling errors can be reasonably expected.

Chromosome analysis and leukocyte alkaline phosphatase score were not requested because of the retrospective character of the study; however, 39 out of 41 patients with chromosome studies had the Philadelphia chromosome.

An arbitrary time criterion has been used to select Group B patients, i.e., patients treated by DBM for more than half the duration of the chronic phase, but previously treated with other drugs and/or radiotherapy. This criterion was a guarantee that these patients had been allocated to DBM therapy while being still in the chronic phase of the disease, and far from the onset of BC. The analysis of this group was thought to give an opportunity to evaluate the possible effect of DBM plus other treatments on the duration of CML. The comparison between the survival curves of Group-A and Group-B patients suggests that no major difference exists.

An attempt has been made to correlate the dose and schedule of DBM with survival. Median survival of patients who received induction treatment with a total dose ranging from 63 to 96 mg/kg of body weight is the same as for the 16 patients treated with higher doses (43·6 vs 41·0 months). Also, there is no apparent relationship between the median survival and the number of induction treatments given during the course of the disease. As to maintenance treatment, median survival has been considerably longer in the patients that had maintenance treatment than in the others (44·8 vs 34·0 months), but the low number of the latter makes this difference insignificant.

The causes of death, in the present series, occur with about the same frequency as reported in previous studies [2, 5, 18, 19]. Most patients (68·7%, and more probably 74·8%) died in BC. Marrow failure appeared in 7 patients, whose mean survival was of 38·1 months. Because of the retrospective character of the study, no information was available on the possible development of myelofibrosis. Drug doses and schedules of these 7 patients are listed in Table 4. None of them had a "standard" regimen. Nevertheless, marrow failure appeared rather late in the course of the disease with respect to induction treatment in all cases but one, so that a clear relationship cannot be shown. However, induction treatments with a

Table 4. DBM doses and schedules in the patients dead because of marrow failure

No. of patients	Previous therapy	No. of induction treatments	Induction treatment (DBM)	Maintenance treatment (DBM)	Duration of the disease (months)
1	—	1	12 mg/kg per day × 6 days (72 mg/kg)	—	4·7
4	—	1	5 mg/kg per day × 42 days (210 mg/kg)	7–9 mg/kg per week	23·2 40·0 43·3 49·4
1	Busulfan dose unknown	8	6 mg/kg per day × 7 days (42 mg/kg)	—	44·0
1	Busulfan 1790 mg	1	5 mg/kg per day × 42 days (201 mg/kg)	12 mg/kg per week	62·3
				Mean	38·1

Table 5

	Years	No. of patients	75% alive at (months)	50% alive at (months)	25% alive at (months)	Treatment
Minot *et al.*	1898–1923	52	22	31	48	None
Reinhard *et al.**	1942–1957	118	20	32	48	^{32}P
Haut *et al.**	1952–1960	30	26	42	—	Busulfan
Medical Res. Council†	1959–1963	54	16	28	32	Radiotherapy
Medical Res. Council†	1959–1963	48	20	40	49	Busulfan
DBM Cooperative Study Group‡	1961–1971	73	26	43	62	Dibromomannitol

*From the estimated onset. †From start of treatment. ‡From diagnosis.

daily dose not higher than 8 mg/kg of body weight and with a total dose not higher than 100 mg/kg of body weight should be recommended.

The main reason for stopping DBM therapy was the onset of BC (37 cases). In 7 patients the drug was discontinued because it was thought to be ineffective, and all these patients died after a short period (65–232 days, mean 129 days) in full BC. These data suggest that the appearance of "resistance" to DBM should be considered as an early sign of BC.

The problem of the role of the treatment in the development of BC is still open to discussion [3], so that the introduction of any new drug for CML should be carefully considered until it is shown that it does not give rise to BC earlier. DBM has been previously shown to be highly effective, to produce a poor and short-lasting haematological toxicity and no toxic side effects clinically recognizable [7, 8, 9, 10, 11, 12, 13, 14], but no data were available neither on its long-term effects nor on its influence on the duration of the disease. The survival data arising from this study and from previous reports [5, 18, 19, 20] are tabulated in Table 5. Minot's patients have been observed between 1898 and 1923, they did not receive any anti-leukaemic therapy nor could profit by any modern support treatment: 75% of them were alive after 22 months. The same percentage of survivors is at 20 months in the Reinhard's series, treated by ^{32}P; at 26 months in the Haut's series treated by busulfan; at 20 months in the Medical Research Council's series, also treated by busulfan. Seventy-five per cent survival in DBM patients is at 26 months. Median survival looks shorter in untreated patients and in patients receiving radiotherapy or ^{32}P. In patients treated by busulfan and by DBM, median survival is about the same: 40–43 months. Twenty-five per cent survival is at 62 months for DBM treated patients, and at 48 months for all the other groups.

The similarity or the curves shown in Fig. 3 for Group A, and Group B, as well as the data reported in Table 5, suggest that DBM does not elicit BC earlier, and that DBM does not affect negatively the course of CML. They also suggest that new strategies should be developed in order to postpone the onset of BC [4, 15, 16, 17].

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A Spectrophotometric Method for the Estimation of the Carcinostatic Agent 6-Chrysenamine (E.O.R.T.C. 116) in Biological Fluids and Tissues*

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Abstract—*An analytical method for the estimation of* 6-*chrysenamine (E.O.R.T.C.* 116) *in biological fluids and tissues is described. Blood and tumor levels were evaluated after a single and a subchronic treatment with* 6-*chrysenamine administered by different routes in* ($C_3H \times O_{20}$) F_1 *mice bearing spontaneous mammary tumor. The results obtained are discussed in terms of their therapeutical significance for experimental chemotherapy.*

INTRODUCTION

6-CHRYSENAMINE is known to inhibit the growth of solid tumors such as spontaneous mammary carcinoma in ($C_3H \times O_{20}$) F_1 mice and rhabdomyosarcoma BA 1112 in inbred Wag/Rij rats [1, 2, 3, 4]. The drug was used in clinical trials and 2 objective remissions were obtained on 32 patients with advanced mammary cancer. Nevertheless, its use was limited by adverse reactions after oral administration [5]. Since biochemical data on the disposition and metabolism of the drug were not yet available, it was of interest to set up an analytical method for the estimation of 6-chrysenamine in biological fluids and tissues. Blood and tumor levels were evaluated after a single and a subchronic treatment with 6-chrysenamine in mice bearing a spontaneous mammary tumor.

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MATERIAL AND METHODS

1. *Animals and tumor*

Tumor-bearing ($C_3H \times O_{20}$)F_1 hybrid mice were purchased from the International Centre for the Provision and Study of Tumor-Bearing Animals (Prof. O. Mühlbock, Amsterdam). Female mice were virgin or force-bred. The males had been castrated at an early age and they were then treated with oestrone added to their drinking water (2 mg/ml). These mice are known to show almost 100% of spontaneous mammary tumors at the age of about 250 days. These tumors occur along the milk crest in both females and castrated males [6, 7, 8], and they are predominantly of the adenocarcinomateous type. It has been established that there is no significant difference in the rate of tumor growth between castrated male or female mice, and that the tumor is not hormone-dependent. In fact, ovariectomy and treatment with androgens do not affect tumor growth at least until they reach a palpable size [9]. The animals used in the experiments were about 9 months old and weighed 25–30 g. They were kept in Makrolon cages (20 × 20 × 13 cm) at room temperature of 22°C and relative humidity of

60% with free access to water and food during the experiments.

2. *Evaluation of the antitumoral activity*

The $(C_3H \times O_{20})F_1$ hybrid mice bearing spontaneous mammary carcinoma were divided into groups; two diameters of the tumors were measured by means of a caliper and tumor growth was observed for a week. After this period, mice that did not show any change in tumor volume were excluded from the experiment. The others were submitted to treatment on days 1, 2, 3, 4, 5, 8, 10, 12. On days 1, 4, 8, 11, 15 the two diameters of the tumors were measured and the total body weight was determined.

If the animals showed more than one tumor at the beginning of the treatment, or if multiple tumors appeared in the course of the experiments, the mean of two diameters measured on the additional tumor was added to the mean of the original tumor just as if the mouse had one single, large tumor. On day 15, the animals were sacrificed and the wet weight of their tumors was recorded. The toxicity of the drug was evaluated by comparing the change of body weight, from the beginning to the end of the treatment, of treated animals with that of controls. The inhibition of tumor growth was evaluated according to the diameter mean and wet weight of the tumor at the end of the treatment. In each group, the sum of individual mean of tumor diameters divided by the number of animals gave the average tumor diameter for the group. The average tumor growth was calculated by subtracting the mean tumor diameter of each animal, recorded at the beginning of the experiments, from the value determined at the end.

3. *Drug, vehicle, route of administration*

6-Chrysenamine micronized in aqueous suspension (kindly supplied by Dr. M. Staquet, Brussels) was administered to mice intravenously (i.v.), intraperitoneally (i.p.) and orally (os) at the dose of 50 mg/kg.

4. *Chemical determination*

The estimation of 6-chrysenamine in biological material involved an extraction at neutral pH in benzol (Merck). The drug in the organic phase (98% recovery from an aqueous solution) was measured spectrophotometrically using a Hitachi-Perkin-Elmer Model 124 double beam greating spectrophotometer.

The compound exhibited two absorption peaks at 280 nm and at 345 nm, respectively. Relationship between the intensity of the two peaks was confirmed by several determinations of 6-chrysenamine in blood. Although at 280 nm the benzene itself showed a certain degree of absorption, it was preferred to operate at this wave-length, instead of 345 nm, because of a higher absorption intensity.

The biochemical determination of 6-chrysenamine was performed as follows: 0·5 ml of heparinized and defibrinized blood or 2 ml of tissue homogenate (1 : 3) in KCl solution 1·15% were transferred into a 15 ml glass tube. Sodium phosphate buffer (0·2 M pH 7·3) was added to the tube in order to reach a final volume of 3 ml.

The extraction was made by the addition of 3 ml of benzol. The tubes were shaken for 20 min and centrifuged. The supernatant organic phase was removed by aspiration and dried over anhydrous sodium sulphate. Two ml of the dried extract were transferred into a 10 mm path length quartz cuvet and the optical density was recorded at 280 nm.

A tissue blank run through the procedure gave optical density which ranged between 0·050 and 0·100 when the benzol was used for the zero setting.

Standards were prepared by handling known amounts of 6-chrysenamine in the same way as the unknown ones.

The optical densities were proportional to the concentrations of 6-chrysenamine ranging from 0·1 to 4 μg/ml.

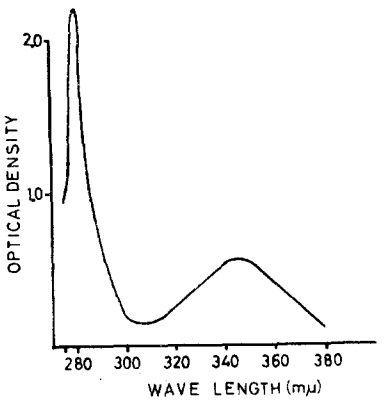

Fig. 1. *u.v. absorption spectrum of* 6-*chrysenamine* (10 μg/ml) *in benzol* (*Merck*).

An optical density of approximately 0·100 was obtained when 1 μg of 6-chrysenamine was run through the procedure. The addition of 6-chrysenamine to biological materials was recovered in 89 ± 5% from the blood and 70 ± 5% from the tumor.

Table 1. Blood (μg/ml ± S.E.) and tumor (μg/g ± S.E.) levels of 6-chrysenamine after intravenous administration in ($C_3H \times O_{20}$) F_1 tumor-bearing mice

	Dose mg/kg	Time after administration						
		0	1 min	30 min	1 hr	3 hr	8 hr	24 hr
Blood	50 × 1	—	36·2 ± 0·4	12·8 ± 0·6	9·8 ± 0·9	12·2 ± 0·9	7·0 ± 1	8·1 ± 0·8
	50 × 8	5·8 ± 1	30·5 ± 0·3*	14·3 ± 2	9·3 ± 2	14·4 ± 1	9·0 ± 0·8	11·5 ± 1·4
Tumor	50 × 1	—	14·6 ± 1·6	15·1 ± 0·7	17·8 ± 0·4	8·3 ± 1·6	< 1	< 1
	50 × 8	< 1	9·3 ± 0·8*	9·3 ± 1·2*	10·7 ± 3†	< 1	< 1	< 1

Each figure is the average of 6 determinations from 6 different animals.
Schedule of chronic treatment: one injection on days: 1, 2, 3, 4, 5, 8, 10, 12. On day 13 animals were further injected for measuring the levels of 6-chrysenamine.
Student's *t*-test: $*P < 0{\cdot}01$ $\dagger P < 0{\cdot}05$.

Table 2. Blood (μg/ml ± S.E.) and tumor (μg/g ± S.E.) levels of 6-chrysenamine after intraperitoneal administration in ($C_3H \times O_{20}$) F_1 tumour-bearing mice

	Dose mg/kg	Time after administration						
		0	1 min	30 min	1 hr	3 hr	8 hr	24 hr
Blood	50 × 1	—	6·8 ± 1·6	10·8 ± 0·6	7·1 ± 0·8	4·9 ± 1·2	7·2 ± 1·3	< 1
	50 × 8	5·2 ± 0·7	3·9 ± 0·4	3·7 ± 0·7*	3·1 ± 0·7*	4·5 ± 1·4	7·7 ± 2·6	2·8 ± 1·4
Tumor	50 × 1	—	< 1	< 1	< 1	< 1	< 1	< 1
	50 × 8	< 1	< 1	< 1	< 1	< 1	< 1	< 1

Each figure is the average of 6 determinations from 6 different animals.
Schedule of chronic treatment: one injection on days: 1, 2, 3, 4, 5, 8, 10, 12. On day 13 animals were further injected for measuring the levels of 6-chrysenamine.
Student's *t*-test: $*P < 0{\cdot}01$.

Table 3. Plasma and red cell distribution of 6 chrysenamine (50 mg/kg IV) in ($C_3H \times O_{20}$) F_1 tumor-bearing mice

Time after injection	% of the total blood concentration of 6-chrysenamine (+S.E.) in		Ratio p/c
	plasma (p)	red cells (c)	
10 min	48 ± 0·5	51 ± 6	0·94
8 hr	32 ± 4	67 ± 1	0·48

Each figure consists of 5 determinations carried out on plasma and red cells of the same blood sample.

RESULTS

1. *Intravenous administration*

6-Chrysenamine was determined in blood and tumor after a single or a repeated treatment. It is evident from Table 1 that after a single or repeated treatment (50 mg/kg) the peak level of 6-chrysenamine was at 1 min in the blood and at about 1 hr in the tumor. However, 6-chrysenamine disappeared in both types of treatment more rapidly from the tumor than from the blood. Repeated treatment resulted in blood and tumor 6-chrysenamine levels lower than after a single treatment.

2. *Intraperitoneal administration*

Table 2 shows that the peak level in the blood was reached at 30 min following the single treatment and only at 8 hr following the repeated one. Levels lower than the limit of sensitivity (1 μg/ml) of the employed method were found in the tumor both after acute and repeated treatments.

3. *Oral administration*

When 6-chrysenamine was given orally at the same dose and schedule as for the intravenous and intraperitoneal treatments, no drug was detected in blood or tumor at 0·5, 1, 3, 8, and 24 hr both after acute and repeated treatment.

4. *Plasma and red cell distribution*

As shown in Table 3, 6-chrysenamine injected intravenously disappeared more rapidly from plasma than from red cells. As a result of this, while the level of 6-chrysenamine were about equal in plasma and in red cells after 10 min, the ratio became 1 : 2 in favour of red cells after 1 hr.

5. *Relation between 6-chrysenamine in tumor and its antitumoral effect*

Table 4 shows that 6-chrysenamine injected intravenously at the dose of 50 mg/kg × 8 times was able to control tumor growth, a fact evident by evaluating the mean of tumor diameters measured during the course of the treatment. This results in a significant inhibition of tumor weight measured at the end of the treatment. Similar data were obtained after intraperitoneal administration of 50 mg/kg of 6-chrysenamine. On the contrary, 6-chrysenamine administered orally seemed to be ineffective in inhibiting tumor growth.

DISCUSSION

The method here described to determine 6-chrysenamine in blood and tumor should be accepted with caution since its specificity is not known as far as the metabolites of 6-chrysenamine are concerned. From the obtained data it is evident that levels of 6-chrysenamine in the blood of $(C_3H \times O_{20})F_1$ mice bearing spontaneous mammary carcinomas are considerably higher when the drug is given intravenously than when it is given intraperitoneally. This may reflect a difficulty in drug absorption from the peritoneal cavity. When the drug is given orally, no blood levels can be detected within the limits of sensitivity of the method. Repeated treatment results, in both intravenous and intraperitoneal administrations, in a reduced blood level of 6-chrysenamine. The explanation of this finding may be related to a reduced absorption of the drug from the peritoneal cavity and/or to an increased rate of biotransformation. As for the first possibility, it should be mentioned that after repeated intraperitoneal treatments there are signs of drug deposition and consequent inflammatory reactions in the peritoneal cavity. Regarding the second possibility, it may be recalled that previous findings showed that 6-chrysenamine was able to enhance the activity of the liver microsomal enzymes [10]. When the oral route is employed, the repeated treatment does not permit to build up measurable blood levels of 6-chrysenamine.

Table 4. Effect of 6-chrysenamine (50 mg/kg) administered by different routes on spontaneous mammary carcinoma $(C_3H \times O_{20})$ F_1 mice

No of Animals ♂	No of Animals ♀	Route of Administration	% body weight change	Mean tumor diameter (mm ± S.E.) 1	4	8	11	15	Av. tumor growth
5	5	—	+16	11·5 ± 1·3	14·6 ± 1·4	16·4 ± 1·7	18 ± 1·2	22·8 ± 3	+9·8 ± 2·5
5	5	IV	−1°	11·1 ± 1·0	11·7 ± 1	11·2 ± 1·5†	11·4 ± 1·2†	12·0 ± 1·2†	+0·1 ± 0·6
5	5	IP	+5*	11·4 ± 0·6	11·7 ± 0·7	11·7 ± 0·8†	12·3 ± 1·1†	14·6 ± 1·6†	+3·2 ± 1·3
5	5	OS	+9	11·7 ± 0·9	11·6 ± 1·1	12·7 ± 1·2	13 ± 1·4	16·5 ± 1·9	+5·7 ± 2·0

Schedule of treatment: one injection on days: 1, 2, 3, 4, 5, 8, 10, 12. On day 15 animals were sacrificed.
Student's *t*-test: *$P < 0.01$. †$P < 0.05$.

The levels in the tumor are consistent with the levels in blood as far as the intravenous route of administration is concerned. However, both for the intraperitoneal and oral routes (acute or repeated treatment) no tumor levels of 6-chrysenamine can be measured. The rate of disappearance from the tumor, in the case of intravenous treatment, is more rapid than from the blood. This finding may be explained by the fact that the drug is stored in the red cells, in a persistent way. Therefore, particularly at long times after administration, the drug in blood is present mostly in the red cells. This preferential storage in red cells may also explain why, in spite of measurable blood concentration no drug is to be found in the tumor after 6-chrysenamine administration by intraperitoneal route.

When the levels of 6-chrysenamine in the tumor are compared with the antitumoral effect of the drug, a lack of correlation is evident. In fact, a similar degree of activity is observed after the intravenous or the intraperitoneal administration even if the concentration of 6-chrysenamine in the tumor are quite different in the two experimental conditions. This finding may be explained in several ways as for instance: (*a*) the antitumoral activity of 6-chrysenamine is linked to levels of the drug below the sensitivity of the method but present in critical sites of the tumor; (*b*) the drug may exert its antitumoral activity by affecting other organs but the tumor; (*c*) 6-chrysenamine does not act *per se* but through the formation of active metabolites. As for the last point, it may be relevant to report that the plasma of animals treated with 6-chrysenamine shows cytotoxic activity against this spontaneous mammary tumor cultivated *in vitro* while 6-chrysenamine added *in vitro* is inactive [11]. Further studies will permit to identify these metabolites.

As far as clinical relevance is concerned, our studies indicate that the clinical trials previously mentioned [5] were performed by giving the drug through a questionable route of administration. Better results in the treatment of mammary tumors may, perhaps, be achieved if the drug could be given by intravenous administration.

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Carcinoembryonic Antigen (CEA): A comparison of the Farr and Z-gel Methods for CEA Detection in Benign and Malignant Diseases*

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Abstract—*Carcinoembryonic Antigen (CEA) was assayed in the serum of 177 patients with the Farr radioimmunoassay and in the plasma of 166 patients with the Z-gel radioimmunoassay. Patients were classified into four groups according to diagnosis: (I: gastro-intestinal (GIT) cancer; II: non GIT cancer; III: benign GIT disease; IV: benign non GIT disease). Abnormal CEA levels (> 2·5 ng/ml) were obtained in 66%, 57%, 39% and 15% with the Farr technique, and in 70%, 44%, 47% and 12% with the Z-gel method in the diagnosis groups I, II, III and IV respectively. The same aliquots of blood was also assayed in 72 patients with both techniques; results agreed in 87% of the cases. Statistical evaluation of the data indicate no difference between the two methods. The range of probability of a false negative CEA value is high no matter which technique is used. This limitation is partially circumvented by raising the considered normal CEA value to higher levels.*

INTRODUCTION

GOLD and Freedman [1, 2], in experiments involving the heteroimmunization of rabbits with normal and malignant human colon tissue, identified a tumor specific antigen found exclusively in entodermally derived malignant tumors and in normal fetal entodermal tissue up to the sixth week of gestation. In view of this dual origin the term of carcinoembryonic antigen (CEA) was given to the antigen. These authors also developed a modification of the Farr radioimmunoassay (RIA) to detect circulating CEA in serum, and found the assay to be highly specific for colo-rectal tumors [3] though more recent studies using the same technique have failed to confirm this specificity [4, 5]. A new simplified RIA using zirconyl phosphate gel (Z-gel) was developed by Hansen *et al.* [6] for CEA detection in plasma. With this technique, elevated levels of CEA were found in a variety of entodermally and non-entodermally derived cancer as well as in non-malignant diseases [7]. We have assayed CEA in the blood of patients with various benign and malignant diseases using the Farr and Z-gel RIA. A comparison of the results obtained by these two RIA methods forms the basis for this report.

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METHODS

CEA was assayed in the serum of 177 patients and in the plasma of 166 patients using the Farr and Z-gel RIA respectively. Aliquots of the same blood samples were also assayed in 72 patients with both techniques affording a more direct comparative evaluation of these techniques.

Serum aliquots were quick frozen and kept

frozen at −20°C until assayed in our laboratory within one week of the time of blood collection. Piasma aliquots were kept frozen at −20°C until shipped, in dry ice, to Hoffmann–LaRoche Cancer Diagnostic Research Laboratory in Nutley, New Jersey, within one week of the time of blood collection. All blood samples were assigned a code number and assayed under blind control.

The Farr RIA, using the method of Thomson [3], was performed as part of a National Cancer Institute of Canada/American Cancer Society collaborative study [5]. Standard inhibition curves were performed weekly, using CEA reagents provided by the Montreal laboratory of Freedman and Gold. Antibody titation curves were done at two month intervals upon receipt of radio-labelled CEA and CEA antiserum. Duplicate serum samples from our patients were also assayed by the Montreal reference laboratory [5] and 87% of our results agreed with theirs.

Plasma samples were assayed at Hoffmann–LaRoche Cancer Diagnostic Research Laboratory in Nutley, N.J., using the Z-gel RIA described by Hansen *et al.* [6, 7].

The upper limit of normal CEA levels for both Farr and Z-gel RIA is considered to be 2·5 nanograms per milliliter (ng/ml) of serum and plasma respectively [3, 7].

Patients were classified into 4 groups according to diagnosis: Group I: gastro-intestinal (GIT) cancer; Ia: colo-rectal cancer; Ib: non colo-rectal cancer; Group II: Non-GIT cancer; Group III: benign GIT disease; Group IV: benign non-GIT disease.

The number of patients of each group tested with Farr, Z-gel, and Farr + Z-gel assays, is shown in Table 1. Histologic confirmation of diagnosis was obtained in all but 6 patients with functional colitis and 2 patients with peptic ulcer. Those patients without histology were examined by upper and lower GIT radiologic studies and sigmoidoscopy. Blood was drawn pre-operatively in all cases except in some patients with metastatic cancer or benign disease in whom a diagnostic biopsy only was performed and in the 8 patients referred to above.

RESULTS

CEA results are shown in Tables 2 and 3. Abnormal antigenemia (CEA > 2·5 ng/ml) were obtained in 66%, 57%, 39% and 15% with the Farr RIA, and in 70%, 44%, 47% and 12% using the Z-gel RIA in the diagnosis groups I, II, III and IV respectively. When the same blood sample was assayed using both techniques in 72 patients, the overall results were in agreement in 87% of the cases (Table 4), with a respective agreement of 95%, 84%, 83% and 82% in disease groups I, II, III and IV.

Table 4 provides a comparison between the Farr and Z-gel techniques. There are 72 individuals whose blood was assayed by both techniques. Of these 31 agree at low CEA levels ($\leq$2·5 ng/ml), 32 agree at high CEA levels (>2·5 ng/ml) and 9 disagree. The question asked is whether the apparent high level of agreement between the two techniques (87%) is consistent with the 9 observations which disagree. That is to say, may it be inferred that the two

Table 1. *Diagnosis groups and number of patients tested for carcinoembryonic antigen*

Diagnosis Groups	Number of patients tested with the indicated method		
	Farr	Z-gel	Farr and Z-gel
I—Gastro-intestinal cancer			
Ia—colo-rectal	77	37	17
Ib—non colo-rectal	9	14	6
II—Non gastro-intestinal cancer	30	41	19
III—Benign gastro-intestinal diseases	48	34	24
IV—Benign non gastro-intestinal diseases	13	40	6
TOTAL	177	166	72

Table 2. Carcinoembryonic antigen levels in cancer patients

Diagnosis groups	*Farr** Number of patients with indicated CEA levels CEA ≦ 2·5 ng/ml	*Farr** Number of patients with indicated CEA levels CEA > 2.5 ng/ml	*Farr** Percentage with CEA > 2·5 ng/ml	*Z-gel*† Number of patients with indicated CEA levels CEA ≦ 2·5 ng/ml	*Z-gel*† Number of patients with indicated CEA levels CEA > 2·5 ng/ml	*Z-gel*† Percentage with CEA > 2·5 ng/ml
I—Gastro-intestinal cancer						
Ia—colo-rectal	22	55	71	9	28	76
Ib—non-colo-rectal						
Stomach	4	0		3	1	
Pancreas	1	2		2	5	
Other	2	0		1	2	
Total	29	57	66	15	36	70
II—Non-gastro-intestinal cancer						
Breast	7	12		17	5	
Lung	0	2		0	3	
Head and neck	2	0		3	1	
Ovary	1	1		1	3	
Edometrium	0	0		2	0	
Prostate	2	1		0	2	
Other	1	1		0	4	
Total	13	17	57	23	18	44

*ng/ml: nanogram per milliliter of serum.
†ng/ml: nanogram per milliliter of plasma.

Table 3. Carcinoembryonic antigen levels in patients with benign diseases

Diagnosis groups	*Farr**			*Z-gel*†		
	Number of patients with indicated CEA levels		Percentage with	Number of patients with indicated CEA levels		Percentage with
	CEA ≦ 2·5 ng/ml	CEA > 2·5 ng/ml	CEA > 2·5 ng/ml	CEA ≦ 2·5 ng/ml	CEA > 2·5 ng/ml	CEA > 2·5 ng/ml
III—Gastro-intestinal						
rectal polyp	3	3		1	3	
villous adenoma	2	1		0	0	
familial polyposis	3	0		1	0	
diverticulitis	3	3		2	3	
functional colitis	4	2		4	2	
peptic ulcer	1	1		2	0	
cholecystitis	3	3		1	3	
Crohn's disease	1	0		3	1	
ulcerative colitis	2	2		1	1	
other	7	4		3	3	
Total	29	19	39	18	16	47
IV—Non-gastro-intestinal						
cystic breast disease	6	2		30	3	
other	5	0		5	2	
Total	11	2	15	35	5	12

*ng/ml: nanogram per milliliter of serum.

†ng/ml: nanogram per milliliter of plasma.

Table 4. Comparison of the Farr and zirconyl phosphate gel radioimmunoassays on aliquots of the same blood samples

Disease group	Total No. of cases	No. of cases with CEA ≦ 2·5 ng/ml with both methods	No. of cases with CEA > 2·5 ng/ml with both methods	Percentage of agreement
I—Total GIT cancer	23	10	12	95
Ia—colo-rectal cancer	17	6	11	100
Ib—non-colo-rectal cancer	6	4	1	82
II—Non-GIT cancer	19	6	10	84
III—Benign GIT disease	24	10	10	83
IV—Benign non-GIT disease	6	5	0	82
TOTAL	72	31	32	87

methods are measuring the same thing? Statistical evaluation of the data indicate that there is no difference between the two techniques.

Tables 2 and 3 summarize the CEA levels obtained by the Farr and Z-gel techniques. For each of the techniques it is possible to consider all cancer and all non-cancer patients and to make inferences about the probability of a false negative and the probability of a false positive, and thus to compare each method on these grounds. Statistical analysis of the data indicate that the range of the probability of a false negative CEA value is between 0·39 and 0·63 with Farr RIA, and between 0·31 and 0·53 with Z-gel RIA. The probability of obtaining a false positive CEA value lies between 0·14 and 0·32 and between 0·18 and 0·40 respectively for the Farr and the Z-gel methods.

DISCUSSION

The lack of specificity of both RIA for GIT malignancies has been previously documented [4, 5, 7]. Our study confirms these findings, and also substantiates a recent report [10] documenting the close agreement between the Farr and Z-gel radioimmunoassays when both techniques are used to detect CEA on the same blood sample. In view of the similarity of the results obtained with both assays, it is probable that the ion sensitive antigentic site [9] measured with the Z-gel RIA is located on the same molecule as the CEA measured with the Farr RIA. When a normal CEA level is reported with the Farr RIA, the chance is between 0·37 and 0·61 that a patient does not have cancer, and between 0·47 and 0·69 with the Z-gel RIA. Thus, the incidence of false negative is high no matter which technique is used. When an abnormal CEA level is reported with the Farr RIA, the chance is between 0·68 and 0·86 that a patient has cancer, and between 0·60 and 0·82 with the Z-gel method. The Farr RIA is slightly superior to the Z-gel RIA in minimizing the number of false positives, but the latter technique offers the advantage of simplicity and rapidity over the Farr procedure. The CEA level is a useful screening device only when it comes out positive (> 2·5 ng/ml).

From a more practical clinical point of view, particularly from the standpoint of differentiating GIT cancer from benign GIT disease, the lack of specificity for GIT cancer and the high incidence of abnormal antigenemia in benign GIT diseases point to some of the difficulties in interpreting an abnormal CEA result. These difficulties can be partially circumvented, however, by raising the considered normal CEA value to higher levels (Table 5). As can be seen, for CEA levels greater than 8 ng/ml, the percentage of "abnormal" CEA in benign GIT disease decreases from 39% to 6% with the Farr RIA and from 47% to 0% with the Z-gel RIA, whereas this percentage decreases to 39% and 41% in GIT cancer, respectively, for both assays. This arbitrary cut-off separates benign from malignant GIT diseases more efficiently than the current accepted "normal" CEA level of 2·5 ng/ml. The gain in specificity thus obtained, though partially offset by a reduced incidence of GIT cancer detection, increases the clinical value of the test, as any patient with CEA levels greater than 8 ng/ml deserves a careful investigation and follow up to rule out the presence of a malignancy.

Martin and Martin [11] have found weak but significant CEA concentrations in non-cancerous

Table 5. Percentage of patients with various levels of carcinoembryonic antigen

CEA levels	Group I gastro-intestinal cancer		Group Ia colo-rectal cancer		Group II Non-gastro-intestinal cancer		Group III benign gastro-intestinal disease		Group IV benign non-gastro-intestinal disease	
	Farr	*Z-gel*	*Farr*	*Z-gel*	*Farr*	*Z-gel*	*Farr*	*Z-gel*	*Farr*	*Z-gel*
> 2·5 ng/ml	66	70	71	76	57	44	39	47	15	12
> 4·0 ng/ml	59	67	61	76	53	24	31	32	8	5
> 8·0 ng/ml	39	41	43	43	37	15	6	0	0	0

colonic mucosa. Differences in circulating CEA levels between benign and malignant GIT diseases are likely to be quantitative rather than qualitative. It is hoped that the availability of immunochemically pure CEA preparations will result in a clearer differentiation between benign and malignant GIT diseases than currently obtained.

Note—Statistical analysis of the data will be furnished upon request to Dr. M. Feldstein, State University of New York at Buffalo, Statistical Laboratory, 4230 Ridge Lea Road, Amherst, New York 14226, U.S.A.

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The Lack of Effect of Mouse Epidermal Growth Factor (EGF) and its Anti-Serum on Human Tissues

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Abstract—*Epidermal Growth Factor (EGF), isolated from adult male mouse submaxillary glands, had a profound effect on mouse tissues. Since this growth promoting effect of mouse EGF is "non-species specific" and enhances the growth of both normal and neoplastic mouse tissues, it was decided to investigate the influence of EGF and its antiserum on human tissues. The* in vitro *growth of human normal and neoplastic tissues was not influenced by either mouse EGF or its antiserum. Similarly, fluorescein-isothiocyanate (FITC) labelled antisera failed to stain any cellular component of the human tissues.*

INTRODUCTION

FACTORS are known to exist which specifically influence the growth of certain tissues. Two such factors have been shown to be present in adult male mouse submaxillary glands. One of them is a Nerve Growth Factor, which has a growth promoting effect on sympathetic and sensory ganglia of a variety of animals [1, 2]. The other is the Epidermal Growth Factor (EGF) which, injected into new-born mice, causes a marked thickening of the epidermis, precocious eruption of the incisors and early opening of the eyelids [3]. Jones [4] found that a crude preparation of salivary gland protein had a stimulating effect on the epithelial cells of several rat organs in organ culture and proposed the term "Epithelial Growth Factor". Turkington [5], using Cohen's own preparation [3] reported that EGF acts as a potent stimulus for cell proliferation in C3H mammary carcinoma.

In view of the "non-species specificity" of the action of mouse EGF, it was decided to investigate the possible effect of mouse EGF on human tissues.

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METHODS

EGF was isolated and assayed according to the method of Cohen [3]. The purified preparation was assayed by injecting varying concentrations into new-born mice. Controls received normal saline.

The procedure for the preparation of antisera in rabbits, gel diffusion and immunoelectrophoresis were the same as described by Kumar *et al.* [6]. The method of Andrews [7] was used to determine the molecular weight of EGF. The tissue culture methods and the preparation of autoradiographs were followed as described previously [8].

Sixteen human tumours comprising 9 breast carcinomas, 4 Wilms' tumours, 2 neuroblastomas and 1 hypernephroma were grown on coverslips in Leighton tubes for 36 hr in the presence or absence of EGF or its antiserum using similar methods to those of Kumar and Steward [9]. Three normal human kidneys and 4 normal human breasts were also cultured in

the presence and the absence of EGF or its antiserum. All cultures were pulse labelled for 2 hr, at the end of the culture period, with ^{3}H-tdR (0·5 μCi/ml, specific activity 5 Ci/mM). The rate of DNA synthesis of cells incorporating radioactivity was measured by immersing some of the coverslip culture successively in 5% trichloracetic acid and methanol and then standing them in scintillation vials to which 12 ml scintillation fluid had been added. Other cultures were saved for autoradiography.

Cryostat sections of freshly obtained biopsies from 32 human breast carcinomas, 5 Wilms' tumours, 1 hypernephroma, 3 normal human kidneys and 4 normal human breasts were used for the direct and indirect immunofluorescent studies [6]. Similarly, cryostat sections of various mouse organs including submaxillary salivary glands were treated with FITC-labelled antisera.

RESULTS

Administration of EGF into new born mice (2 μg/g body weight) resulted in stunted growth, loss of hair, wrinkling of skin, precocious eruption of incisors and early opening of eyelids, thickening and keratinization of epidermis (Figs. 1, 2 and 3). This showed the EGG to be biologically active. Using the calibrated sephadex G100 method the activity was in a single peak with an indicated molecular weight of 15,000.

A single precipitation line was obtained in both gel-diffusion and immunoelectrophoresis using the antisera raised in rabbits (Fig. 4).

Fluorescent labelled anti-EGF serum specifically stained tubular nuclei situated in the basal part of the cells of the mouse submandibular gland (Fig. 5). The fluorescence was blocked by pre-treatment with unlabelled antiserum and did not occur with FITC conjugated sera from rabbits which had received injections of Freund's adjuvant or no injections of any sort. FITC-labelled antiserum failed to stain any cellular component of the 32 human breast carcinomas, 5 Wilms' tumours, 1 hypernephroma, 3 normal kidneys and 4 normal breasts.

In organ cultures purified EGF (0·5–2·0 μg/ml) resulted in thickening of the mouse skin epidermis and kidney capsule. However, in the presence of EGF, the labelling indices and uptake of ^{3}H-tdR (scintillation counting) of all the 16 human tumours and 7 normal tissues tested remained unchanged compared with the controls. Similarly, addition of anti-EGF serum to the culture medium of 6 breast carcinomas, 2 Wilms' tumours, 1 hypernephroma, 3 normal kidneys and 4 normal breasts produced no change in the labelling index and uptake of ^{3}H-tdR.

DISCUSSION

The growth stimulatory effect of mouse EGF is not species specific in the sense that it is capable of promoting the growth of both normal mouse and normal rat organs [3, 4]. Furthermore, the growth enhancing effect of EGF is not limited to normal rodent tissues, but it markedly influences the growth of mouse mammary carcinoma [5].

However, the results of the present study indicate that the *in vitro* growth of human breast carcinoma, Wilms' tumour and hypernephroma, or normal human breast and kidney, are not influenced by either mouse EGF or its antiserum. Similarly, FITC-labelled antisera failed to stain any cellular components of human tumours and normal tissues. Therefore, it seems likely that the growth enhancing effect of mouse EGF may be limited to the rodents tissues.

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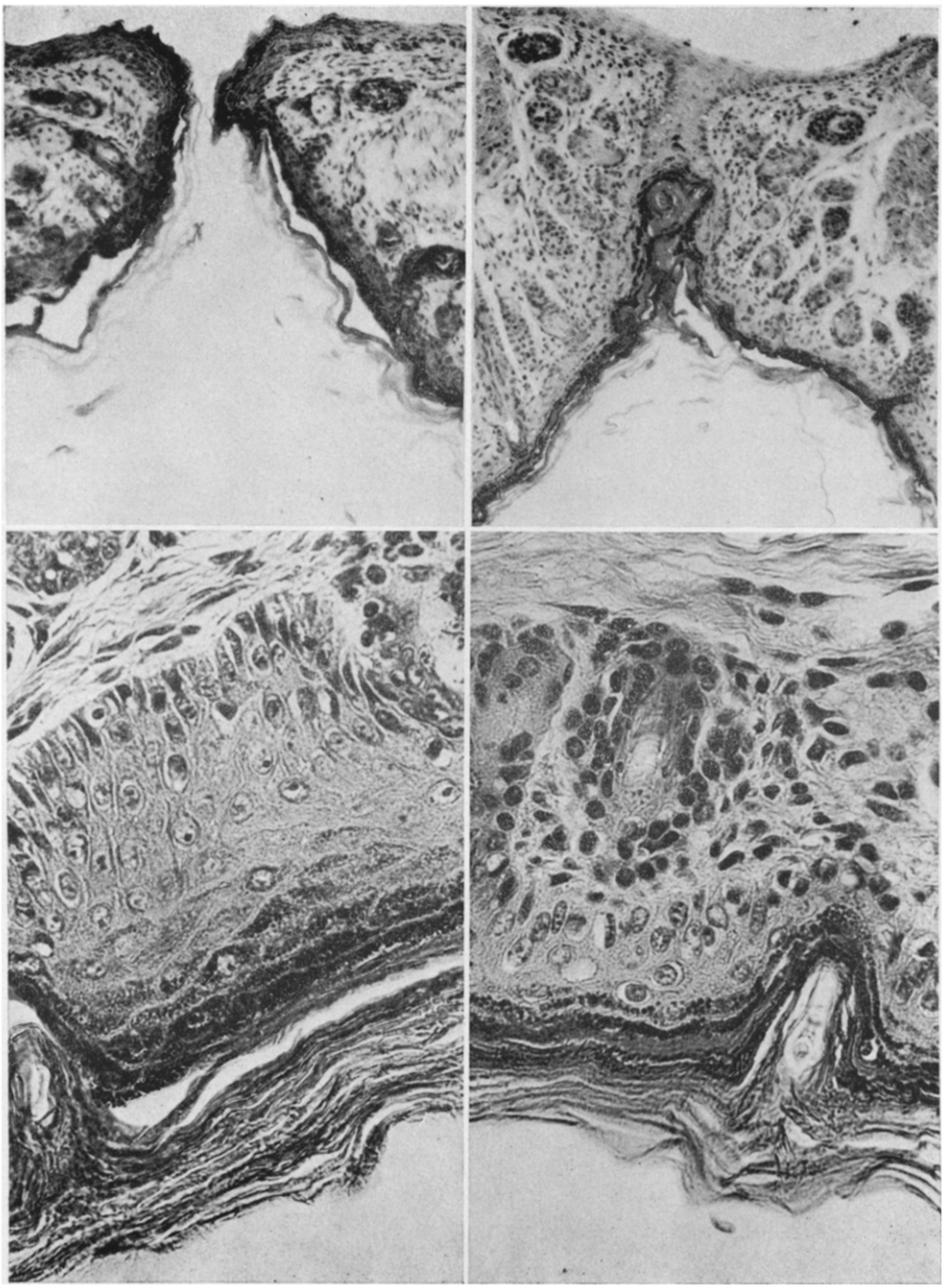

Fig. 3. *The effects of daily injections of purified EGF into new-born mice. Top left a histological section showing early opening of eyelids (experimental), control litter mates received normal saline only (top right). The injections of EGF also resulted in thickening of epidermis (bottom left), compare it with histological section of litter mate who received normal saline (bottom right).*

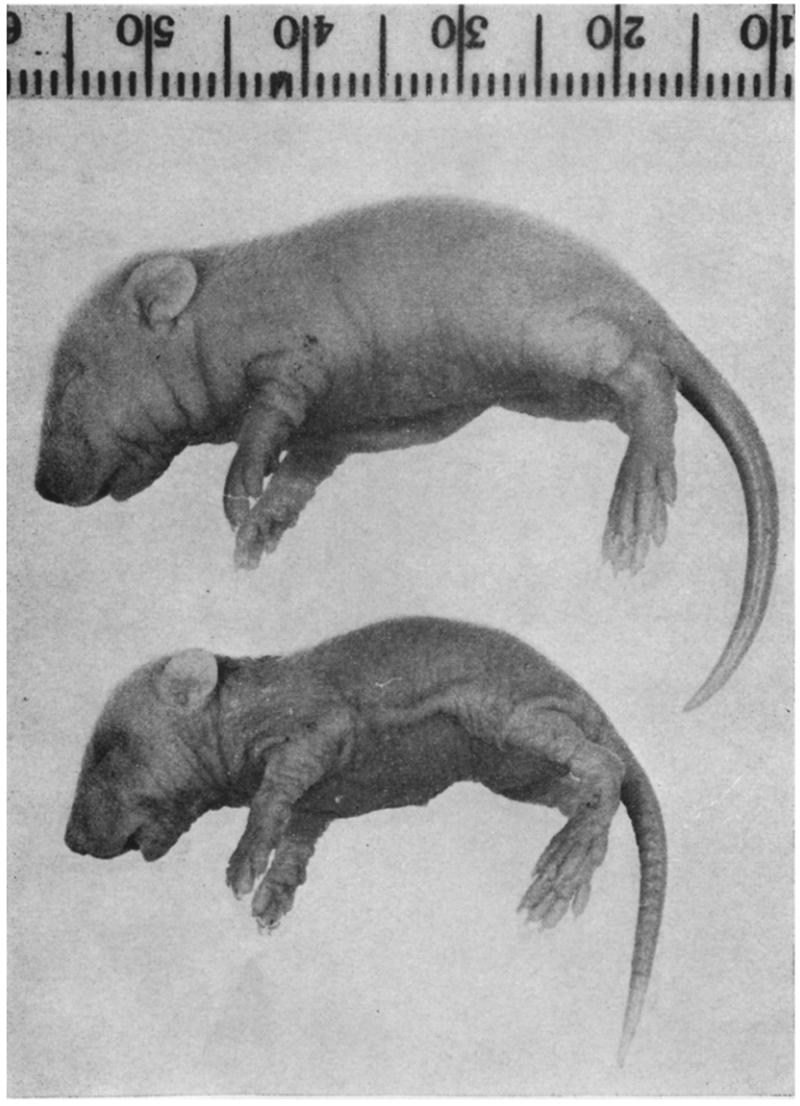

Fig. 1. *Gross appearance of a new-born mouse that was daily injected with purified (2μg/g body weight) for 7 days. Note stunted growth, wrinkling of skin and loss of hair in experimental mouse (bottom), control litter mate received normal saline (top).*

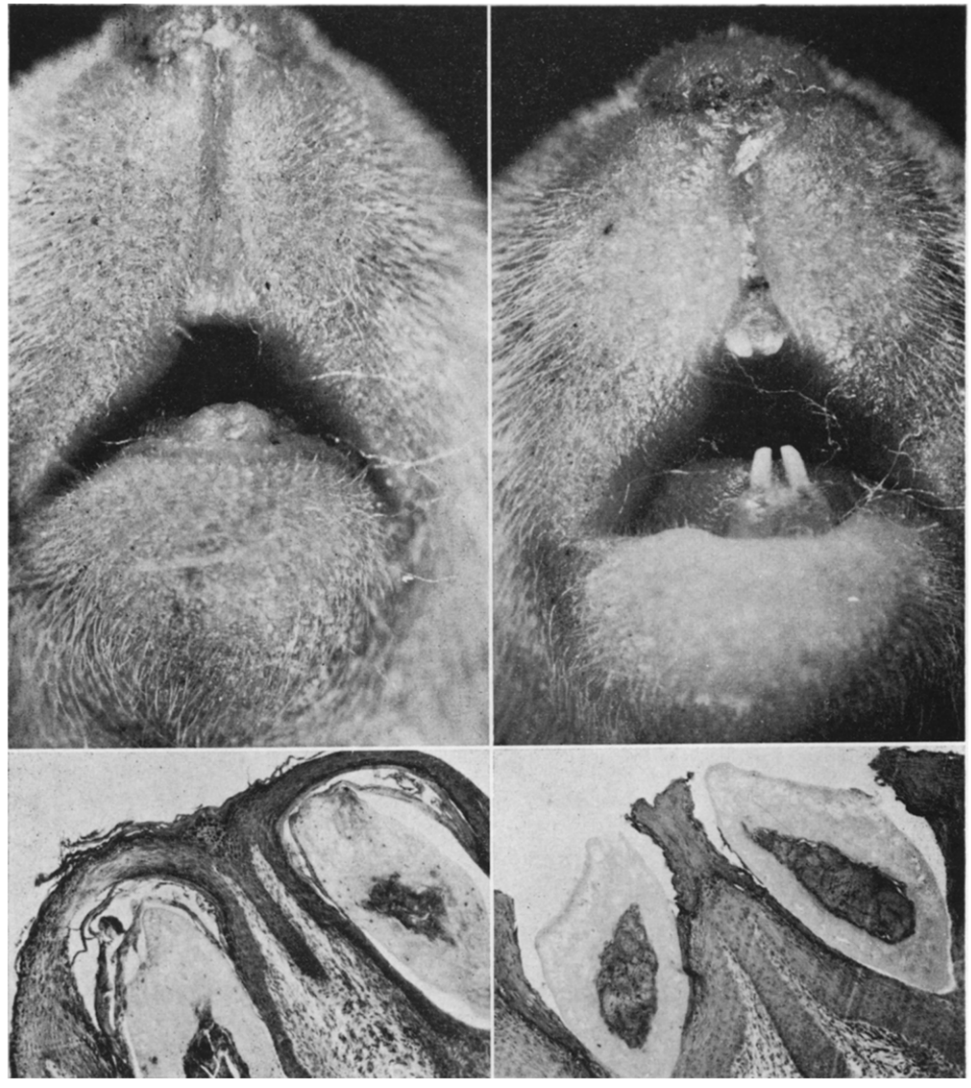

Fig. 2. *The effects of daily injections of purified EGF into new-born mice for 7 days. Experimental mice showed precocious eruption of upper and lower incisors (top right and lower right—histological section). Compare them with control litter mates who were injected with normal saline (top left and lower left—histological section).*

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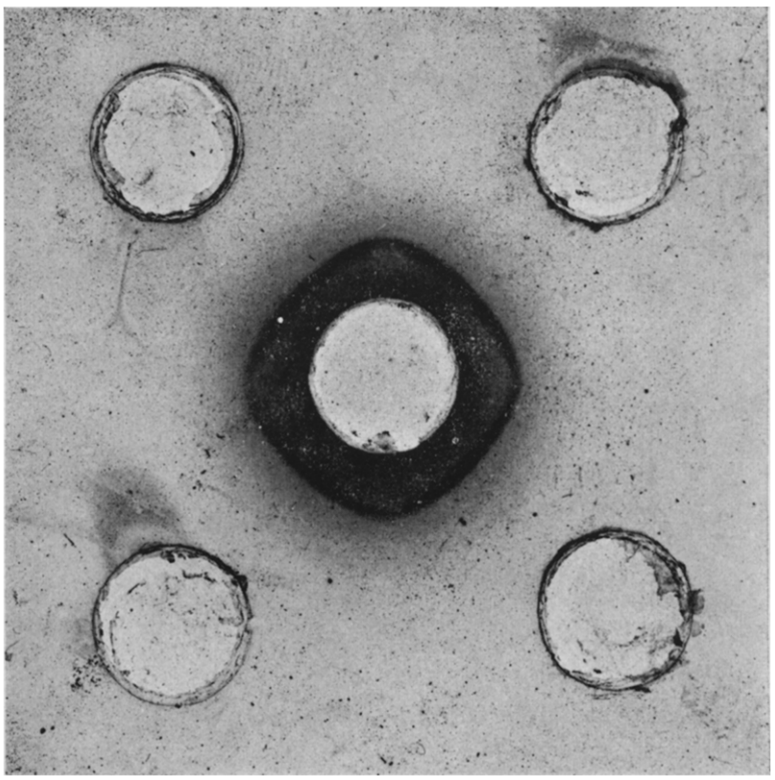

Fig. 4. In gel-diffusion only one line of precipitation was obtained, when purified EGF (peripheral wells) was confronted with its antiserum raised in rabbits (Central Well).

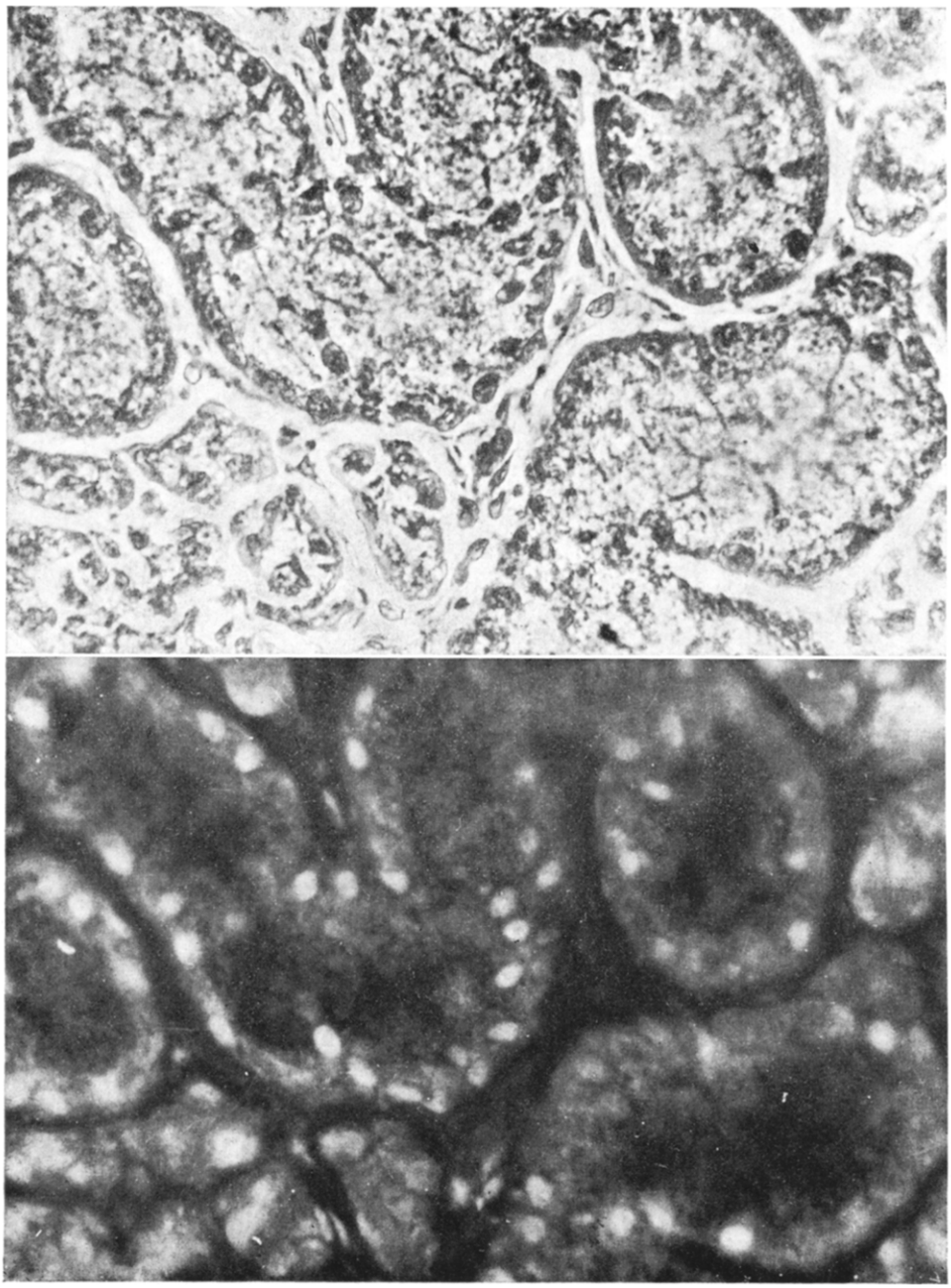

Fig. 5. Cryostat section (5μ) of an adult male mouse submaxillary salivary gland treated with anti-EGF serum conjugated with fluorescein isothiocyanate (FITC). Top phase contrast and bottom showing staining of tubular nuclei.

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The Specificity of the Cytoplasmic Antigen in Human Malignant Melanoma*

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Abstract—*It has been established that antibody to cytoplasmic antigen in malignant melanoma cells is present in the sera of patients with early or localized malignant melanoma. This study was performed to find out if antibody to cytoplasmic antigen is present in the serum of patients with skin lesions other than malignant melanoma. No antibody could be demonstrated in any of these cases, thus confirming the specificity of cytoplasmic antigen in melanoma cells to raise antibodies only in those patients with early or localized malignant melanoma.*

INTRODUCTION

DURING recent studies into immunological host reactions in patients with cutaneous malignant melanoma [1, 2], circulating tumour-specific antibodies were found in those patients with early or localized tumours, but not in those patients whose tumour was widely disseminated [3]. These antibodies were demonstrated by complement-dependent cytotoxicity tests [1, 4], immunofluorescent microscopy [2, 5–8] and radioactive isotope uptake studies [9], and have been confirmed recently by a variety of immunodiffusion and immuno-electrophoretic techniques [10, 11].

Immunofluorescent microscopical methods demonstrated two distinct types of antigen in melanoma cells. They were situated in:

1. the cytoplasm [3, 5–8] of snap-frozen malignant melanoma cell smear preparations and short term cultured melanoma cells. The cell cytoplasm stained positively with a fluorescein labelled antiglobulin after treatment with positive serum from the same or another patient.
2. the membrane [12, 13] of living melanoma cells. Significant titres of antibody were seen in the sera only of individual patients against their own tumour cells [7, 14].

Nuclear immunofluorescence [5, 8] has also been observed in acetone-fixed smears of malignant melanoma cells from freshly biopsied specimens, which have been incubated with fluorescein-labelled specific antiglobulin, but not in other studies [3, 7, 11, 14, 21] where the smears have been snap-frozen in liquid nitrogenisopentane and incubated with a specific antiglobulin.

This study was performed to examine the specificity of cytoplasmic antigen in melanoma cells by incubating sera from patients with a wide range of benign and malignant, pigmented and non-pigmented skin lesions against melanoma cells, and to determine its value as a screening test in cases of suspected malignant melanoma.

MATERIAL AND METHODS

Blood was taken from a consecutive and unselected series of patients attending the skin out-patient department at the Royal Devon and Exeter Hospital, Exeter, for biopsy or excision of a wide range of skin lesions. The sera

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were separated on receipt in the laboratory at Exeter and stored at −18°C to await transport to London. At Westminster Hospital the sera were examined by the techniques used for the demonstration of cytoplasmic antigen in melanoma cells by two of us (P.G.E. and B.T.) as follows: [7, 15]. Smears of melanoma cells obtained from fresh biopsy specimens were snap-frozen in liquid nitrogen–isopentane at −160°C for 2 minutes and then dried in air, after which they were washed in phosphate-buffered saline to remove adherent isopentane. The test sera, together with known positive and negative controls as well as normal human serum, and a conjugate control in phosphate-buffered saline were then diluted 1:4 with phosphate-buffered normal saline.

The object of including a phosphate-buffered saline conjugate control was to ensure a correct washing dilution of, and to test the reliability of the fluorescein-labelled goat anti-human γ globulin.* The diluted serum was incubated with the melanoma smears for 30 min at 20°C in a moist chamber and then washed for 30 minutes with phosphate-buffered saline. Then fluorescein labelled goat anti-human γ globulin† [16–19] was added and left in contact with the melanoma cells for 30 min at 20°C. The smears were then washed with phosphate-buffered saline for 1 hr and mounted in 1:1 phosphate-buffered saline–glycerol mixture at pH 7·6. The smears were then examined under incident ultra-violet illumination using a Leitz Orthoplan microscope equipped with a HBO 200 mercury vapour light source. Ground glass Schott UG1, BG12 and KP490 primary filters were used in the light path, together with a K530/510 secondary filter and a 45° inclined dichroic mirror setting of 3. [20] Strict criteria were applied. Positive results were recorded only when 25% or more of the total number of cells on the smear showed fluorescent staining of the cytoplasm at a titre of 1:4 or greater in an average of five fields at ×400 magnification. The endpoint of the titration was taken to be the last dilution of the patient's serum that showed these stringent requirements. The baseline titre of 1:4 was found to be necessary to exclude the possibility of non-specific reactions, which were found even in the control sera when applied to the cell smears either neat or in a dilution of 1:2 [7, 21]. All positive sera were re-tested and titrated as outlined.

*Obtained from the Wellcome Foundation Limited, Temple Hill, Dartford, Kent, England DA1 5AH.

RESULTS

The sera from 152 patients were examined, and in every case the diagnosis was confirmed histologically by one of us (P.R.G.N.) independently at Exeter. The histopathology reports were not forwarded until the tests had been completed in London. The skin lesions were divided into pigmented and non-pigmented on the basis of whether or not there was melanin present in the lesions or the overlying skin. Cytoplasmic staining was negative in 150 patients in whom the following histopathological diagnoses were made:

Pigmented lesions
- Squamous cell carcinoma
- Basal cell carcinoma
- Seborrhoeic warts
- Squamous cell papilloma
- Epidermoid cysts with overlying hyperpigmentation of epidermis
- Histiocytoma with deeply pigmented overlying epidermis
- Intradermal cellular naevus
- Activated junctional naevus

Non-pigmented lesions
- Squamous cell carcinoma
- Squamous cell papilloma
- Molluscum sebaceum
- Verruca vulgaris
- Epidermoid cysts
- Histiocytoma
- Molluscum contagiosum
- Neurofibroma
- Angioma
- Calcifying epithelioma of Malherbe
- Glomangioma
- Mycosis fungoides
- Non-specific vasculitis
- Chronic dermatitis

Serum from two patients in this study wh skin tumours were subsequently diagnosed malignant melanoma were also tested. T serum from one of these patients gave posi cytoplasmic staining but no intranuclear st ing, as did all our positive control sera. T patient fulfilled the criteria for acceptance Stage I case clinically [3], and is at present and free of metastases, 2½ years after exci of her primary tumour. The serum from other patient with malignant melanoma sho no antibodies to cytoplasmic antigen. weeks after excision of the primary tumou was found to have widespread metastases. patient was most probably a Stage III clinically at the time of excision of his prir

tumour although clinical evidence of metastases was not evident at the time.

DISCUSSION

The common cytoplasmic antigen in malignant melanoma has already been established by specific immune blocking and absorption studies [3, 7] to be tumour specific in early or localized malignant melanoma. It has been shown in a recent study [21] into the immunological relationships between the halo naevus and malignant melanoma that antibody to cytoplasmic antigen is also present in the blood of patients with halo naevi but not in those patients who had had their halo naevi excised 3–5 months previously.

Our investigation was performed to find out whether antibody to cytoplasmic antigen could be demonstrated in the blood of patients who presented with a wide range of pigmented lesions that could be misdiagnosed as malignant melanoma clinically; a number of patients with non-pigmented lesions were also included in this study. In every case, apart from one of the two cases with malignant melanoma, no antibody to cytoplasmic antigen could be demonstrated, thus establishing the fact that antibody is present only in those patients who have early or localized malignant melanoma, or who have halo naevi [21]. A study [21] on a smaller number of cases with pigmented lesions that could be misdiagnosed as malignant melanoma clinically has confirmed our findings; none of their patients' sera induced positive cytoplasmic staining of melanoma cells.

We believe that the examination of the sera of patients with suspected malignant melanoma for antibody to cytoplasmic antigen in melanoma cells is of value as a screening test which is quick and easy to perform, and which has been shown to be reliable in one other survey using a large number of patients.

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Europ. J. Cancer Vol. 9, pp. 611–612. Pergamon Press 1973. Printed in Great Britain

Introduction

IT SO HAPPENED that in the Spring of 1973 a certain number of manuscripts were ready for publication from the Service of Medicine and Laboratory of Clinical Investigation of the Institut Jules Bordet. This year happens also to be the 20th anniversary of the creation of this service and its laboratories. The idea was proposed to publish these manuscripts together and make them into a special issue of the European Journal of Cancer: hence this Anniversary Number. However, two conditions were put by the Editor before the suggestion was effected. The first was of course that the authors should agree to submit their papers for publication in the European Journal of Cancer instead of sending them to the various journals in which they usually publish their work. Such acceptance was immediately given and gratefully received. The second was a natural and necessary condition also immediately agreed upon: all manuscripts should be submitted to referees in the usual manner adopted by the Journal since its inception, and subjected to the rigorous editorial screening and revision which is the policy of the European Journal of Cancer. Clearly an Anniversary Number should not be an excuse or a pretext for the publication of articles by-passing the usual editorial criteria of the Journal.

These two conditions being fulfilled, the Editor believes that there probably should be no objection to the publication of an issue assembling a series of articles representative of a significant part of the research activity of a single medical service. This publication, because of the date it commemorates, certainly has sentimental value to the members of the staff. However, a better reason may be that some readers, not well acquainted with the functions and orientations of a medical service in a Cancer Center, will perhaps find it interesting to observe the increasing role played by the disciplines of internal medicine in the care of cancer patients and in cancer research. Twenty years ago, such a participation of internal medicine was at best tolerated in many Cancer Centers in this and other countries in Europe and in America. Now, twenty years later, internal medicine is recognized as indispensable, along with the radiotherapeutic and surgical approaches, to insure patients' total care, and to explore and develop "medical" types of treatment in a field formerly considered to belong exclusively to the radiotherapist and the surgeon.

Perhaps we, internists in cancer medicine, may say here with the appropriate modesty that what seemed to some to be an adventure fraught with uncertainty one generation ago has now, with the successful creation and development of medical oncology services and specialists, turned out to be an important deployment of the logistics of cancer medicine. Although this is now well recognized by the great majority of those responsible for the administration of existing Cancer Centers and the planners of new centers to be created here and abroad, there occasionally persists in certain cancer therapeutic units the anachronic absence of adequately represented and structured medical oncology. In Brussels, we have to be thankful to the "Université Libre de Bruxelles" for having favored from the beginning the development of a complete Cancer Center in the country, in collaboration with the "Commission d'Assistance Publique" of the City of Brussels.

Much of the work published in this issue could not have been done without the stimulation and collaboration provided by the other services of the Center: Radiotherapy (Professor Henry), Surgery (Professor Smets), Pathology (Professor Gompel), Radiology (Professor Jeanmart) and Anesthesiology (Professor Reinhold).

The European Organization for Research on Treatment of Cancer (EORTC) has always provided the most stimulating intellectual environment for our Center. We are thankful to the members of this organization who are also our best and faithful friends. They are also the founder members of the European Journal of Cancer.

Much credit is due to the National Cancer Institute of the National Institutes of Health, Bethesda, Md, for active collaboration with the EORTC in joint programs of cooperative clinical trials and screening of new agents.

The most important support for cancer research in Belgium in recent years has come from the "Fonds Cancérologique de la Caisse Générale d'Epargne et de Retraite" or "Algemene Spaar en Lijfrentekas". The generosity and vision of this Foundation, its Scientific Committee and Administrative Board deserve unmitigated praise from cancer research workers in this country and the public.

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Mode of Action of Chemotherapy *in vivo* on Human Acute Leukemia—II. Vincristine*

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Abstract—*The action of VCR* in vivo *on the survival of circulating leukemic blast cells was studied in one case of adult acute lymphoblastic leukemia. Providing certain assumptions are made, this study indicates a cytolytic effect of VCR on the blood blast cells. This effect was seen despite the absence of mitosis in the blood. It is concluded that VCR, at clinically useful doses, may destroy G_1 leukemic blasts and that its action is not always dependent on the passage of cells through mitosis.*

INTRODUCTION

IT HAS BEEN shown that the effect of many antitumor agents depends on the cell-cycle phase wherein the exposed cells are engaged and on the proliferative activity of the concerned cell population. For instance, vincristine (VCR), an alkaloid extracted from the periwinkle plant *Vinca rosea* Linn, is known to block proliferative cells in metaphase [1, 2, 3] by interfering with the formation of the microtubules of the mitotic spindle [4]. Although the drug seems capable of entering the cell during phases other than mitosis [5], many authors consider VCR a cell-cycle-phase-dependent agent blocking or killing cells during mitosis [6].

By itself, administered once a week, VCR is capable of inducing a complete remission in more than 50% of patients with acute lymphoblastic leukemia (ALL) [7, 8, 9]. Such a therapeutic effect is difficult to understand by a strictly phase-dependent action in view of the following facts:

(*a*) the peak of metaphase figures observed after VCR administration is reached within 24 hr [10, 11]. Thereafter, telophases are again observed [5].

(*b*) 7–18% of the blast cells in the bone marrow do not enter DNA synthesis phase, within a period of 8 to 10 days, as shown by Clarkson [12]. The percentage of blast cells not entering mitosis must thus be of the same order.

In order to test the postulated cell-cycle phase dependency of VCR, a study was undertaken regarding its action on blood blast cells. Since blast cells in the blood enter mitosis only occasionally and are essentially G_1 cells [13], any immediate effect of VCR on these cells would exclude it from the category of strictly cell-cycle-dependent drugs.

MATERIAL AND METHODS

One male patient, 43 years old, suffering

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from acute lymphoblastic leukemia was studied. The bone marrow contained approximately 50% lymphoblasts. The white blood cells (183,000/mm^3) were essentially lymphoblasts (94%). The study was performed as soon as the diagnosis was made and prior to the administration of any antileukemic drug. The method used was essentially the same as the one described by Clarkson [14]. Five hundred milliliters of blood were withdrawn in a plastic bag (Fenwal®) containing 75 ml of ACDA solution. Five hundred μCi tritiated cytidine (Amersham, S.A. 26·8 Ci/mM) were injected into the bag. The suspension was incubated for 1 hr at 37°C and thereafter autotransfused to the donor within 12 min. Seventy-three hours after the autotransfusion of labeled blasts, one dose of VCR (2 mg/m^{2}BSA) was injected i.v. and it was thereafter continued at the same dose once a week. Fifteen days after the beginning of the treatment, prednisone (40 mg/m^2 BSA per day) was added. A complete remission was observed in the bone marrow on day 21.

The leucocytosis, the differential count, the absolute number of blast cell/mm^3, and the percentage of blasts labeled were determined immediately after transfusion, and at least twice a day thereafter for one week.

Forty-six hours after the autotransfusion of labeled blasts a sample of bone marrow was taken at the sternum. Since the labeling index of the marrow blasts with ^{3}H cytidine was only of 0·7%, a second labeling by incubating the marrow cells *in vitro* for 1 hr with ^{3}H thymidine (Nuclear Center, Amersham, England, SA 5 Ci/mM) at room temperature made it possible to determine the fraction of marrow blasts in DNA synthesis. A similar ^{3}H thymidine labeling was not performed for the blood blasts since it was expected that the ^{3}H thymidine (*vitro*) labeling index would have been of the same order of magnitude as the ^{3}H cytidine labeling index (*vitro–vivo*) and therefore impossible to determine on the same autoradiography. Therefore the fraction of circulating blasts in DNA synthesis was estimated by 2 other methods. Firstly, smears made of the blood incubated with ^{3}H cytidine before the autotransfusion were dipped in perchloric acid 5% at 4°C for 5 hr in order to extract the RNA [15]. Only the cells which were in DNA synthesis while in the presence of ^{3}H cytidine and had incorporated ^{3}H cytidine in their DNA remained labeled after perchloric acid extraction. Autoradiographies of these smears made it possible to determine a labeling index representing the fraction of blasts in DNA synthesis. Secondly, the fraction of cells containing between 2 and 4 n DNA in their nucleus was determined by a Barr and Stroud microdensitometer after Feulgen staining on 100 circulating blasts 67, 81 and 106 hr after autotransfusion, i.e., respectively before and 8 and 33 hr after the injection of VCR.

Methanol-fixed blood smears were immersed in a solution of ^{3}H actinomycin D 10^{-5} M (Schwarz Bioresearch Orangeburg, New York) (S.A. 3·8 μCi/mM) for 1 hr at room temperature in order to evaluate on autoradiographies the capacity of the DNA of the blood cells to bind actinomycin before, 8 hr and 33 hr after the injection of VCR.

All the smears were fixed in methanol for 30 min and dipped in a photographic emulsion (Kodak NTB2) diluted with distilled water (1:1, v/v). The slides labeled by ^{3}H cytidine (1 μCi/ml) were exposed in the dark for 53 and 131 days, those also labeled by ^{3}H thymidine (10 μCi/ml) were exposed for 20 days, while those incubated in the presence of ^{3}H actinomycin were exposed for only 20 hr.

The ^{3}H cytidine labeling indices were determined by observing 5000 to 10,000 leukemic blasts. The ^{3}H thymidine labeling index of the bone marrow was determined on 1000 leukemic blasts. The mean grain count of the cells exposed to ^{3}H actinomycin D was obtained by counting the number of silver grains overlying the nuclei of 100 leukemic blasts.

RESULTS

Figure 1

This shows on semilogarithmic paper the variation of the absolute number of leukemic blasts/mm^3 of blood, the origin of time being the end of the autotransfusion of labeled blasts. The total number of blasts remained essentially constant up to the first 24 hr following the injection of VCR. Thereafter, the total number of leukemic blasts in the blood decreased dramatically in an exponential fashion.

Figure 2

This shows on semilogarithmic paper the variation of the absolute number of labeled blasts/mm^3 of blood as a function of time, the origin of time being the same as in Fig. 1. For more than 90 hr, an apparently exponential decrease was observed with a T1/2 of approximately 60 hr. As a consequence of VCR being injected at 73 hr and after a delay of approximately 20 hr, the absolute number of labeled blasts in the blood decreased much faster. The

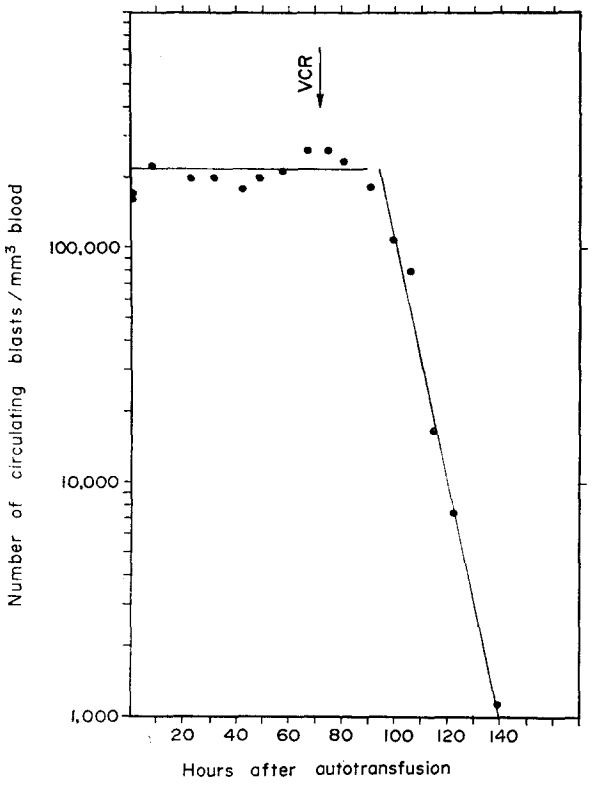

Fig. 1. The absolute number of blasts/mm³ of blood as a function of time from the end of the autotransfusion of labeled cells. The arrow (VCR) indicates the injection of vincristine.

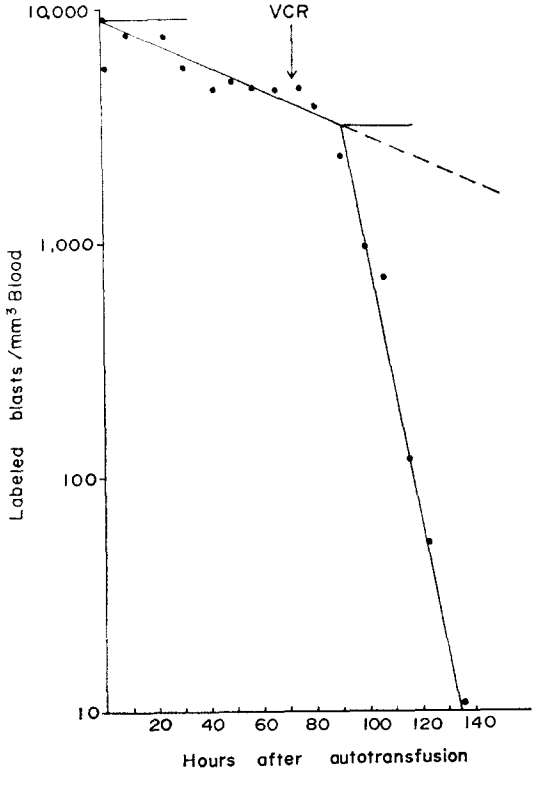

Fig. 2. The absolute number of labeled blasts/mm³ of blood as a function of time from the end of their autotransfusion. The arrow (VCR) indicates the injection of vincristine.

decrease remained exponential but the T1/2 was only 5 hr.

Figure 3

This shows, on semilogarithmic paper, the changes of the percentage of labeled blasts in the circulation as a function of time, from the end of the autotransfusion of labeled cells on. The variation of the percentage of labeled blasts was exponential and was not modified by VCR. These data were obtained on autoradiographies exposed in the dark for 53 days. The mean grain count per cell from the beginning of the study till the end decreased by a factor of almost 3 from 41 to 14. However, since a cell was considered as labeled when more than 3 grains only were seen on its surface it seems unlikely that the loss of the label was responsible for the decrease of labeled cells. In order to rule out this possibility, additional autoradiographies were made but exposed in the dark approximately 3 times longer (131 days instead of 53). Under these conditions, the decrease remained exponential and the slope of the exponential decrease was not modified by VCR indicating that the decrease observed was really due to a disappearance of cells but not to a disappearance of the labeled RNA from the cell by the normal process of RNA turnover.

Figure 4

This shows an histogram of marrow and blood blasts according to their DNA content just before, and 8 and 33 hr after the injection of VCR. In the bone marrow, the percentage of blasts containing more than 2n DNA was 12%. This value, determined on 100 cells, is in agreement with an ^{3}H thymidine labeling

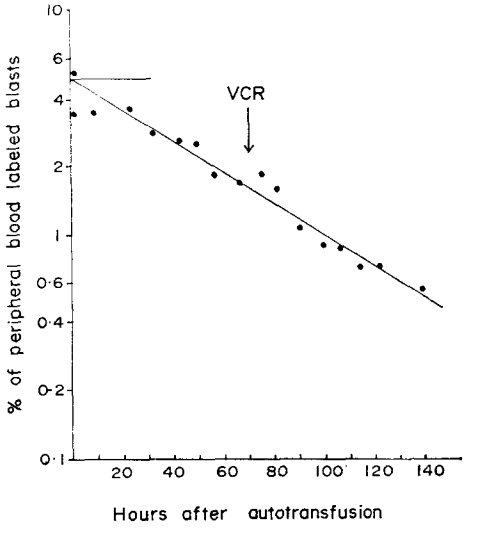

Fig. 3. The $^{3}HTdR$ labeling index of blood leukemic blasts as a function of time from the end of their autotransfusion. The arrow (VCR) indicates the injection of vincristine.

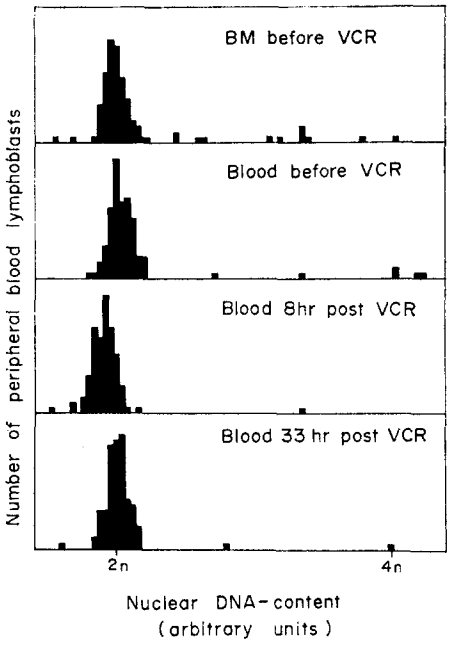

Fig. 4. Distribution of blood and marrow lymphoblasts with respect to nuclear DNA content: 2 n, diploid DNA content; 4 n, tetraploid DNA content. In the marrow, before vincristine 11 cells are situated between 2 n and 4 n. In the blood, 2–4 cells are situated between 2 and 4 n.

index of 13·9%. On the other hand, by microdensitometry of the blood blasts it was shown that around 5% of the cells contained more than 2 n DNA, a value which is close to 7·6% of cells still labeled by ^{3}H cytidine after perchloric acid extraction. The values obtained later (1 or 2%), respectively 8 and 33 hr after the injection of VCR, were certainly not higher.

The binding of ^{3}H actinomycin D on DNA was evaluated on autoradiographies. The mean grain count of the circulating blasts was lower (5·5 and 17 respectively at 8 and 33 hr) after VCR than before (22·7).

DISCUSSION

Many investigators have shown that the proliferative activity of the leukemic cells is low in the blood as compared to the bone marrow [16, 17]. This was true also for the patient who is the subject of the present study. This apparently results from a preferential release of slowly proliferating cells from the bone marrow into the blood [18]. Such a selective passage allows one to observe, in a particular patient, at the same moment in different sites of the organism, cells belonging to the same population, but with different proliferative activities. One can thus study the effect of VCR on a cell population which only rarely enters mitosis (the blood leukemic cells) and compare it to the effect on a more intensively proliferating population (the bone marrow leukemic cells). The observation of the kinetics of blast cells is however complicated by the existence of a continuous flux of these cells from the marrow into the blood and from the blood to the tissues [19]. Therefore the study of the effect of a drug on blood blast cells requires to begin with a knowledge of these fluxes in the "natural" condition, i.e., prior to the administration of any drug.

For the purpose of describing the observation results and of achieving some insight into the action of VCR, a mathematical model is constructed. Accordingly, two compartments are defined, one corresponding to the blood and one corresponding to the marrow. In Fig. 5, the model is illustrated by indicating the paths that blast cells in the system were assumed to follow.

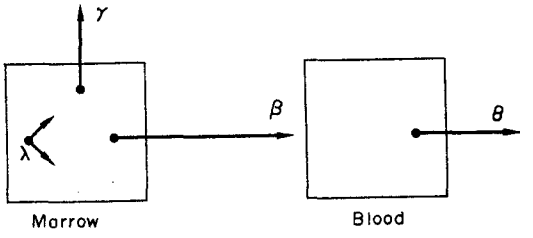

Fig. 5. Model for blood and bone marrow blasts. Kinetics before VCR. The parameters γ, λ, β, θ are defined in the appendix.

There are studies indicating that, in the absence of treatment, there is no cell return from the blood to the marrow or at least that such a return is of negligible order [20]. Therefore, before the injection of VCR, the model is specified only by the four parameters λ, γ, β, θ indicated in Fig. 5 and defined in the appendix.

At time t_1, VCR was introduced and this resulted in a change in the parameter values. There was apparently about a 24-hr period of instability before again the system became simple to describe. This period of instability is indicated observationally by the fact that the largest departures from the expected straight line in Fig. 3 appeared immediately after VCR was introduced. In Fig. 6, the situation is described allowing 24 hr for the system to stabilize (t_2) after the injection of VCR. Since several published reports on the effect of VCR on the rate of human leukemic cell division have indicated that this effect does not last much longer than 24 hr [10, 11] it may be suggested that $\lambda' = \lambda$ once the system has stabilized. Moreover, arguments exist[17] to suggest that the effect of chemotherapy on the

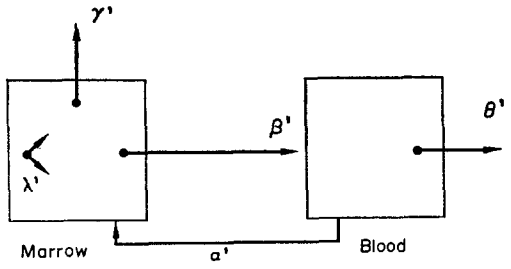

Fig. 6. Model for blood and bone marrow blasts. Kinetics after VCR. The parameters γ', λ', β', θ' and α' are defined in the appendix. The parameter α' has been introduced in the model to account for the possibility that blasts return from the blood to the bone marrow. Such a return was demonstrated however only in one leukemic patient treated with methotrexate [21].

release of blasts from the marrow to the blood is an immediate consequence of its effect on cell division. Thus when $\lambda' = \lambda$ after t_2, it would seem reasonable that we would also have $\beta' = \beta$.

As an alternative to cell kill, Killmann *et al.* [21] have proposed that the dramatic effect of some chemotherapic agents on the leukemic cells of the blood might be explained by a return of the blast cells of the blood to the bone marrow, as it was observed by them in one patient with ALL after an injection of MTX. In other words, the increase of the rate of disappearance of blasts from the blood would represent in this hypothesis a redistribution of these cells in the body rather than their destruction. This possibility cannot be ruled out by the present study but seems unlikely in this patient who entered rapidly into complete remission and moreover received VCR instead of methotrexate. To account for the uncertainty of a possible cell return from the blood to the marrow once VCR had been added, an additional parameter, α', has been introduced into the model.

As a special case of equation 11 (see Appendix) we may consider the probable event that there was no cell return from the blood to the marrow after VCR was added, i.e., $\alpha' = 0$. In this case, equation (11) says that the effect of VCR on the marrow compartment was to increase the exit rate by the same quantity δ as that of the increase in the exit rate from the blood compartment.

We have seen that the effect of VCR was to increase the exit rate from both compartments by the same quantity providing there was no cell return from the blood to the marrow. The most likely explanation for this observation is that the increases are due to the cytocidal effect of VCR.

The process of cell death observed on the blasts of the peripheral blood exerts itself independently of the passage of the cell through mitosis, since mitotic figures were seen only exceptionally during the observation of more than 50,000 circulating leukemic blasts after the administration of VCR. It is possible, however, that circulating blasts during the process of mitosis, while remaining in the vascular tree, could stick to the wall of a small vessel and therefore were never seen in blood samples taken in a large vein. This is a possibility since a few DNA synthesizing blasts (Fig. 4), although scarce, were seen in the blood. Considering a DNA synthesis time of the order of 13–20 hr [27], the fraction of DNA synthesizing blasts in the blood was too low to account for the killing during mitosis of more than 99% of these cells in 48 hr. One could still hypothesize, as a first effect of VCR, a recruitment of non-proliferating blasts into the cell cycle which would amplify its killing effect in mitosis. Although "recruitment" after chemotherapy is generally considered as a consequence of cell kill and not as a primary effect of chemotherapy [22], such a recruitment was suggested by the existence of a lag period of approximately 24 hr before an effect of VCR on the peripheral blood could be observed. Figure 4 indicates that no recruitment of cell in S could be observed at the time the effect of VCR was clearly demonstrable on the peripheral blood. The binding of ^{3}H actinomycin D on DNA did not increase as a consequence of the injection of VCR. This observation is an additional argument to rule out a recruitment of "dormant cells" into the cell cycle. As a matter of fact it has been shown that an increased binding of actinomycin D on DNA presumably reflects necessary changes for the transformation of inactive chromatin into active template for the RNA synthesis which precedes DNA synthesis [23].

These observations lead to the conclusion that, in this particular case, VCR destroyed leukemic blasts in the blood independently of their passage through the S and M phases of the cell cycle. Most if not all the blasts affected were in a non-proliferative state, a prolonged G_1 or G_0 phase.

There are studies [24, 25] indicating that the lethal action of the *Vinca* alkaloids is not limited as far as cell viability is concerned to a point at or close to mitosis. For Vinblastine (VLB) which differs from VCR by only a N methyl group [26] it appears clearly from these studies that an interphase cytolytic effect on the cells exposed in late G_1 can be observed independently of the passage through mitosis when high doses are used. Plageman [25], in a study conducted on rat hepatoma cells growing

in suspension culture, found that low doses (0·001–0·1 mM) caused only metaphase arrest whereas higher doses (0·2–0·6 mM) caused no metaphase arrest but inhibition of ^{3}H-uridine incorporation into the RNA and a cytotoxic effect objectivated by the dissolution of cell nuclei leading to cell death. It is concluded therefore that VLB may have a direct cytotoxic effect independent of its action during metaphase.

In contrast, the lethal effect of VCR at an equivalent dose (0·1 μg/ml) on synchronously growing HeLa and Chinese hamster cells appears to be limited to those cells which were exposed to it during their passage in the DNA synthesis phase [24]. In our experiment, the exponential decrease of the number of labeled blasts over more than 2 log 10 indicates that more than 99% of the blasts were identically affected by the drug. This observation rules out the possibility that only the blasts in S phase in the blood were destroyed since only around 5% were in S in the blood at the time of VCR injection. Madoc Jones and Mauro showed moreover that a lethal dose of VCR (0·1 μg/ml) which is very close to the dose used in our patient, given to synchronized cells in S does not appear to affect the rate of progression through the cycle to the next mitosis at which a lethal effect finds expression as metaphase arrest [24]. This observation is not in agreement with the lytic effect seen in the absence of mitotic figures in our study. On the other hand, the observation made by Rosner *et al.* [28] that VCR at 0·056 μg/ml kills stationary populations of human leukocytes growing in culture is more compatible with our observation.

When certain assumptions are made, the constancy of the ratio of the number of cells in the marrow to the number in the blood (m/n) was not affected by VCR. This indicates that marrow and blood blasts were equally affected by the drug. The ^{3}HTdR labeling index in the bone marrow determined just before VCR injection was 13·9% which is higher than the fraction of blast cells in S in the blood at the same moment as determined by their DNA content. This suggests that, in this particular case, the effect of VCR was not significantly affected by the proliferative activity of the blast population it was applied on. The final conclusion is that the blasts of this particular patient were probably very sensitive to VCR. Therefore, presumably, all the phases of the cell cycle were affected by the drug. This conclusion does not contradict the concept that certain phases of the cell cycle and that active proliferation render cell more sensitive to chemotherapy but it indicates that in certain conditions of high sensitivity, the above parameters may become relatively unimportant. This high sensitivity made it possible to demonstrate that VCR is able to destroy G_1 cells.

APPENDIX

The time origin is taken to be the time at which the labeled blast cells were injected into the blood compartment. Let the times $0\ t_1$, t_2 be defined where VCR is given at t_1, the interval (t_1, t_2) refers to a transient response, and the time t_2 or later is a stable response. Let

$n(t)$ = number of blast cells (labeled and unlabeled) in the blood at time t;
$m(t)$ = number of blast cells in the marrow at time t;
$n_L(t)$ = number of labeled blast cells in the blood at time t

and define the proportion of labeled blast cells in the blood compartment to be

$$P_L(t) = n_L(t)/n(t). \qquad (1)$$

Before the injection of VCR, the model is specified by the four parameters $\lambda, \gamma, \beta, \theta$ indicated in Fig. 5. For the present, these hazards are taken as functions of t which allows the possibility that the nature of the transfers is changing with time. We shall later assume that these are all constant parameters and independent of time. Thus, up to terms of $0(\Delta t)$ we have

(i) $\theta(t)\Delta t$ is the probability that a blast cell chosen at random in the blood at time t died or was leaving the blood to the tissues in $(t, t+\Delta t)$.

(ii) $\gamma(t)\Delta t$ is the probability that a blast cell chosen at random in the marrow at time t died in $(t, t+\Delta t)$.

(iii) $\beta(t)\Delta t$ is the probability that a cell chosen at random in the marrow at time t left the marrow compartment for the blood compartment in $(t, t+\Delta t)$.

(iv) $\lambda(t)\Delta t$ is the probability that a cell chosen at random in the marrow at time t went through mitosis adding an additional cell to the marrow in $(t, t+\Delta t)$.

It is assumed that the number of cells involved in the system was sufficiently large that $n(t)$ and $m(t)$ can be viewed as continuous functions of time and that the process can be viewed as essentially deterministic so that $n(t)\theta(t)\Delta t$ is the number of cells leaving the blood compartment in the interval $(t, t+\Delta t)$. Alternatively, our calculations may be taken to apply to the expectations of the functions $n(t)$, $m(t)$ and $n_L(t)$.

With these definitions, it is a simple matter to define differential equations. Clearly

$$n(t+\Delta t) = n(t) - \theta(t)n(t)\Delta t + \beta(t)m(t))\Delta t + 0(\Delta t).$$

Dividing through by $n(t)\Delta t$ and taking the limit as $\Delta t \to 0$ gives

$$\frac{d}{dt}\log n(t) = -\theta(t)+\beta(t)m(t)/n(t). \quad (2)$$

Since no labeled cells entered the blood from the marrow compartment, the equation for $n_L(t)$ may be obtained from (2) as

$$\frac{d}{dt}\log n_L(t) = -\theta(t). \quad (3)$$

Implicit in these derivations is the assumption that the action of labeling the cells did not affect the rate of exit.

Figure 2 gives the observed relation between $\log n_L(t)$ and t. The close conformity of this plot to that of a straight line suggests that $\theta(t) = \theta$ was a constant function of time up to the introduction of VCR. Under this assumption, a simple calculation from the slope of the straight line gives an estimate of the parameter θ.

The differential equation for $P_L(t)$ is obtained by taking logarithms of (1), differentiating and using results (2) and (3). This gives

$$\frac{d}{dt}\log P_L(t) = -\beta m(t)/n(t) \quad (4)$$

The observed data on $P_L(t)$ are summarized in the points plotted in Fig. 3. Here the close conformity to a straight line suggests that

$$\beta m(t)/n(t) = \text{constant}.$$

Thus we have

$$\frac{m(t)}{n(t)} = \frac{m(0)}{n(0)} = c,\ t < t_1 \quad (5)$$

i.e., the ratio of the number of cells in the marrow to the number in the blood was a constant up to the time VCR was added.

Taking all of the parameters in Fig. 5 to be constant functions of time, substitution of (5) in (2) gives

$$n(t) = n(0)\exp[(\beta c-\theta)t]$$

and from (5) again

$$m(t) = c\,n\,(0)\exp[(\beta c-\theta)t].$$

A differential equation for $m(t)$ can be obtained directly and gives

$$\frac{d}{dt}\log m(t) = \lambda-\gamma-\beta \quad (6)$$

so that the parameters in Fig. 5 are related as

$$\beta c-\theta = \lambda-\gamma-\beta \quad (7)$$

At time t_1, VCR was introduced and this resulted in a change in the parameter values. In Fig. 6, an additional parameter α' has been introduced into the model to account for a possible cell return from the blood to the marrow once VCR has been added. The parameters β', θ', λ', γ' are defined in a manner analogous to the previous case and again θ' is indicated to be a constant function of time. From this point on, all of these parameters are assumed constant.

From the model specification, differential equation for $n(t)$, $n_L(t)$ and $P_L(t)$ can be obtained for $t > t_2$ where t_2 is the time at which stability is reached. The observed change in the slope of $\log n_L(t)$ after t_2 implies that the combined exit rate from the blood and rate of cell return to the marrow had changed. The parameter sum $\theta'+\alpha'$ is estimated by the slope of this line ($t > t_2$) and the difference between the slopes before and after t_2 is an estimate of $\delta = (\theta'-\theta)+\alpha'$. Note that if $\alpha' = 0$, then δ is the increase in the exit rate from the blood.

The slope of $\log P_L(t)$ is given by $-\beta' m(t)/n(t)$ for $t > t_2$ and the observational result indicates that this slope is the same after VCR as before. The constant slope suggests that

$$\frac{m(t)}{n(t)} = \frac{m(t_2)}{n(t_2)} = c',\ t > t_2 \quad (8)$$

and the equality with the previous slope suggests that

$$\beta c = \beta' c'. \quad (9)$$

The interpretation of (9) must be made with care, however, since there was a period of instability after VCR was added ($t_2 > t_1$). Since under assumption, the probability that a cell in the marrow enters the blood was the same before and after the injection of VCR, the ratio of the number of cells in the marrow to the number in the blood also remains unchanged.

After VCR, we now find that

$$\lambda'+\alpha' c''-\beta'-\gamma' = \beta' c'-\theta'-\alpha' \quad (10)$$

where $c'' = (1-/c')$. This was obtained by the same method used in deriving (7). The difference between (10) and (7) gives

$$(\gamma'-\gamma) = \delta+\alpha' c'' \quad (11)$$

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Early Diagnosis of Brain Metastases in an Unselected Population of Cancerous Patients*

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Abstract—*In a series of* 50 *unselected cases in whom brain metastases were later demonstrated by pathological examination, early clinical signs and symptoms, as well as paraclinical abnormalities were studied. These included electroencephalogram, brain scan, angiography, and analysis of cerebrospinal fluid.*

Two groups of patients could be distinguished. In the first group (22 *patients*) *brain metastases were heralded by focal neurological symptoms. In these patients electroencephalogram, brain scan or angiography demonstrated focal lesions in* 83%. *In the second group* (28 *patients*), *diffuse symptoms of involvement of central nervous system were first observed. In these patients focal lesions were shown by the additional diagnostic procedures in only* 42%.

Determinations of proteins, LDH and GOT enzymes in the cerebrospinal fluid were of limited help. Neoplastic cells were found in only 10% *of examined fluids. A striking increase of lipids was observed in* 20% *of the cerebrospinal fluids. This determination was not previously performed in a series of patients with brain metastases. The physiopathological signification of changes in lipid concentration of cerebrospinal fluid is discussed.*

INTRODUCTION

BRAIN metastases are found with an increasing frequency. Recent epidemiological studies revealed that they may account for as much as 41% of the total brain tumors [1] and systematic pathological examination of patients who died with a generalized neoplasm showed secondary brain tumors in 11–24% [2, 3, 4]. Particularly high rates were found for carcinoma of breast (25%), lung (42%) and melanoma (51%). The treatment of brain metastases is disappointing although transitory improvements have been observed after steroid administration [5, 6], chemotherapy [7], radiation therapy [8, 9] and, in selected cases, surgery [10, 11]. Therapeutic success depends in part of early diagnosis.

Several comprehensive studies have been devoted to diagnosis of brain metastases [10, 12, 13]. But they were performed in departments of neurosurgery or neurology where patients had been referred primarily because of a neurological deficit.

The purpose of this study is to establish criteria for early recognition of brain metastases in an unselected population of cancerous patients.

MATERIAL AND METHODS

Over 300 patients with recent neurological symptoms or mental abnormalities admitted at the Institut Jules Bordet between 1969 and 1972 were examined for the presence of brain metastases. Fifty consecutive patients with brain metastases eventually confirmed by a pathological examination are reported in this study.

Investigations to demonstrate brain metastases consisted of a clinical neurological examination and complementary diagnostic procedures such as electroencephalogram (EEG), radioisotopic brain scan using iodinated serum albumin or mercury-97, bilateral carotid angiography or vertebral angiography, examination of optic fundi, and lumbar puncture. These

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diagnostic procedures were performed in all patients unless not compatible with good patient care.

Several chemical measurements were made in the cerebrospinal fluid (CSF) by the following methods: protein [Lowry *et al.*, 14], lactic dehydrogenase, LDH [Wroblewski, 15], glutamic transaminases, GOT [Wroblewski, 16] and glucose using glucostat reagent. Phospholipids were extracted from the CSF with 19 volumes of chloroform methanol 2:1 (v/v) after centrifugation at 1000 rev./min. for 5 min. Lipid-bound phosphorus was determined by the method of Bartlett [18]. Thin-layer chromatography of the lipids was performed on silica gel G plates. The solvents used were chloroform–methanol–water 65:25:4 (v/v/v) and for the two-dimensional chromatography chloroform–methanol–7N ammonia 60:35:5 (v/v/v) and chloroform–methanol–7N ammonia 35:60:5 (v/v/v) [18]. Pellets obtained after centrifugation of the CSF at 1200 rev./min. for 10 min were examined for the presence of neoplastic cells.

RESULTS

Brain metastases were observed primarily in lung carcinoma (48%), breast carcinoma (34%) and melanoma (10%) (Table 1).

In the 24 patients with lung carcinoma all brain metastases appeared within the first year following the diagnosis of the primary neoplasm, the mean interval being 5 and a half months. In one third of these patients brain metastases were the only metastatic lesion.

In the remaining 26 patients the mean interval between the diagnosis of primary cancer and that of brain metastases was 55 months. In this group 17 patients had radiological evidence of pulmonary and/or pleural metastases when the first symptoms of brain metastases were observed. In 4 patients brain metastases were the only secondary localization.

Early symptoms of brain metastases

The early clinical symptoms and signs are summarized in Table 2. The results of EEG, brain scan, angiography, and optic fundi examinations are given in Table 3.

In 22 patients the first clinical manifestation pointed to a focal cerebral lesion. Fifteen had hemiparesis, 3 Jacksonian seizures, 3 hemianopsia, aphasia was noted in 2, and parietal lobe syndrome in one patient. EEG was pathological in all patients of this group. In 15 cases

Table 1. *Distribution of cases*

Primary cancer	Number of cases	%	Interval between diagnosis of primary cancer and BM Months Mean ±	σ	Survival after BM diagnosis Weeks Mean ±	σ	Metastases outside the brain Lung	Others	None
Lung	24	48	5·5	4·0	2·5	3·5	—	16	8
Breast	17	34	55·7	46·0	3·0	2·8	12	3	2
Melanoma	5	10	56·0	24·0	4·8	4·8	3	1	1
Miscellaneous*	4	8	50·3	49·0	1·6	1·6	2	1	1

*One of each: hypernephroma, carcinoma of thyroid, reticulo-sarcoma and epithelioma of neck.

Abbreviations: BM: brain metastases;
σ: standard deviation to the mean.

Table 2. *Early manifestation of brain metastases*

Primary cancer	Number of cases	Changes of personality and/or awareness	Focal neurological symptoms	Headaches and/or ICHT*
Lung	24	9	11	4
Breast	17	4	8	5
Melanoma	5	2	1	2
Miscellaneous	4	0	2	2
Total	50	15	22	13

Abbreviation. *ICHT: intracranial hypertension.

Table 3. Results of special investigations

First clinical manifestations	Number of cases	EEG N	EEG AN F	EEG AN D	EEG NP	Brain scan N	Brain scan AN U	Brain scan AN M	Brain scan NP	Angiography N	Angiography AN U	Angiography AN M	Angiography NP	Papilledema Present	Papilledema Absent
Focal symptoms	22	0	15	7	0	6	12	2	2	8	8	4	2	2	20
Personality and/or awareness changes	15	0	5	10	0	5	7	1	2	8	5	0	2	3	12
Headaches and/or ICHT	13	0	4	6	3	4	5	1	3	4	4	1	4	5	8
Total	50	0	24	23	3	15	24	4	7	20	17	5	8	10	40

Abbreviations. N: normal; AN: abnormal; U: single lesion; M: multiple lesions; F: focally; D: diffusely; NP: not performed.

focal abnormalities were present in the hemisphere where brain metastases were suspected on clinical grounds. In 7 patients EEG changes were diffuse. Brain scan and bilateral carotid angiography, performed in 20 patients, were normal in 6 and 8 cases respectively, demonstrated a single lesion in 12 and 8, and multiple lesions in 2 and 4 patients. In patients where EEG was altered unilaterally, the abnormalities of brain scan and angiography were present in the same hemisphere as the changes of EEG. Only 2 patients of this group had papilledema.

In 15 cases, brain metastases were heralded by changes of personality or awareness which were found one week to three months before the appearance of focal neurological signs. These changes were characterized by a mild confusion, lack of interest and reduced spontaneous activity.

EEG was diffusely abnormal in 10 patients and unilaterally in 5. Brain scan and bilateral carotid angiography showed a single lesion in 7 and 8 patients respectively. Focal unilateral changes of EEG, brain scan, and angiography were always located in the same hemisphere. Bilateral focal lesions were present on angiography in only one patient who had normal brain scan and diffusely pathological EEG. Three patients had choked disks.

Headaches led to the discovery of brain metastases in 13 patients. The five patients with posterior fossa metastases were in this group and complained, in addition, of nausea, vomiting, drowsiness and somnolence. All 5 had papilledema. In these patients EEG was characterized by bilateral burst of delta waves with marked frontal predominance. A space-occupying lesion in posterior fossa could be demonstrated by at least one of the following procedures: brain scan using mercury-97, vertebral angiography or iodoventriculography. In the remaining 8 patients of this group, 4 had unilateral hemispheric lesion on EEG and brain scan, two of them showed, in addition, focal lesion on angiography. Four others had diffusely abnormal EEG, normal brain scan and, in all but one normal angiography.

Analysis of CSF

Lumbar punctures were performed in all patients except those who had papilledema; the results of CSF analysis are given in Table 4.

Proteins were higher than 45 mg per 100 ml in 19 patients, but only in 3 were the levels higher than 90 mg per 100 ml.

The levels of LDH were increased in 18 patients ranging from 45 to 220 units. GOT

Table 4. Abnormalities of CSF

First clinical manifestations	Number of cases (analyzed)	Protein <45 mg %	Protein >45 mg %	LDH <40 U	LDH >40 U	GOT <10 U	GOT >10 U	Neoplastic cells Present	Neoplastic cells Absent
Focal neurological signs	20	9	11	11	9	18	2	1	19
Personality and/or awareness changes	12	9	3	7	5	12	0	2	10
Headaches and/or ICHT	8	2	6	4	4	7	1	1	7
Total	40	20	20	22	18	37	3	4	36

were normal in 37 cases and moderately increased (18–30 U) in 3 patients.

Neoplastic cells were found in 4 cases.

In 16 patients with brain metastases the levels of lipid-bound phosphorus varied from 12·0 to 35·9 μg per 100 ml with a mean of 22·9 ± 5·8 μg. Corresponding values in the control ranged from 7·0 to 24·6 with a mean of 17·5 μg ± 4·9 (Fig. 1). No statistical difference could be demonstrated between these two groups by the Student's *t*-test ($T = 0{\cdot}48$). However, in four other patients with secondary brain tumors very high values of lipid phosphorus were noted: 71·6, 127·9, 185·5 and 224·5 μg per 100 ml. No relation was found between the elevated levels of phospholipid, clinical symptoms, type of primary cancer or subsequent clinical evolution of the neurological disease.

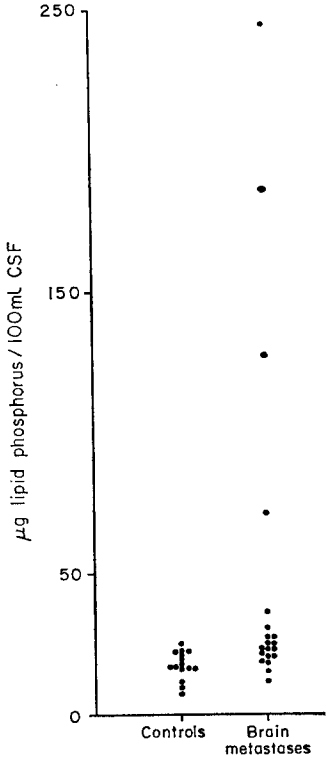

Fig. 1. Values of total phospholipids phosphorus in patients with brain metastases and controls. Each point is a result of one determination.

In cases where high values of phospholipids were found, bidimensional thin-layer chromatography allowed in each case an easy separation, identification and determination of major lipids (Fig. 2). The identified phospholipids were lysophosphatidylcholines (LPC), sphingomyelins (Sph), phosphatidylcholines (PC) and phosphatidylethanolamines (PE). All of them were found in the 4 analyzed cases with the exception of LPC, which was absent in one case. PC (38·2 to 46·3%) appeared as the major phospholipid, Sph (18·1 to 31·2%) and PE (22·4 to 35·6%) accounted for about the same percentage, while LPC (0–7·6%) was a minor compound.

Two other major lipids were found in addition to neural lipids and phospholipids. The first migrated to the same position as authentic cerebrosides on thin-layer chromatography and gave characteristic blue staining with anthrone spray. These lipids identified as cerebrosides were present in the 4 cases examined. Their concentration ranged from 240 to 550 μg per 100 ml. A second lipid spot was found in 3 cases out of four, and 2 cases where the test was performed this lipid gave a positive reaction with orcinol spray. This group of lipids was tentatively identified as gangliosides.

DISCUSSION

Two series of clinical factors have to be considered in the diagnosis of brain metastases: the origin and the spread of the primary tumor on one hand and the neurological findings on the other.

As in many previous studies [5, 6, 10, 11], our observation has confirmed that brain metastases are associated primarily with melanoma, lung and breast carcinoma. When primary neoplasm was located outside the lung, brain metastases were more likely to occur when pulmonary metastases were present. From 26 such patients reported here lung metastases could be demonstrated by standard chest X-ray in 17 (65%), while brain metastases were the only secondary localizations in only 15%. In patients with primarily lung carcinoma metastases outside the nervous system were present in 66% when the first symptoms of brain metastases appeared.

Early neurological symptoms of brain metastases were polymorphic and were not related to the nature of the primary tumor. Their differential diagnosis often raised difficult problems although the presence of an extraneural neoplasm was established in all patients.

In 7 out of 22 cases, where the first clinical manifestation was a focal deficit, the onset of neurological symptoms was acute, mimicking a vascular stroke. In 2 of them EEG, brain scan or angiography helped to establish the presence of brain metastases by demonstrating multifocal lesions. In the remaining 5 patients, however, definite diagnosis was based on subsequent clinical evolution or pathological examination. In the group of 28 patients with diffuse neurological symptoms, focal cerebral lesions were

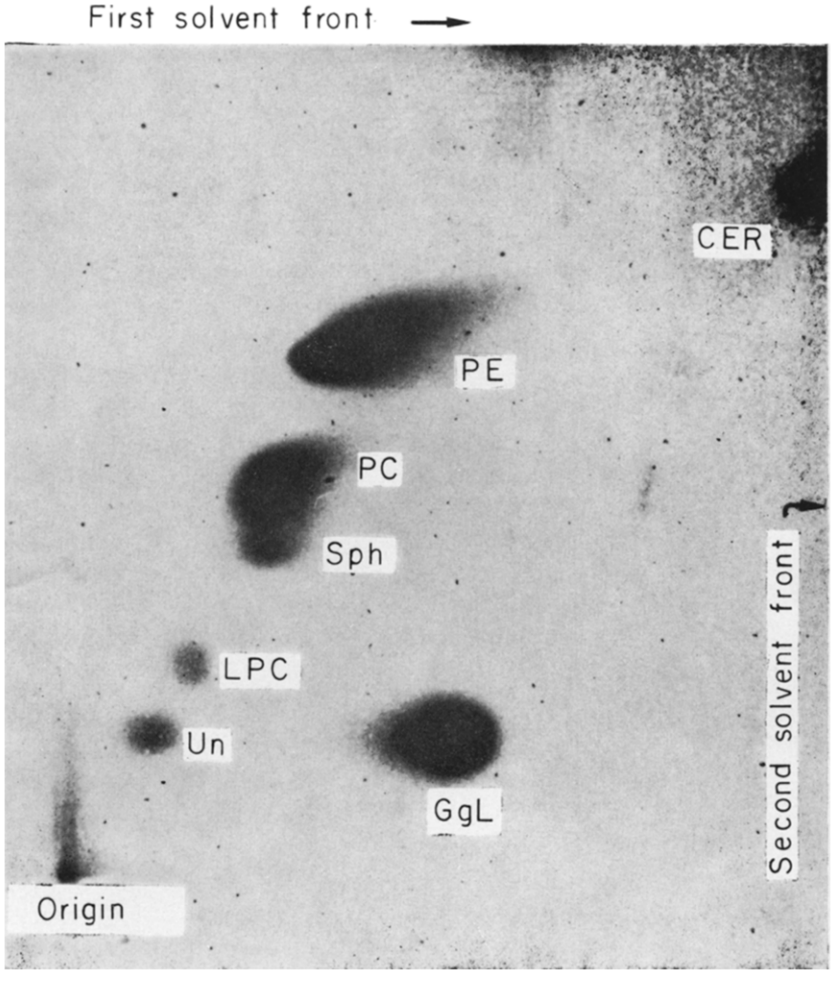

Fig. 2. Two-dimensional thin-layer chromatogram of the lipid extract from case 2, performed as described in the methods. Lipids were revealed by sulfuric acid spray. The following abbreviations are used: LCP (lysophosphatidylcholines), Sph (sphingomyelins), PC (phosphatidylcholines), PE (phosphatidylethanolamines), Cer (cerebrosides), GgL (gangliosides), NU (non identified product). The neutral lipids, not seen on the figure, migrated very close to the solvent front.

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Combination Chemotherapy with 1-(2-Chloroethyl-3-cyclohexyl-1-nitrosourea) (CCNU), Vincristine and Methotrexate in Primary and Metastatic Brain Tumors— A Preliminary Report*

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Abstract—*Thirty-eight patients, 15 with recurrent primary brain tumors, 8 with breast carcinoma and 15 with lung carcinoma brain metastases were treated by a combination of 1-(2-chloroethyl-3-cyclohexyl-1-nitrosourea) (CCNU): 100 mg/m² every 6 weeks; vincristine: 2 mg/m² and methotrexate: 24 mg/m² both given once a week for 4 weeks and subsequently once every 4 weeks. Dexamethasone: 10 mg/m² per day was initiated concomitantly with chemotherapy and administered for 4 weeks. Chemotherapy was well tolerated. Objective remission, defined as clinical improvement present 6 weeks after complete discontinuation of dexamethasone and lasting for 4 weeks or more, was observed in 11 patients: 6 with primary brain tumors, 4 with breast carcinoma and 1 with lung carcinoma brain metastases. In 7 patients, the remissions were characterized by a remarkable functional recovery. EEG and brain scan appeared both as useful complementary examinations in the estimation of tumor size modifications.*

INTRODUCTION

SURGERY, often followed by irradiation, is accepted as a standard treatment of brain tumors despite its failure to produce satisfactory survival [1–4]. The necessity for new therapeutic approaches has been recognized since a long time. Large number of chemotherapeutic agents and several routes of administration have been tested in the last decade [5]. However, the results were often discouraging and sometimes even contradictory.

Recent renewal of interest for chemotherapy of malignant brain tumors was stimulated by promising and reproducible results achieved with nitrosourea derivatives. Several studies have demonstrated that BCNU (1,3-bis (2-chloroethyl)-1-nitrosourea) was able to produce high rates of objective remissions in patients with recurrent brain tumors whether used alone [6, 7] or in association with vincristine [8]. It was also shown in a controlled and randomized study that the association of radiation therapy and BCNU was able to prolong significantly the survival time [9]. CCNU (1-2-chloroethyl)-3-cyclohexyl-1-nitrosourea) has been tested less extensively in brain tumors. A marked functional improvement in all 3 patients treated with

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CCNU was reported by Hansen *et al.* [10], and Fewer *et al.* confirmed that CCNU was effective in recurrent primary brain tumors and metastases [11].

Our purpose is to report primary results of a phase II study with a combination of CCNU, vincristine (VCR) and methotrexate (MTX) in primary and metastatic brain neoplasms.

VCR was added to CCNU because their mode of action and toxicity are quite different. In addition, favorable effect of VCR has been reported in primary brain tumors [12] and this drug was used in combination or alone in breast cancer [13]. MTX was chosen because it can possibly act on cerebral neoplasm when given intrathecally while its systemic toxicity is prevented by administration of citrovorum factor. For practical reasons, however, the intrathecal administration of MTX had to be abandoned, and to preserve as much as possible the initial schedule, we continued to give citrovorum factor 2 hours after intravenous administration of MTX.

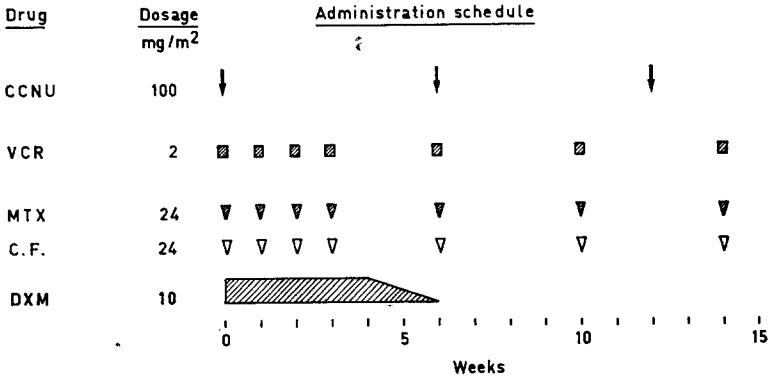

Fig. 1. *Chemotherapy schedule.*

MATERIAL AND METHODS

On 42 patients selected for the study, 38 are reported: 15 had primary brain tumors, 8 breast cancer and 15 lung cancer brain metastases. Three patients with brain gliomas and one with a metastatic tumor were lost during the follow-up, and discarded.

All patients with primary brain tumors had undergone surgery followed within two weeks by radiation therapy (6000 rad). Chemotherapy was initiated at least 2 months after completion of the radiations when there was clinical evidence of recurrences. Only patients with established histological diagnosis and expected survival of two months or more were admitted to the study. Patients with extraneural neoplasms had an expected survival of 4 weeks or more. The diagnosis of brain metastases was based on clinical criteria and complementary examinations reviewed elsewhere [14]. None of the 38 patients had previously received antitumoral chemotherapy.

Administration of chemotherapy

Drugs were given following the schedule shown in Fig. 1. CCNU was administered orally every 6th week: 100 mg/m^2; VCR intravenously: 2 mg/m^2 on days 1, 8, 15 and 22 and then every 4th week; MTX was given first intrathecally: 24 mg/m^2, at the same time as VCR. In most patients, however, MTX was subsequently given intravenously. Citrovorum factor was injected intramuscularly 2 hr after MTX: 24 mg/m^2. Dexamethasone (DXM) was given orally or intramusculary for 4 weeks: 10 mg/m^2, then decreased gradually and stopped within 2 weeks. Corticosteroids were resumed if necessary.

CCNU and MTX were discontinued when platelets and/or leukocytes were respectively lower than 100·000 and 3·000/mm^3. VCR was discontinued when signs of peripheral neuropathy other than transitory paresthesia or loss of tendon reflexes were observed or when paralytic ileus appeared.

Chemotherapy was administered until death.

Evaluation of objective remission

Objective remission was defined as an improvement of one or several neurological signs without new neurological deficits being observed. The improvement had to be present 6 weeks after complete discontinuation of corticosteroids and to last 4 weeks or more. EEG and brain scan were performed before the treatment and when a clearcut improvement or relapse were observed.

RESULTS

1. *Antitumoral effect*

The rate of objective remissions was related to the nature of the neoplasm: primary brain tumors and breast cancer metastases responded much better than lung carcinoma metastases.

Primary brain tumors. In this group, 6 of 15 patients responded to the treatment (Table 1). In 3 patients, the functional improvement was remarkable. These patients were hemiplegic and bedridden prior to chemotherapy; they improved gradually, were able to walk again without assistance and to follow their treatment as outpatients. Except 3 patients who were already on steroids when chemotherapy was initiated, all the patients responded to DXM within 48 hr. Thus objective remission could be appreciated only at the 12th week of chemotherapy, i.e. six weeks after the complete discontinuation of steroids. In 3 patients, however, the beginning of an objective remission was observed after an even longer delay of: 15 (case 4), 20 (case 5) and 21 (case 1) weeks. Although 6 patients of this group are still alive, the survival appeared to be much longer in patients who responded favorably to the treatment (mean 33 weeks, range 18 to 56^+) as compared to those who did not (mean 14 weeks, range 1 to 36^+).

Metastases of breast carcinoma. Objective improvement of the neurological status was observed in 4 patients on 8 (Table 2). The recovery was dramatic in all 4, who were able to walk after being bedridden, while aphasia present in 3 patients regressed completely. The improvement lasted only 10 and 12 weeks after complete discontinuation of DXM in 2 patients (cases 20 and 21); however, it was considerably longer in 2 other (cases 17 and 19). The survival of the 4 patients who did not respond to chemotherapy ranged from 4 to 10 weeks (mean 6 weeks) whereas it varied from 28 to 43 weeks (mean 34 weeks) in patients with objective remission. In patients 20 and 21, brain metastases were the only detected neoplastic lesion. In case 17, the size of the primary mammary tumor, which has not been removed, regressed markedly after chemotherapy; in case 19, the X-rays aspects of bone metastases remained unchanged throughout the treatment.

Metastases of lung carcinoma. Only one patient (case 34) on 15 responded to the treatment, the remission lasted more than 5 weeks. In this patient, standard chest X-rays showed an objective regression of the primary tumor. The survival, in the 14 patients who did not respond, was particulary short (mean 7 weeks). Only 5 survived long enough to allow the discontinuation of DXM (Table 3). However, in all of them, with the exception of case 34, it was necessary to resume the administration of steroids because of the recurrence of neurological deficits.

2. *Correlation between clinical examination, EEG and brain scan in patients with objective remission*

Although the remission was defined as clinical neurological improvement, EEG and brain scans were repeated during the treatment.

EEG recorded prior to chemotherapy was focally abnormal in all the 11 patients who subsequently responded to chemotherapy. In 9 cases (1, 4, 6, 13, 17, 19, 20, and 34), the recordings improved in parallel to the clinical recovery. In cases (4, 17, 19 and 21) where the recurrence of neurological deficit was observed, EEG worsened again. This evolution of EEG is illustrated in Figures 2 and 3. In 2 patients, EEG remained unchanged during the improvement of neurological symptoms (cases 5 and 15).

Brain scans performed before the administration of chemotherapy, available in 10 patients, were normal in 2 (cases 20 and 21). In the 8 remaining cases, brain scans improved together with the clinical recovery in 4 (cases 4, 6, 17 and 19) and remained unchanged in 4 others (1, 5, 15 and 34). In 3 patients whose brain scan initially improved (cases 4, 17 and 19), an extension of the hyperactive focus was demonstrated when the relapse of the brain tumor occurred. An example of such changes of the brain scan is shown in Fig. 4.

3. *Drug toxicity*

Blood examination, liver tests and determination of serum creatinine and urea were performed twice a week in hospitalized patients, and before each drug administration in outpatients. Neurological examinations were controlled following a similar schedule. Although toxic manifestations of the treatment were frequent, no patient died from drug toxicity.

Bone marrow toxicity. Platelet depression was observed in all patients 3–4 weeks after the administration of CCNU, but figures lower than 100·000 per mm^3 were found only in 16 cases: 5 with primary tumors, 6 with breast carcinoma metastases and 5 with lung cancer metastases. Five of them reached levels below 30·000 per mm^3.

Table 1. Patients with primary brain tumors

Case	Sex	Age	Histological diagnosis	Location		Objective remission: Symptoms	Objective remission: Delay (weeks)	Objective remission: Duration	Chemotherapy (mg): CCNU	Chemotherapy (mg): VCR	Chemotherapy (mg): MTX	Toxicity	Survival (weeks)
1	M	44	Astrocyt gd. I	R	F	Marked regression of L. hemiplegia and mutism	21	15+	5×200	7×3	7×40	Thp, An, Gp	36+
2	M	37	Astrocyt. gd. II	L	T	None	—	—	6×200	8×3	10×40	Mw	36+
3	F	69	Astrocyt. gd. IV	L	F	None	—	—	2×180	6×2·5	6×40	none	15
4	M	42	Astrocyt. gd. I	L	F	Marked regression of R hemiplegia and partial regression of aphasia	15	41	7×200	13×3·5	15×40	none	56+
5	F	16	Ependymoma	R	PO	Decrease of headaches improvement of gait	20	7+	3×180	5×2·5	5×40	none	27+
6	F	20	Astrocyt. gd. III	L	T	Partial regression of headaches. Disappearance of shocked dics	12 or less	24+	6×180	2×2·5	10×40	Pi, Thp	40+
7	F	69	Glioblast. multif.	L	TO	None	—	—	3×180	6×2·5	6×40	none	13
8	M	70	Glioblast. multif.	L	FP	None	—	—	200	4×3	4×40	Thp	6
9	M	42	Glioblast. multif.	L	T	None	—	—	200	3×3	3×40	none	3
10	M	46	Astrocyt. gd. IV.	L	F	None	—	—	3×200	6×3	6×40	Thp	15
11	M	55	Astrocyt. gd. IV.	R	PT	None	—	—	3×200	6×3	6×40	none	16+
12	F	47	Glioblast. multif.	L	PT	None	—	—	3×200	6×3	6×40	none	21
13	M	49	Glioblast. multif.	L	F	Complete regression of hemiparesis and drowsiness	12 or less	11	2×200	5×3	5×40	none	23
14	F	12	Astrocyt. gd. III	R	FP	None	—	—	160	2·5	30	none	1
15	F	41	Glioblast. multif.	R	T	Regression of hemiparesis	12 or less	6	2×200	5×3	5×40	Thp, An, Gp	18

Abbreviations:
M: male, F: female, R: right, L: left.
F: frontal, P: parietal, T: temporal, O: occipital.
Thp: thrombocytopenia, Gp: granulopenia, An: anemia.
VCR: vincristine, MTX: methotrexate.
Pi: paralytic ileus, Mw: muscular weakness.

Table 2. Patients with metastases of breast carcinoma

Case	Sex	Age	Location	Objective remission: Symptoms	Objective remission: Delay (weeks)	Objective remission: Duration (weeks)	Chemotherapy: CCNU	Chemotherapy: VCR	Chemotherapy: MTX	Toxicity	Survival (weeks)
16	F	62	L T	None	—	—	180	4 × 2·5	4 × 40	Thp, An	4
17	F	59	L P	Marked regression of hemiparesis and aphasia	12 or less	19	5 × 180	7 × 3	8 × 40	Thp, Gp, An, Mw	33
18	F	69	L FP	None	—	—	120	4 × 2	4 × 30	Thp, Gp	6
19	F	59	L T	Regression of drowsiness, hemiparesis and aphasia	12 or less	33	6 × 180	7 × 3	8 × 40	Thp, Gp, An Mw	45
20	F	54	L FT	Regression of hemiplegia and aphasia	12 or less	4	4 × 180	7 × 3	7 × 40	Thp, Gp, An	28
21	F	38	Multiple	Complete regression of gait unsteadiness and drowsiness	12 or less	6	5 × 180	7 × 3	7 × 40	Thp	33+
22	F	68	R TO	None	—	—	180	4 × 3	4 × 40	None	6
23	F	52	L F	None	—	—	2 × 160	5 × 2·5	5 × 35	None	10

Abbreviations are the same as for Table 1.

Table 3. Patients with brain metastases of lung carcinoma

Case	Sex	Age	Histological diagnosis	Location	Objective remission: Symptoms	Objective remission: Duration (weeks)	Chemotherapy (mg): CCNU	Chemotherapy (mg): VCR	Chemotherapy (mg): MTX	Toxicity	Survival (weeks)
24	M	52	Epidermoid	R F	None	—	200	3 × 3	3 × 40	Thp	3
25	M	66	?	R F R P	None	—	200	2 × 3	2 × 40	None	2
26	M	58	Indifferentiated	L F	None	—	200	4 × 3	4 × 40	Thp	6
27	M	52	Epidermoid	L FP	None	—	2 × 200	4 × 3	4 × 40	Thp	6
28	M	61	Glandular	Multiple	None	—	200	2 × 3	2 × 40	None	1
29	M	68	Oat cell	L FT	None	—	200	4 × 3	4 × 40	Thp, Gp	6
30	M	62	Epidermoid	L P	None	—	2 × 200	4 × 3	4 × 40	None	6
31	M	74	Indifferentiated	L FP	None	—	2 × 180	5 × 3	5 × 40	None	15
32	M	52	Glandular	L T	None	—	2 × 200	5 × 3	5 × 40	Thp	10
33	M	41	Epidermoid	R P	None	—	2 × 200	5 × 3	5 × 40	None	9
34	M	67	Indifferentiated	L T	Complete regression of aphasia	5+	3 × 200	6 × 3	6 × 40	None	17+
35	M	62	Epidermoid	Multiple	None	—	2 × 200	5 × 3	5 × 40	None	6
36	M		?	L T	None	—	2 × 200	5 × 3	5 × 40	None	11
37	M	42	Epidermoid	R P	None	—	200	3	40	None	1 day
38	M	63	Oat cell	R F	None	—	200	3	40	None	2

Abbreviations are the same as for Table 1.

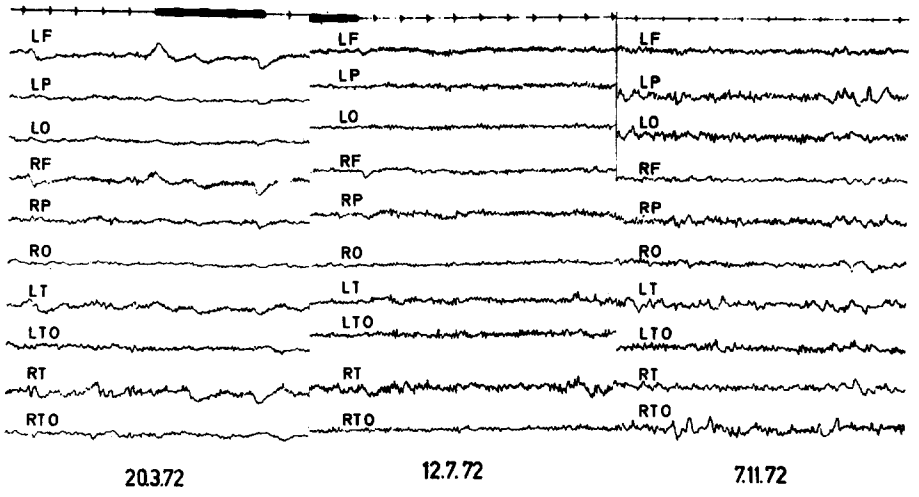

Fig. 2. Case 19. EEG recorded before the administration of chemotherapy (20.3.72) during the remission (12.7.72) and after the recurrence. Amplification and time constant are the same for the 3 recordings.

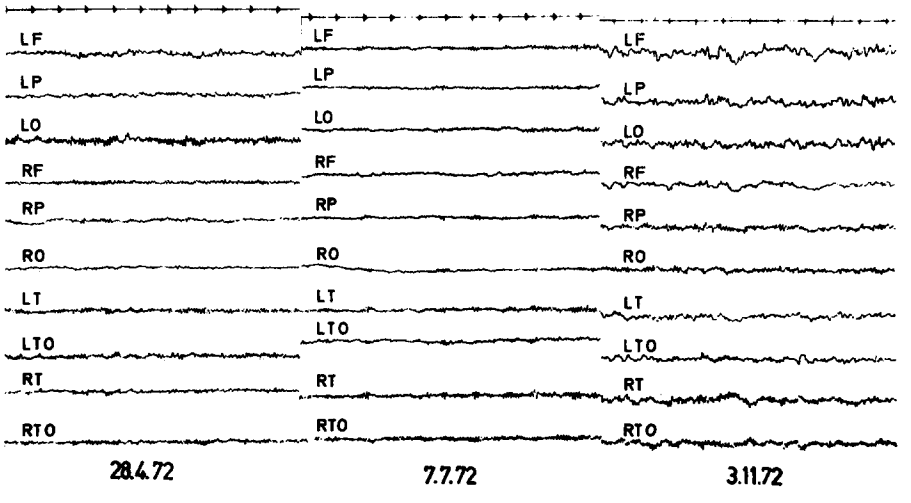

Fig. 3. Case 17. EEG recorded before the administration of chemotherapy (28.4.72) during the remission (7.7.72) and after the recurrence (3.11.72). Amplification and time constant are the same for the 3 recordings.

Fig. 4. Case 4. Brain scan obtained before the administration of chemotherapy (1.2.72) during the remission (7.11.72) and after the recurrence.

White blood cells lower than 3·000 per mm^3 were seen in 7 patients, only one (case 1) had less than 1·000 per mm^3.

A certain degree of anemia was found in all patients who received 3 cures of CCNU or more. In six patients hemoglobin was lower than 10 g per 100 ml, 3 had less than 8 g per 100 ml and were transfused.

Cumulative toxicity was observed in most patients after 3 cures of CCNU. Bone marrow toxicity was usually more frequent and more severe in patients in the brain metastases as compared to those with brain glioma.

Neurological toxicity. One patient experienced a severe paralytic ileus after administration of 5 mg of VCR.

All patients who received 9 to 12 mg of VCR complained of transitory distal paresthesia and lost tendon reflexes. Total dosages of VCR rarely exceeded 20 mg; the administration of this drug was limited by the apparition of distal symmetrical limb weakness. This symptom of peripheral neuropathy was usually reversible; however, in 3 patients (cases 4, 17 and 19), a more severe and durable weakness could not be avoided by discontinuing the drug.

DISCUSSION

Corticosteroids produce an improvement of neurological signs and symptoms in a high percentage of patients with brain tumors [15]. Thus the administration of dexamethasone could not be omitted in this trial. In order to distinguish the effect of corticosteroids who act primarily on brain edema from that of anti-tumor agents, we considered as responses to chemotherapy only the cases where the neurological improvement persisted 6 weeks after discontinuation of DXM. Indeed the effect of steroids is sustained only if their administration is maintained [16]. This procedure, however, has two drawbacks.

First, the delay of the response to chemotherapy is masked by the corticosteroid effect. In our experiment, this delay could be determined only in 3 patients who responded after 15 to 21 weeks of treatment.

The second disadvantage was that short remissions, ending within 12 weeks of treatment, could have been overlooked. Despite this, tumor regressions were observed in 11 patients (29%). Changes of tumor size were appreciated by neurological examination and, as other authors who used nitrosourea derivatives [7, 8, 10, 11], we were impressed in most of our patients who responded to the treatment by the importance of functional recovery. In addition, modifications of EEG correlated well with clinical changes and, in about half of the cases, variations of tumor size were also demonstrated by brain scan during the remission and at the final relapse.

Metastases of lung carcinoma responded less frequently than breast carcinoma metastases or primary brain tumors. This relative resistance of lung neoplasms to chemotherapeutic agents, and particularly to CCNU, has been also observed in other studies [17].

Although our study was not designed to evaluate the survival, it appeared that patients with objective remission survived longer than those who failed to respond. For instance, in the breast carcinoma group, the mean survival was 34 weeks for patients who responded to chemotherapy and only 6 weeks in those who did not. The last figure is comparable to that found in this hospital for 17 consecutive and unselected patients with breast carcinoma brain metastases [14].

The drug combination used in this trial was well tolerated. Bone marrow toxicity was reversible in all patients. It appeared, however, that to avoid major peripheral nerve toxicity total dosages of VCR should not exceed 20 mg.

We have already considered the rationale for the drug association. However, since this study was initiated, it has been shown by Fewer *et al.* that the association of a nitrosourea derivative (BCNU) and vincristine did not give better results than BCNU alone in primary and metastatic brain tumors [8]. On the other hand, in most of our patients, MTX was given intravenously and its administration followed within 2 hr by citrovorum factor, which possibly reduced its therapeutic effect.

We thus incline to consider that the remissions observed are primarily due to the administration of CCNU. A randomized study comparing the action of CCNU alone to that of the tested association will be undertaken.

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Effect of AC-3579, a Diazafluoranthen Derivative, on Murine Leukemia P388 and L1210—I. *In Vivo* Study*

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Abstract—*AC*-3579 (*NSC*-170561), *a diazafluoranthen derivative, was given intraperitoneally* (100 *to* 300 *mg/kg per day*) *to BDF*$_1$ *mice with P*388 *or L*1210 *leukemia from day* 1 *to* 9 *or until death. Its administration produced an increase of the survival time over the controls of* 44 *to* 66% *in P*388 *and* 23 *to* 28% *in L*1210. *A* 5*-day administration of the drug* (100 *mg/kg per day*) *resulted in a* 3*-fold decrease of the number of cells per ascites. Electron microscopic examination of cells remaining in the peritoneal cavity after the treatment failed to demonstrate any striking modification. Biochemical determination showed a* 70% *increase of total cellular phospholipids and a marked enhancement of* [^{32}P]*-orthophosphate incorporation into total phospholipids. Specific activities of ATPase and* 5′*AMPase as well as the labeling index with tritiated thymidine were not affected by the treatment* in vivo. *Both morphologic and chemical data indicated that the antitumor action of AC*-3579 *could differ from its effect on rat hepatocytes which has been previously elucidated.*

INTRODUCTION

IT WAS recently shown in our laboratories that AC-3579§, a diazafluoranthen derivative (Fig. 1), produces in rat hepatocytes a rapid and marked hypertrophy of smooth endoplasmic reticulum (SER) and formation of myelin-like figures regarded as lysosomes overloaded with lipidic material [1]. This experimental model was used to elucidate the biochemical composition and the mechanism of formation of such subcellular changes. We have demonstrated that the material which accumulates in the hepatocytes corresponds primarily to SER membranes [1] and that this overloading is due to an inhibition of phospholipids degradation without any stimulation of their biosynthesis [2]. Morphological and biochemical changes induced by AC-3579 were both completely reversible [1] and, despite the extent of intracellular changes, the drug altered minimally

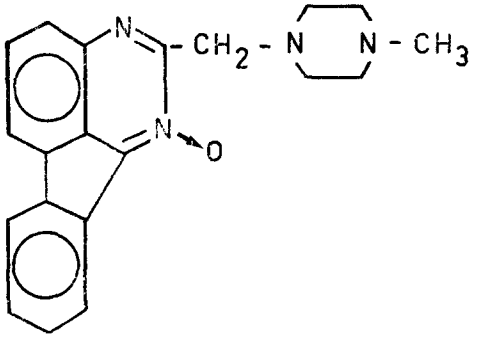

Fig. 1. *AC*-3579 (*NSC*-170561): *2-N-methylpiperzinomethyl*-1,3*-diazafluoranthen* 1*-oxide.*

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§A. C. Christiaens Laboratories, Brussels, Belgium. AC-3579 was registered as NSC-170561.

the liver function and was tolerated at relatively high dosages [3].

Assuming that AC-3579 could have a similar action on other cells, particularly the neoplastic ones, and possibly alter their growth, the drug was administered to leukemic mice. Preliminary but encouraging results of this study are reported in the present paper.

MATERIAL AND METHODS

BDF_1 male mice (Charles River Breeding Laboratories) weighing 19 to 24 g were inoculated with P388 or L1210 leukemia by intraperitoneal injection of respectively 10^6 or 10^5 cells. AC-3579 was given intraperitoneally as freshly prepared suspension containing 4 mg of the drug per ml of physiological serum.

Quantitative removal of leukemic ascites for cell counts and chemical determinations was achieved by washing the peritoneal cavity with 2 ml of physiological serum. Heparin was added to prevent cell agglutination.

Cell counts were performed, after proper dilution, in a counting chamber of Thomas.

Total lipids were extracted with 19 volumes of chloroform methanol (2/1) and washed by the method of Folch, Lees and Sloane-Stanley [4]. Phosphorus was determined according to the procedure of Fiske and Subbarow [5] and the concentrations of phospholipids obtained by multiplying the phosphorus figures by 25. [^{32}P]-orthophosphate (specific activity from 5 to 20 μCi per μgP) was from I.R.E., Mol, Belgium. Phospholipid radioactivity was measured in toluene scintillation fluid.

Tritiated thymidine ([^{3}H]TdR) was from the Radiochemical Center, Amersham, England. A quantity of 20 μCi of [^{3}H]TdR, dissolved in 0·5 ml of physiological serum, was given by intraperitoneal injection 45 min before the sacrifice of the animals. The technique used for the study of the labeling index by autoradiography was detailed by Stryckmans *et al.* [6].

Proteins were determined by the method of Lowry *et al.* with bovine albumin standards [7]. ATPase was determined by the method of Emmelot and Bos [8] and 5'AMPase as previously reported [9].

For electron microscopy ascites were quickly removed, centrifuged at 1000 rev/min and the pellet was fixed in 2·5% glutaraldehyde. Other steps were performed as previously described [1]. Over 100 micrographs of treated and control P388 cells were examined.

RESULTS

1. *Effect of AC-3579 on the survival time and number of leukemic cells*

Administration of 100 mg/kg per day or more of AC-3579 produced a significant ($P<0·001$) prolongation of the survival time (Table 1). In mice with P388 leukemia, the increase ranged from 44 to 66% as compared to the controls (10·3 days $\pm$ 0·9). The results were slightly better when the drug was given until death. Little or no benefit was gained by changing the total dosage or the schedule of administration during the 9 first days of the leukemia.

In L1210 leukemia, the increase of the survival time was shorter but still significant ($P<0·001$).

Table 1. *Effect of AC-3579 on the survival time of mice with P388 or L1210 leukemia*

Leukemia	Number of animals	AC-3579: Dosage per administration (mg/kg)	AC-3579: Daily administrations	Duration of treatment (days)	Survival: Mean increase over controls (%)	Survival: *t*-test	Toxicity: Mean loss of weight (g)	Toxicity: Deaths before 5th day
P388	6	25	1	1 to 9	none	—	0·6	0
P388	6	50	1	1 to 9	none	—	0·8	0
P388	7	75	1	1 to 9	none	—	1·2	0
P388	5	100	1	1 to 9	45	$P<0·001$	2·2	0
P388	7	100	1	1 to death	66	$P<0·01$	0	0
P388	6	150	1	1 to 9	55	$P<0·001$	3·5	2
P388	7	100	2	1 to 9	55	$P<0·001$	2·6	0
P388	7	100	2	1 to death	66	$P<0·001$	2·7	0
P388	7	50	3	1 to 9	44	$P<0·001$	1·6	0
P388	6	50	3	1 to death	44	$P<0·001$	0·4	0
P388	9	100	3	1 to 10	50	$P<0·001$	4·2	1
L1210	7	100	1	1 to 9	23	$P<0·001$	0·7	0
L1210	9	100	3	1 to 10	28	$P<0·001$	4·2	0

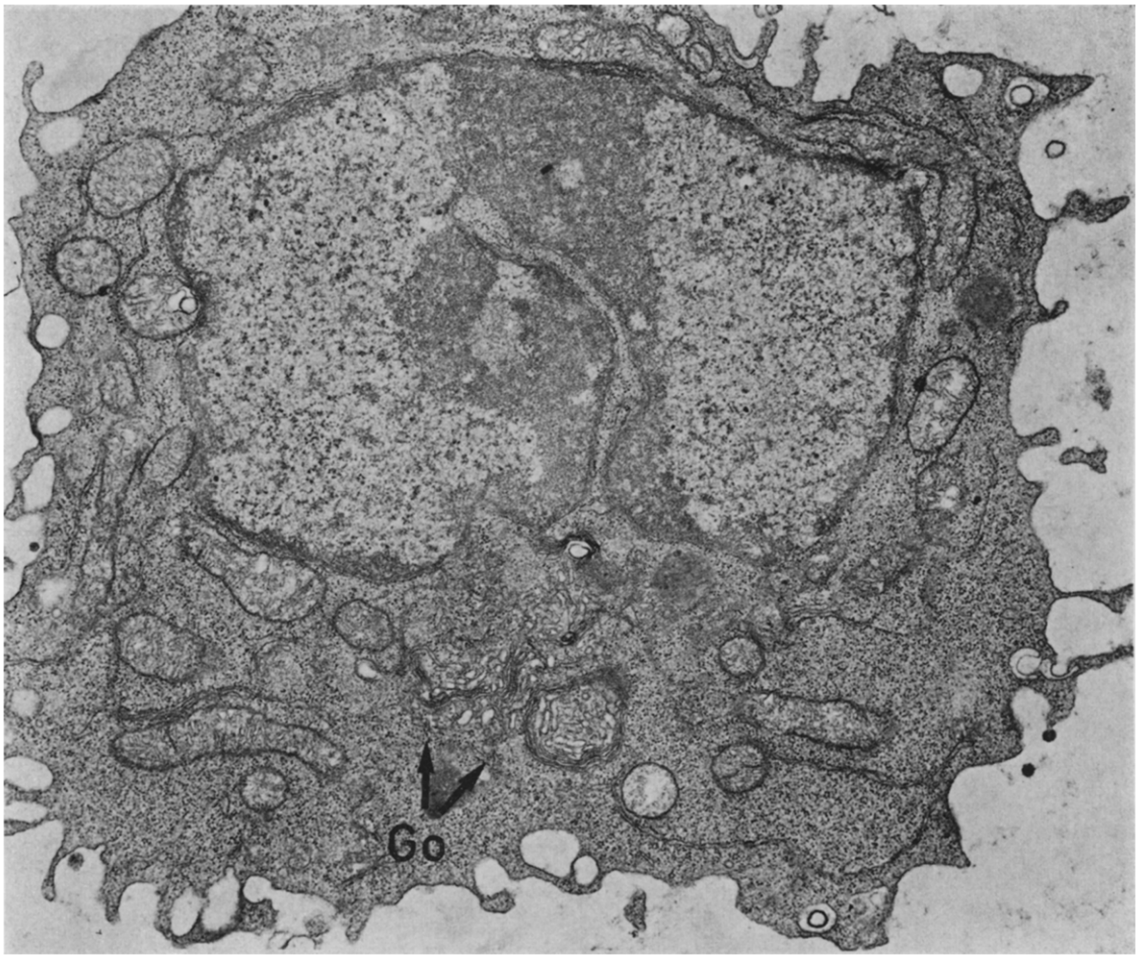

Fig. 2. P388 cell after an 8-day administration of AC-3579 (100 mg/kg per day). There is no increase of endoplasmic reticulum membranes nor formation of lamellate cytosomes. Golgi apparatus (Go) appears well developed (× 13,500).

(to face p. 636)

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Antipseudomonal Drugs: Comparative Study of Gentamicin, Sisomicin and Tobramycin *in Vitro* and in Human Volunteers

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Abstract—*Gentamicin, sisomicin and tobramycin were compared* in vitro *against a variety of micro-organisms isolated from patients who have been admitted to a cancer hospital. No major differences of activity between these antibiotics have been observed, however, sisomicin appeared the most active drug on* E. coli, *Klebsiella, Proteus strains and* Staphylococcus aureus *whereas tobramycin was the most active compound on* Pseudomonas aeruginosa.

Sera of volunteers, treated with these drugs in a cross-over fashion, did not exhibit significantly different activity against the strains tested, although gentamicin appeared more active on E. coli *and tobramycin was more effective on* Pseudomonas aeruginosa.

Cross resistance between gentamicin, sisomicin and tobramycin was frequently found, although not constantly.

The combinations of gentamicin, sisomicin and tobramycin with carbenicillin were found frequently synergistic in vitro *against* Pseudomonas aeruginosa *strains. Under these conditions relatively low concentrations of the antibiotics tested were effective in inhibiting most strains tested in this study, even when the microorganism had been found resistant to one of several aminoglycoside drugs used alone.*

The present study leads to the conclusion that combinations of gentamicin, sisomicin and tobramycin with carbenicillin or related antibiotics, should be evaluated in the treatment of clinical infections, especially those caused by Pseudomonas aeruginosa.

INTRODUCTION

TODAY, the most commonly used parenteral aminoglycoside antibiotics are kanamycin and gentamicin. The latter drug has an outstanding anti-Pseudomonas advantage over kanamycin, and this property is clinically important since *Ps. aeruginosa* continues to be a major cause of mortality and morbidity in hospitalized patients, especially in those with impaired natural resistance to infections. Patients with leukemia, lymphoma and other malignant tumors are frequently afflicted with bacterial infection which is often caused by *Pseudomonas aeruginosa.* Owing to the peculiar properties of this type of microorganism, the morbidity and mortality of infections caused by Pseudomonas strains remain distressingly high.

Recently, two additional parenteral aminoglycoside antibiotics have been described: these are sisomicin and tobramycin. Several studies have compared gentamicin with tobramycin [1, 2, 3, 4]; and have indicated that tobramycin might have an anti-Pseudomonas advantage over gentamicin, *in vitro* at least. Clinical studies

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are needed to asses whether these differences, found *in vitro*, have clinical significance.

Comparative activity of sisomicin, gentamicin and tobramycin has been studied by Waitz and co-workers [2] *in vitro* and in animals. These investigators found that *in vivo* tests failed to confirm all specificities of *in vitro* tests and emphasized the need for further *in vivo* and clinical investigations to properly assess the role of each antibiotic in human therapy. The present study offers another approach of an evaluation of these drugs in man.

It should be emphasized that gentamicin alone has not always been very effective in the treatment of severe infections caused by *Ps. aeruginosa* [5]. Synergistic and additive effects of tobramycin and carbenicillin, similar to those existing between gentamicin and carbenicillin have been reported [6]. In the present investigation, we have studied in a comparative fashion the combination of sisomicin with carbenicillin with those of tobramycin and gentamicin with carbenicillin.

MATERIAL AND METHODS

Tobramycin was supplied by Eli Lilly and Co. (Dr. A. Jaunau), and gentamicin and sisomicin by Schering Corp. (Dr. A. Velghe and Dr. G. Twichi). Fresh dilutions were prepared daily in sterile medium or buffer.

The bacteria tested were isolated from patients hospitalized at the Institut Jules Bordet, which is the clinical center for cancer therapy of the Brussels University. All were recent isolates. They included *Ps. aeruginosa* (56), *Escherichia coli* (122), Klebsiella–Enterobacter (82), *Proteus mirabilis* (86), indole positive Proteus species (14), Paracolon sp. (22), Providence (17) and *Staphylococcus aureus* (75).

Minimum inhibitory concentrations (MIC) of tobramycin, gentamicin and sisomicin were determined by the inocula-replicating method [7] using Mueller–Hinton agar (Difco) and an overnight bacterial suspension in trypticase soy broth (Difco) diluted to a final concentration of 10^8 viable micro-organisms per milliliter in tests involving staphylococci and of 10^5 in those relative to Gram-negative bacilli. For 56 strains of *Ps. aeruginosa*, we have tested these organisms simultaneously on Mueller–Hinton agar containing twofold dilutions of a mixture of carbenicillin (800 μg/ml) and gentamicin, tobramycin or sisomicin (25 μg/ml). These relative concentrations were chosen because they are multiples of maximal levels of these antibiotics obtainable in the blood during therapy with usual doses. The lowest concentration of antibiotic showing complete macroscopic inhibition of growth after overnight incubation at 37°C was considered as the bacteriostatic endpoint.

A combination was considered to show synergism or antagonism when there was a fourfold (2-dilution) increase or decrease of activity of both agents when combined. No instance of antagonism has been detected in this series; thus in cases where no synergism has been detected, the combination was labeled indifferent.

Standard killing curves were done on representative strains of *Ps. aeruginosa* on which carbenicillin and one other aminoglycoside antibiotics were synergistic, and these tests confirmed the occurrence of synergism for the strains for which the agar dilution tests had suggested it.

Cross-over studies using human volunteers were performed in 5 male patients hospitalized at the Institut Jules Bordet, each of whom had a malignant tumor without evidence of metastases. Age ranged from 56 to 72 years. None showed any signs of impaired renal or hepatic function. The patients were given a single dose (2, 5 mg per kg) of tobramycin, gentamicin or sisomicin intramuscularly on 3 consecutive days. Two patients received the antibiotics in this sequence; 1 received sisomicin, then gentamicin and tobramycin, and 2 patients received tobramycin, sisomicin and gentamicin.

Blood was obtained 60 min after the administration of the drugs; serum was separated after the blood had clotted and was stored at −20°C until used. The period of storage did not exceed 10 days. Assays for tobramycin, gentamicin and sisomicin were performed by the disc plate method described by Davis and Stout [8] *Bacillus subtilis* (Difco spore suspension) was used as the indicator organism and Muller–Hinton agar was used throughout.

Antibacterial activity (bacteriostatic and bactericidal) of serum was determined by serial twofold dilutions of the 1-hr serum specimens. These tests were performed on 5 strains of *E. coli*, 5 Klebsiella, 5 *Proteus mirabilis*, 5 indole positive Proteus strains and 5 *Ps. aeruginosa* that have been chosen at random from isolates newly obtained from clinical material. The sensitivity of these strains to gentamicin, tobramycin and sisomicin ranged between 0·15 and 0·70 μg per milliliter, as tested by the inocula-replicating method.

Plastic plates containing 96 wells were used, the bacteria were added in a final concentration of 10^5 micro-organisms per milliliter in a

volume of 25 μl of a twofold dilution of the serum to be studied with pooled human serum.

The plates were incubated at 37°C for 18hr. The highest dilution that did not contain visible growth was considered as the maximum inhibitory dilution (MID) and the highest dilution that did not contain viable bacteria as the maximum bactericidal dilution (MBD).

RESULTS

As indicated in Fig. 1, sisomicin proved to be more active *in vitro* than tobramycin and gentamicin against strains of *E. coli*, Klebsiella inhibited by 12 μg/ml. These observations are in accord with studies by Waitz and co-workers as far as the apparent superiority of tobramycin against *Ps. aeruginosa* strains is concerned [2]. This hospital has limited experience with Serratia strains, but Providence bacilli have been occasionally responsible for severe sepsis in our institution. Tobramycin, sisomicin and gentamicin appeared similarly active on strains of the latter; the median MIC was comprised between 1·0 and 1·5 μg/ml; all the Providence strains tested here could be inhibited by 12 μg/ml of all the 3 antibiotics studied. As could be expected, strains of *Staphylococcus aureus* (whether oxacillin-sensitive or oxacillin-

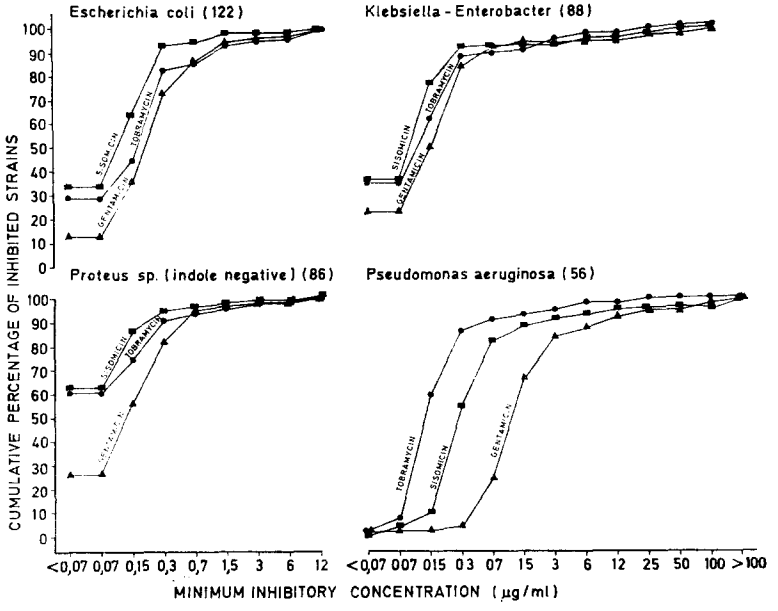

Fig. 1. *Cumulative percentage of strains of* E. coli, *Klebsiella*, Proteus mirabilis *and* Pseudomonas aeruginosa *inhibited by gentamicin, sisomicin and tobramycin.*

and indole negative Proteus. The median MIC for these 3 antibiotics was very similar, and the inhibition of more than 90% of these strains was achieved with a concentration of 1·5 μg/ml of each antibiotic. These differences in susceptibility to antibiotics that can be shown *in vitro* are probably of no clinical significance.

Differences in susceptibility of *Ps. aeruginosa* to sisomicin, gentamicin and tobramycin were more pronounced. The median MIC was 0·10 μg/ml for tobramycin, 0·3 μg/ml for sisomicin and 1·0 μg/ml for gentamicin. At higher concentrations, the differences between these drugs were less striking: over 90% of the strains of *Ps. aeruginosa* tested here were resistant) were found sensitive *in vitro* to all 3 antibiotics tested. As illustrated in Fig. 2, only minor differences in susceptibility of these micro-organisms to sisomicin, gentamicin and tobramycin could be detected.

Table 1 shows the respective numbers and percentages of *Ps. aeruginosa* strains which could be inhibited by 0·7 or 3·0 μg of gentamicin, tobramycin or sisomicin and by 25 or 100 μg of carbenicillin. The number of strains inhibited by the various combinations of these agents is also shown in that figure. The data do not bear upon the issue of synergism but they are presented in order to establish whether the combinations offer a broader coverage against

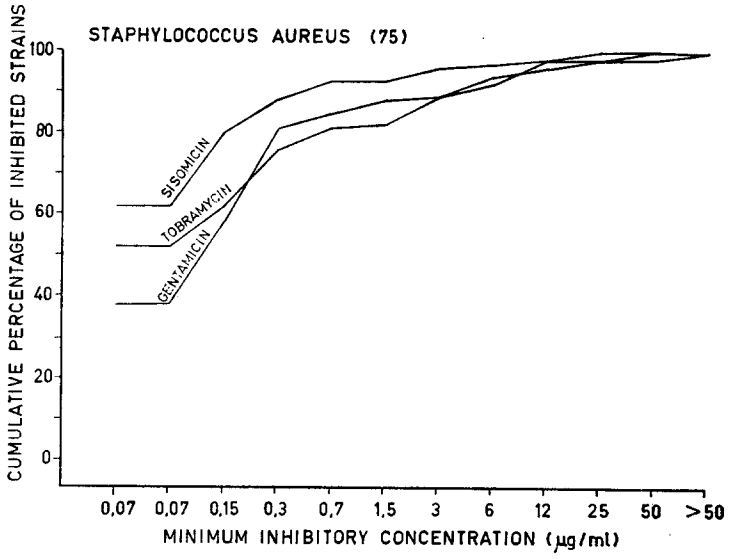

Fig. 2. Cumulative percentage of strains of Staphylococcus aureus *inhibited by gentamicin, sisomicin and tobramycin.*

Table 1. *Comparison of the inhibitory action of tobramycin, sisomicin and gentamicin and their combination with carbenicillin against* 56 *strains of* Ps. aeruginosa

	Tobramycin		Sisomicin		Gentamicin		Carbenicillin		Tobramycin + Carbenicillin		Sisomicin + Carbenicillin		Gentamicin + Carbenicillin	
Concentration (µg × ml)	0·7	3·0	0·7	3·0	0·7	3·0	25	100	0·7 + 25	3·0 + 100	0·7 + 25	3·0 + 100	0·7 + 25	3·0 + 100
No. strains inhibited	51	53	46	51	14	47	38	51	56	56	54	56	54	56
% strains inhibited	91	95	82	91	25	83	68	94	100	100	96	100	96	100

Ps. aeruginosa than one agent alone, when each is given in therapeutic dose.

As already indicated in Fig. 1, most strains of *Pseudomonas* could be inhibited by relatively low concentrations of tobramycin and sisomicin; the activity of gentamicin was somewhat less dramatic. Nevertheless, the addition of carbenicillin to these aminoglycoside antibiotics resulted in an increased antibacterial action. Carbenicillin has been added in a 100:3 weight: weight proportion to the aminoglycosides in order to realize a proportion of these drugs which can be usually achieved in the serum of treated patients.

As indicated in Table 1, the combination of carbenicillin with tobramycin, sisomicin and gentamicin inhibited 100% of the strains tested when 100 µg/ml of carbenicillin and 3 µg/ml of the aminoglycoside antibiotic were used. Tobramycin and carbenicillin used in combination, inhibited 100% of the strains at lower concentrations (0·7 µg/ml and 25 µg/ml respectively).

Figures 3 and 4 illustrate graphically the reduction of the concentration of the aminoglycoside antibiotic or of carbenicillin when these drugs were employed in combination with a fixed proportion of carbenicillin or aminoglycoside drug respectively.

These data suggest that when used with carbenicillin relatively lower amounts of gentamicin and sisomicin are needed to inhibit similar proportions of strains. Conversely, the addition of a fixed proportion of gentamicin,

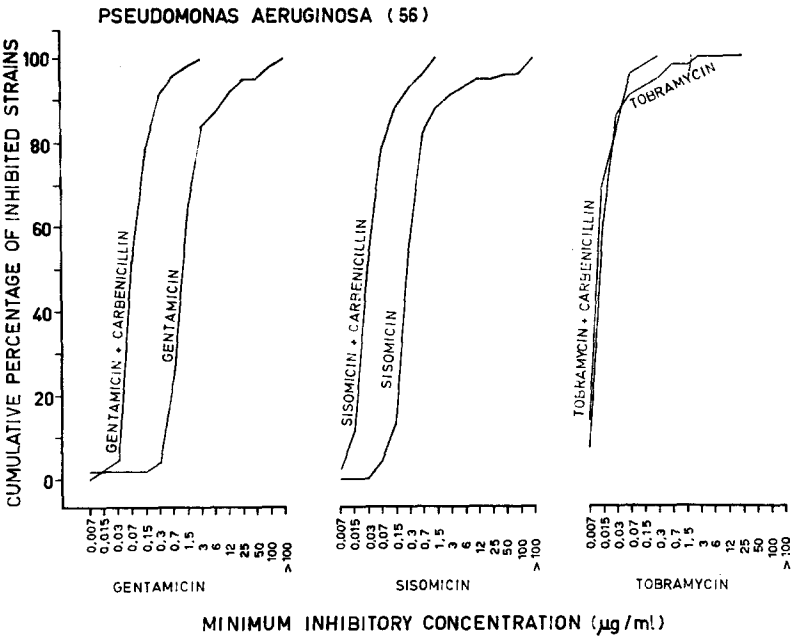

Fig. 3. *Sensitivity of* Ps. aeruginosa *strains to gentamicin, sisomicin and tobramycin used alone or in combination with carbenicillin.*

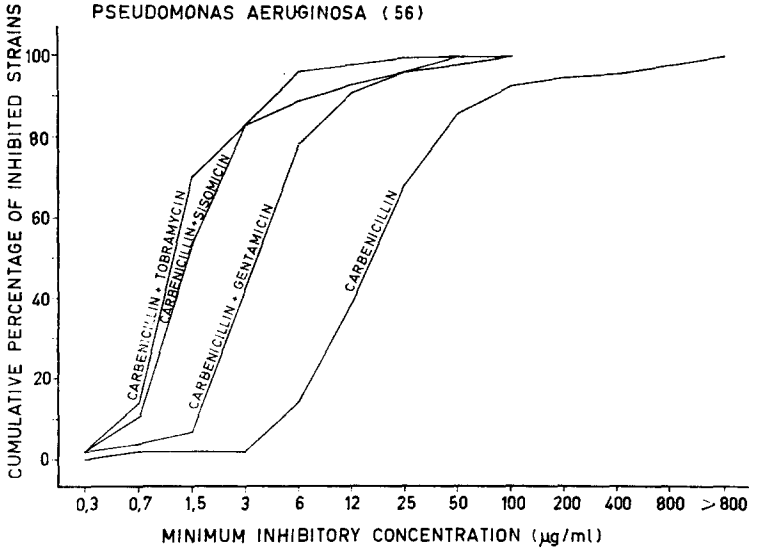

Fig. 4. *Sensitivity of* Ps. aeruginosa *strains to carbenicillin used alone or in combination with gentamicin, sisomicin or tobramycin.*

sisomicin and tobramycin to carbenicillin, allowed a marked reduction of the dose of carbenicillin needed to obtain a given effect.

Synergism was defined as a fourfold increase of activity of the combination over the activity of each drug alone. This happened for 49 (87·5%) of the strains exposed to tobramycin and carbenicillin, 50 (89·2%) of the strains exposed to sisomicin and carbenicillin and for 41 (73·2%) of those that were treated with gentamicin and carbenicillin. Selected strains were tested for synergism *in vitro*, using the classic killing-curve method, which is considered by some authors as the most demanding test for *in vitro* synergism [9]. For all strains tested it has been possible to confirm that when synergism had been suggested by the dilution tests, increased rate of bactericidal activity of the combination over each drug used alone could be demonstrated.

Table 2. Cross resistance of Ps. aeruginosa *to gentamicin, sisomicin and tobramycin*

Strain	Minimal Inhibitory Concentration (μg/ml) Gentamicin (G)	Sisomicin (S)	Tobramycin (T)	Carbenicillin (C)	G+C	S+C	T+C
1	100	50	0·15	50	0·7 + 25	1·5 +50	0·03× 1·5
2	100	100	0·3	100	1·5 + 50	0·7 +25	0·15+ 6
3	100	100	25	12	0·15+ 6	0·03+15	0·15+ 6
4	25	6	3	50	0·7 + 25	0·7 +25	0·7 +25
5	12	3	1·5	25	0·3 + 12	0·15+ 6	0·15+ 6
6	12	1·5	0·7	800	0·3 + 12	0·3 +12	0·15+ 6
7	12	12	6	12	0·15+ 6	0·15+ 6	0·3 +12
8	6	1·5	0·7	100	0·7 + 25	0·3 +12	0·15+ 6
9	6	1·5	0·7	400	3 +100	1·5 +50	0·15+ 6
10	3	3	6	200	0·15+ 6	0·07+ 3	0·03+ 1·5

Table 3. Reciprocal of maximum inhibitory dilution (MID) and maximal bactericidal dilution (MBD) of sera of patients treated with gentamicin, tobramycin and sisomicin

Patient	Drug*	*E. coli* (5) MID	MBD	Klebsiella (5) MID	MBD	Proteus sp (indole −) (5) MID	MBD	Proteus sp (indole +) (5) MID	MBD	*Ps. aeruginosa* (5) MID	MBD
1	G	4	4	8	8	4	4	2	2	1	1†
	T	4	4	4	4	2	2	2	2	4	4
	S	4	4	4	4	4	4	2	2	2	2
2	G	8	8	8	8	4	4	4	2	2	2
	T	4	4	8	8	4	4	2	2	8	4
	S	8	8	8	8	4	4	4	2	4	4
3	G	8	8	8	8	4	4	4	4	8	4
	T	4	4	8	8	4	4	4	2	8	8
	S	4	4	8	8	4	4	4	4	8	8
4	G	8	8	8	4	8	8	4	4	4	2
	T	4	4	4	4	4	4	4	4	8	8
	S	4	4	4	4	4	4	4	4	4	4
5	G	8	8	8	8	4	4	4	4	4	2
	T	4	4	8	8	4	4	4	4	8	4
	S	4	4	4	4	4	4	4	4	8	4
Mean‡	G	8	8	8	8	4	4	4	4	4	2
	T	4	4	8	8	4	2	4	2	8	8
	S	4	4	4	4	4	4	4	4	4	4

* = G: gentamicin; T: tobramycin; S: sisomicin.

† = In circumstances in which the twofold diluted serum was not inhibitory nor bactericidal, we have arbitrarily considered the MIC and MBD as 1/1; 2 = ½ dilution; 3 = ⅛ dilution; etc.

‡ = approximated to the lower MID or MBD.

Cross-resistance between gentamicin, sisomicin and tobramycin against *Pseudomonas aeruginosa* has been investigated, since this can be of major importance in clinical conditions. As shown in Table 2, we have found 10 strains for which MIC of gentamicin was equal or greater than 3 μg/ml. Seven of these strains were similarly resistant to sisomicin and 4 were resistant to tobramycin. It is clear therefore that cross-resistance between these 3 drugs exists, although it cannot be assumed that complete cross-resistance is common place. This observa-

tion has already been made by Crowe and Sanders [10]. This suggests that complete *in vitro* evaluation of the susceptibility of microorganism should be made before substituting one drug for the other. *In vitro* testing of one of the drugs that have been studied here would not appear to be adequate to estimate the very sensitivity of a given strain.

It is noteworthy that when employed in combination with carbenicillin, none of the "resistant" strains to gentamicin, sisomicin or tobramycin has been found really resistant: relatively low concentrations of any of the aminoglycoside drugs used in combination with carbenicillin were adequate to inhibit these strains, as indicated in Table 2.

The studies relative to the antibacterial activity of sera of tobramycin-, gentamicin- and sisomicin-treated volunteers following the Finland–Jones method [11] are summarized in Table 3.

These data indicate that tobramycin might have an antibacterial advantage over the 2 other drugs against *Ps. aeruginosa*. This has been already established by previous work from this laboratory and elsewhere [1, 6]. Gentamicin appeared more active than tobramycin and sisomicin on *E. coli*. Gentamicin and tobramycin were more active on Klebsiella than sisomicin. On Proteus strains (indole-negative and indole-positive) the effectiveness of all three drugs appeared to be quite similar.

Our data were analyzed for statistical significance but, owing presumably to the relatively small numbers of strains that were tested, no significant differences could be observed. This does not imply that, with the use of much more numerous strains, significant differences could not have been observed; it might be suspected, however, that the actual differences between the activity of these drugs would not be considerable and therefore of dubious clinical significance.

DISCUSSION

The introduction of a new drug into the antimicrobial armamentarium should be justified by some important and original features of the new antibiotic to be used. Waitz and co-workers have emphasized the need for *in vivo* tests and clinical studies rather than extensive *in vitro* comparisons to evaluate the place of a new antibiotic in human therapy [2].

However, *in vitro* tests are useful to predict clinical effectiveness of antimicrobial agents: numerous studies have established that strains which are inhibited by low concentrations of antibiotics are usually more easily eradicated *in vivo* than more resistant strains [12, 13]. On the other hand, experimental work in animals has not been systematically correlated to the clinical outcome in diseased patients. This is not to say that other forms of investigation of antimicrobial drugs than plain *in vitro* studies are to be neglected: the level of antibiotics in tissues might be worthy of more extensive investigation since important differences in concentrations in tissues of drugs that look at first glance quite alike—such as gentamicin and tobramycin—can be demonstrated [14]. The antimicrobial activity of sera of treated patients has a definite prognostic significance in the treatment of infectious diseases (Klastersky *et al.*, studies to be published). Therefore, the method for comparing antimicrobial drugs that has been proposed by Jones and Finland [11] and which has been used here can be a valuable approach to the evaluation of antibiotics.

Using this method, we have not been able to establish significant differences between gentamicin, sisomicin and tobramycin against selected strains of gram-negative bacilli. These observations do not exclude a possible advantage of one of these drugs over another on occasional strains. In previous studies, on *Ps. aeruginosa*, we have found some superiority of tobramycin as compared to gentamicin [1]. In addition, complete cross-resistance between the 3 antibiotics does not seem to exist, although for most strains the sensitivity to one of them is predictive of the sensitivity to the others. These observations suggest that differences between gentamicin, sisomicin and tobramycin do exist, although in most instances these differences are minimal. Clinical studies have to assess whether these differences could be of clinical significance.

The demonstration of *in vitro* synergism between antibiotics against Gram-negative strains has a definite clinical significance for the treatment of infectious diseases [15]. Therefore, we have studied the antimicrobial activity of gentamicin, tobramycin and sisomicin combined to carbenicillin on *Pseudomonas aeruginosa* strains. We have found that the addition of carbenicillin to the aminoglyciside drugs studied here has a distinct antimicrobial advantage over the use of one of these antibiotics alone against *Ps. aeruginosa*.

This might be important since aminoglycosides alone—namely gentamicin—has not always been very effective in granulocytopenic patients [5].

To conclude, gentamicin, tobramycin and sisomicin have been found similarly effective—

with minor differences—on clinical isolates obtained from patients admitted to a cancer hospital. Studies on volunteers have confirmed these observations, showing that sera of patients treated with these drugs were about equally active.

More important has been the demonstration of a frequent potentiation of anti-Pseudomonas activity of gentamicin, tobramycin and sisomicin by carbenicillin. The effectiveness of these various combinations should be tested in clinical trials, since, this is the only way to establish adequately whether one drug is really superior to the other in the treatment of human disease.

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Fever of Unexplained Origin in Patients with Cancer*

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Abstract—*Long standing fever, with no obvious origin, has been found in* 47 (0·7%) *of* 6,880 *patients admitted to a cancer hospital. Among these patients* 27 (57·4%) *had leukemia or lymphoma, although this type of illness did not represent a majority among the patients admitted to this hospital.*

Infection caused the fever in 27 (57·4%) *patients and was most often due to localized infections. Gram-negative rods were most frequently responsible for it.*

Fever was attributed to the cancer itself in 18 (38·3%) *patients:* 5 *had acute leukemia in relapse,* 6 *had extensive lymphomas,* 7 *presented widespread metastatic carcinomas, and, in* 6 *of them, large liver metastases were present.*

FEVER has been a well-known manifestation of neoplastic disease for many years. Briggs studied 238 malignancies and found fever to be present in 38%. However, in his series, only 7 patients (2·9%) has sustained fever [1].

Petersdorf and Beeson, in their study of 100 consecutive cases of fever of unexplained origin, gathered prospectively in a medium sized city in the north-eastern United States between 1952 and 1958, found 19 patients in whom the neoplastic lesion was thought to have played a major role in the pathogenesis of fever [2].

Boggs and Frei studied a group of patients with leukemia, lymphoma, and carcinoma of the lung, who have been admitted to a cancer hospital, and found that 56% of the febrile episodes in their patients were unassociated with infection and were presumably due to cancer. These authors concluded also, from data found in the literature, that fever not associated with infection was present in 66% of the neoplasms of the colon and rectum, 49% of the squamous carcinoma of the skin, 47% of the cancers of the lung, 44% of the neoplasms of the gall-bladder and liver, 41% of the tumors of the stomach, 36% of the uterine malignancies, 35% of the neoplasms of the oesophagus and 33% of the neoplasms of the breast [3]. On the basis of these studies, one should conclude that fever is an extremely common manifestation of malignant disease.

Browder, Huff and Petersdorf studied the significance of fever in neoplastic disease in 343 patients with cancer admitted to a general hospital over a 1-year period [4]. These authors found also a high incidence of fever in patients with cancer, but their data strongly suggest that infection or localized obstruction produced by the tumor is responsible for the fever which, in most instances, disappears spontaneously. These authors have concluded that fever due to cancer itself is rare: in 5·4% of their patients.

Differences between the results obtained by the various investigators can probably be explained by the variable selection of their patients and by differences in criteria used to define a fever of unexplained origin.

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This is the reason why we have decided to investigate the problem of fever of unexplained origin in patients with cancer, as Boggs and Frei did [3], but to use for the definition of fever criteria which have been employed to define a fever of undertermined etiology by Petersdorf and Beeson [2]. The patients with cancer and fever of unexplained etiology have been gathered prospectively over a 3-year period, and all these patients have undergone a standardized work-up.

From the practical point of view, these observations may have some value since the precise importance of cancer itself and of other causes in the genesis of long-standing fever in patients with neoplasia is not completely known.

MATERIAL AND METHODS

From January 1970 to December 1972, 47 patients who have been hospitalized at the Institut Jules Bordet were found to present a fever of unknown etiology, responding the following criteria: (1) febrile illness of more than 3-week duration; (2) fever higher than 38°C on several occasions and (3) cause of fever uncertain after one week of study in the hospital.

During the same period of time, 6,880 patients have been admitted to the hospital. Fever of unknown origin, as defined earlier, has thus been found in 0·7% of the patients admitted to the oncology services of the Institut Jules Bordet.

At our institution, the usual "fever work-up" includes aerobic and anaerobic blood cultures (3 consecutive samples are usually taken over a few hours period), urine culture and urinalysis, culture of bronchial secretions obtained by a transtracheal aspiration, and culture of stools. Urine, sputum, and stools, are also investigated for the presence of acid-fast organisms and fungi by direct examination and culture.

Throat swabs and stools are inoculated on cell-cultures for viral culture. Intermediate strength PPD is performed. Complete hematologic analysis, a chest roentgenogram and serologic investigation for antibodies to common viruses (herpes simplex, herpes zoster, adenovirus, cytomegalovirus, respiratory syncytial, influenza, parainfluenza, psittacosis group, mycoplasma pneumoniae, and Australian antigen) are performed. Serologic evidence of candida and aspergillus infection is looked for. A complete physical examination is performed and, according to it, other bacteriological, serological, and radiological investigations, are undertaken: this is why lumbar puncture and intravenous pyelograms were obtained in most patients in this series. Most patients in this series underwent this type of work-up several times during the febrile period. In granulocytopenic (i.e. granulocyte count $< 1{\cdot}500$ per mm^3) patients therapy with carbenicillin (30 g daily), cephalothin (9 g daily) and gentamicin (5 mg/kg per day) was usually started after completion of the initial bacteriological studies. In non-granulocytopenic patients antibiotics were usually withheld until presumptive diagnosis of infection, on clinical grounds at least, had been made. In the patients in this series antibiotics were usually discontinued 5 or 6 days after the onset of therapy, if no effect on fever and clinical signs attributed to infection had been observed.

In patients with prolonged fever and no obvious etiological diagnosis, therapy with isoniazid (10 mg/kg) and ethambutol (25 mg/kg) was started after multiple cultures of sputum, gastric content and urine for acid fast bacilli had been taken. These patients underwent a bone marrow or/and a liver biopsy prior to the treatment. Empiric anti-tuberculous therapy was usually discontinued after 3 weeks if no defervescence and no changes in the clinical status had occurred.

Clearly, in many patients in this series, all of whom presented a serious underlying disease and were often undergoing aggressive surgical or medical therapies, short bouts of febrile illness were caused by minor infections or other inflammatory conditions such as phlebitis. These episodes and the fevers associated with transfusion of blood products were not considered here. On a clinical and bacteriological evidence, which has been obtained prior to death or at autopsy, we have decised which type of infection has been involved to explain at best the febrile course. Causes of fever other than infections were established on circumstantial evidence and on lack of demonstration of sepsis.

Obviously, we cannot be completely certain that in all the cases studied here a single cause has been responsible for prolonged fever.

RESULTS

As indicated in Table 1, we have considered that fever was caused in this series by infection in 27 (57·4%) patients, presumably by the cancer itself in 18 (38·4%) and by other causes in 2 (4·2%). The ratio of the neoplastic versus infectious origin of fever was clearly different in

Table 1. *Presumed causes of fever in* 47 *patients with cancer and protracted pyrexia of obscure etiology*

	Total	Presumed causes of fever: Neoplasia	Infection	Other*
Leukemia	17	5	11	1
Chronic lymphocytic	1	0	1	0
Acute lymphoblastic	6	3	3	0
Acute myeloblastic	5	2	3	0
Chronic myelogenous	1	0	0	1
Myelosclerosis–Polycythemia	2	0	2	0
Acute monoblastic	2	0	2	0
Lymphomas	10	6	3	1
Hodgkin's disease	6	4	1	1
Chediak–Higashi's disease	1	1	0	0
Reticulum cell sarcoma	2	1	1	0
Multiple myeloma	1	0	1	0
Solid tumors	20	7	13	0
Breast	9	5	4	0
Bronchus	3	0	3	0
Larynx–Pharynx	3	1	2	0
Prostate	1	0	1	0
Melanoma	1	0	1	0
Cervix uteri	1	0	1	0
Oesophagus	1	0	1	0
Kidney	1	1	0	0
Total	47	18	27	2

*Busulfan lung.
Multiple pulmonary emboli.

patients with lymphomas (6/3) and in those with leukemias (5/11) and solid tumors (7/13). It has been known since a long time that fever without infection is a common manifestation of certain lymphomas, especially Hodgkin's disease, and our observations are in accordance with these observations [5].

In 2 patients fever was presumably caused by factors other than neoplasia or intercurrent infection. One patient with controlled chronic myelogenous leukemia and inactive pulmonary tuberculosis died after a prolonged illness characterized by respiratory insufficiency and fever. At autopsy no infection was found, but the pulmonary tissue had all the features of the "busulfan" lung. Fever has been previously associated with pulmonary changes caused by busulfan [6].

The other patient had had Hodgkin's disease and a carcinoma of the breast but no tumor could be found at autopsy. She had been admitted for fever and thrombophlebitis; at autopsy pulmonary infarcts were found. This patient had in addition a bone marrow aplasia with thrombocytopenia and died as a result of diffuse pulmonary hemorrhages. Meningeal hemmorrhage and a pituitary cyst were also found in this patient.

Table 2 summarizes the infections that were considered as responsible for the febrile episodes in this series. The most frequent type of infection was a localized pyogenic abscess or infection and this has been found in 17 (62·9%) of the 27 patients who were considered to have presented infection as a course of protracted fever. These abscesses were similarly common in patients with leukemia or lymphoma and those with metastatic tumors. In each group about half of these localized infections resulted in septicemia and death; a total of 9 (52·9%) of these 17 infections were considered to have been fatal. Table 3 indicates the anatomic localization of the abscesses or focal infections and the nature of the microorganisms responsible for them. Neoplasia was the underlying site in 5 cases (2 neoplasms of the floor of the mouth, 2 bronchial carcinomas and a neoplastic pleural effusion caused by a metastatic carcinoma of the breast). In all cases, the extent of the neoplasia was masking the underlying infectious process which has been recognized only late; indeed, in 3 cases, the infectious

Table 2. Number of patients with infections (bacterial, mycobacterial, fungal and viral) responsible for fever of unknown origin

	Total number of patients	Fatal infections† Leukemia Lymphoma	Solid tumor	Non fatal infections Leukemia Lymphoma	Solid tumor
Endocarditis*	2	0	2	0	0
Localized infections or abscesses*	17	4	5	3	5
Tuberculosis (pulmonary)	1	0	0	0	1
Fungal infections	5	4	1	0	0
Viral infections	2	1	0	1	0
Total number of infections	27	9	8	4	6

*Bacterial infections.
†Infection was the predominant or an important associated factor for the fatal outcome.

Table 3. Bacteriological etiology of infections responsible for prolonged fever of uncertain etiology in patients with cancer

Type of infection	Number	Offending microorganism	Septicemia	Death†
Endocarditis	2	*Str. viridans*	+	+
		Str. faecalis	+	+
Abscess with neoplasia	4	*Ps. aeruginosa*	+	+
		Str. faecalis + *Str. pyogenes*	−	−
		Bacteroides	−	+
		Bacteroides	−	+
Abscess post surgery	2	*P. mirabilis*	−	−
		Klebsiella	+	−
Splenic abscess	2	*E. coli*	+	+
		P. mirabilis	+	+
Rectal abscess	2	*E. coli* + *P. mirabilis* + Bacteroides	−	−
		P. mirabilis	+	+
Breast abscess	1	*E. coli*	+	+
Dental abscess	1	?	−	−
Cholecystitis	1	?	−	−
Phlebitis	1	*E. coli*	+	+
Pyelonephritis	1	Klebsiella	+	−
Osteomyelitis	1	*Staph. aureus*	+	−
Empyema*	1	*Staph. aureus*	−	+

*Complicating a carcinomatous pleurisy.
†Infection considered as the principal or contributing cause of death.

nature of the process was only recognized at autopsy. In 2 patients, abscesses developed at the site of previous surgery: in one patient the procedure was an axillary radical dissection for metastatic melanoma and in the other a splenectomy in a patient with acute lymphoblastic leukemia.

Abscesses of the spleen were found at autopsy in 2 other patients (one with polycythemia and the other with acute monoblastic leukemia) in whom septicemia had been documented and has been cured with antibiotics a few weeks before the onset of the actual febrile episode. Pain in the splenic area had been documented during the initial bacteremic episode in one of these patients.

Both patients with rectal abscesses had acute leukemia. The importance of that localization of infection in leukemics has been stressed recently by Schimpff and co-workers [7].

In addition, 2 patients with bacterial endocarditis were seen in this series and this infection was the cause of hospitalization in both cases. Both patients were treated and have been presumably cured from their bacterial infection, but both died as a result of widespread metastatic disease. Six other patients presented with a localized infection as indicated in Table 3.

In Table 3 are also summarized the etiologic agents responsible for bacterial infection in this series. In 2 patients, no bacterial micro-

organisms have been identified although the infection was considered to be caused by bacteria: one was a leukemic patient with severe thrombopenia and granulocytopenia who developed a dental abscess, the other patient had a breast cancer presented a cholecystitis. Both infections responded well to antimicrobial therapy alone. Gram-positive organisms were responsible for 5 infections in this series, including the 2 cases of endocarditis. All the other bacterial infections were caused by Gram-negative rods making the Gram-negative etiology responsible in 12 (70·5%) of the cases of bacterial infection that were proved bacteriologically. This high frequency of Gram-negative bacilli in infections in patients with severe underlying diseases is in accordance with recent reports from this hospital and from elsewhere [8, 9]. Anaerobic Gram-negative rods (Bacteroides sp.) were isolated in pure culture from 2 cases and in mixed culture with other Gram-negative rods in 1 case.

Only 1 patient in this series had long standing fever caused by tuberculous infection of the lungs, and in 2 other patients the cause of fever was considered to have been a viral infection caused by cytomegaloviruses. In a case of acute leukemia, the viral infection was found to be disseminated at post mortem examination. In the other, a long standing febrile pneumonia was found, eventually associated with a rise of antibodies from 0 to 1/32.

Fungal infection, as summarized in Table 4, was encountered in 5 patients and was fatal in 2 cases. The fungal infection involved the lungs in all 5 cases but has been considered to originate from elsewhere, a phlebitis and a peritonitis respectively in 2 cases in whom the infection was found to be also disseminated in many organs other than the lungs. An increase of the morbidity and mortality due to fungal pulmonary infections in compromized hosts has been recently reported [10].

In 18 patients, no other cause than the neoplasia has been found to explain the fever; especially no infectious illness has been detected.

As indicated in Table 5, 6 patients had a lymphoma, 5 had acute leukemia and 7 presented disseminated carcinoma. A striking feature in these patients was the extension and the progressive nature of the neoplasm: all the lymphomas presented visceral involvement and all the cases of leukemia were in relapse or during their first evolutive stage. Solid tumors were widely metastatic in 6 cases out of 7 and, in all of these 6 patients, extensive liver metastasis was present. In 5 out of 6, the breast was the original neoplasm. The patient whose initial tumor was the larynx with large cervical metastasis which consisted of necrotic and sterile material; in addition that patient had extensive metastasis to the liver.

The only solid tumor with only 1 known metastasis was a carcinoma of the kidney, and this type of tumor has been considered as the prototype of tumor frequently associated with fever, although the actual frequency of febrile patients with renal cell carcinoma seems relatively low (3%) [11]. Hodgkin's disease is also frequently associated with fever unrelated to infection and this has been found to be the case for 6 of the 10 patients with lymphoma in this series. In patients with lymphoma a

Table 4. Fungal diseases responsible for prolonged fever of unknown origin

Underlying neoplasm	Presumptive source and Type of infection	Type of microorganism	Death*
Hodgkin's disease	Lungs Pneumonitis	*Aspergillus fumigatus*	–
Ca oesophagus	Lungs Pneumonitis	*Aspergillus* sp.	–
Chronic myelogenous leukemia	Lungs Pneumonitis	*Candida albicans*	–
Acute lymphoblastic leukemia	Phlebitis Disseminated	*Candida albicans*	+
Reticulum cell sarcoma	Peritonitis Disseminated	*Candida* sp.	+

*Death caused presumably by the fungal infection itself.

Table 5. Type of tumor and extension in patients with unexplained fever

Type of tumor	Extension of the neoplasia	Leukopenia	Response to Therapy*	Death
Lymphomas				
Chediak–Higashi	lymph nodes (? visceral)	+	−	+
Hodgkin's disease	stage IV (lungs, liver)	−	+	+
Hodgkin's disease	stage IV (liver)	−	+	−
Hodgkin's disease	stage IV (lungs)	−	+	−
Hodgkin's disease	stage IV (lungs)	+	+	−
Reticulum cell sarcoma	liver, GI tract, heart	−	−	+
Leukemias				
A.M.L.	1st evolution	+	+	−
A.M.L.	2nd relapse	+	+	−
A.L.L.	1st evolution	+	+	−
A.L.L.	3rd relapse	+	+	−
A.L.L.	1st evolution	+	+	−
Solid tumors				
Breast	skin, liver	−		−
Breast	lungs, liver	−		−
Breast	liver	−		−
Breast	lymph nodes, liver	−		+
Breast	bones, liver	−		−
Larynx	lymph nodes, liver	−		+
Kidney	bone	−		−

*Corticoids or cytostatic therapy.

response of fever and of other manifestations to chemotherapy was observed in 4 patients suggesting the neoplastic origin of the fever. Fever unrelated to infection was seen in 5 patients with acute leukemia (29%) in this series. All these patients were severely granulocytopenic during the febrile episode and, in all of them, fever disappeared as granulocytopenia subsided and remission of the leukemic process took place.

The characteristics of fever of undetermined etiology (presumably the fever of neoplastic disease) were in many respects similar in patients with fever caused by infections. These observations are in accordance with previous studies [3] and suggest that "fever of cancer" is best considered typeless. Similarly, in the patients whose fever was caused by infections, no clear information could have been obtained from how high the fever did go and how long it lasted.

DISCUSSION

Long-standing fever, with no obvious origin, has been found in 0·7% of the patients admitted to a cancer hospital. The use of strict criteria, according to Petersdorf and Beeson [2], eliminates self-limited febrile illnesses which frequency and spontaneous subsidence has been documented by Browder and co-workers [4]. Although the majority of the patients admitted to our institution have some kind of disseminated solid tumor, 27 (57·4%) of the 47 patients studied here had a lymphoma or a leukemia; this suggests that a major factor which influences the frequency of protracted fever of undetermined origin, as defined earlier, is the type of the neoplastic disease. This factor influences also the etiology of that type of fever: frequently no other cause for pyrexia than cancer itself was found in patients with lymphoma but infections were found to be responsible more commonly in patients with leukemia or solid tumors. On the whole, infection caused the fever in nearly 60% of the cases studied here and most often was due to bacterial abscesses or localized infections, the diagnosis of which was often made more difficult by extensive neoplastic involvement of various organs. In this series, most cancers in which fever of undetermined etiology has been found were extremely extensive and all the patients in whom fever has been attributed to cancer itself were in this category.

It is, therefore, concluded that fever presents a diagnostic problem in patients with cancer relatively rarely and that, in most of these instances, infection remains still the most common cause of it. Fever caused by cancer itself appears exceedingly rare, which is in accordance with previous work from this hospital, showing that bacterial infection, especially that caused by Gram-negative infections, is the leading cause of death in patients with all kinds of neoplastic diseases [12].

The mechanism of fever in patients with cancer and without infection has been the subject of much speculation, but no definite explanation has been found until now. It has been shown that fever in febrile but uninfected patients with Hodgkin's disease was presumably due to a pyrogenic substance that can be recovered in the urine and that has the biological properties of endogenous pyrogen [13]. This suggests that in neoplastic diseases as in infections endogenous pyrogen serves as the final common pathway in producing fever [14]. Whether the pyrogen comes from necrotic tumor cells, from granulocytes, from macrophages infiltrating the tumor, or from some other place, remains a matter of controversy. It should be emphasized, however, that infiltration of tumors by macrophages is relatively common and might have some implications for the immunological resistance of the host to malignant tumors [15].

Whether patients with hepatic metastases are more susceptible to present fever unrelated to infection remains to be investigated. In several studies the frequency of fever unrelated to infection seems high among patients with liver metastasis [1, 4, 16]. In this series, 6 of 7 patients with solid tumor and fever caused presumably by cancer had extensive hepatic metastasis. The possible relationship between tumors of the liver and fever is unknown. Several explanations have been discussed by Browder, Huff and Petersdorf [4] and certainly deserve further studies.

A granulocytopenia associated with evolutive leukemia or its therapy was found to be associated with fever which subsided when cytopenia disappeared and remission occurred. However, in leukemics, fever was due more often to infection than to the leukemic process itself. These observations are in accordance with those made previously by Raab and co-workers [17] and it can be said that, in most cases, appearance of high fever in a patient with leukemia signifies infection or progression of the disease.

In conclusion, it seems at the present time that the classical differential diagnosis of fever of unexplained origin has not greatly changed during the past years, at least in patients who are admitted to a general hospital [18]. However, in other categories of patients, such as children, patients who have recently travelled abroad, and patients who present serious underlying diseases and who are treated by aggressive medical and surgical therapies, the causes of prolonged pyrexia may be quite different. This is the reason why the present investigation has been undertaken. We have tried to delineate the principal cause for protracted fever in patients with cancer admitted to a specialized hospital. It is recognized that, in such complex patients as those with widespread cancer who are undergoing cytostatic or radiation therapy, multiple causes for fever may co-exist or cause successive bouts of pyrexia. Nevertheless, it appears from our study that the cause of fever in patients with cancer can often be recognized and treated. The diagnostic problem of a significant fever of obscure etiology which lasts for a sufficient period appears only relatively rare.

The present study indicates that long-lasting type of fever in patients with cancer is caused chiefly by infection, but that in a few patients with lymphoma, evolutive acute leukemia and metastatic cancer fever might be presumably due to the cancer itself.

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Effects of 2-Br-α-Ergocryptine, L-Dopa and Cyclic Imides on Serum Prolactin in Postmenopausal Women*†

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Abstract—*The effects of cyclic imide derivatives (CG* 603 *and CG* 809*), of* L-*dopa and of* 2-*Br-α-ergocryptine (CB* 154*) on serum prolactin levels were studied in postmenopausal women.*

A single oral dose of L-*dopa* (500 *mg*) *or CB* 154 (5 *mg*) *was followed by a significant decrease in serum prolactin levels. After* L-*dopa, this decrease lasted an average of* 4 *hr and was followed by a rebound at* 8 *hr. After CB* 154*, the decrease lasted at least* 8 *hr. Single doses of CG* 603 (660 *mg*) *or CB* 809 (2 *g*) *failed to influence prolactin levels.*

The effects of chronic oral administration of either L-*dopa or CB* 154 *were also studied. The pretreatment circadian rhythm of serum prolactin levels, as determined on samples taken every* 2 *hr, served as control: it was characterized by a high and broad peak occurring during the night.* L-*dopa,* 500 *mg given every* 6 *hr, produced a saw-toothed effect with short-lasting falls after each dose, followed by sharp rebounds. The mean* 24*-hr levels on days* 9 *and* 14 *of treatment were* 94% *and* 72% *of the mean control value. CB* 154, 5 *mg three times daily, induced a profound and sustained decrease in prolactin levels, with complete abolition of the circadian rhythm. The mean* 24*-hr levels on days* 13 *and* 17 *of treatment were* 27% *and* 33% *of the mean control value.*

These experiments show that CB 154 *is an effective prolactin-inhibiting agent although complete suppression was not achieved.* L-*dopa was effective though definitely less so than CB* 154*. Reported successes of the former and failures of the latter to induce remissions in advanced breast cancer are discussed with regard to the present data.*

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INTRODUCTION

WHEREAS prolactin dependence is a well-established property of the dimethylbenz[a]-anthracene (DMBA)-induced mammary tumor of the rat [1, 2], a possible influence of prolactin on the growth of human breast cancer is a much debated matter [3]. Selective pharmacological interference with prolactin secretion might give insight into this problem and possibly also provide a new approach to the treatment of breast cancer.

Various compounds are known to more or less selectively inhibit prolactin secretion. They act by various mechanisms.

2-Br-α-ergocryptine (CB 154) is an ergot alkaloid which inhibits prolactin secretion in the rat and other animal species [4, 5, 6]. It is effective in young healthy women [7] as well as in patients with pathologic elevation of their blood prolactin levels [7, 8]. Ergot alkaloids act by increasing the prolactin inhibiting factor (PIF) content of the hypothalamus [9] and by a direct effect on the secretion of prolactin by the pituitary cell [10].

L-dopa [β(3-4-dihydroxyphenyl)-L-alanine] inhibits prolactin secretion in the rat [11] and in man [12, 13, 14, 15], presumably by stimulating PIF secretion in hypothalamic centers [16].

The cyclic imide 1-(morpholinomethy)-4-phtalimidopiperidindione-2, 6 (CG 603) inhibits prolactin secretion in the rat, by a mechanism as yet unknown [17, 18]; its parent compound the cyclic imide 4-phtalimidopiperidindione-2, 6 (CG 809) is probably the active part of the molecule [19].

Based on the observation of the therapeutic efficacy of CB 154 [5, 20, 21] and of CG 603 [22, 23] in the DMBA-induced rat mammary tumor system, clinical trials were undertaken with these compounds in postmenopausal women suffering from advanced breast cancer. Both failed to effect any significant tumor regression [24, 25]. In contrast, it was reported last year that L-dopa induced objective tumor regression in 2 out of 7 patients tested [26] and a third case was recently reported [27].

Interpretation of these conflicting results is difficult since the effectiveness of these compounds as inhibitors of prolactin secretion in postmenopausal women is not known. Success or failure might be due to different degrees of prolactin suppression. Alternatively, the effective compound might well act through mechanisms other than interference with prolactin secretion.

The purpose of the present paper was to elucidate these questions by assessing the inhibitory effect of CG 603, CG 809, L-dopa and CB 154 on prolactin secretion in postmenopausal women and to derive information on their respective potential value in the treatment of breast cancer and possibly other neoplastic diseases.

MATERIAL AND METHODS

Drugs

Tablets of CG 603 and CG 809 were kindly supplied by Chemie Grünenthal GmbH (Stolberg, West Germany), and capsules of CB 154 by Sandoz Ltd (Basel, Switzerland). L-dopa was given as tablets of Larodopa®, Hoffmann La Roche Ltd. (Basel, Switzerland).

Patients

Forty-six women, in-patients of the geriatric hospital (Centre de Gériatrie, de Traumatologie et de Réadaptation) in Montignies-le-Tilleul, Belgium, were admitted to this study. Their median age was 78, ranging from 44 to 90. All patients were more than two years postmenopausal, except for one patient receiving CG 809 and whose menopause occurred 6 months before. A routine examination, including fasting blood glucose, T_3 and T_4 uptake test and plasma 11-OH corticosteroids, measured in the morning and in the afternoon, revealed no evidence of diabetes, nor disturbance of the thyroid or adrenocortical function.

Prolactin assay

Human prolactin was measured by a radioimmunoassay developed for ovine prolactin [28] and later extended to the measurement of human prolactin. In this system, there is no interference with human growth hormone [29]. Extensive validation of this assay will be published elsewhere [30]. Slight modifications were introduced as previously reported [31, 32]. Serum prolactin levels were expressed as milliunits (mU) per ml: 1·0 mU is by definition the amount of immunoreactive prolactin contained in 1·0 μl of the laboratory standard, which is a pool of human sera rich in prolactin. In this assay, 1·0 μl of the laboratory standard has been found to be equivalent to 2·3 milliampoules of the Research Standard 71/167 (MRC, Division of Biological Standards, Great Britain) which is a standard serum. As the distribution of prolactin values appeared to be log normal, the logarithms of these values were used for statistical calculations. The latter were carried out by an analysis of the variance.

Experimental design

The present experiments involved serum prolactin determinations both after a single dose of the various compounds and during chronic drug administration.

(a) *Single dose administration.* Patients were randomly allocated to 5 treatment groups. One capsule of lactose was given as placebo to 8 patients, 660 mg of CG 603 to 6 patients, 2 g of CG 809 to 5 patients, 5 mg of CB 154 to 8 patients and 500 mg of L-dopa to 7 patients. These doses were selected as the highest which could supposedly be given without major side-effects. Each compound was administered around 8·30 a.m. together with a light breakfast, in order to avoid gastric intolerance.

Blood samples were collected through an indwelling venous catheter at various times before and after drug administration.

(b) *Chronic drug administration.* Six patients received increasing doses of L-dopa starting at 125 mg three times a day on day 1, to reach a full dosage on day 9. From that day on, 550 mg was given 6-hourly, starting at 6 p.m.

Five patients received increasing doses of CB 154, starting at 2 mg three times a day on day 1, to reach a full dosage of 5 mg three times a day (namely at 8 a.m. and 6 p.m.) from day 10 to day 27. The full dosages of L-dopa and CB 154 were those used in the clinical trials referred to above [24, 26].

Blood samples were collected through an indwelling venous catheter every two hours for 24 hr, starting at 5.30 p.m., on days zero, nine and fourteen in the L-dopa experiment, and on days zero, thirteen and twenty-seven in the CB 154 experiment. The indwelling needles were inserted about 90 min before starting blood collection.

The patients had their meals as usual at 7.30 a.m., 11.45 a.m. and 5.30 p.m. Occasional nausea and vomiting were controlled with metoclopramide (Primperan®).

RESULTS

(a) *Single dose administration*

Figure 1 depicts the changes in mean serum prolactin levels in the control group (Fig. 1A) and in four groups of subjects after administration of cyclic imides (Fig. 1B), L-dopa (Fig. 1C), or CB 154 (Fig. 1D).

In the control group (Fig. 1A), there was a slight apparent decline of the observed values from 60 to 240 min after placebo administration. However, the overall mean of the values from −60 min to +30 min is not significantly different at the 0·05 level from the mean of the values from +60 to +240 min.

In patients receiving cyclic imides (Fig. 1B) none of the mean values was lower after than before drug administration, with one exception at 30 min in the CG 603 group. This relatively low mean is due to a single low value in one patient, which could correspond to an experimental error.

In the group receiving L-dopa (Fig. 1C), there was an obvious decrease in the mean prolactin levels from +60 to +240 min. The overall mean for these values is about 40% lower than the mean of the earlier values ($P < 0{\cdot}05$). There was a sharp increase between 240 and 480 min, which was not observed in the control group.

The effect of CB 154 (Fig. 1D) was most conspicuous and consisted of a marked decrease in mean prolactin levels starting 60 min after administration and progressing during the whole experimental period. Incidentally it should be noted that the mean pretreatment values were higher in the CB 154 group than in the others.

No side effects were recorded after cyclic imide administration. Nausea and perspiration were frequent in the L-dopa group; vomiting occurred in one patient. Nausea and vomiting were often observed in the CB 154 group.

(b) *Chronic administration*

Because L-dopa and CB 154 administration was followed by a drop in the mean prolactin level after administration of a single dose, whereas similar drops were not observed after the cyclic imides, the former drugs were subjected to further study during chronic administration.

In these experiments, the patients served as their own controls. The 2-hourly schedule of blood sampling during 24-hr periods was selected since wide circadian variations of serum prolactin levels exist in human beings, under various physiological conditions [32, 33], with highest values occurring during the night.

The results are given in Fig. 2. In both treatment groups, the pretreatment values displayed a characteristic circadian rhythm of serum prolactin concentrations. There was a distinct elevation between near midnight and 7 a.m.

The results of the L-dopa experiment are given in Fig. 2(A) and 2(C). On the first day of full dosage (day 9), each dose of the drug except one (at 12 a.m.) induced a marked drop in prolactin levels, which lasted a minimum of 210 min. It was followed by a peak occurring at 330 min; however, in view of the short half-life of serum prolactin, about 10–15 min [34], earlier determinations could have disclosed higher peak values. On the sixth day of full dosage (day 14), the pattern of effect of L-dopa was basically the same as on the first day, although most peaks and intermediate values were lower.

The mean values of the 24-hr determinations (omitting the last 5.30 p.m. determination which in fact duplicates the first one), on days 9 and 14 of L-dopa administration, were 94% ($P > 0{\cdot}05$) and 72% ($P < 0{\cdot}001$) of those found prior to treatment.

After CB 154, the serum prolactin concentrations were markedly depressed and remained roughly at the same level during the whole 24-hr period of sampling (Fig. 2B and

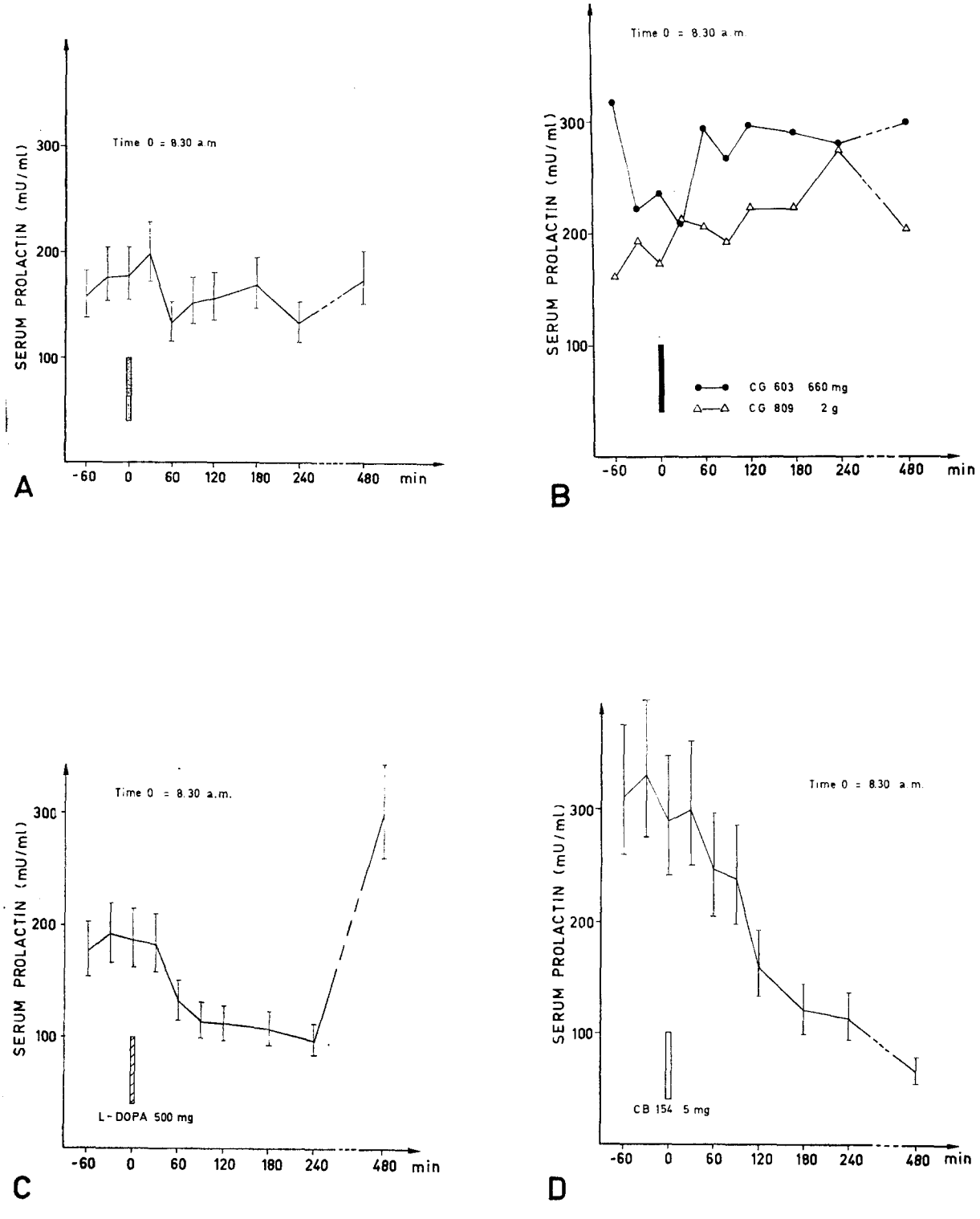

Fig. 1. *Prolactin serum concentration in five groups of post-menopausal women after oral administration of a placebo (Fig.* 1*A),* 660 *mg of CG* 603 *or* 2 *g of CG* 809 *(Fig.* 1*B),* 500 *mg of* L*-dopa (Fig.* 1*C), and* 5 *mg of CB* 154 *(Fig.* 1*D). The bars represent the standard errors of the means. For clarity, they are omitted in Fig.* 1*B in view of the obvious lack of inhibitory effects of cyclic imides.*

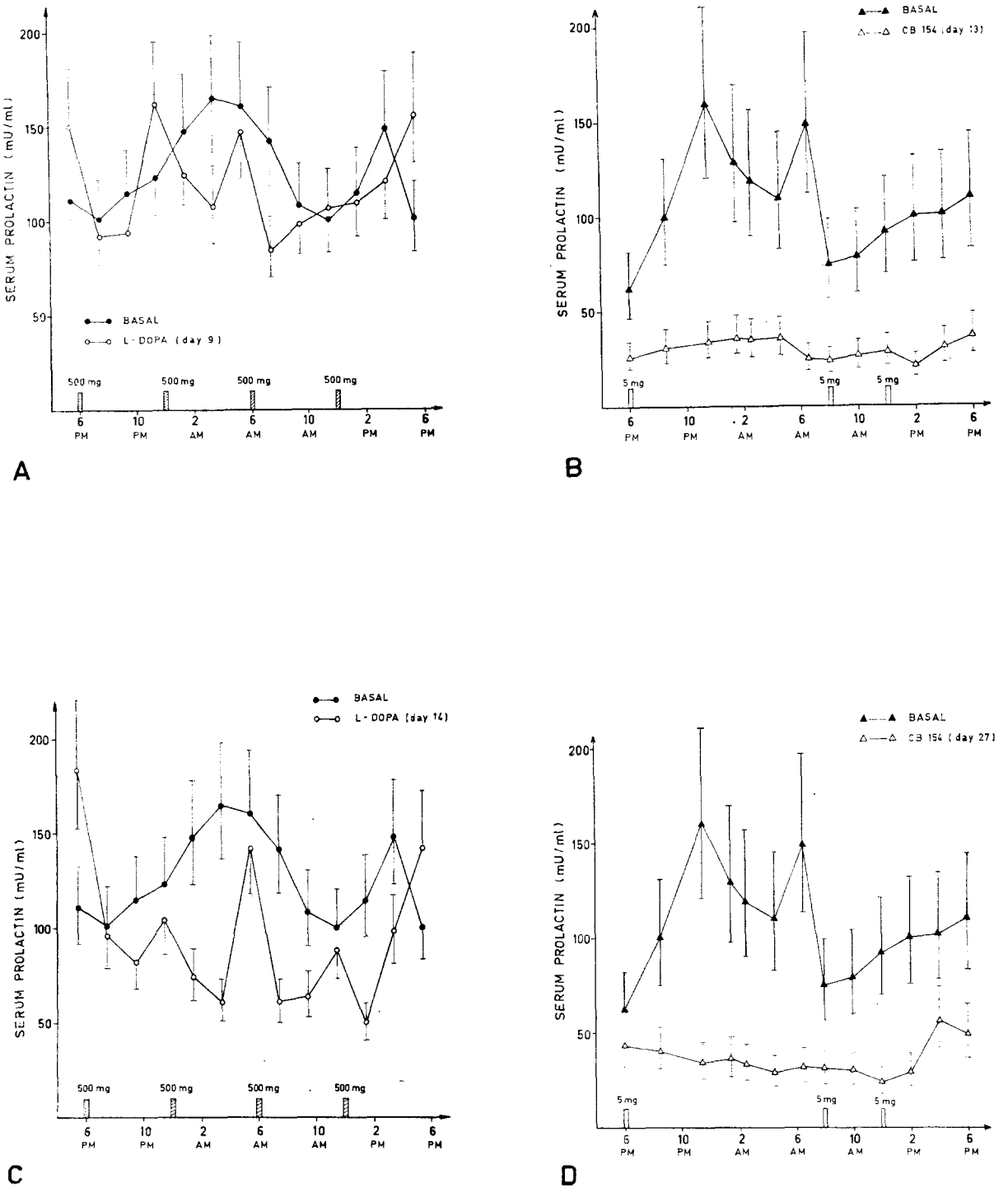

Fig. 2. Circadian periodicity of serum prolactin concentration in two groups of postmenopausal women before L-*dopa* (●——●) *and CB* 154 (▲——▲) *treatment. Effects of oral* L-*dopa* (500 *mg four times daily*) *on days* 9 (*Fig.* 2*A*) *and* 14 (*Fig.* 2*C*) *of administration* (○——○) *and of oral CB* 154 (5 *mg three times daily*) *on days* 13 (*Fig.* 2*B*) *and* 27 (*Fig.* 2*D*) *of administration* (△——△). *The bars represent the standard errors of the means. For clarity, overlapping bars are omitted.*

2D). The pretreatment circadian rhythm was abolished. A reduction of the mean prolactin 24-hr level down to about 27% ($P < 0.001$) and 33% ($P < 0.001$) of the control levels obtained before treatment was achieved on days 13 and 27 of the CB 154 experiment.

DISCUSSION

The present data indicate that, in postmenopausal women as in normally menstruating women, serum prolactin levels display wide circadian variations with a high and broad peak during the night.

The observation of these variations has important implications for the design of experiments studying potential inhibitors of prolactin secretion. Single drug administration studies carried out during day time are of limited value because prolactin levels are lower in the morning and even possibly still declining from the nocturnal peak. In addition, the imposed stress may elevate the initial values [35] and be followed by a progressive decline, falsely ascribed, in an acute experiment, to a drug effect. Nevertheless, such single dose experiments may be of indicative value and helpful in the screening of potential inhibitors.

In contrast, studies of drug effects on the circadian rhythm, according to the experimental design selected here for L-dopa and CB 154, are more informative and less sensitive to stress factors. They give information on the duration of the effect of individual doses, on the drug effect on the whole 24-hr prolactin secretion, and finally on a possible escape from, or conversely progressive increase of, drug effect during the course of treatment.

After a single oral dose, both L-dopa and CB 154 significantly inhibited prolactin secretion. There was however a difference in the duration of the inhibition since with L-dopa the mean value at +480 min was significantly higher than the basal values, while with CB 154 the mean level was still declining at +480 min. This is in agreement with previous reports [7, 15].

Our studies during chronic drug administration indicate that L-dopa given at an oral dose of 500 mg every 6 hours, is a poor inhibitor of prolactin secretion, decreasing only slightly the overall prolactin output. For therapeutic purposes, a more frequent schedule of administration, which might be more effective in lowering prolactin levels, would be largely inpractical. The use of L-dopa administered in combination with inhibitors of decarboxylase might be more promising.

In contrast, CB 154 produced a profound, steady and long-lasting decrease in serum prolactin levels with abolition of the circadian variations. It seems however that prolactin secretion was not completely suppressed. This may account for the reported failure of CB 154 to achieve objective tumor regression in advanced breast cancer [24]. However, alternative explanations for this failure are (*a*) that prolactin is involved in only few breast cancers, and no such patient happened to be included in this trial, or (*b*) that prolactin plays no part in the maintenance of human breast cancer.

Indeed, the part played by prolactin in human breast cancer is still highly controversial and prolactin dependence cannot at present be proven, nor dismissed on firm grounds [3]. It is true that Salih *et al.* [36] claimed they had directly demonstrated "prolactin dependence" in 32% of fifty human breast cancers. They reached this conclusion by comparing the pentose shunt activity of the fresh-frozen tumor to that of slices kept 24 hr in culture media with and without added prolactin. However, their observations simply suggest that metabolic pathways, possibly linked to differentiation, may be sensitive to prolactin in some tumors. Equating such sensitivity to "prolactin dependence" is an oversimplification, the danger of which is well illustrated by the fact that prolactin inhibits growth of a prolactin-sensitive mammary tumor of the rat [37].

Whatever part prolactin actually plays in human breast cancer, L-dopa seems unlikely, in view of the present data, to inhibit prolactin production to such an extent as to induce objective regression of breast cancer. Pearson's report of 3 successes [26, 27] thus raises the possibility of L-dopa acting in breast cancer by a mechanism independent of prolactin production. This possibility is supported by Friesen's contention, based on indirect evidence, that L-dopa might inhibit prolactin effects at the target tissue level [15].

Conclusions as to the potential value of prolactin inhibitors in the treatment of breast cancer must await a better knowledge of the alleged "prolactin dependence" of this tumor or a clear-cut, reproducible demonstration of objective tumor regression achieved by specific inhibitors of prolactin secretion of known efficacy.

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Estrogen Receptors in Human Breast Cancer*

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Abstract—*Samples from 77 primary and 65 metastatic human breast cancers were assayed for specific cytoplasmic estrogen receptors. Cytosol preparations were incubated with increasing amounts of ^{3}H-estradiol-17β. The unbound radioactivity was removed by charcoal-coated dextran. Saturable high affinity binding sites were detected in 56% of the primary and 37% of the metastatic tumors. The binding constants of these receptor sites for estradiol were quite variable; most ranged from 1·0 to $20 \times 10^{-10}M$, but some were larger (up to $108 \times 10^{-10}M$). The concentrations of binding sites were distributed within a continuous range from 5 to 1330 femtomoles per mg protein. This wide range was not ascribable only to variations in amounts of contaminating serum proteins. Receptors were detected in only 8% of cytosol preparations containing less than 2 mg protein per ml; in contrast they were detected in 53% of cases with higher protein concentration. This indicates that at low protein concentration, false negative results are likely to occur. Detectable amounts of receptors were not found in sera or samples from normal mammary gland, nipple, areola, skin and non-invaded lymph nodes. The reliability of a simplified procedure based on isotopic dilution of the labelled estradiol, to be used with very small tumor tissue samples, was studied. It compared quite well with the other one except in case of very low concentrations of receptor. No relationship was found between the occurrence of receptor and the age of the patient or the histological type of the tumor. There was also no relationship between the occurrence of receptors in the primary tumor and presence or absence of metastatic axillary lymph nodes. However, when present, the latter had the same characteristics with respect to the receptor as the corresponding primary, with only one exception. In cases of metastatic tumors, no correlation was observed with the free interval.*

INTRODUCTION

IT IS KNOWN that estradiol-17β and other estrogens are selectively retained in their target tissues, such as the uterus and vagina [1]. This retention is due to a non-covalent, high-affinity binding of the hormone to macromolecules which have essentially the structure of proteins. They are called estrogen receptors and are located both in the cytoplasm and nuclei of the target tissues. They are supposed to play an essential part in the mechanism of action of these hormones [2].

In respect to the human breast cancer, Folca *et al.* [3] demonstrated a selective retention of ^{3}H-hexestrol injected *in vivo*, in the tumor tissue of patients responding to adrenalectomy. Sander [4] and Johansson *et al.* [5] showed that biopsies of breast cancer concentrate ^{3}H-estradiol-17β *in vitro* in a certain proportion of cases. These observations suggested that such tumors contain specific estrogen receptors similar to those present in target tissues of the estrogens. This hypothesis was confirmed by Korenman and Dukes [6], and by Jensen *et al.* [7] who demonstrated the

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presence of proteins with high affinity for estradiol-17β in some but not all breast cancer specimens.

In agreement with recent reports from various laboratories [8–14], this paper describes the presence of estrogen receptors in primary and metastatic breast cancers in the women. It adds information on the affinity of these receptors for estradiol-17β and their concentration in the tumor tissue. It critically evaluates a simplified procedure to be applied to very small tissue samples. Finally it studies the significance of the presence of estrogen receptors in the primary breast cancer in terms of propensity to metastasize to axillary lymph nodes, characteristics of the latter in regard to estrogen receptors, and free interval.

MATERIAL AND METHODS

Tumors

Samples of primary breast cancers and of invaded axillary nodes were obtained in the operating room at the time of mastectomy. They were processed within less than one hour by dissecting out adipose and necrotic tissues, and by washing in pH 7·3 Krebs–Ringer–Henselheit glucose buffer (KRH buffer) in order to remove blood, albumin and debris. They were then stored for a maximum of one month in liquid nitrogen until biochemical study. This treatment does not significantly change the binding capacity of the receptor [15]. Biopsies from metastatic tumors, mammary non-tumor tissues and skin specimens were obtained and treated in exactly the same manner.

Cytosol

The tumor tissue was homogenized in 3 to 4 volumes (w/v) of buffer (0·01 M Tris-HCl, pH 7·5, containing 1·5 mM EDTA, disodium salt, and 6 mM β-mercaptoethanol). Homogenization was carried out by means of a whole-glass homogenizer kept in melting ice operated at about 5 rev/sec for runs of 15 sec., repeated three times at 45-sec intervals. The homogenate was centrifuged at 105,000 $\times g$ for 90 min at 2°C. The supernate was decanted to yield the cytosol fraction.

Binding parameters of the receptors

Method 1. The method used was a modification of those described by Korenman [16] and Mester *et al.* [17] for study of the uterine receptor. Aliquots of 100 μl of cytosol were mixed with the same volume of homogenization solution containing increasing amounts ot ^{3}H-estradiol-17β to reach final concentrations ranging from $4{\cdot}5 \times 10^{-11}$ to $4{\cdot}5 \times 10^{-9}$ M or from 5×10^{-10} M to 5×10^{-9} M. A minimum of six concentrations of estradiol were usually used; when only a small amount of cytosol was available five or four concentrations were used. The tubes were incubated at 18°C for 30 min with gentle shaking in order to obtain the maximum of binding [8]. Furthermore, it was found in one experiment that equilibrium was obtained at 30 min. At this time, 250 μl of a suspension of dextran-charcoal (0·005–0·5%) was added in order to remove unbound estradiol. In some experiments, dextran–charcoal (0·0025–0·25%) was added in amounts of 500 μl. The mixture was incubated with shaking at 18°C for 10 min. The adsorption of low-affinity, non-specific binding being virtually complete at this time, the tubes were centrifuged to remove the dextran–charcoal. Bound estradiol was estimated by measuring the radioactivity of the supernate and by subtracting that of blanks run in parallel. Unbound estradiol was calculated as the difference between the radioactivity of total and bound estradiol. The binding parameters were estimated on Scatchard plots [18]; the number of binding sites was expressed as femtomoles per mg of protein.

Method 2. One hundred μl of cytosol were incubated with the same volume of homogenization solution containing ^{3}H-estradiol-17β to reach a final concentration of 2×10^{-9} M. In a second tube, an isotopic dilution was achieved by adding unlabeled estradiol-17β at a final concentration of 10^{-8} M. Incubation, removal of unbound estradiol and evaluation of the bound ^{3}H-estradiol were performed as in Method 1. Bound ^{3}H-estradiol was expressed as counts/min per mg of protein.

Plastic tubes (polystyrol) were generally used for the incubation step. Lack of adsorption of ^{3}H-estradiol on these tubes was verified. At 4°C and 18°C no adsorption of the labeled steroid occurred even after an overnight incubation.

Assay for protein and radioactivity

Total protein concentration was measured either by the method of Lowry *et al.* [19], or by the biuret reaction [20], using bovine serum albumin as standard. Contents of serum proteins were estimated by measuring the content of albumin which was assayed by radial immuno-diffusion test (21) (Partigen plates from Behring). The radioactivity was determined by scintillation counting, using Bray's solution [22].

Reagents

[2,4,6,7]-^{3}H-estradiol-17β, 100 Ci/mM, was purchased from the Radiochemical Center, Amersham, England; estradiol-17β, from the Sigma Chemical Company, St. Louis, Mo.: bovine serum albumin fraction V, from the same Company; charcoal from Merck, Darmstadt; Dextran, T70, from Pharmacia, Uppsala, Sweden.

RESULTS

One hundred and forty-two samples of breast cancer were investigated; 77 were from primary tumors, 65 from metastic tumors. From the primary breast cancers, 65 samples were ductal carcinoma, 7 were lobular carcinoma, 3 were Paget disease, 1 was a medullary carcinoma and 1 was a comedocarcinoma; 9 of the ductal carcinomas and 1 of the lobular carcinomas contained significant amounts of hyperplastic mammary epithelium. Metastatic tumors were either invaded lymph nodes (2 samples) or skin metastases (63 samples). In addition to cancer tissue, 2 samples of benign mammary dysplasia, 2 of normal mammary gland, 2 of areola, 4 of nipple, 7 of non-invaded axillary lymph nodes and 5 of skin were investigated as controls.

1.1. *Presence of high-affinity binding sites in tumor samples.* Method 1 was applied to all samples for the detection of estrogen receptors. A saturation curve (Fig. 1) for ^{3}H-estradiol-17β was obtained in a certain proportion of samples from both primary and metastatic tumors, suggesting the presence of saturable receptors.

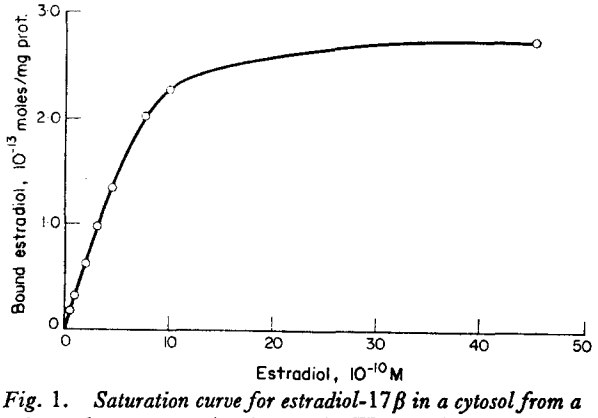

Fig. 1. Saturation curve for estradiol-17β in a cytosol from a primary breast cancer (patient HP). The protein concentration in the cytosol was 5 mg/ml.

The data were plotted according to Scatchard. Cytosols containing high affinity binding sites yielded straight lines in these coordinates, with few exceptions. Thus, convexity was sometimes apparent near the y axis (Fig. 2). In addition, 2 samples yielded non-linear functions

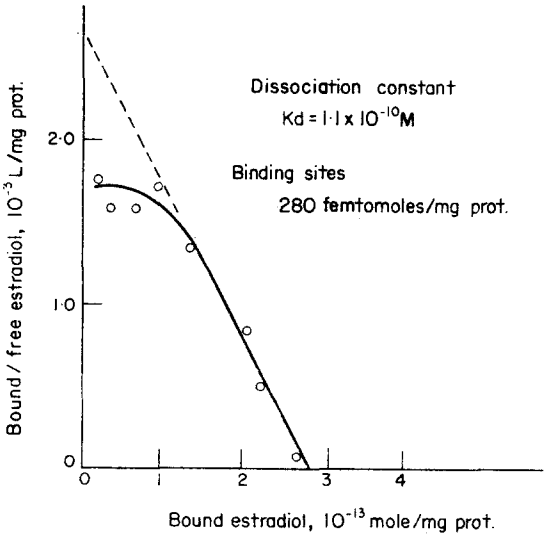

Fig. 2. Scatchard plot of the data of Fig. 1. The dissociation constant is measured by the reverse of the slope of the curve; the number of binding sites is determined by the intersect of the slope with the abscissa.

indicating the presence of more than one binding component. In these cases, the curve was linear at low concentrations of bound estradiol but tended toward horizontal at high concentrations.

Analysis of the Scatchard plots revealed that estrogen receptors were present in 56% of the primary tumors (43/77) and in 37% of the metastatic tumors (24/65). It should be pointed out that the proportion of positive cytosols varied considerably depending upon the concentration of protein in the cytosol (Fig. 3): only 8% were positive for protein concentrations lower than 2 mg/ml while 53% were positive for protein concentrations of at least 2 mg/ml. This indicates that at low protein concentrations, false negative results are likely to occur by the present assay method.

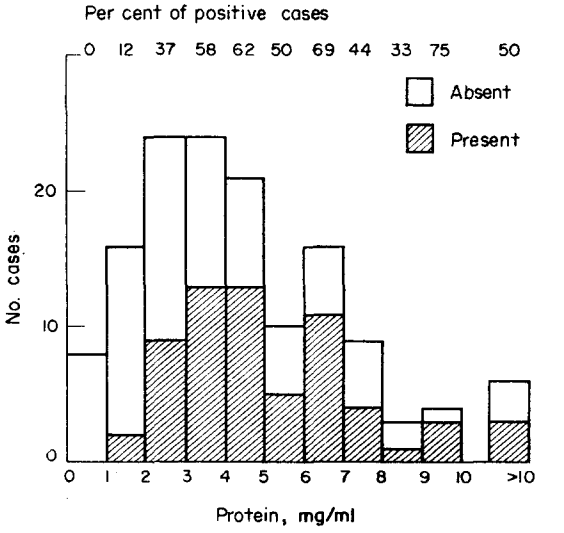

Fig. 3. Occurrence of estrogen receptors in tumor samples as a function of protein concentration in the cytosol.

Table 1. Occurrence of estradiol receptors in tissues other than breast cancer

Tissue	No. of receptor positive over total No. of samples
Normal mammary gland	0/2
Benign mammary dysplasia	0/2
Fibroadenoma	0/3
Nipple	0/4
Areola	0/3
Non-invaded axillary lymp node	0/7
Skin	0/6

Table 2. Binding parameters of the estrogen receptors from primary breast cancer

Patient	Dissociation constant $\times 10^{-10}$M	Number of binding sites femtomoles per mg of prot.
DD	6·5	1·040
BG	6·3	900
BrR	3·6	620
BR	1·3	600
VC	5·3	510
SJ	3·1	510
CR	1·7	377
LD	17·8	365
DJ	4·5	365
Va	12·8	320
HP	1·1	280
BM	3·2	223
FB	10·0	185
HM	6·7	168
BJ	7·0	165
VS	1·3	165
HS	11·5	162
BE	2·5	160
MP	10·6	150
BE	13·0	146
VM	2·3	123
WyP1	1·7	90
MM	1·8	79
RL	65·2	78
SA	3·3	78
VML	9·8	76
HuS	4·9	75
DLC	48·0	73
WD	1·0	64
LL	3·5	61
BM	2·0	58
SM	2·7	53
KB	14·0	46
DC	2·5	46
BA	20·0	43
ML	4·0	36
WyP2	34·4	32
LY	20·0	23
HJ	108·0	21
RN	2·7	20
SM	3·5	18
PV	2·0	6
Cec	6·0	5
34 patients	—	0

Estrogen receptors were not detected in any of the various tissues other than breast cancer (Table 1).

The binding parameters of the estrogen receptors were determined on the Scatchard plots. The dissociation constants of the estradiol-17β-receptor complex (Kd) and the number of binding sites (n) are given in Tables 2 and 3. In the two samples showing non-linear functions (primary cancers LY and HJ, the binding parameters were determined by the extrapolating procedure of Mester *et al.* [17]. Analysis of Tables 2 and 3 shows that most samples had dissociation constants comprised between 1·0 and 20×10^{-10} M. These values are in the same range as those found in the uterus (23) and in the DMBA-induced rat mammary tumors [24]. However, 4 of the 42 samples of primary and 5 of the 31 samples of metastatic cancers had higher Kd's, reaching as high values as 108×10^{-10} M. The largest Kd's were not associated with inaccurate or non-linear Scatchard functions (Fig. 4). It may be that they reflected the presence of non-specific serum contaminants such as the sex hormone binding globulin [25, 26]. This possibility is suggested by the high content of serum albumin in many

Table 3. Binding parameters of estrogen receptors from metastatic breast cancer

Patient	Dissociation constant $\times 10^{-10}$M	Number of binding sites femtomoles per mg of prot.
He	1·2	1·330
PC	1·7	1·080
V	4·9	960
TS	3·1	757
VE	2·6	473
DVC	9·2	420
PR	6·1	307
KR	5·9	257
LL	22·0	170
MV	5·0	98
DA	15·1	94
LS	2·5	92
M	15·8	82
LT	10·9	55
FB	10·0	54
CE	2·2	45
V	16·2	30
VM	12·5	24
DC	34·0	24
BM	70·9	22
GS	30·1	16
GM	73·5	11
RJ	7·5	9
SJ	1·5	5
41 patients	—	0

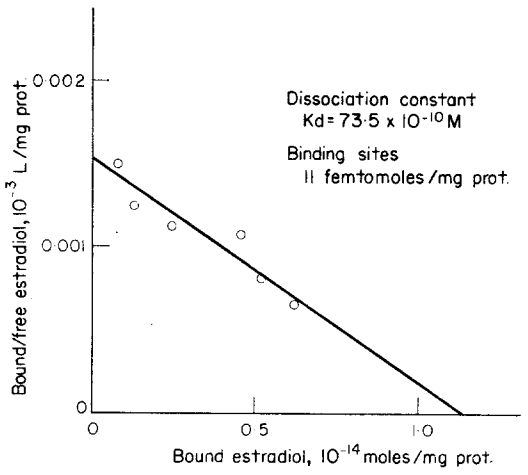

Fig. 4. Scatchard plot of a saturation curve for estradiol-17β in a cytosol from metastatic breast cancer (patient GM). The protein concentration in the cytosol was 10·4 mg/ml. The dissociation constant and number of binding sites were determined as in Fig. 2.

cytosols. Its concentration measured in 47 cytosols ranged from 10 to 30% of the total protein concentration. However, this hypothesis is made somewhat unlikely in view of the fact that 19 sera, 7 from premenopausal and 12 from postmenopausal women, did not contain any binding activity detectable by the present assay method.

In primary as well as in metastatic cancer, the concentration of binding components was extremely variable from patient to patient, ranging from 5 to 1330 femtomoles per mg protein. This variability was not due to variations in amounts of contaminating serum proteins in the cytosol preparations, as shown by its persistence after the results have been corrected for these proteins (Table 4).

Table 4. Number of binding sites (n) expressed as femtomoles per mg of either total or tissue protein in the cytosol*

Patient	Number of binding sites	
	Femtomoles per mg total prot.	Femtomeles per mg tissue prot.
PC	1·080	1·330
BG	900	1·270
TS	757	1·850
VC	510	990
SJ	510	860
CR	377	870
DJ	365	607
PR	307	465
KR	257	455
BM	223	318
DA	94	133
SA	78	175
RL	78	168

*The tissue protein concentration was calculated by the following equation:

$$\text{tissue protein} = \text{total protein} - \frac{\text{serum albumin} \times 100}{60},$$

assuming an average albumin concentration of 60% in the serum contaminating the cytosol preparation.

1.2. *Detection of estrogen-receptor in very small samples of tumor tissue. Evaluation of a method of analysis based on isotopic dilution.* As shown in section 1.1, Method 1 necessitates a minimum concentration of about 2 mg protein per ml. Because Method 1 requires 5–6 determinations on a single cytosol, this critical concentration cannot be reached with very small samples of less than 100 mg wet weight. Method 2 which necessitates only 2 determinations per cytosol can then be used. This method is based on the assumption that a 5-fold isotopic dilution of a nearly saturating concentration of ^{3}H-estradiol should produce a considerable decrease in the bound radioactivity when the cytosol contains a specific saturable receptor. This assumption was tested by comparing the results obtained in parallel with Methods 1 and 2 on a series of primary and metastatic tumor samples. The results are shown in Tables 5 (primary tumors) and 6 (metastatic tumors).

In both series, it appears that cytosols containing receptors as detected by Method 1 could also be interpreted as positive by Method 2, inasmuch as they fulfilled two criteria: detectable binding of labelled estradiol and substantial reduction of this binding effected by the radioactive dilution. However, one case in each series (CM in Table 5, RI in Table 6) was negative by Method 1 but should be interpreted as positive by method 2 when using the criteria defined above.

2. *Additional observations on estrogen receptors in breast cancer.* In nine cases of mastectomy the primary tumor was analysed together with the invaded axillary nodes (Table 7). Except in one case, both tumor sites were always either positive or negative with respect to estrogen receptors. The only exception (RL) consisted of a positive primary tumor with one positive and two negative metastatic nodes. Whether these are methodological artifacts or mestatases with modified properties cannot be decided from these data.

Table 8 shows that tumors with or without estrogen receptors have exactly the same propensity to metastasize to the axillary lymph nodes.

No relationship was observed between the presence or absence of receptors and the age of the patient. There was also no correlation

Table 5. Comparison of methods 1 and 2 in cytosol preparations from primary breast cancer

Patient	Method 1 Number of binding sites		Method 2†	
			$^xE_2$	$^xE_2E_2$
	Femtomoles per mg prot.	CPM per mg prot.	CPM per mg prot.	CPM per mg prot.
DJ	365	9,735	6,356	1,162
HM	168	4,218	3,677	583
BJ	165	4,865	3,563	709
WyP1	90	4,178	3,016	627
HuS	75	3,484	3,016	702
DLC	73	3,279	1,043	316
SM	53	2,890	1,526	369
BA	43	1,921	1,011	254
WyP2	32	1,583	865	386
CM	—	—	470	170
RI	—	—	100	83
BA	—	—	131	135
SL	—	—	375	487
TH	—	—	−55	−400

†$^xE_2$: Bound ^{3}H-estradiol-17β.
$^xE_2E_2$: Bound ^{3}H-estradiol-17β in presence of an excess of unlabeled estradiol-17β (isotopic dilution = 1:6)

Table 6. Comparison of methods 1 and 2 in cytosol preparations from metastatic breast cancer

Patient	Method 1 Number of binding sites		Method 2*	
			$^xE_2$	$^xE_2E_2$
	Femotomoles per mg prot.	CPM per mg prot.	CPM per mg prot.	CPM per mg prot.
V	960	46,082	36,800	7,963
VE	473	21,784	20,173	6,686
BE	82	3,694	1,955	647
DLC	74	3,344	2,435	596
LT	55	2,940	1,164	235
BM	22	1,053	387	211
GS	16	774	482	226
GM	11	564	218	139
RI	—	—	465	142
PV	—	—	−36	−90
BR	–	—	62	114
DK	—	—	523	613
PE	—	—	144	211
OG	—	—	63	−139
Va	—	—	−85	−155
DI	—	—	−959	−735
BB	—	—	−1,312	−275

*: for abbreviations, see Table 5.

Table 7. Estrogen receptor in primary breast and invaded axillary lymph nodes

Patient	Primary tumors		Invaded lymph nodes					
			Lower		Middle		Upper	
	Kd $+10^{-10}$M	*n* femtomoles per mg prot.	Kd $\times 10^{-10}$M	*n* femtomoles per mg prot.	Kd $\times 10^{-10}$M	*n* femtomoles per mg prot.	Kd $\times 10^{-10}$M	*n* femtomoles per mg prot.
Ly	20.0	23	13.5	50		—		—
MP	10.6	150		—		—	5.3	51
BE	13.0	146	18.5	82		—		—
DLC	48.0	73	8.3	74		—		—
BM	3.2	223	5.3	132		—	2.5	228
DJ	4.5	365	6.5	263		—		—
RL	65.2	78		0	1.1	43		0
Ry		0		0		—		—
WN		0		0		—		0

Table 8. Occurrence of estrogen receptors in primary tumors with and without invaded axillary lymph nodes

	Presence of invaded axillary lymph nodes	Absence of invaded axillary lymph nodes
Presence of estrogen receptors	13	30
Absence of estrogen receptors	8	26

with the histological type of the tumor or the presence of benign hyperplastic mammary epithelium in the tumor sample.

In regard to metastatic cancers there was no correlation between the occurrence of receptors and the free interval (Fig. 5).

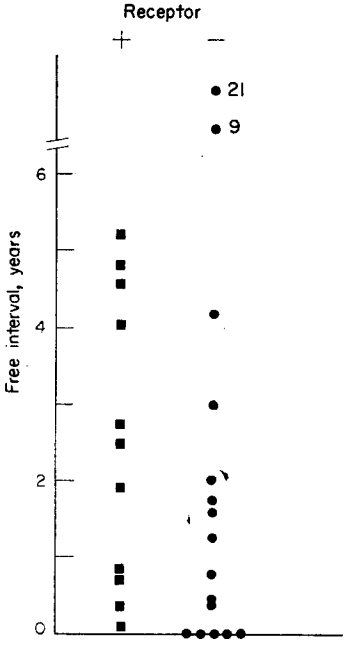

Fig. 5. Relationship between the occurrence of estrogen receptor in metastatic tumors and the free interval.

DISCUSSION

The present paper reports that human breast cancer contains specific estrogen receptors as detected by the presence of high-affinity binding sites for estradiol-17β in the cytosol preparations. This finding is in agreement with other publications [8, 10, 14] studying either cytosol fractions or whole homogenates. The assay was performed by measuring the bound radioactivity after addition of increasing amounts of ^{3}H-estradiol-17β into the cytosol and removal of the unbound radioactivity by dextran-coated charcoal. Graphic representation of the results according to Scatchard yields accurate estimates of the dissociation constant (Kd) of the estradiol-receptor complex and of the number of binding sites (*n*). Both are valuable information relating to the specificity and concentration of the receptors.

Whereas in most samples, the dissociation constants were in the same range as those reported for the uterine estrogen receptors [23], higher values were found in rare samples. We have no explanation to account for these abnormally higher values. Although we presented indirect evidence against it, the possibility cannot be ruled out that they reflect the presence of binding component of blood serum contaminants; other binding components of low affinity might also play a part. Other possible explanations for the unusually high apparent Kd's are the following: presence of endogenous estrogens or other substances acting as competitive inhibitors; presence of variable concentration of protein in the cytosols [23, 27]; underestimation of the "blanks"; presence of altered receptors in some tumors. Whatever the true explanation is, it might be cautious to group these cases in a separate class.

In the present series, the number of binding

sites (n), namely the concentration of receptors, varied considerably from tumor to tumor within a very wide range. This should be borne in mind when receptors are interpreted for clinical purposes and suggests, as will be stressed below, that the mere distinction between presence and absence of receptors, though informative, will not be sufficient.

Detectable amounts of estrogen receptors were found in 43 of the 77 primary breast cancers (56%). This proportion is similar to those reported by Korenman and Dukes (7/15) [6], Johansson *et al.* (14/31) [5], Jensen *et al.* (39/84) [7] and McGuire (24/43) [14]. It is larger than those found by Wittliff *et al.* (29/89) [13] and Maass *et al.* (72/174) [11] but lower than those obtained by Feherty *et al.* (36/52) [10] and Korsten and Persijn (28/42) [12]. These differences could be due to sampling variations or to differences in methodology. In particular it should be stressed that the latter two groups were due to assaying whole homogenates instead of cytosol fractions. Microsomes which have recently been shown to contain estrogen receptors [28], were present in whole homogenates and could therefore contribute to positive assays in same cases. In metastatic tumors, estrogen receptors were detected in 24 of the 65 samples studied (37%). This proportion is similar to those reported by Jensen *et al.* (12/40) [7] and Maass *et al.* (7/20) [11]. It is lower than those reported by McGuire (14/21) [14] and by Korsten and Persijn (11/14) [12]. The higher proportion obtained by the latter authors could be due to assaying whole homogenates, as discussed above.

Notwithstanding these differences, it appears from these reports that not all tumors contain estrogen receptors. However, based on the apparently continuous distribution of concentrations of binding sites in the positive tumors, from high values to extremely low levels, an alternative interpretation may be that the tumors are not qualitatively different with regard to estrogen receptors but that the latter are not detected in "negative tumors" because of insufficient sensitivity of the presently available methods or of partial or total destruction of the receptors during the various steps of the experimental procedure. The importance of processing the tissue quickly and in the cold has recently been stressed [29]. It is not unlikely that technical improvements will yield a much higher proportion of positive cases.

An interesting observation which is in agreement with other reports [5, 6, 13], is the lack of detectable amounts of receptors in normal mammary tissue, benign mammary dysplasia, areola and nipple. This observation is in apparent contradiction with the reported finding that human breast tissue concentrates and retains intravenously injected ^{3}H-estradiol-17β to an extent which is not much smaller than breast cancer tissue [30]. Our data do not provide an explanation for this discrepancy. However they do indicate that the tissues listed in Table 1 probably do not contribute any receptor activity in biopsies of breast cancer admixed with these tissues. Nevertheless, this might not hold true in tumors containing a high proportion of benign hyperplastic mammary epithelium. This stresses the necessity for a thorough histological examination of the tumor tissue which is submitted for analysis to the biochemist.

The assay method used in this paper requires a rather large amount of tissue. For very small tissue samples, a simplified method was tested here. It compares quite well with the other one, except in 2 cases of borderline values. However, it does not provide such valuable information as the dissociation constant of the estradiol-receptor complex and number of binding sites. This simplified method is therefore of limited value and any possible effort should be made to obtain large enough samples of tissue for complete analysis.

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Specific Estrogen Receptor of the DMBA-Induced Mammary Carcinoma of the Rat and its Estrogen-Requiring Molecular Transformation*

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Abstract—*Presence of specific estrogen receptors was observed in* 11 *cytosol preparations from DMBA-induced mammary tumors. These receptors are characterized by a high affinity for estradiol-17β similar to the one reported for the receptors of the estrogen-target tissues. Incubation of cytosol with* ^{3}*H-estradiol-17β at* 18°*C yielded* 3·5 *S and occasionally* 7·5 *S receptor in low-ionic sucrose gradients. On the contrary, a predominant* 7·5 *S receptor was found when non-incubated cytosol samples were fractionated by ultracentrifugation and then labelled with the hormone. Conversion of the* 7·5 *S to the* 3·5 *S receptor on incubation was obtained only in the presence of estradiol.*

INTRODUCTION

ONE CHARACTERISTIC of the estrogen-target tissues, such as the uterus or vagina, is their capacity to concentrate and retain ^{3}H-estradiol-17β. Considerable evidence has now accumulated, suggesting that specific receptors are responsible for this retention [1]. Mobbs [2] and Jensen *et al.* [3] reported that, among dimethylbenz(a)anthracene-induced rat mammary tumors, those which regress after ovariectomy concentrate ^{3}H-estradiol-17β to a greater extent than those which do not regress. This observation suggested that the former behave as a normal estrogen-target tissue. This was confirmed by the demonstration of estrogen receptors in the cytosol of these tumors [4–6]. These receptors are apparently similar to those present in the uterus; they are characterized by a very high-affinity binding for estradiol-17β and an 8 S sedimentation velocity in low-ionic-strength sucrose gradient.

King *et al.* [5] and McGuire and Julian [6] reported the presence of specific 4 S receptors in addition to the 8 S receptors in these tumor cytosols. Since tumor cytosols contain a significant amount of 4 S components that bind estradiol non-specifically, it was of interest to assess again the specificity of these receptors. The data reported here confirm this specificity and suggest that the 4 S component is derived from the major 8 S receptor on incubation in the presence of estradiol.

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MATERIAL AND METHODS

Reagents

(2,4,6,7) ^{3}H-estradiol-17β (100 Ci/mmole) was purchased from the Radiochemical Center, Amersham, England; yeast alcohol dehydrogenase and horseradish peroxydase from Boehringer, Mannheim, Germany; dextran T70 from Pharmacia, Uppsala, Sweden; charcoal from Merck, Darmstadt, Germany and 7,12-dimethylbenz(a)anthracene (DMBA) from

Eastman Organic Chemicals, Rochester, N.Y. The compounds U11,100A and U23,469* were kindly supplied by the Upjohn Cy, Kalamazoo, Michigan. All reagents were analytical grades.

Animals—preparation of tumor extracts

Female Sprague–Dawley rats, 50 days old, were given 20 mg of DMBA by gastric instillation. Tumor-bearing rats were killed by cervical dislocation. Their tumors were rapidly excised, carefully trimmed and washed in cold pH 7·3 Krebs–Ringer–Henselheit—glucose buffer. These tumors were carcinomas. They should be considered hormone-dependent on the basis of our extensive experience showing that in this type of DMBA mammary carcinomas of recent appearance, nearly 100% of tumors are hormone-dependent. They were rinsed in pH 7·5, 10^{-2} M Tris–HCl buffer containing 1·5 mM EDTA and 6 mM β-mercaptoethanol and homogenized in 2 to 3 vol (w/v) of this buffer. Homogenization was carried out at 0°C by means of an all-glass homogenizer [7] and centrifuged at $105{,}000 \times g$ for 90 min at 2°C. The supernates were decanted to obtain the cytosol fractions.

Kinetic analysis of estradiol binding

Aliquots of 100 μl of cytosol were mixed with the same volume of homogenization buffer containing increasing amounts of ^{3}H-estradiol-17β to reach final concentrations ranging from $2{\cdot}5 \times 10^{-10}$ M to 5×10^{-9} M. The tubes were incubated at 18°C for 30 min. At this time, 250 μl of a suspension of dextran-charcoal (0·005–0·5%) in the homogenization buffer was added. The mixtures were incubated with gentle shaking at 18°C for 10 min. They were then centrifuged at $3500 \times g$ for 10 min. Bound estradiol was estimated by measuring the radioactivity of the supernate and subtracting that of blanks run in parallel [7, 8].

Sucrose density gradient analysis

Two hundred microliters of cytosol added with trace amounts of yeast alcohol–dehydrogenase (ADH) or horseradish peroxydase (PER) were layered on 4 ml of a linear sucrose gradient, 10–30%, in the homogenization buffer. After 15 hr of centrifugation at $308{,}000 \times g$ in a Spinco SW 56 rotor at 2°C, the gradient was divided into 4 drop fractions. Sedimentation velocities of estradiol-binding components were assigned relative to the migration of PER or ADH standards.

Assay for protein and radioactivity

Protein concentration was measured by the method of Lowry *et al.* [9] using bovine serum albumin as standard. The radioactivity was determined by liquid scintillation counting, using Bray's solution [10].

RESULTS AND DISCUSSION

The presence of estrogen receptors in cytosol preparations from 11 tumors was demonstrated by assessing their binding affinity for ^{3}H-estradiol-17β at 18°C. Saturation curves were obtained in each. Scatchard plots [11] yielded straight lines indicating that only one class of binding sites was present (Fig. 1). The dissociation constants of the binding reactions varied from 2 to 26×10^{-10} M, a range close

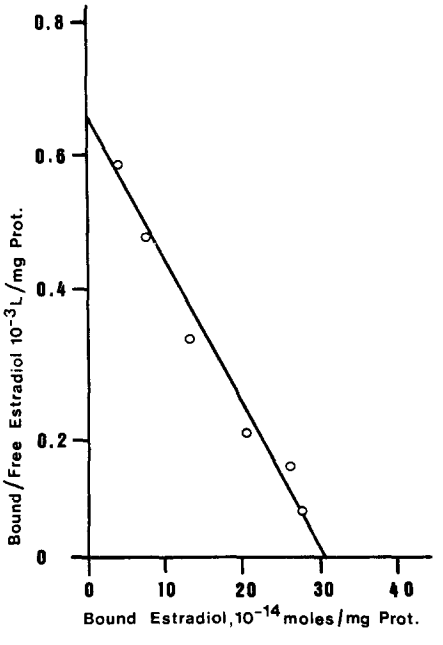

Fig. 1. Scatchard plot of a binding reaction. The slope of the line is equal to $-1/Kd$, where Kd is the dissociation constant. The value of Kd obtained from this plot is $4{\cdot}7 \times 10^{-10}$ M.

to that reported for the uterine estrogen receptors [11, 12]. Specificity was further established by incubating cytosol fractions with increasing amounts of ^{3}H-estradiol-17β in the presence and absence of antiestrogens U11,100A and U23,469 [13]. The saturation curves for ^{3}H-estradiol obtained under these conditions were plotted on a double reciprocal plot (Fig. 2). In these coordinates, a typical pattern of competitive inhibition was observed indicating that the binding components had in

*U11,100A is 1-[2-(*p*-(3,4-dihydro-6-methoxy-2-phenyl-1-naphthyl)-phenoxy)-ethyl] pyrrolidine; U23,469 is 3-[*p*-(1,2,3,4-tetrahydro-6-methoxy-2-phenyl-1-naphtyl)-phenoxy]-1,2 propanediol.

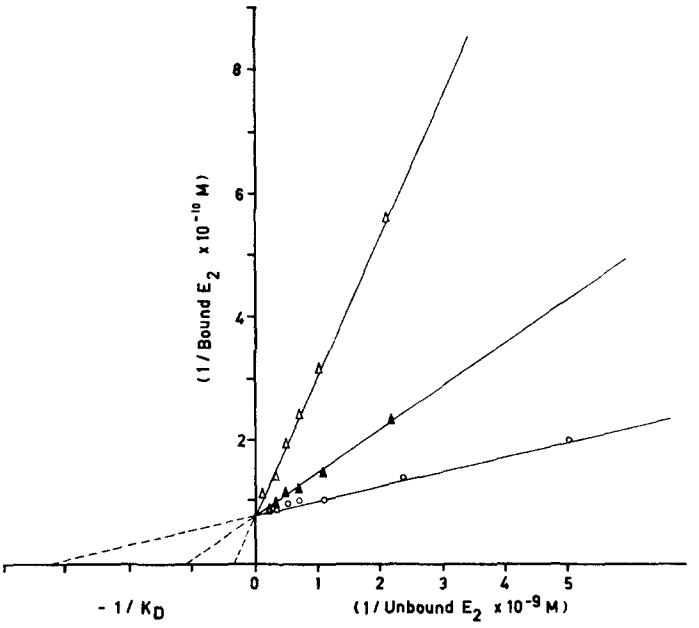

Fig. 2. Competitive inhibition of the binding of ^{3}H-estradiol to the receptor by antiestrogens. Cytosol fractions were incubated with increasing amounts of ^{3}H-estradiol (range: 5×10^{-10} M to $4{\cdot}5 \times 10^{-9}$ M) in the absence (○—○) or presence of $2{\cdot}5 \times 10^{-7}$ M U11,100A (△—△) or U23,469 (▲—▲). The apparent dissociation constants of the binding reactions were respectively: $3{\cdot}0 \times 10^{-10}$ M in the absence of an antiestrogen compound; $9{\cdot}2 \times 10^{-10}$ M in the presence of U23,469; 29.1×10^{-10} M in the presence of U11,100A.

this regard properties similar to those of the specific estrogen receptors from the uterus [14].

In order to determine the sedimentation velocity of the receptor, the cytosol preparations were incubated in the presence of ^{3}H-estradiol, either overnight at 4°C or at 18°C for 30 min and centrifuged in low ionic sucrose gradient. A major radioactive peak was always found in the 3·5 region (Fig. 3). In three of the cytosols a second peak was observed in the 7·4 S region (Fig. 4). The specificity of these two peaks was established by the fact they persisted after removal of the non-specifically bound radioactivity by the charcoal–dextran procedure before the centrifugation (Fig. 3 and 4). Although this procedure is not universally considered as a definite proof of specificity, the latter was confirmed by the kinetics and competitive binding with antiestrogens (Fig. 2). Furthermore, partial or complete suppression of the 3·5 and 7·4 S peaks respectively by pretreatment of one cytosol by the antiestrogen U11,100A (Fig. 4) was another indication of their specificity [15]. Location of peaks in the sucrose gradient being the same whether the samples were pretreated or not with charcoal–dextran (Figs. 3 and 4) indicates that this treatment does not modify the sedimentation velocities of the two binding components. This observation is similar to that reported by Sanborn *et al.* [12] for the 8 S uterine receptor.

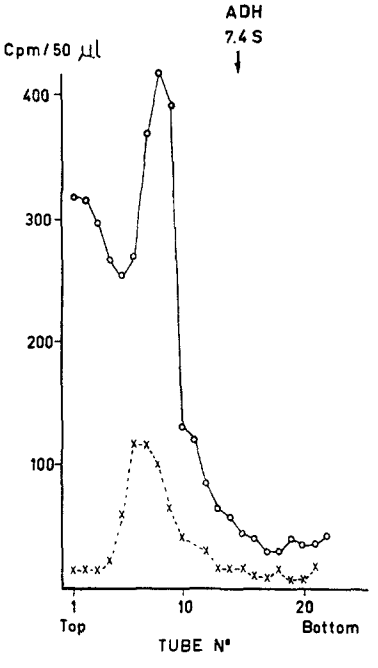

Fig. 3. Samples of a same cytosol were incubated with 2×10^{-9} M ^{3}H-estradiol at 18°C for 30 min. One sample was then extracted with an equal volume of charcoal–dextran, the other being left untreated. Both were centrifuged on low-ionic-strength sucrose gradients. Without charcoal–dextran extraction (○—○); after charcoal-dextran extraction (×—×).

The decrease in bound radioactivity observed after treatment with charcoal–dextran can be explained by the dilution brought about by this procedure and by a possible adsorption of

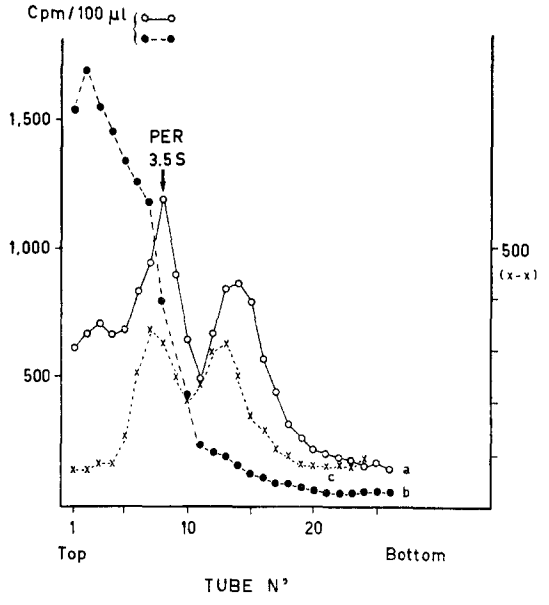

Fig. 4. Cytosol samples were subjected to the following experimental conditions. Sample a *was incubated for* 30 *min at* 18°C *with* ^{3}*H-estradiol-*17β *(final concentration* 2×10^{-9} *M). Sample* b *was incubated for* 15 *min at* 18°C *with U*11,100*A (final concentration* $2{\cdot}5 \times 10^{-7}$ *M) before incubation with* ^{3}*H-estradiol-*17β *for* 15 *min at* 18°C. *Sample* c *was incubated with* ^{3}*H-estradiol-*17β *for* 15 *min at* 18°C *and then extracted with charcoal–dextran* (200 µl:250 µl). *Two hundred microliters of each sample, added with horseradish peroxidase (PER), were then sedimented on a sucrose density gradient.*

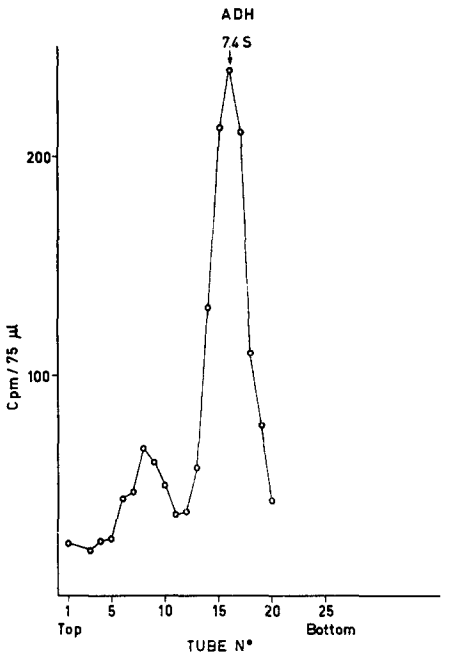

Fig. 5. Cytosol was sedimented on a low-ionic-strength sucrose gradient. Alcohol dehydrogenase (ADH) was added as a marker. After fractionation of the gradient, 100 µl *of each fraction was added with* 10 µl *of* 5×10^{-8} *M* ^{3}*H-estradiol-*17β *and incubated at* 18°C *for* 30 *min. At this time,* 100 µl *of charcoal–dextran was added and incubated further at* 18°C *for* 10 *min. Bound radioactivity was then measured in one aliquot of* 75 µl *after removal of the charcoal–dextran.*

some specifically bound radioactivity on the charcoal–dextran [16].

In the case of the uterine cytosol, it is not unusual to observe estrogen receptors with 8 S as well as 4 S sedimentation velocities [15, 17–20]. The 8 S receptor is the major form described; the 4 S receptor appears to be produced under various experimental conditions [17–20]. King *et al.* [5] and McGuire and Julian [6] reported the presence of similar 8 S and 4 S estradiol-binding components in the cytosol from the rat mammary tumor. It is likely that the 3·5 S and 7·4 S values reported here are equivalent to the values reported by these authors who were using different sedimentation standards. It was attempted to determine whether these sedimentation velocities were those of the "native" cytosol components or rather of components modified by the preincubation procedure. "Native" receptor present in non-preincubated cytosols from two different tumors were fractionated by ultracentrifugation in sucrose gradient. The fractions were then labelled with a nearly saturating concentration of ^{3}H-estradiol-17β and their binding components were measured by the charcoal procedure. Under these conditions, a large 7·4 S and a small 3·5 S peak were obtained (Fig. 5) The same cytosols were then incubated for 30 min at 18°C with and without ^{3}H-estradiol-17β. The samples incubated with estradiol were extracted with charcoal–dextran prior to centrifugations. Both samples were then subjected to a sucrose gradient centrifugation and fractionation. The fractions from the unlabelled cytosols were then studied as in Fig. 5. The sedimentation profiles for one cytosol are given in (Fig. 6(a)) and (b). They show that a 7·4 S peak was present in the sample preincubated without estradiol (Fig. 6(a)). After preincubation with estradiol the major peak was in the 3·5 S region (Fig. 6(b)). It should be noted that about 2/3 of the binding components was lost in the absence of estradiol. This was probably due to sedimentation of aggregates, as suggested by an experiment involving a longer preincubation where considerable amounts of such binding components were recovered from the bottom of the tube. Similar results were obtained with the other cytosol.

These experiments lead to the conclusion that the freshly prepared cytosol from the rat mammary tumors contain a predominant 7·4 S binding component. It is transformed into a

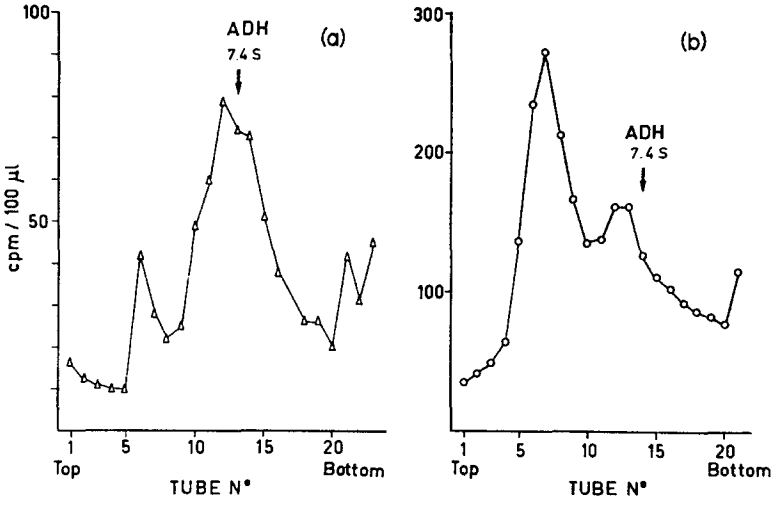

Fig. 6. Cytosol samples were incubated for 30 *min at* 18°C *without* (*a*) *and with* (*b*) 2×10^{-9} *M* ^{3}*H-estradiol-17*β; *the subsequent experimental procedures were as in Fig. 5 and 4c respectively.*

3·5 S component after preincubation provided estradiol is present. This suggests that the latter is required either to affect the molecular conversion or to stabilize the converted form if this was due to limited proteolysis [21]. Further studies will establish whether this conversion is related to molecular transformations described in other estrogen-receptor systems [1, 17–21].

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Interaction of Activated Estradiol-Receptor Complex and Chromatin in Isolated Uterine Nuclei*

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Abstract—*Exposure at 4°C and 22°C of purified uterine nuclei to uterine cytosol preincubated with estradiol-17β causes a significant increase in their ability to bind ^{3}H-actinomycin D. This increase reaches almost its maximum within 15 minutes following exposure. It does not occur at 4°C when the nuclei are exposed to cytosol added with estradiol-17β immediately before mixing rather than preincubated with it. It does not occur either when nuclei are exposed to any of the following solutions: cytosol preincubated with estrone, cytosol alone, buffer added with estradiol-17β or estrone, or buffer alone. Exposure of nuclei from uterus and from diaphragm to cytosols from these tissues increases the nuclear binding of ^{3}H-actinomycin D only when uterine nuclei are exposed to uterine cytosol.*

These data suggest that a change in chromatin structure occurs in isolated nuclei solely from estrogen-target tissue as a specific effect of exposure to estradiol-"activated" uterine cytosol. They are consistent with the hypothesis that the "activated" form of the estrogen receptor induces metabolic changes in target tissue through a direct effect at the chromatin level.

INTRODUCTION

ESTRADIOL-17β has been reported to induce an increase of RNA synthesis within minutes following an injection of 17β-estradiol to immature or ovariectomized mammals [1–4]. This stimulation is abolished by actinomycin D [2], a molecule that blocks the transcription of RNA on a DNA template. Teng and Hamilton [5] reported an enhancement of the template activity of the uterine chromatin within 30 min following the injection of estradiol-17β to ovariectomized rats. By molecular hybridization competition experiments between RNA and DNA, Church and McCarthy [6] provided evidence that the steroid induces the synthesis of a new population of DNA-like RNA in the uterus. Leroy *et al.* [7], using autoradiographic techniques [8], showed that *in vivo* administration of estradiol increases the binding of ^{3}H-actinomycin D by nuclei of uterine cells. Similar observations were made on the epithelial component of the mammary gland [9]. These data support the hypothesis that the early steps of the hormone stimulation involve transcription of new populations of RNA through unmasking of DNA transcription sites.

Numerous studies have shown that the cytosol of estrogen-target tissues contain specific binding proteins. These proteins, called "estrogen receptors" [10, 11], play an essential part in the mechanism that allows the steroid-induced stimulation of RNA synthesis [10–13]. It is now established that an important function of estradiol-17β is to promote the conversion of the receptor to an "active form" [13–15]

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which can enter the nucleus and increase its ability to synthesize RNA [13].

Preliminary observations suggested that an activation of RNA polymerase accounted for this stimulation of RNA synthesis [13]. In contrast, this paper presents evidence in favor of a direct effect at the chromatin level. This evidence is derived from studies measuring the amount of ^{3}H-actinomycin D which binds to purified nuclei and the effect of their exposure to hormone-added cytosol under various conditions.

MATERIAL AND METHODS

1. *Material*

In these experiments, mature female Sprague–Dawley rats were used instead of castrated ones, since King *et al.* [16] observed that ovariectomy decreased the ability of uterine nuclei to accept receptor-bound estradiol. The animals were killed by cervical dislocation. The uteri or the diaphragms were removed and washed in pH 7·3 Krebs–Ringer–Henselheit, glucose buffer.

2. *Preparation of the tissue fractions*

Cytosol. Pooled uteri or diaphragms were homogenized in 3 to 4 volumes of buffer (10^{-2} M Tris-HCl, pH 7·5, containing 2·2 M sucrose and 6 mM β-mercaptoethanol). Homogenization was carried out at 4°C by means of a whole-glass homogenizer. Three or 4 consecutive 15 sec runs were used. The homogenates were centrifuged at $105{,}000 \times g$ for 90 min to obtain the cytosol fractions.

Nuclei. Pooled uteri or diaphragms were homogenized at 0°C in 10 volumes of 10^{-2} M Tris-HCl buffer, pH 7·5, containing 250 mM sucrose and 3 mM $CaCl_2$. Homogenization was performed in the same manner as for preparing the cytosol fraction. The homogenates were filtered through a gauze mesh to eliminate debris and centrifuged at $800 \times g$ for 10 min. The pellets were suspended in 10^{-2} M Tris-HCl buffer, pH 7·5, containing 2·2 M sucrose and 3 mM $CaCl_2$, filtered through a gauze mesh and centrifuged at 27,000 rev/min for 30 min in a Spinco rotor 65. The pellets consisted of nuclei which were free of myofibrils and cell debris on histological examination; the integrity of this nuclear fraction was checked by Unna staining [17].

3. *Incubation of purified nuclei in various solutions*

Five hundred μl of a suspension of freshly prepared nuclei were mixed and incubated with hormone-added or hormone-free buffer or cytosol under various conditions. At the end of incubation, the nuclei were separated by centrifugation at $3{,}500 \times g$ for 10 min at 0°C, resuspended in buffer and smeared on microscopic slides. The smears were fixed for 10 min. at 0°C in ethanol–acetic acid 3:1, rinsed with 70% ethanol and air dried.

4. *Radioautography after labelling with ^{3}H-actinomycin D*

The smears were dipped for 1 hr at room temperature in a solution of 10^{-5} M ^{3}H-actinomycin D. They were rinsed overnight in running tap water, washed in distilled water and air dried. They were covered with K_2 Ilford emulsion and exposed for 3 days in the dark room at 4°C. They were then developed 20 min at 14°C by the amidol procedure and stained with Unna. Grain counts were carried out on 100 nuclei in each preparation.

5. *Chemicals*

^{3}H-actinomycin D, 4·8 Ci/mmole was purchased from Schwartz/Mann Bioresearch Inc., Orangeburg N.Y.; estradiol-17β and estrone from the Sigma Chemical Company, St. Louis Mo.

RESULTS

1. *Time course of the effect of incubation of uterine nuclei with various solutions on their binding of ^{3}H-actinomycin D*

Figure 1 shows the amounts of ^{3}H-actinomycin D bound to the purified nuclei after their incubation for 5, 15 and 35 min at 4°C or 22°C with each of the following solutions: uterine cytosol added with 5×10^{-9} M estradiol-17β or estrone and left overnight at 4°C, cytosol without added hormone, buffer containing 5×10^{-9} M estradiol-17β or estrone, and buffer alone.

Increase in bound ^{3}H-actinomycin D occurred only in nuclei incubated with cytosol added with estradiol-17β and left overnight with the hormone. This increase was not present after 5 min of incubation, but reached almost its maximum within 15 min, with a small though non-significant, rise during the subsequent 20 min. The effect was larger at 4°C that at 22°C, possibly due to thermic degradation of part of the system. No increase of bound ^{3}H-actinomycin D occurred in the nuclei incubated with either estrone-containing cytosol, estradiol- or estrone-containing buffer, cytosol alone or buffer alone.

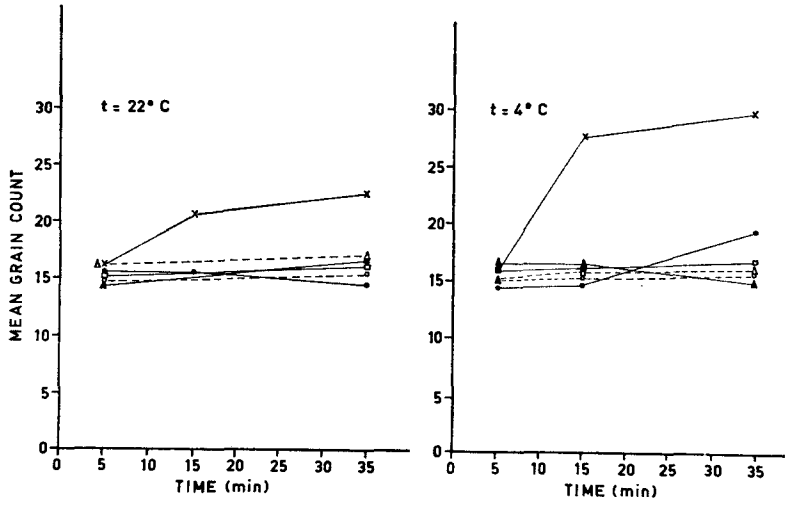

Fig. 1. Nuclear binding of ^{3}H-actinomycin D after exposure of purified uterine nuclei to various solutions: uterine cytosol preincubated overnight at 4°C with 5×10^{-9}M estradiol-17β (×—×), 5×10^{-9}M estrone (○—○), cytosol (●—●), homogenization buffer (△----△), buffer containing 5×10^{-9}M estradiol-17β (▲—▲) or 5×10^{-9}M estrone (□—□). The temperature of incubation was 4°C or 22°C. Each value is the mean count of 100 nuclei.

2. *Influence of preincubation of the cytosol with estradiol*

Table 1 compares the values of ^{3}H-actinomycin D binding in nuclei incubated 30 min at 4°C with cytosol alone, cytosol added with estradiol-17β immediately before incubation or cytosol preincubated at 4°C overnight with estradiol-17β. Under the latter condition only was there a significant increase in the nuclear-binding of ^{3}H-actinomycin D.

Table 1. Influence of preincubation of the cytosol with estradiol upon the increased nuclear binding of ^{3}H-actinomycin D

Uterine nuclei were incubated at 4°C for 30 min with the various uterine cytosol preparations. Each value is the mean count of 100 nuclei.

	Mean number of grains/nucleus ± S.E.
Cytosol	12·2 ± 0·57
Cytosol + 5×10^{-9}M estradiol-17β	12·6 ± 0·45
Cytosol + 5×10^{-9}M estradiol-17β (overnight preincubation at 4°C)	16·3* ± 0·57

*This value is larger than all others; $P < 0{\cdot}05$, Student's *t*-test.

3. *Incubation of cytosol and purified nuclei from uterus and diaphragm in various combinations*

Cytosols from uterus and diaphragm were incubated overnight at 4°C with estradiol-17β. They were then mixed with purified nuclei from uterus and diaphragm in the four different combinations for 30 min at 4°C. Cytosols without added hormone were included as controls. Table 2 shows that an increase in the nuclear binding of ^{3}H-actinomycin D occurred only when both the hormone-added cytosol and the nuclei were from uterus.

Table 2. Effect of incubation of nuclei from uterus or diaphragm with cytosols from uterus or diaphragm on nuclear binding of ^{3}H-actinomycin D

Cytosols were incubated overnight with or without 5×10^{-9}M estradiol-17β before being incubated with the purified nuclei for 30 min at 4°C. Each value is the mean count of 100 nuclei.
Mean number of grains/nucleus ± S.E.

Cytosol		Nuclei: Diaphragm	Nuclei: Uterus
Diaphragm	$+E_2$	10·1 ± 0·32	10·6 ± 0·45
	−	10·0 ± 0·44	11·0 ± 0·52
Uterus	$+E_2$	10·6 ± 0·36	16·3* ± 0·53
	−	10·8 ± 0·35	12·2 ± 0·57

*This value is higher than all others, $P < 0{\cdot}05$, Student's *t*-test.

DISCUSSION

An important function of estradiol-17β is to promote the conversion of the uterine cytoplasmic receptor to an "active form" which then enters the nucleus and increases its ability to synthesize RNA. *In vitro*, this conversion is temperature-dependent; it requires more than

150 min at 4°C [14], half an hour at 25°C [13], and about 7 min at 37°C [15]. In contrast, estrone was reported by Jensen *et al.* [18] not to affect this conversion. Furthermore, the estrone-receptor complex failed to enhance RNA synthesis in isolated nuclei [13]. However, Jensen's data were later reevaluated and it was found that estrone might also be a weak promoter of the conversion of the receptor and that it does induce the same uterine protein as estradiol, although with least efficacy [19–22].

Brachet and Fick [8], and Hulin [9] presented evidence that the amount of ^{3}H-actinomycin D that binds to nuclei is related to the number of DNA sites available for transcription. The results reported here show that exposure of purified uterine nuclei to uterine cytosol preincubated overnight at 4°C with estradiol causes a significant increase in their ability to bind ^{3}H-actinomycin D. This increase, which occurs both at 4°C and 22°C, reaches its maximum within 15 minutes following exposure. It does not arise in nuclei incubated either with cytosol alone, cytosol preincubated with estrone or buffer added with estradiol or estrone, nor when purified nuclei are incubated at 4°C with cytosol added with estradiol immediately before mixing. These observations indicate that, under the conditions used here, the presence of the receptor and its preincubation with estradiol are both required to increase the nuclear binding of ^{3}H-actinomycin D. They also indicate that estrone is unable to induce such modification under these conditions. They suggest that the conversion of the receptor to its activated form, which is supposed to have occurred here only after preincubation with estradiol, is the necessary event which induces a modification in chromatin structure, as revealed by the increased nuclear binding of ^{3}H-actinomycin D. Because the exact significance of the changes in such binding in terms of transcriptional availability of DNA is unknown, no attempt was made to draw quantitative correlations between the present data and others related to changes in RNA polymerase activity under similar experimental conditions [13].

Incubation of nuclei and cytosol from uterus and diaphragm in the four possible combinations showed that an increase of nuclear binding of ^{3}H-actinomycin D occurs only when the cytosol and nuclei are both from uterus. This observation is in agreement with the one reported by Mohla *et al.* [13] who showed that the "active form" of the cytoplasmic estrogen-receptor complex does not enhance the RNA synthetic capacity of liver or kidney nuclei. It is also consistent with the observations that *in vitro* the estrogen-receptor complex interacts with chromatin extracted from uterus but not from non-target tissues for estrogens [23]. These data support the hypothesis that nuclear acceptor sites for the receptor are present only in the target cells. In contradiction with this view, it has been recently reported that preincubated estradiol-receptor complex does bind to isolated nuclei from non-target tissues [24]. However, no data are available as to whether metabolic effects result form this binding, nor as to its real specificity.

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Adrenal Secretion of Estrogens, Glucocorticosteroids and Mineralocorticosteroids in the Dog*

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Abstract—*The secretion rates of cortisol, corticosterone, aldosterone, estradiol and estrone by isolated adrenal glands perfused* in situ *were compared to each other. The following conclusions are drawn:*

1. *The canine adrenal gland is capable of secreting estrogens but the secretion is extremely low even under intense ACTH stimulation. By extrapolation, this suggests that most of the estrogens produced by castrate or postmenopausal women might derive from a peripheral conversion of the adrenal androgens.*
2. *In nephrectomized dogs the adrenal cortex behaves as a single functional unit with respect to cortisol, corticosterone and aldosterone secretion in response to various degrees of ACTH stimulation. This observation stresses the non-specific dependence of aldosterone on ACTH.*

INTRODUCTION

BILATERAL adrenalectomy is known to produce a remission in approximately 30% of the patients with disseminated mammary carcinoma [1]. This frequency is comparable to that obtained in selected series with various other endocrine treatments [2]. Thus, the remission results presumably from some modification in the patient's endocrine status. The nature of this modification is uncertain, but in view of the sensitivity of experimental mammary carcinoma to estrogens [3] the adrenalectomy might be effective by suppressing a source of estrogens; the latter, directly or indirectly [4], might stimulate the proliferation of the neoplastic cells. An argument which supports this hypothesis is that a good correlation seems to exist between remission after bilateral adrenalectomy and persistence of an estrogen receptor in the tumor [5].

The purpose of the work reported here is to measure the adrenal secretion of estrogens and to compare it with the secretion of the glucocorticosteroid and mineralocorticosteroid hormones in a preparation of isolated canine adrenal glands. Indeed, if it is well-known that the adrenal glands are capable of producing estrogens [6], the relative importance in this production of the peripheral conversion of the adrenal so-called androgens and of direct secretion is still unsettled [7]. On the other hand, this study provides an opportunity to compare the sensitivity of the two main glucocorticosteroids and of aldosterone to various degrees of ACTH stimulation in an hypophysectomized and nephrectomized animal.

MATERIAL AND METHOD

Four male and one female dogs (Beagle) weighing between 10·0 and 15·3 kg were anesthetized with pentobarbital, 30 mg/kg of body

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weight. Transbuccal hypophysectomy was performed by the method of McLean [8]. Dexamethasone (1 mg i.v.) was given during the surgical procedure and pursued (1 mg i.m. per day) until 24 hr before the experiment.

After a delay varying from 1 to 12 days, the dogs were anesthetized with thiopentone, nitrous oxide and halothane. Both adrenal glands were isolated *in situ* and perfused according to Hilton *et al.* [9] and to Urquhart [10]. The right renal artery was cannulated for perfusion and the perfusion line was attached to the right carotid artery through a perfusion pump (Holter micro-infusion roller pump, laboratory model—RL 175). The ligatures of the aortic pouch were not tied until the perfusion was started so that the arterial blood supply to the adrenal glands was never interrupted. Arterial blood temperature was maintained by passage of the line through a heater. Adrenal vein blood was obtained through a catheter in the vena cava. Blood samples were collected for periods of 15–30 min. A sample of peripheral blood (periph. bl.) was taken for control before the start of the adrenal perfusion. The operated dogs were transfused during the operation with blood obtained from dogs hypophysectomized 1–7 days prior to sacrifice. The adrenal blood flow was adjusted by the perfusion pump to 5 ml/minute. Solutions of test substances could be added to the perfusion line as described by Urquhart [10] at a rate of 0·5 ml/min. Saline solutions of synthetic ACTH (Cortrosyn: 24 first aminoacids) were freshly prepared from lyophilized powder and kept at 0°C during the experiment.

Cortisol (F), corticosterone (B), and aldosterone (aldo) were measured by acetylation with tritiated acetic anhydride (Amersham SA. 100 $\mu C/\mu$ mole) according to the method of Kliman and Peterson [11] as modified by Davis, Burwell, Casper and Bartter [12], [13].

Estradiol and estrone were measured by the radio-ligand assay of Tulchinsky and Korenman [14] which is specific for the estrogens.

RESULTS

The secretion rates of cortisol, corticosterone, aldosterone, estradiol, and estrone obtained in the five different experiments are shown in Table 1.

As can be seen, the hypophysectomy was total in only two experiments, experiments 1 and 5, where the control secretion of cortisol and corticosterone was negligible. In the other

Table 1. *Adrenal steroids secretion rates*

Exp. No.	Sex and body wt. (kg)	Adrenal wt. (g)	ACTH concn, (μU/ml)	F (μg/min)	B (μg/min)	Aldo (μg/min)	F/B	B/Aldo	Estradiol (pg/min)	Estrone (pg/min)
1	F 12·5	1·067	0	0·045	—				40	85
			5	1·055	0·545		1·9		100	110
			10	1·210	0·455		2·7		135	60
			50	3·175	1·755		1·8		135	125
			100	3·910	2·315		1·7		195	300
2	M 15·3	1·142	(periph.bl.)		(0·012)*	—			(0)†	(0)†
			0		1·645	0.080		21	0	0
			10		1·860	0.110		17	6	76
			50		2·320	0·210		11	30	70
			250		5·275	0·265		20	68	89
3	M 14·0	1·025	(periph.bl.)		(0·007)*	—			(0)†	(2)†
			0		1·125	0·080		14	—	0
			10		1·455	0·065		22	127	0
			50		1·690	0·150		11	126	84
			250		2·060	0·205		10	721	168
			1000		3·325	0·265		13	600	172
4	M 11·5	1·090	(periph.bl.)	(0·066)*	(0·002)*	(0·0004)*			(2)†	(4)†
			0	2·220	1·550	0·065	1·4	24	—	21
			10	2·510	1·980	0·080	1·3	25	—	44
			50	3·815	2·105	0·110	1·8	20	112	52
			250	4·420	3·145	0·380	1·4	8	221	103
			1000	3·715	3·080	0·420	1·2	7	216	99
5	M 10·0	1·200	(periph.bl.)	(0·002)*	(0·001)*	(0·0008)*			(2)†	—
			0	0·040	0·030	—			11	0
			5	1·360	0·785	0·070	1·7	11	7	0
			10	1·990	1·175	0·140	1·7	8	12	12
			50	3·855	2·005	0·170	1·9	12	13	0
			250	6·495	3·585	0·265	1·8	14	41	46

*Peripheral blood levels, μg/ml.

†Peripheral blood levels, pg/ml.

three experiments the hypophysectomy was only subtotal but the addition of ACTH to the adrenal perfusion could still double or triple the control glucocorticosteroid secretion; besides, if extrapolated to man on a body-weight basis, the control glucocorticosteroid secretion in these partially hypophysectomized animals would still be in the normal physiological range.

A first observation shown in Table 1 is that despite a progressive increase in glucocorticosteroid secretion with increasing levels of ACTH stimulation, the ratio of cortisol to corticosterone remains remarkably constant as if the two hormones were produced by the same cell. Indeed, in the fourth experiment, this ratio varies only between the values of 1·2 and 1·4 with one single exception; in experiments 1 and 5 it varies from 1·7–1·9 with again one single exception in experiment 1. More unexpected, however, is the finding of a similar constancy for the ratio of corticosterone to aldosterone in three of the four dogs in which this ratio could be evaluated, and interestingly enough, in experiment 4, where this ratio is not constant, it is aldosterone secretion which increases fastest with increasing degrees of ACTH stimulation.

A second observation shown in Table 1 is that some secretion of estradiol and of estrone can be detected in the adrenal effluent plasma at various levels of ACTH stimulation. However, this secretion is extremely low, the highest figures measured corresponding to a daily secretion of 1γ of estradiol and 0·4γ of estrone under intense ACTH stimulation; furthermore, when compared to the secretion of cortisol plus corticosterone, the estrogen secretion is from ten thousand to several hundred thousand times smaller.

DISCUSSION

Two main conclusions are drawn from the present investigation: first, that the canine adrenal gland is capable of secreting estrogens but that this secretion is extremely low; second, that in nephrectomized dogs the adrenal cortex can behave as a single functional unit with respect to cortisol, corticosterone and aldosterone secretion in response to various degrees of ACTH stimulation.

The first conclusion is not so unexpected since small amounts of estradiol and estrone have already been found in the adrenal effluent plasma of the ovariectomized Rhesus Monkey under conditions of maximal ACTH stimulation [15]; on the other hand, it is well-known that the estrogen production in castrate or postmenopausal women is very low and that much of this production can be accounted for by the peripheral conversion of the so-called adrenal androgens [7]. Our study in the dog indicates that an adrenal secretion of estradiol and of estrone can be detected at various degrees of ACTH stimulation and confirms that this secretion is extremely low when compared with that of the glucocorticosteroid hormones; actually, the adrenal secretion of estradiol is at best ten thousand times lower and up to several hundred thousand times lower than the adrenal secretion of cortisol plus corticosterone, a difference which is comparable to that previously found in the monkey. If one extrapolates this difference to humans [16], the adrenal secretion of estradiol should vary between 2γ and less than 0·1γ per day, i.e. should be well below the daily production of 25 to 10γ measured in castrate or postmenopausal women [7]. A similar extrapolation could be made for estrone. While such extrapolations have definite limitations, they suggest that most of the estrogens produced by the adrenal glands which are removed for the treatment of advanced mammary carcinoma derive from a peripheral conversion of the adrenal androgens rather than from a direct adrenal secretion.

The second conclusion, relative to the glucocorticosteroid and mineralocorticosteroid secretion, is perhaps more unexpected. It is remarkable indeed that the secretions of cortisol, of corticosterone, and of aldosterone increase almost identically in response to increasing concentrations of ACTH. This indicates that the cells which produce the three different hormones are very similar to each other, at least at the level of ACTH action, i.e. of cyclic AMP activation [17]. In other words, variable degrees of ACTH stimulation do not disclose different populations of cells with respect to cortisol, to corticosterone, or to aldosterone secretion, and the zona glomerulosa is as sensitive to ACTH as the zonas fasciculata or reticularis. This observation is not incompatible, of course, with the existence within the zona glomerulosa of an additional and distinct mechanism of regulation, concerning more precisely aldosterone, depending on angiotensin [18] and not involving cyclic AMP [19], but it stresses the non-specific dependence of aldosterone on ACTH. Such a close dependence explains some recent observations in man indicating that aldosterone can be secreted in bursts which are synchronous with those of cortisol [20].

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Direct Fibrinogenolytic and Fibrinolytic Activity in the Human Prostate*

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Abstract—*Previous work had demonstrated a direct proteolytic activity of human prostatic extract on a substrate of casein but not of fibrinogen or fibrin. In the experiments reported here, with a substrate of fibrin or fibrinogen having a higher concentration and specific activity than in previous experiments, it became possible to demonstrate such a direct proteolytic activity on these substrates.*

In addition, the presence of a trypsin and plasmin inhibitor in prostatic extract could be demonstrated.

By ammonium sulphate fractionation, a fraction can be separated from the extract, containing the factor responsible for the direct proteolytic activity and the inhibitor.

As REPORTED previously, *in vitro* analysis of crude human prostatic extracts showed the presence of three different proteolytic factors [1]. The first of these factors, a direct proteolytic activity (DPA), different from plasmin is capable of proteolyzing casein without prior activation; the second factor, plasminogen proactivator (PPA) is able to transform plasminogen into plasmin in the presence of streptokinase or urokinase; the third factor, plasminogen activator (PA), is capable of activating directly human plasminogen into plasmin but is inactive on bovine plasminogen.

The properties of DPA were further investigated. Although little or no activity could be demonstrated on a substrate of fibrinogen or fibrin in our previous studies, modification of the method used for measurement of its proteolytic activity allowed us to demonstrate a definite activity on fibrinogen and fibrin as well as on casein. The present report indicates the methods used for fractional precipitation of the human prostatic extract, allowing to separate from the other proteolytic factors a fraction containing DPA and a proteolytic inhibitor (PI), as well as some properties of both constituents of this fraction.

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MATERIAL AND METHODS

Detailed description of methods of preparations and measurements has been described in a previous paper [1]. In the present study following new procedures and following modifications of already described techniques were applied.

Preparation of prostatic tissue extracts

For the fractional precipitation procedure, the supernatant obtained after centrifugation at 900 *g* was again centrifuged at 25,000 *g* for 2 hr. The resulting supernatant (S1) was either used immediately or quick frozen and kept at −30°C until used.

Fractional precipitation of prostatic tissue extracts

This is a 3-step procedure (Fig. 1).

First step. One volume of fresh or thawed supernatant (S1) contained in a sealed dialyzer tubing (No. 4465–A2, Arthur H. Thomas Company, Philadelphia, U.S.A.) was immersed in 9 volumes of a $(NH_4)_2SO_4$ solution. Con-

centration of the whole 10-volume solution was 50% of a $(NH_4)_2SO_4$-saturated solution. This procedure was carried out, using a mechanical stirring device, at 4°C for 3 hr. At the end of this period, the sample was centrifuged at 4°C during 30 min at 1,800 *g*. The supernatant (S2) was stored at −30°C and the precipitate (P2) was dissolved in one volume phosphate buffer M/15, pH 7·4.

Second step. Starting with the dissolved precipitate (P2) an identical procedure to the first step was followed, except that the $(NH_4)_2SO_4$ concentration was 40% instead of 50% of saturation, yielding the S40 supernatant and the P40 precipitate.

Third step. Supernatants obtained during both first steps (S2 and S40) were pooled and precipitated as described above at a $(NH_4)_2SO_4$ concentration equal to 75% of saturation. Resultant supernate and precipitate were respectively called S75 and P75.

At the end of the fractional precipitation procedure, all the fractions were salted out by dialysis. This was performed against phosphate buffer M/15, pH 7·4 for the study of the proteolytic activity or Tris/HCl buffer 0·05 M pH 7·4 for the study of the coagulation properties.

Proteolytic enzymes

Plasmin. Human plasmin Kabi (Stockholm, Sweden) was used. This preparation is obtained by glycerol activation of plasminogen. The vials were stored at −30°C and the lyophilized powder was dissolved in phosphate buffer M/15, pH 7·4 before the experiment. The concentration of the solutions varied from 0·02 to 0·25 Remmert–Cohen unit per ml.

Trypsin. Crystalline bovine trypsin (Sigma Chemical Company, St. Louis, Mo, U.S.A.) was dissolved in hydrochloric acid 0·001 N. A stock solution containing 1 mg per ml was prepared and stored at −30°C.

Activator

L-*Cysteine.* This compound (Koch–Light Laboratories Ltd., Colnbrook, England) was used as a $1{\cdot}5\ 10^{-2}$ M solution in phosphate buffer M/15, pH 7·4.

Inhibitors

Soybean Trypsin Inhibitor (SBTI) from British Drug Houses Ltd., Poole, England, was used as a 3 mg per ml solution.

Epsilon-aminocaproic acid (EACA) was prepared as described previously and used as a 20% solution.

Iodoacetamide (Koch–Light Laboratories Ltd., Colnbrook, England) was also dissolved in phosphate buffer M/15, pH 7·4. Concentration of the solution was $1{\cdot}5\ 10^{-2}$ M.

Parachloromercuribenzoic acid sodium salt. This compound (Schuchardt, Chemische Fabrik, München, Germany) was dissolved in phosphate buffer M/15, pH 7·4, at a 10^{-4} M concentration.

Trasylol. This preparation (Bayer, Leverkusen, Germany) was used at the concentration of 10,000 KI units per ml.

Substrates

The substrates of fibrinogen and fibrin used had a much higher specific activity, $4\ 10^4$ counts/sec per ml. This was realized by tagging the fibrinogen molecule by means of a carrier free solution of $Na^{125}I$ in phosphate buffer (M/15) pH 7·4, containing 50–100 mC per ml. As compared to our previously ^{131}I fibrinogen preparations [1], the present preparation had 10 times more specific activity. The concentration of the substrate was increased to 1 per cent (w/v).

Measurements of proteolytic activity

The incubation of prostatic extract and P75 fraction was carried out in the presence of radioactive fibrinogen or fibrin. Precipitation of undigested substrate was obtained by the addition of 10% trichloroacetic acid solution in amounts of 2 ml for a total reaction mixture of 3 ml containing 1 ml of radioiodinated substrate.

Protein concentration measurement

This was carried out in the homogenate and its fractions using the Folin technique [2].

Coagulation studies

For these studies the crude human prostatic extract and its fractions were suspended in Tris/HCl buffer 0·05 M, pH 7·4. Human fibrinogen (Kabi, Stockholm, Sweden), at least 90% clottable, dissolved in the same buffer was used at a 0·2% concentration. Clotting properties were studied at 37°C adding to 0·2 ml of the fibrinogen solution, volumes of prostatic extract or fraction from 0·2 ml to 0·8 ml; experiments were also performed adding to the mixture 0·025 ml of a $CaCl_2$ 0·02 M solution.

Thromboplastic properties were studied, using a one-step prothrombin-time technique, the thromboplastin being replaced by the prostatic extract or a fraction. To 0·1 ml human plasma, 0·1 ml of a $CaCl_2$ 0·02 M solution and 0·2 ml of the prostatic extract or fraction were added. Clotting times were determined using a Fibrometer Coagulation timer (Baltimore Biological Laboratory Division, B–D Laboratories, INC, U.S.A.).

RESULTS

Presence of a proteolytic inhibitor (PI) in the crude human prostatic extracts

As previously suspected [1], the prostatic extract contained a proteolytic inhibitor (PI). Using radioactive casein and fibrin as substrates, addition of increasing amounts of crude prostatic extracts resulted in a marked inhibition of the proteolytic activity of trypsin.

Separation of proteolytic factors from prostatic tissue extracts

The fractional precipitation procedure allowed the separation of some proteolytic factors of the human prostate (Fig. 1). The P40 fraction contained the plasminogen proactivator (PPA) and the plasminogen activator (PA).

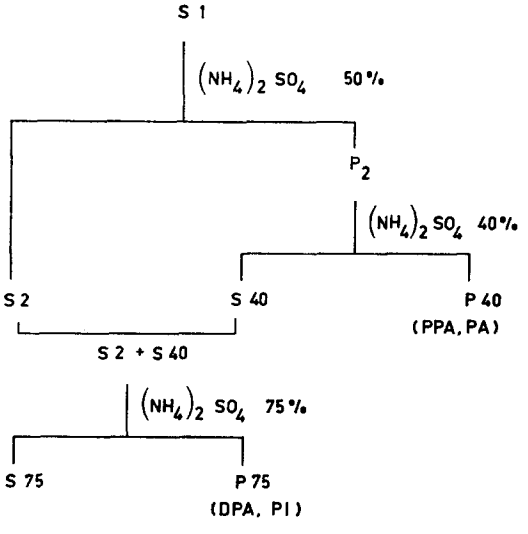

Fig. 1. Fractional precipitation of prostatic tissue extracts.

At the end of the first step of the fractionation, the bulk of the direct proteolytic activity (DPA) appeared in the S2 supernate while the P2 precipitate regularly contained, in addition to PPA and PA, some DPA. As far as the proteolytic inhibitor (PI) is concerned, an identical situation was found, the S2 fraction having stronger proteolytic inhibitory properties than the P2 fraction. Further fractionation of P2 resulted in complete separation of DPA and PI from the plasminogen proactivator and activator. Performing an additional precipitation on the pooled S2 and S40 fractions resulted in recovery of DPA and PI in the P75 precipitate. This latter fraction contained from 50 to 55% of the total protein amount of the crude human prostatic extract and from 90 to 95% of the direct proteolytic activity. On the other hand, PI seemed to be sensitive to this precipitation procedure, a variable part of it being degraded, according to our different experiments.

Direct fibrinogenolytic and fibrinolytic activity of DPA (crude extract and P75 fraction)

Modification of our previous methods of measurement of proteolytic activity on radioactive fibrinogen and fibrin [1], used in the present study, allowed us to observe a definite proteolytic activity of DPA on these substrates. This proteolysis was demonstrated using the crude human prostatic extract or the P75 fraction which contained as sole proteolytic factor the DPA (Fig. 2).

Figure 3 shows that, with these modified substrates, the proteolytic activity increased with increasing concentrations of substrate in the presence of a fixed amount of the P75 fraction. This was true whether the precipitating agent was trichloroacetic acid (Fig. A) or whether it was thrombin (Fig. B). In this last case, proteolysis was only detected when the concentration of the substrate was higher than 0·5%.

Absence of proteolysis of fibrinogen degradation products by DPA (P75 fraction)

Further experiments were performed to determine if DPA was able to proteolyze the fibrinogen degradation products resulting from the action of plasmin. If this were the case one should expect a very important increase of proteolysis after addition of P75 to a radioactive fibrinogen and plasmin mixture preliminary incubated for one hour. As shown in Fig. 4, this resulted in a rather small increase of proteolysis due to further digestion of the fibrinogen substrate by both enzymatic activities.

Effect of cysteine on DPA (P75 fraction)

Addition of L-cysteine to the P75 fraction did not significantly affect the proteolytic activity of the enzymatic preparation (Fig. 5).

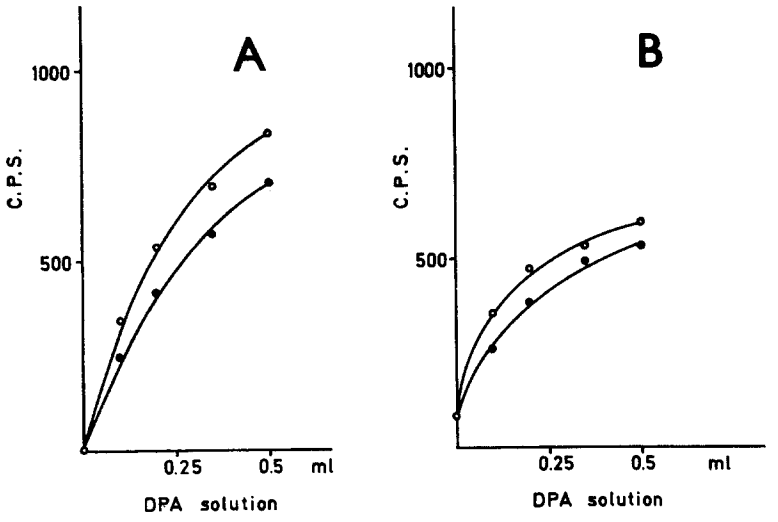

Fig. 2. *Effect of increasing amounts of DPA tested on human fibrinogen (A) or human fibrin (B). Precipitation by TCA.* ○—○ *Crude extract.* ●—● *P75 fraction. One milliliter of a* 1% *radioiodinated human fibrinogen solution;* 0·05 *ml of a* 300 *units per ml-bovine thrombin solution;* 2 *ml of a* 10% *TCA solution. Digestion:* 1 *hr at* 40°C.

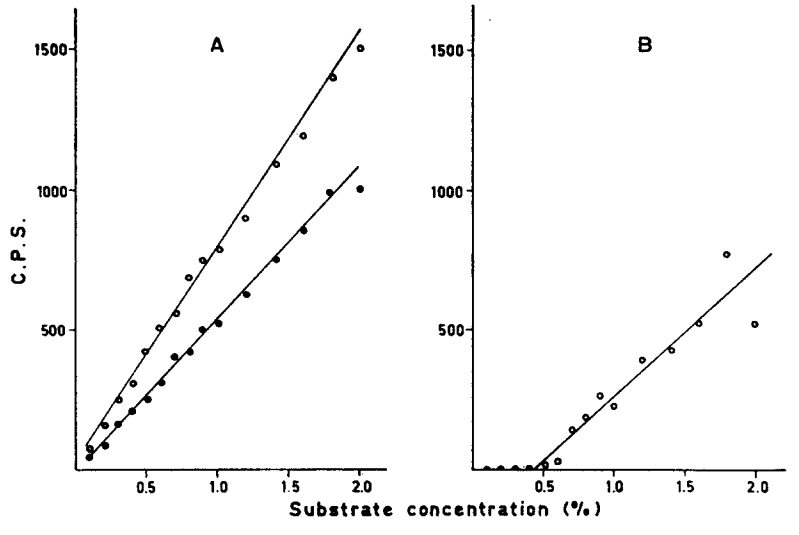

Fig. 3. *DPA activity in the presence of increasing amounts of substrate. (A)* ○—○ *tested on human fibrinogen, precipitation by TCA;* ●—● *tested on human fibrin, precipitation by TCA. (B) Tested on human fibrin, precipitation by thrombin. One half milliliter of P75 fraction;* 1 *ml of a* 1% *radioiodinated human fibrinogen solution;* 0·05 *ml of a* 300 *units per ml bovine thrombin solution;* 2 *ml of a* 10% *TCA solution. Digestion:* 1 *hr at* 40°C.

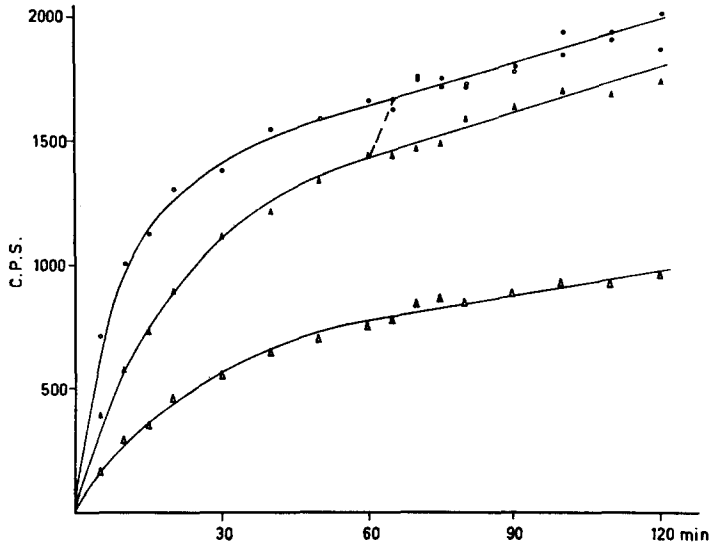

Fig. 4. Effect of DPA on fibrinogen degradation products. △—△ *Effect of DPA on fibrinogen;* ▲—▲ *effect of plasmin on fibrinogen;* ○—○ *effect of DPA + plasmin on fibrinogen;* ●—● *DPA added to a mixture of plasmin + fibrinogen digested for* 1 *hr at* 40°*C. One half milliliter of P*75 *fraction;* 0·5 *ml of a* 0·25 *Remmert–Cohen unit per ml plasmin solution;* 1 *ml of a* 1% *radioiodinated fibrinogen solution;* 2 *ml of a* 10% *TCA solution. Digestion at* 40°*C.*

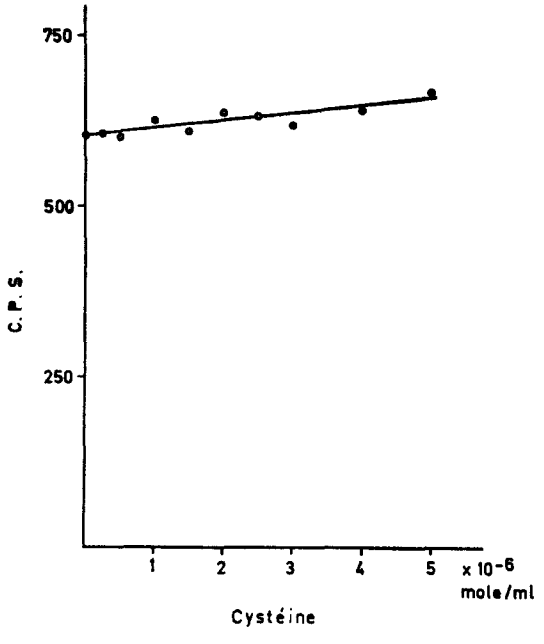

Fig. 5. Effect of increasing amounts of L-*cysteine on DPA.* 0.5 *ml of P*75 *fraction;* L-*cysteine:* 1·5 10^{-2} *M solution;* 1 *ml of a* 1% *radioiodinated human fibrinogen solution;* 2 *ml of a* 10% *TCA solution. Digestion:* 1 *hr at* 40°*C.*

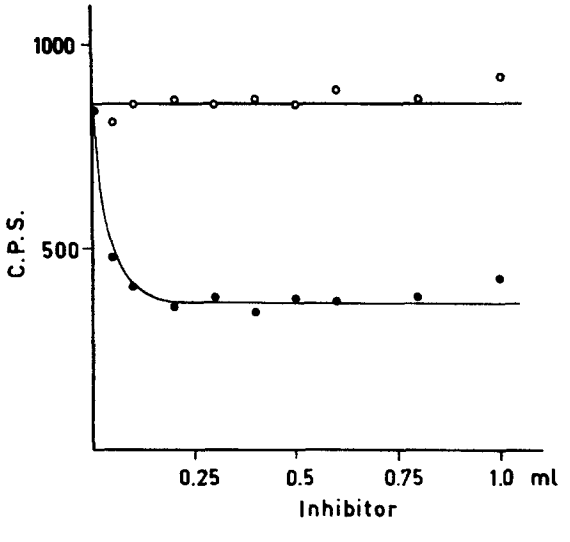

Fig. 6. Effect of increasing amounts of EACA and SBTI on DPA. ○—○ *Effect of SBTI* (3 *mg per ml solution);* ●—● *effect of EACA* (200 *mg per ml solution). One half milliliter of P*75 *fraction;* 1 *ml of a* 1% *radioiodinated human fibrinogen solution;* 2 ml *of a* 10% *TCA solution. Digestion:* 1 *hr at* 40°*C.*

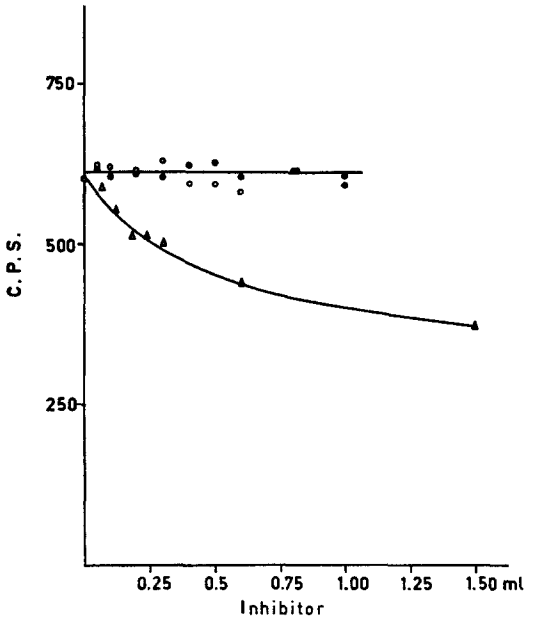

Fig. 7. Effect of increasing amounts of iodoacetamide, p-chloromercuribenzoic acid sodium salt and trasylol on DPA. ○—○ *Effect of iodoacetamide* ($1{\cdot}5\ 10^{-2}$ *M solution);* ●—● *effect of* p*-chloromercuribenzoic acid sodium salt* (10^{-4} *M solution);* △—△ *effect of trasylol* (10,000 *KI units per ml solution). One half milliliter of P75 fraction; 1 ml of a* 1% *radioiodinated human fibrinogen solution; 2 ml of a* 10% *TCA solution. Digestion: 1 hr at* 40°*C.*

Effect of various inhibitors on DPA (P75 fraction)

As indicated in Figs. 6 and 7, DPA was not inhibited by the soybean trypsin inhibitor (SBTI), iodoacetamide and parachloromercuribenzoic acid sodium salt. On the other hand, epsilon-aminocaproic acid and trasylol produced a marked but incomplete inhibition of DPA.

Presence of a proteolytic inhibitor (PI) in the P75 fraction

This fraction utilized in a volume as small as 0·1 ml produced a marked inhibition of the proteolytic activity of trypsin on casein and on fibrin. When tested with plasmin, a much smaller inhibition was produced; this occurred only when fibrin was used as a substrate, no inhibition being observed on casein (Fig. 8, A and B).

Coagulation studies

Addition of crude human prostatic extract to human fibrinogen failed to induce coagulation. Even after 24 hr, no fibrin clot was apparent in the tubes. In the presence of $CaCl_2$, no clot appeared. This happened also when S2, the pooled S2 and S40 fractions, or P75 were tested, with or without $CaCl_2$. At the end of the 24-hr incubation period, some fibrinogen was still

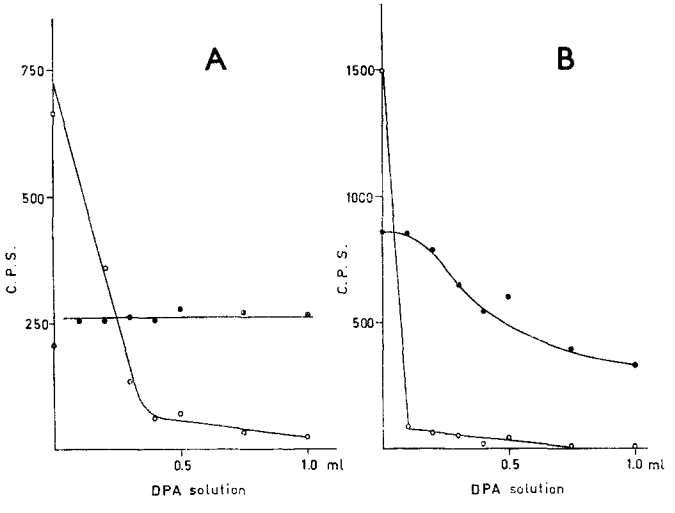

Fig. 8. Effect of PI on trypsin and plasmin. ○—○ *Effect on trypsin;* ●—● *effect on plasmin.*

(A) Tested on radioiodinated casein; P75 fraction. One half milliliter of a 0·0125 *mg per ml trypsin solution;* 0·5 *ml of a* 0·2 *Remmert–Cohen unit per ml plasmin solution;* 1 *ml of a* 2% *radioiodinated casein solution;* 2 *ml of a* 10% *TCA solution. Digestion:* 1 *hr at* 40°*C.*

(B) Tested on radioiodinated fibrin; P75 fraction. One half milliliter of a $3\ 10^{-3}$ *mg per ml trypsin solution;* 0·5 *ml of a* 0·02 *Remmert–Cohen per ml plasmin solution;* 1 *ml of a* 0·35% *radioiodinated fibrinogen solution;* 0·05 *ml of a* 100 *units per ml bovine thrombin solution. Digestion:* 1 *hr at* 40°*C.*

present in the mixture, as demonstrated by the clotting produced by the addition of a bovine thrombin solution.

As shown in Table 1, the prostatic extract possessed thromboplastic activity. This activity was found in the P2 fraction but could not be separated during the next step of the fractional precipitation procedure. Nevertheless the thromboplastic activity present in the S40 fraction was precipitated during the last step of the procedure and recovered in the P75 fraction.

Table 1. *Thromboplastic properties*

Test reagent	Clotting time (sec)
Commercial thromboplastin	12·1
Prostatic extract	14
S2	> 150
P2	16·6
S40	12·7
P40	18·7
S2 + S40	30·4
S75	> 150
P75	19·4
Tris–HCl buffer	> 150

DISCUSSION

Several methods of fractional precipitation were used for separation of the 3 proteolytic activities reported in the human prostatic extract [1]. Incomplete separation of these activities or degradation of one of them (PPA) was observed. Best results were obtained using $(NH_4)_2SO_4$ as precipitating agent at the various concentrations mentioned above, the prostatic homogenate being suspended in phosphate buffer M/15, pH 7·4 at a concentration of 300 mg/ml (Fig. 1). The plasminogen proactivator and the plasminogen activator present in the P40 fraction could not be separated by further fractional precipitation with $(NH_4)_2SO_4$. The direct proteolytic activity (DPA) of the prostatic extract completely separated from these 2 other proteolytic factors was isolated in the P75 fraction which contained about 50% of the total protein amount of the initial homogenate; purification of this DPA by further precipitation with $(NH_4)_2SO_4$ was unsuccessful.

A previously suspected proteolytic inhibitor [1] was demonstrated in the prostatic extract. This inhibitor (PI) was precipitated along with DPA during the fractional procedure and was found in the P75 fraction. This PI is a powerful inhibitor of trypsin, whereas it produces a much smaller inhibition of human plasmin (Fig. 8, A and B). In our experiments, the inhibition of plasmin by PI could only be detected using a 0·35% fibrinogen substrate precipitated by thrombin, which is a much more sensitive method than the caseinolytic one. As a matter of fact, on fibrinogen a much smaller amount of plasmin was used, the ratio PI/plasmin being much higher than the one used in the experiments on casein. The yield in DPA of the P75 fractions obtained from several fractionations was rather constant while their PI activity was variable. Therefore it seems probable that this proteolytic inhibitor is not active on the direct proteolytic activity of the human prostate.

In this study, a digestion of fibrinogen and of fibrin by DPA was observed. This occurred using the crude prostatic extract as well as the P75 fraction. This action on fibrinogen and fibrin was not observed in our previous experiments [1] because the demonstration requires a higher concentration and higher specific activity of these substrates than the demonstration on casein. It should be stressed that the activity of DPA could be demonstrated on fibrinogen and fibrin only when these radioactive methods were used. As a matter of fact, when measurement of the radioactivity of the supernates indicated clear-cut proteolysis, no proteolysis could be detected on these supernates by measurement of the optical density.

Because of the small proteolytic activity of DPA on fibrinogen, two possible properties of this enzymatic preparation were tested: DPA failed to induce coagulation of the fibrinogen molecule, even in the presence of $CaCl_2$ [3], and to digest further on the plasmin-resistant fibrinogen degradation products (Fig. 4). No significant effect on DPA resulted from the addition of L-cysteine, while no inhibition occurred in the presence of iodoacetamide and parachloromercuribenzoic acid sodium salt, indicating that sulfhydryl groups are not essential for this enzymatic activity [4]. Like plasmin, DPA was inhibited by EACA and Trasylol. Nevertheless, as it was not inhibited by SBTI, the proteolytic activity observed in the crude prostatic extract and in the P75 fraction could not be attributed to the presence of small amounts of plasmin.

As it is considered at the present time that enzymes other than thrombin may be involved in the pathogenesis of the so-called defibrination syndrome, the study of the properties of this enzymatic activity of the human prostate, is of interest in order to define its possible role in the hemorrhagic diathesis of prostatic diseases [5].

Finally it should be stated that the thromboplastic activity of the prostatic homogenate was found in the P40 and P75 fractions, indicating that this procoagulant activity is not bound to the enzymatic property of the P75 fraction.

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Letter to the Editor

Growth-Stimulating Effect of Nafoxidine on Rat Mammary Tumor after Ovariectomy*

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THE POTENT antiestrogen Nafoxidine§ has been shown to inhibit growth of the dimethylbenz[a]anthracene (DMBA)-induced mammary tumor of the rat [1, 2]. Like in the uterus, it probably acts by preventing the tissue uptake of 17β-estradiol [3], which in turn is likely due to inhibition of the binding of 17β-estradiol to the cytosol estrophiles [4]; kinetics of the latter reaction are characteristic of a competitive inhibition [5].

However, in contrast to the almost complete regression of the rat mammary tumors usually observed after oophorectomy, inhibition of tumor growth by Nafoxidine was only partial; some tumors regressed while others continued to grow. Failure to achieve complete tumor regression could be due either to incomplete inhibition of uptake of the endogenous estrogens or to an intrinsic growth-stimulating effect of Nafoxidine. The latter hypothesis is supported by the known uterotrophic effect of Nafoxidine [3, 6, 7] which has been interpreted as the expression of a weak estrogenic activity. The purpose of the present paper was to test this hypothesis.

Eighteen female Sprague–Dawley rats bearing one to three DMBA-induced mammary tumors [8] were castrated and paired according to number of tumors and total tumor surface per rat. They were randomized into two groups, one receiving subcutaneous injections of Nafoxidine suspended in sesame oil, 1 mg per kg daily (6 days a week), the control group receiving 0·2 ml of oil only. The animals were killed after 6 weeks and the remaining tumors were subjected to histological examination.

The results are shown in Fig. 1. After ovariectomy (control group), all tumors rapidly regressed; only 6 out of 19 were still visible after

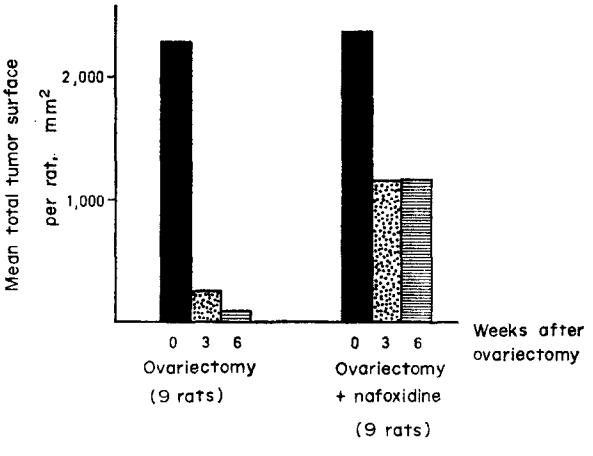

Fig. 1. Effect of Nafoxidine (1 mg per kg daily, subcutaneously) on regression of rat mammary tumors after ovariectomy. Inhibition of regression is statistically significant ($P < 0{\cdot}01$, Wilcoxon test on the differences between paired rats).

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§Nafoxidine = 1-[2-[p-(3,4-dihydro-6-methoxy-2-phenyl-1-1-naphthyl) phenoxy]ethyl]-pyrrolidine, hydrochloride lot no. 8053-CRM-25-2.

3 weeks, and 4 after 6 weeks. In the Nafoxidine-treated ovariectomized rats, 2 tumors continued to grow, 3 were newly formed, 1 remained static, while the others partially or completely regressed; 15 tumors out of 19 were still present after 3 weeks, and 14 after 6 weeks. Mean tumor regression per rat was significantly lesser ($P < 0{\cdot}01$) in the treatment group (52%) than in the control group (89%). During the experimental period, the rats, in the treated group, lost an average of 4% of their initial weight while, in the control group, they gained an average of 15%. Histological examination of the tumors revealed a typical regressive pattern of all tumors in the control group whereas, in the treated group, foci of healthy tumor cells, sometimes surrounded by regressive areas, were most often observed.

These data clearly indicate that Nafoxidine partially prevents the near-complete regression of the rat mammary tumors after ovariectomy and therefore displays intrinsic growth-stimulating activity on these tumors. This activity is even more striking in view of the definite weight loss of the treated animals, which by itself inhibits mammary tumor growth [9, 10]. This growth-stimulating activity of Nafoxidine on the DMBA-induced rat mammary tumor should probably be ascribed to the weak estrogenic properties it is known to exert on other target tissues [3, 6]. It probably accounts, at least in part, for the failure of Nafoxidine to achieve complete tumor regression in non-ovariectomized rats.

This growth-stimulating property should be kept in mind when Nafoxidine is used in the treatment of hormone-sensitive human breast cancer; aggravation is likely to occur in some patients and indeed Nafoxidine-induced hypercalcemia was reported in one patient [11].

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Letter to the Editor

Hodgkins' Disease with Massive Hepatic Involvement and Uninvolved Spleen*

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IN STUDIES on large series of patients subjected to systematic laparotomy and splenectomy in Hodgkin's disease, no instance of liver involvement was noted without concomitant splenic involvement [1, 6]. The case reported here is in contradiction with these observations.

CASE REPORT

This 59-year old male patient was admitted in December, 1963, to another hospital with left submaxillary lymphadenopathy. Biopsy of the node was described by the pathologist as showing a "marked reticular reaction" that was suspected of being Hodgkin's disease. No treatment was given. A second biopsy of a left cervical lymph node was taken in March 1966; it was reported by the same pathologist as showing a moderate granulomatous reaction with eosinophils and Sternberg–Reed cells. A definite diagnosis of Hodgkin's disease was made and the patient was treated with radiotherapy and chemotherapy. He received 2400 rads (200 kV) on left cervical and axillary fields and afterwards, unknown doses of vinblastine. The slides of these two biopsies were not available for review.

In May 1971, the patient was first admitted at this Institute. On clinical examination, there was an enlarged lymph node, 3 cm in diameter, in the left inguinal region. No other lymphadenopathy was found. The liver and spleen were not palpable. No symptoms or other signs were present. The blood hematology was normal. The erythrocyte sedimentation rate was elevated (68 mm/hr). The usual liver function tests were normal, including the bromsulphalein retention; the only exception was a slight elevation of the alkaline phosphatase (5·3 Bessey Lowry units). The lymphangiography showed massive involvement of the iliac and lumbar lymph node chains on both sides.

A biopsy of the left inguinal lymph node disclosed the characteristic scleronodular form of Hodgkin's disease with cellular nodules circumscribed by thick birefringent collagen bundles. Typical, hyperlobulated Sternberg–Reed cells with prominent acidophilic nucleoli were numerous; the cellular proliferation showed mixed infiltration with lymphocytes, plasma cells, polymorphonuclear neutrophils and eosinophils (Fig. 1). A laparotomy with splenectomy was made with liver biopsy by both wedge section and needle techniques.

PATHOLOGICAL DESCRIPTION AFTER LAPAROTOMY

At laparotomy, the liver was not enlarged but showed massive infiltration by confluent nodules measuring about one millimeter in diameter. The lymph nodes of the porta hepatis were not enlarged. The lomboaortic nodes were macroscopically enlarged but no biopsy was taken. Microscopic examination of the liver biopsies showed a cellular proliferation centered on portal spaces, but also massively invading the lobules (Fig. 2). There was a mixture of fibrocytes, lymphocytes, reticular cells, polymorphonuclear neutrophils

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and eosinophils, and a few plasma cells. The reticular cells were often atypically hypertrophied, with vesicular nuclei and prominent nucleoli. Some of them contained a large oval nucleus with delicate chromatin bridges between the nucleolus and the nuclear membrane; the cytoplasm was amphophilic with indistinct boundaries (Fig. 3a). They should be interpreted as neoplastic mononuclear cells with nuclear features of Sternberg–Reed cells. A few had a plurilobulated nucleus with multiple nucleoli and were interpreted as typical Sternberg–Reed cells (Fig. 3b). The presence in the liver of these characteristic infiltrations containing a few typical Sternberg–Reed cells and numerous mononuclear variants leads to the unquestionable diagnosis of hepatic involvement by Hodgkin's disease.

In contrast to the extensive cellular infiltration of the liver, the spleen appeared macroscopically normal and weighed only 170 g; its capsule was smooth and tense. The organ was cut into thin slices which were fixed and carefully examined for abnormal nodules and macroscopic involvement. The colour was dark red and neither abnormal lymphoid nodules nor infiltration was observed. Forty blocks were taken at random for microscopic examination; many sections from each block were scrutinized. Microscopic examination showed congestion of the sinuses with marked atrophy of the germinative centers. Many microscopic foci of hemosiderin pigment were noted. No microscopic granulomatous lesion was found, even of the epitheloid cell type described by Kadin *et al.* [7] and by Brincker [8]. There was no infarct, necrosis or any lesion in relation to a previous involvement by Hodgkin's disease. It was felt that the number of inclusion blocks examined was large enough to lead to the conclusion that there was no splenic involvement.

DISCUSSION AND CONCLUSION

This case of Hodgkin's disease presented itself in 1963 with an enlarged left submaxillary lymph node. The patient was first treated by excisional biopsy followed in 1966 by radiotherapy and chemotherapy (vinblastine) for local recurrence. Stage IV was diagnosed in May, 1971, with massive hepatic involvement without detectable splenic involvement. Enlarged iliac and lomboaortic lymphadenopathy was also present.

In their reviews of patients who underwent systematic laparotomy and splenectomy, Kadin *et al.* [2], Glatstein *et al.* [1], Rosenberg [3], Aisenberg *et al.* [4], Prosnitz *et al.* [5] and Zarembok *et al.* [6] point out that they found no single instance of liver involvement without concomitant splenic involvement. It should be mentioned that Ripault and Dumont [9], in a series of 51 laparotomies for splenectomy and liver biopsies in cases of Hodgkin's disease, reported hepatic involvement with unaffected spleen in one case. However, they did not give any detail as to how extensively the spleen had been examined. It is concluded that the present paper establishes the fact that, in contradiction with all other large reported series, massive liver involvement by Hodgkin's disease can occur in the absence of concomitant spleen involvement.

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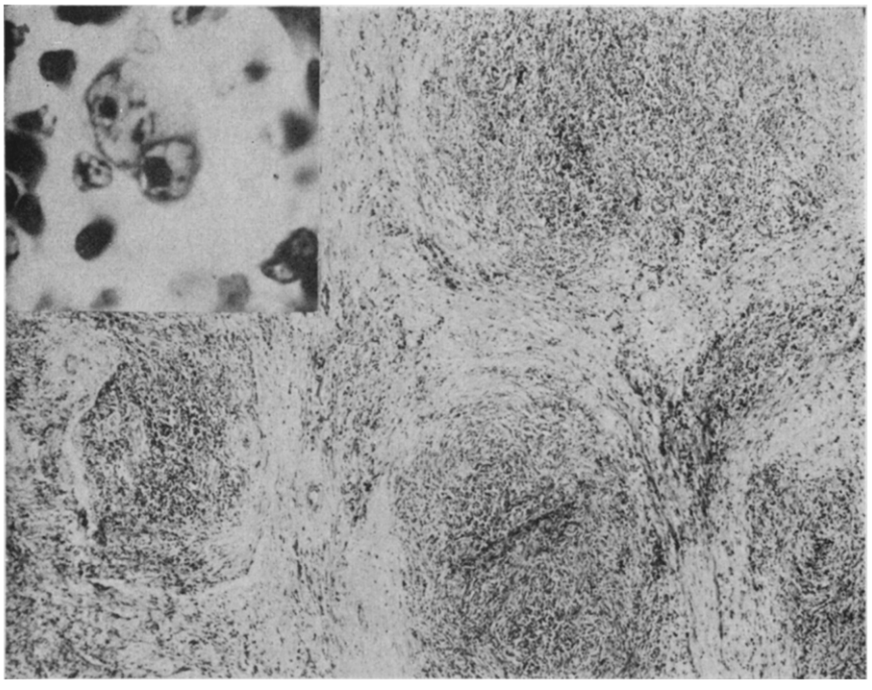

Fig. 1. *Biopsy section from left inguinal node showing involvement by Hodgkin's disease, scleronodular type. Hematoxylin–Erythrosin–Safran,* × 1160.

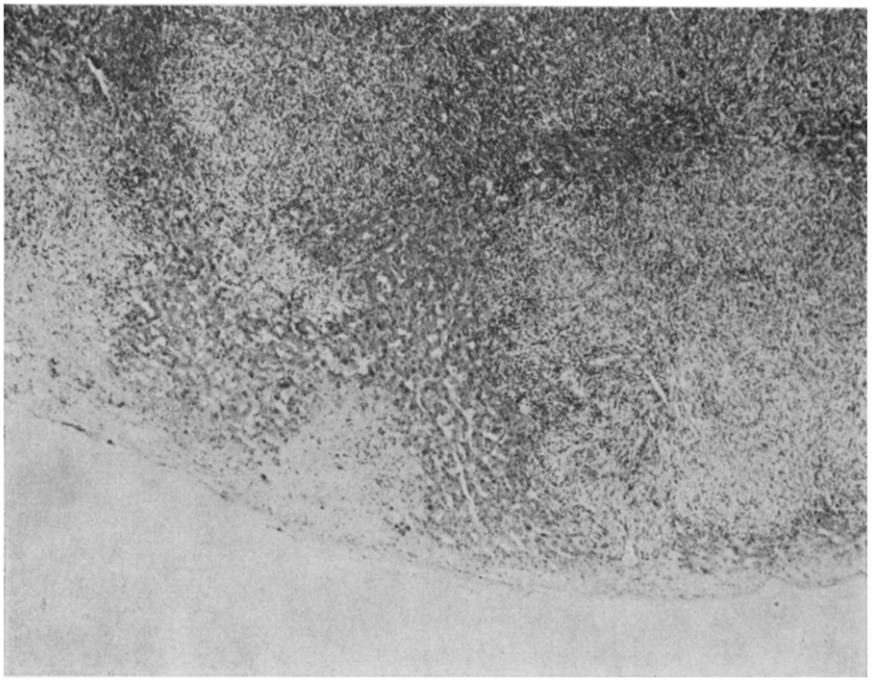

Fig. 2. *Hodgkin's disease of the liver. Hematoxylin–Erythrosin–Safran,* × 40.

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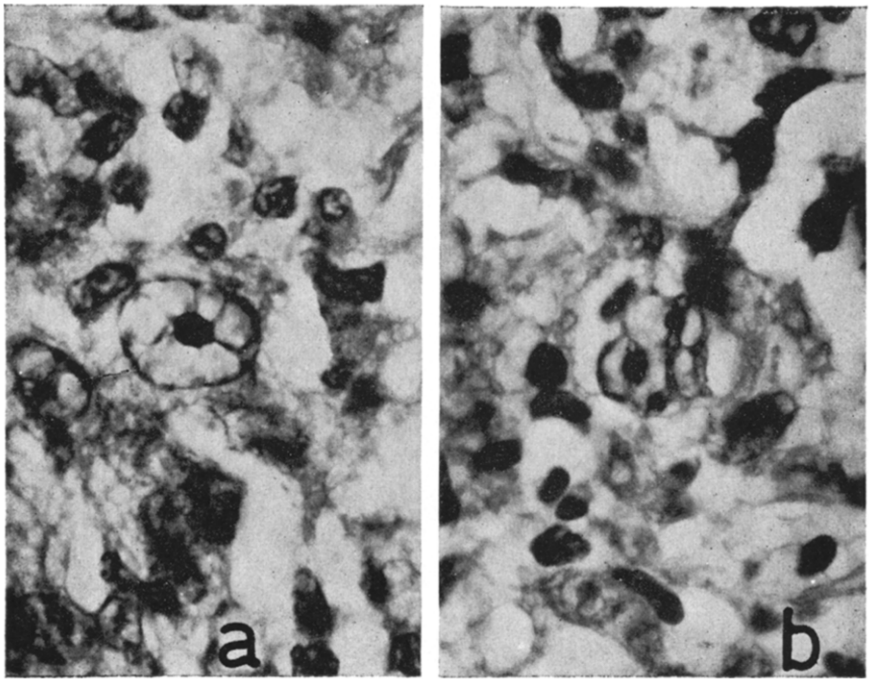

Fig. 3. *Hodgkin's disease of the liver.* (a) *Neoplastic mononuclear cell with nuclear features of Sternberg–Reed cells.* (b) *Sternberg–Reed cell. Hematoxylin–erythrosin–safran,* × 1300.

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Some Findings on Movement and Contact of Human Normal and Tumour Cells *In Vitro*

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Abstract—*In order to investigate differences in the contact inhibition of movement between normal and tumour cells* in vitro, *we studied cell populations from* 17 *normal and* 29 *neoplastic human tissues. The contact phenomena were quantitatively analysed by means of the nuclear overlapping ratio (Abercrombie and Heaysman). The study revealed differences in the extent of the loss of contact inhibition between the normal and neoplastic populations. These differences could be shown to be statistically significant (U-test by Mann and Whitney). We concluded that the contact inhibition of the populations of normal cells examined varies within biological limits, whereas the considerable variation in the distribution of overlapping values for the populations of neoplastic cells examined appears to suggest an individuality of the tumours.*

INTRODUCTION

DURING the last few years, time-lapse microcinematography, interference microscopy [1] surface contact microscopy [2] and stereoscan [3] have enabled us to observe details of the nature and principles of cell movement *in vitro*. Thus the phenomenon of contact inhibition of movement was discovered and analysed. As this phenomenon was observed in freshly isolated chicken and mice fibroblasts, but not, however, in sarcoma cells of mice, Abercrombie *et al.* [4, 5, 6] proposed the hypothesis that malignancy implies loss or reduction of contact inhibition. On the other hand, Sanford *et al.* [7], found no evidence that the virus-induced morphologic alteration observed in the growth pattern of mouse and hamster cells resulted from loss of contact inhibition of locomotion. They suggested that the morphological change seemed to reflect a proliferative response accompanied by decreased adhesion of ce to glass. In 1970, they added that reduced surface substrate dependency for membrane spreading may account for the growth pattern change [8]. Curtis [9] defined contact inhibition as a cell property, controlled by the factors in the culture medium, such as the embryo extract content and probably by the consequences of aging of the medium in contact with the cells. It is independent of the rate of cell population increase but is dependent to some degree on the local density of population.

No completely uniform explanation of contact inhibition and its relation to malignancy has been offered as yet. Obviously, a very complex phenomenon [10] covering many biochemical and biophysical parameters is involved.

Research groups who have so far been dealing with movement and contact of cells, obtained their material mainly from chicken, hamster embryos, mice and rats. They also used established cell lines and transformed cells, or cells from induced animal tumours. So far, relatively few results on the contact phenomena of adult normal and neoplastic human cells in primary and short-term cultures are available.

For this reason, and in view of the potential practical importance which a characterization of human tumours might have with regard to their invasiveness on the basis of their motility and their contact behaviour *in vitro*, we considered it justifiable to extend the studies on this question by quantitative investigations on human material.

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It would be desirable to observe the behaviour of cell populations of the same histological type of tumour and the corresponding normal tissue. Unfortunately, it is often very difficult to fulfil this demand for practical reasons. Another very important point in the evaluation of the results is the influence of the *in vitro* environmental conditions on the cell behaviour. We have studied these because some results have been published, which suggest that the expression of contact inhibition of movement and of division depends to a certain extent on the culture conditions of the cells [11, 12, 13].

MATERIAL AND METHODS

To obtain cultures from adult human tissues we used both the cell culture technique and the explant culture technique.

Cell cultures

Isolated tissue material was washed in Hanks' salt solution, minced and digested in fractions with 0·25% trypsin (Difco 1 : 250) at 37°C. The cells separated by centrifugation (maximally 156 *g*) were repeatedly washed with Hanks, suspended in Parker's medium 199 with 20% calf serum and subsequently explanted into culture vessels.

Explant cultures

Pieces of tissue 1–1·5 mm^3 in size were washed in medium and immobilized by means of perforated cellophane (Serva)* on the bottom of the culture vessels at a distance of about 1 cm from one another. The culture vessels used were culture chambers according to Tanneberger and Mohr [14] and Tissue Culture Flasks 3012 (Falcon Plastics, Los Angeles).

In both kinds of culture we renewed the medium when the pH dropped below 7·0. The cultures were fixed when an average cell density from 40 to 200 cells per microscope field was reached. This is a prerequisite for counting the overlaps of nuclei from cells not influenced by insufficient space for movement. Cultures were fixed in 5% formalin and stained with hematoxylin eosin.

For the quantitative evaluation of the contact inhibition of movement the following data were recorded:

1. We measured 20 cell nuclei per culture and ascertained the mean nuclear radius from the values for length of the two axes, since the nucleus is often elliptical.
2. The cell nuclei and cell nuclear overlappings of mononuclear cells in microscope fields of 0·16 mm^2 (40–200 cells) were counted (0 = observed overlaps of nuclei). Altogether, our counting covered 1000 cells of each population.

   For this quantitative estimation the following had to be considered:

   We evaluated only cells in the true monolayer area of the outgrowth from one explant, that means, we used only portions within a distance of 20 μ around cell patches and explant islets. The most reliable conditions for exact determination of the contact inhibition reaction or its absence are present only in such culture regions where free space for a potential cell movement still exists.
3. We calculated, via the mean values of the individual data obtained, according to the formula by Abercrombie and Heaysman [5], the number of overlaps which would have to be expected if the cells were distributed at random (E = expected overlaps of nuclei).

RESULTS

1. *Experiments concerning the influence of the* in vitro *conditions on the contact behaviour of cells*

We examined some *in vitro* factors, which possibly could influence the results of our experiments. We cultured the human cells on glass and plastic substrates (Table 1), we compared the results from explant cultures with those of cell cultures (Table 2). In both investigations we did not find significant differences [15]. Besides these experiments we cultured 9 human tissue tumours in medium, supplemented with 5, 10 and 20% serum. Four were grown in one concentration only, 2 were grown in two concentrations and, in 3 cases, the tumour cells were grown in all three concentrations tested. The results we obtained with these three different serum concentrations are demonstrated in Table 3. No evidence was obtained that serum concentrations, under our culture conditions, effects the O/E-value in this investigation.

2. *Investigation of* 17 *normal and* 29 *neoplastic cell populations*

The mean value of the number of cells and the effective nuclear areas $\pi(2r)^2$ [5], as well as the O/E-values, have been compiled for normal cells in Table 4 and for tumour cells in Table 5, according to rising O/E-values. If one compares the results in both tables, it is obvious that quite a number of the tumour cell populations show

*Serva (16415), Entwicklungslabor Heidelberg.

Table 1. *Comparison of the O/E-mean values from cultures on glass and plastic*

Tissue	Glass No.	Glass O/E	Plastic No.	Plastic O/E	$t$calculated	$t$table	f	Significance
Normal	10	8·6	7	11·5	1·450	2·13	15	n.sign.
Neoplastic	8	23·3	21	24·4	0·126	2·05	27	n.sign.

Table 2. *Comparison of the O/E-mean values from cell and explant cultures*

Tissue	Cell No.	Cell O/E	Expl. No.	Expl. O/E	$t$calculated	$t$table	f	Significance
Normal	6	11·0	11	9·1	0·837	2·13	15	n.sign.
Neoplastic	18	21·9	11	27·5	0·713	2·05	27	n.sign.

Table 3. *O/E values from cultures with different serum concentrations*

Culture	Serum concentration 5%	10%	20%
233 struma	11·03	8·33	11·79
250 ascites	33·14	32·34	34.30
262 pleural effusion	7·53	11·59	8.87

O/E-values which are comparable with those observed in the normal cells. A considerable number of the tumour cell populations, however, show markedly increased values. Whereas the normal cell populations evaluated have an O/E-mean value of $9{\cdot}0 \pm 4$, the corresponding value of neoplastic populations amounts to $24{\cdot}1 \pm 20$.

3. *Demonstration of the results*

For the sake of demonstration, we combined the individual overlaps (O/E-values) to categories of 4 units each (0–4; 4–8; 8–12% etc.), so as to demonstrate their frequency distribution. It should be noted that cultures from different normal tissues did not show significant differences in their O/E-values so that it is permissible to compare them as one group with the group of neoplastic cultures (Fig. 1).

Table 4. *Data for calculation of overlaps (O/E) of normal cells*

No. of exper.	Type of tissue	Number of cells	Effect. nucl.	E	O	O/E (%)
71	Skin	116·0	0·35	23·9	0·8	3·4
43	Kidney	75·3	0·37	10·5	0·4	3·8
161	Thyroid	42·0	0·22	1·9	0·1	4·2
70	Skin	125·0	0·33	26·3	1·5	5·7
185	Thyroid	92·3	0·23	7·8	0·6	7·7
92	Skin	92·3	0·33	14·5	1·2	8·3
52	Lung	144·0	0·33	33·8	3·0	8·9
190	Lung	137·0	0·10	7·5	0·7	9·3
81	Skin	96·8	0·33	15·9	1·5	9·4
7	Thyroid	74·6	0·17	4·8	0·5	10·4
160	Thyroid	55·1	0·50	7·6	0·8	10·5
112	Skin	91·5	0·33	14·2	1·6	11·3
89	Skin	64·9	0·31	6·6	0·8	12·1
78	Skin	90·5	0·31	12·8	1·6	12·5
158	Lung	104·0	0·18	9·5	1·4	14·7
189	Lung	128·0	0·16	10·3	1·6	15·5
180	Thyroid	146·0	0·09	7·5	1·4	18·7

No. of exp. = number of the experiment.
Effect. nucl. = effective nuclear area.
E = expected number of overlaps.
O = observed number of overlaps.

Table 5. Data for calculation of overlaps (O/E) of neoplastic cells

No. of exper.	Type of tissue	Number of cells	Effect. nucl.	E	O	O/E (%)
146	Papillary ovary carcinoma	125·0	0·18	14·1	0·3	2·1
150 b	Lymphoma	56·5	0·18	4·5	0·1	2·2
168	Thymoma	127·0	0·29	18·2	0·6	3·3
124	Granulosa cell tumour	127·0	0·39	31·9	1·5	4·7
193	Krukenberg tumour	88·4	0·37	11·2	0·6	5·4
177	Lymph node (Breast carc.)	76·6	0·54	12·6	0·7	5·6
200	Fibrosarcoma	53·6	0·20	2·3	0·2	8·7
198	Osteogenic sarcoma	197·0	0·16	24·5	2·3	9·4
152	Papillary ovary carcin.	196·0	0·54	81·2	8·0	9·9
102 b	Fibrosarcoma	137·0	0·29	16·0	1·9	11·9
201	Fibrosarcoma	65·3	0·19	3·2	0·4	12·5
104	Granulosa cell tumour	142·0	0·33	33·8	4·6	13·6
93	Lung carcinoma	71·7	0·37	9·8	1·6	16·3
203	Granulosa cell tumour	122·0	0·23	13·7	2·3	16·8
178	Ovary carcinoma	142·0	0·57	44·7	7·8	17·4
197	Osteogenic sarcoma	104·0	0·16	6·8	1·3	19·1
127	Undifferentiated sarcoma	116·0	0·15	10·1	2·2	21·8
179	Ovary carcinoma	113·0	0·42	21·0	4·6	21·9
171	Ovary carcinoma	131·0	0·13	8·5	1·4	22·4
150	Ovary carcinoma	135·0	0·20	14·3	4·9	34·3
119	Undifferentiated sarcoma	60·2	0·39	9·4	3·3	35·1
167	Thymoma	68·7	0·22	4·1	1·5	36·6
188	Granulosa cell tumour	169·0	0·14	15·1	5·7	37·7
184	Leiomyosarcoma	89·7	0·31	9·6	4·2	44·0
11	Hypernephroma	49·4	0·18	2·2	1·0	45·5
181	Sarcoma	77·5	0.29	6·8	3·1	45.6
100	Lung carcinoma	48·2	0·37	4·5	2.1	46·7
154	Ovary cystoma	40·5	0·25	1.6	0.9	56·3
170	Follicular adenocarcinoma	52·5	0.10	1·1	1·0	90.9

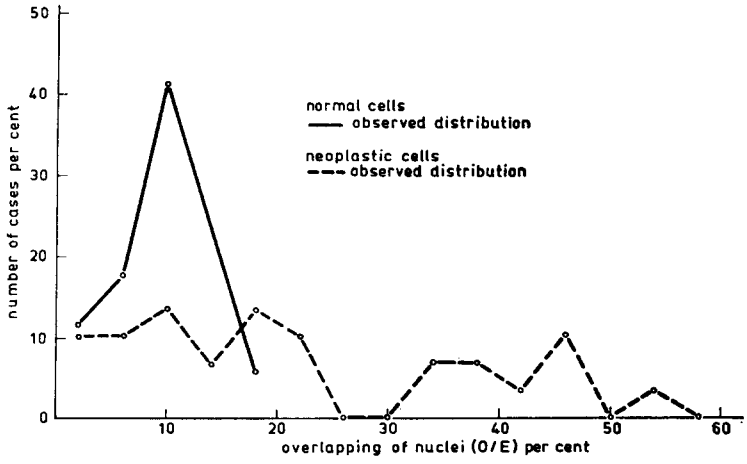

Fig. 1. Distribution of frequency of overlap-categories from normal (17) and neoplastic (29) cell populations.

DISCUSSION

The present paper studies and compares the contact inhibition of movement in cultivated cells of human non-neoplastic and neoplastic tumour tissues.

The method of Abercrombie and Heaysman can yield reliable, quantitative results, if the previous items stated in "Material and Methods" are considered. In the course of the last few years this method has been used in studies by different authors [9, 16]. Also Abercrombie and co-workers [17] investigated different fibroblast populations by comparing the overlaps observed with the overlaps expected, obtaining the data on nuclear area from a point-quadrat method (Chalkley, 1943).

The O/E-individual findings of the two independent random sample groups (non-neoplastic and neoplastic populations) demonstrated in Tables 1 and 2 proves to be statistically different according to the 1%-rejection level with the U-test by Mann and Whitney [18]. The O/E-values of the non-neoplastic populations show a reasonably symmetrical distribution about some central value, whereas neoplastic cells manifest a great variation and cannot be classified to any known statistic distribution. High O/E-values are only shown by tumour cell cultures. It should be emphasized, however, that a large proportion of tumour cell populations do not differ in their O/E-values from the normal cell cultures.

Actually there is no basic explanation for the different behaviour of non-neoplastic and neoplastic cells. No evidence was obtained in our studies that a different adaptation of non-neoplastic and neoplastic cells to the standardized *in vitro* system could be responsible. Therefore it should be more probable that the different contact behaviour reflects differences in the biophysical properties of non-neoplastic and neoplastic cells. Since we did not study corresponding neoplastic and non-neoplastic matter, no further interpretation can be given.

The great variation of the O/E-values of the neoplastic populations and the uncertain distribution of the values demonstrated offer two alternative interpretations:

(1) Multiplicity of results may be due to differences between histologically different tumours.

(2) Equally, these results might be due to the individuality of each tumour.

A more precise answer as to which of these interpretations is more accurate, will be rendered possible only after more comprehensive testing of tumour material.

Irrespective of which alternative will in the future prove to be the correct interpretation, we would like to assume the following on the basis of the results obtained:

The more or less marked loss of the contact inhibition of movement reflects changes in the cells, which occurred to a widely differing extent during carcinogenesis. This phenomenon suggests a considerable individuality either of histologically different tumours, or, which is more likely, of each individual tumour.

Of course in the interpretation of the individual behaviour of neoplastic cell populations differences also in adaptation to the *in vitro* conditions could be discussed. With respect to our results concerning differences in adaptation of non-neoplastic and neoplastic cells we do not believe that this phenomenon will play an important role in this connection, but further studies in this field should be carried out.

If the differences observed in the contact behaviour of different neoplastic cells in fact do result from a biological individuality of human tumours [17], the determination of motility and of contact inhibition of movement might be a useful aid for clinical oncology. An exact knowledge of the characteristics of human tumours is required both for therapy [20] and diagnostics [21].

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Comparison of the Effects of Methotrexate and Pyrimethamine on L5178Y Lymphoblasts in Cell Culture*

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Abstract—*The effects of methotrexate and pyrimethamine were compared against methotrexate sensitive and resistant sublines of L5178Y lymphoblasts. Against the sensitive subline methotrexate on a molar basis was more effective in reducing cell viability. Against a subline that was resistant to methotrexate by virtue of impaired transport of the drug, pyrimethamine proved superior to methotrexate in reducing cell viability.*

INTRODUCTION

PYRIMETHAMINE (5-P-chlorophenyl (2,4-diamino-6-ethylpyrimidine or Daraprim® is a potent anti-folate agent which has activity against a variety of bacterial and protozoal organisms [1]. Its principal use in man has been in malarial prophylaxis [1, 2], and it has also been used to treat toxoplasmosis [1, 3]. Early anti-tumour screening tests indicated activity against certain experimental tumours [4, 5], but limited trials in man were disappointing [6]. Pyrimethamine has been used with benefit, in polycythemia *vera* [7], and, more recently, was reported to be effective in controlling meningeal leukemia in man [8]. Its effects in meningeal leukemia were attributed to the drug's ability to cross the blood brain barrier, and also suggested that it had under certain circumstances at least, significant anti-tumour properties in man.

In this report we have compared the effects of pyrimethamine and methotrexate against L5178Y lymphoblasts in cell culture. Our results indicate that pyrimethamine produces a similar dose response curve to that of methotrexate (MTX) against methotrexate sensitive cells, being less effective on a molar basis than methotrexate itself. However, pyrimethamine was shown to have superior activity to methotrexate when tested against a methotrexate resistant subline of L5178Y cells.

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MATERIAL AND METHODS

Chemicals and reagents

Methotrexate was obtained as 5 mg vials from Lederle Products Ltd. Montreal, Quebec. Folinic acid was obtained as calcium leucovorin

from Lederle Products Ltd., Montreal, Quebec. Pyrimethamine was obtained from the Burroughs Wellcome Co. Ltd., thymidine and adenosine were obtained from Sigma Biochemicals Ltd. Radioactive methotrexate 3', 5' T sodium salt, specific activity 250 m/Ci per min. mole was obtained from the Radiochemical Centre, Amersham, England. Nicotinamide adenine-dinucleotide-phosphate reduced (NADPH) was obtained from Boehringer GmBH, agar and Hanks' tissue culture medium was supplied by Difco Laboratories, Detroit, Michigan. Fischer's medium for Leukemic Mouse Cells was obtained from Grand Island Biological Supplies Ltd., N.Y., and Fetal Bovine serum was supplied by Flow Laboratories Ltd., Maryland, U.S.A. Dihydrofolate reductase was prepared according to the method of Futterman (as modified by Friedkin *et al.*) [9, 10].

Cells

The cell lines employed in these studies were L5178Y lymphoblasts. Two sublines were used: the methotrexate sensitive line being inhibited by continuous exposure to 10^{-8} molar methotrexate, and a MTX resistant subline which had been produced by continuous exposure to sublethal concentrations of the drug and which was capable of growing continuously in 10^{-6} molar methotrexate, but was inhibited by 5×10^{-6} molar methotrexate. Both cell lines had similar growth rates having a doubling time of approximately 36 hr during exponential phase and reaching stationary phase at a cell concentration of approximately 6×10^5 cells per ml. The cells were grown in suspension culture in Fischer's medium supplemented with 10% fetal bovine serum in Falcon clear (polystyrene) tubes 17 × 100 mm. (Falcon Plastics Ltd.). Growth curves of the cell lines were obtained by removing small aliquots from the culture tubes and doing a direct cell count with a hemocytometer. Cell viability assays were performed by exposing exponential phase cultures of the cells to varying concentrations of the drugs for periods of 24, 48 and 72 hr. Following drug exposure the cells were centrifuged and washed in Fischer's media three times, counted and then added at a concentration of 100 cells/ml to Falcon tissue culture tubes which contained cloning media. The cloning media was prepared by supplementing the tissue culture media used for suspension cultures with heavily irradiated (1500 rads) L5178Y cells at a concentration of 10^4 cells/ml. These heavily irradiated cells functioned as "feeder" cells to increase cloning efficiency. The media was further supplemented by dilute agar to a concentration of 0·15%. A total of 10 ml of the cell suspension was added to each tissue culture tube. The tubes were sealed and then incubated at 37°C for 8 days. The resultant macroscopic colonies were then counted using dark field illumination and a stereo microscope. Only colonies with a size greater than 100 cells were scored. The cloning efficiency of the cells varied with each experiment from 40 to 70%. Back ground counts from irradiated feeder cells alone were negligible. The cloning efficiency of the untreated control cultures were set at 100% of normal. Survival curves were obtained by computing the means and standard deviations of the colony counts of five replicate cultures and expressing these as a percentage of the control.

Uptake of methotrexate

L5178Y cells (methotrexate sensitive) in logarithmic growth were counted and separated by centrifugation at 350 ***g*** at room temperature for 5 min. The cell pellet was resuspended in Hank's solution pH 7·4 into which was incorporated 10% foetal calf serum to give a final cell concentration of 5×10^5/ml. 10 ml aliquots of the cell suspension were preincubated at 37°C and 0·01 ml tritiated (3H) methotrexate added to each sample giving a final drug concentration of 4×10^{-6} molar and a radioisotope concentration of 1 μCi/ml. Pyrimethamine was added immediately prior to the methotrexate to the appropriate sample. The cells were incubated with the drugs at 37°C with gentle shaking for periods between 0 and 30 min. At the end of the incubation the cells were cooled rapidly in ice, spun at 350 ***g*** at 4°C for 5 min, washed three times with ice-cold phosphate buffered saline (PBS) and dissolved in 0·5 ml 12·5% tetraethylammonium hydroxide. Duplicate 0·2 ml aliquots were counted in a Packard Tri-Carb Scintillation counter model No. 3320.

Estimation of dihydrofolate reductase

L5178Y cells in logarithmic growth were counted, centrifuged at 350 ***g*** for 10 min at 4°C, and the cell pellet washed twice with ice-cold PBS. The cells were suspended in 0·05 molar citrate buffere pH 5·9 to a final concentration of 1×10^8/ml. The cells were ruptured by sonic oscillation for 30 sec at 2kc/sec, (MSE Ultrasonicator) and centrifuged at 3,000 ***g*** for 20 min at 4°C. Dihydrofolate reductase activity

in the supernatant solution was determined by the method of Osborn and Huennekens using a Gilford Reaction Kinetics Spectrophotometer [11]. Protein was estimated by the method of Lowry *et al.* [12].

RESULTS

We first sought to determine whether, in our system, pyrimethamine was functioning as a true biological folic acid antagonist. Growth curves were set up for both the sensitive and resistant cell lines and pyrimethamine at a concentration of 10^{-6} molar was added (Fig. 1a, b). With pyrimethamine alone, significant

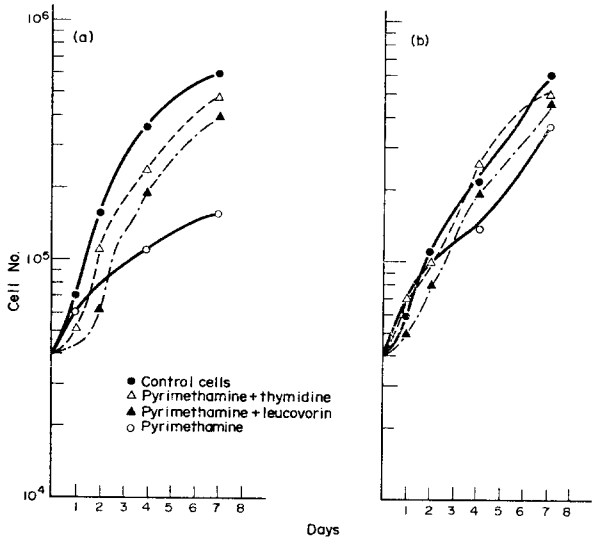

Fig. 1(*a*). *Growth curve of L5178Y lymphoma cells (methotrexate sensitive) exposed to continuous concentration of* $10^{-6}M$ *pyrimethamine,* $10^{-6}M$ *pyrimethamine* + $4 \times 10^{-6}M$ *thymidine, and* $10^{-6}M$ *pyrimethamine* + $10^{-5}M$ *leucovorin (folinic acid).*

Fig. 1(*b*). *As in (a), except methotrexate resitant L5178Y cells employed.*

suppression of cell growth occurred in the sensitive cell line. Some suppression of cell growth was noted with the resistant cell line but this was less marked than with the sensitive cells. This suppression was very largely prevented by the simultaneous addition of either folinic acid at a concentration of 10^{-5} molar or thymidine at a concentration 4×10^{-6} molar. Adenosine, at the same concentration as the thymidine, did not prevent or enhance the growth inhibition.

Figure 2 shows the effects of various concentrations of pyrimethamine on the activity of a crude preparation of dihydrofolate reductase from L5178Y cells. It can be seen that pyrimethamine at relatively low concentration is a relatively effective inhibitor of the enzyme *in vitro*. Fifty per cent inhibition occurred at a

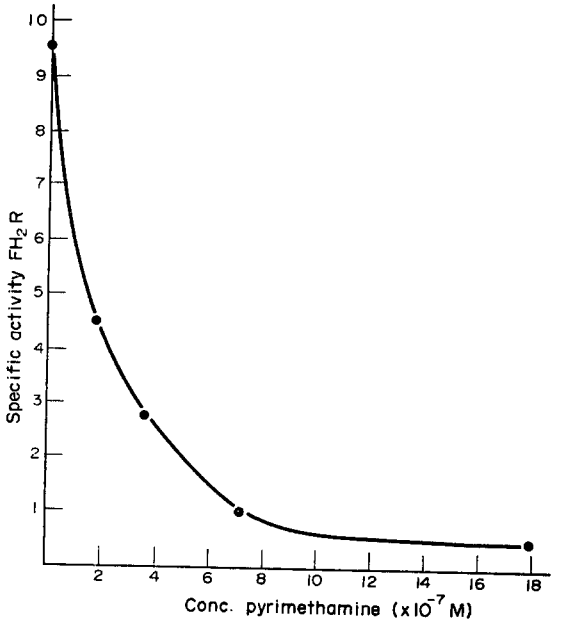

Fig. 2. *Titration of dihydrofolate reductase (FH_2R) from L5178Y cells against increasing concentrations of pyrimethamine. Specific activity of dihydrofolate reductase = mμ moles NADPH oxidised/min per mg protein. Enzyme concentration in cuvette under assay conditions described = 3·2 mμ moles per ml.*

concentration of $1{\cdot}8 \times 10^{-7}$ molar while a concentration of $1{\cdot}8 \times 10^{-6}$ molar produced almost complete inactivation of the enzyme. These findings were felt to be consistent with an anti-folate effect by the drug, with inhibition of dihydrofolate reductase and consequent suppression of thymidine biosynthesis.

Next we compared the effects of pyrimethamine and methotrexate on the viability of the L5178Y methotrexate sensitive cells. Figure 3(a) shows the effects of increasing concentrations of methotrexate on the cells following 24 and 48 hr exposure to the drug. The survival curve shows a steep initial fall followed by a plateau. The 48 hr exposure to methotrexate had a significantly greater effect on cell viability than the 24 hr, though the shape of the curves is similar. Figure 3(b) shows the effect of pyrimethamine on the same cells. With a 24 hr exposure to the drug there was an initial fairly steep reduction in viability followed by a plateau. This is similar in over all shape to that seen with the methotrexate but the initial fall in viability is not so steep nor is the fractional cell kill as great. The 48 hr exposure produced a nearly straight line fall with a considerably greater reduction in cell viability than with the 24 hr exposure. No evidence of a plateau was seen at the limit of sensitivity of the assay.

The effect of the two drugs on the viability of the methotrexate resistant cell line was then

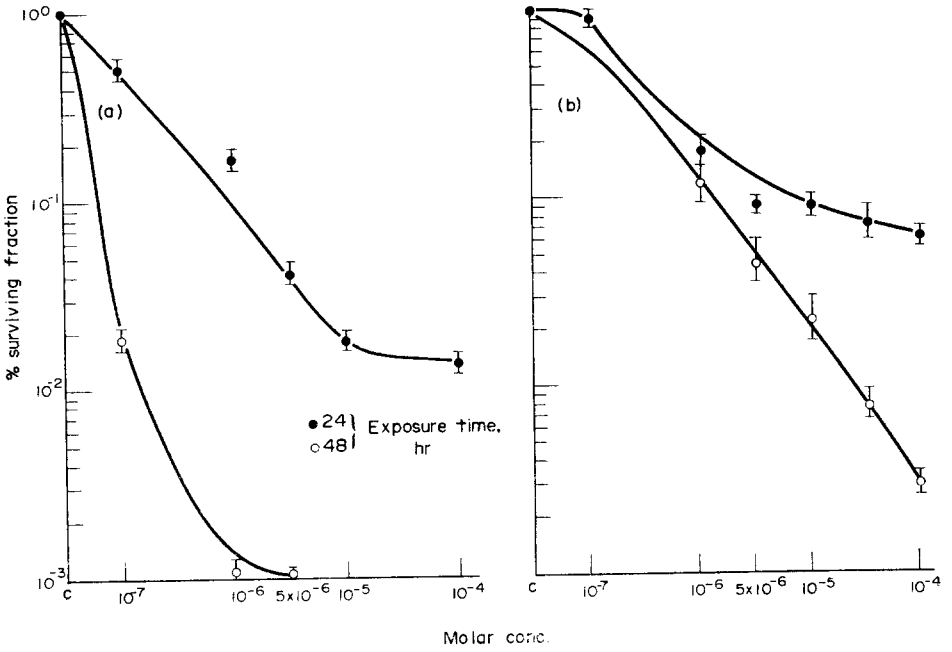

Fig. 3(a). Effect of 24 and 48 hr exposures to increasing concentration of methotrexate on L5178Y cells (methotrexate sensitive). Percentage of surviving fraction plotted against drug concentration in moles.

Fig. 3(b). Effect of 24 and 48 hr exposures to increasing concentration of pyrimethamine on L5178Y cells (methotrexate sensitive). Percentage of surviving fraction plotted against drug concentration in moles.

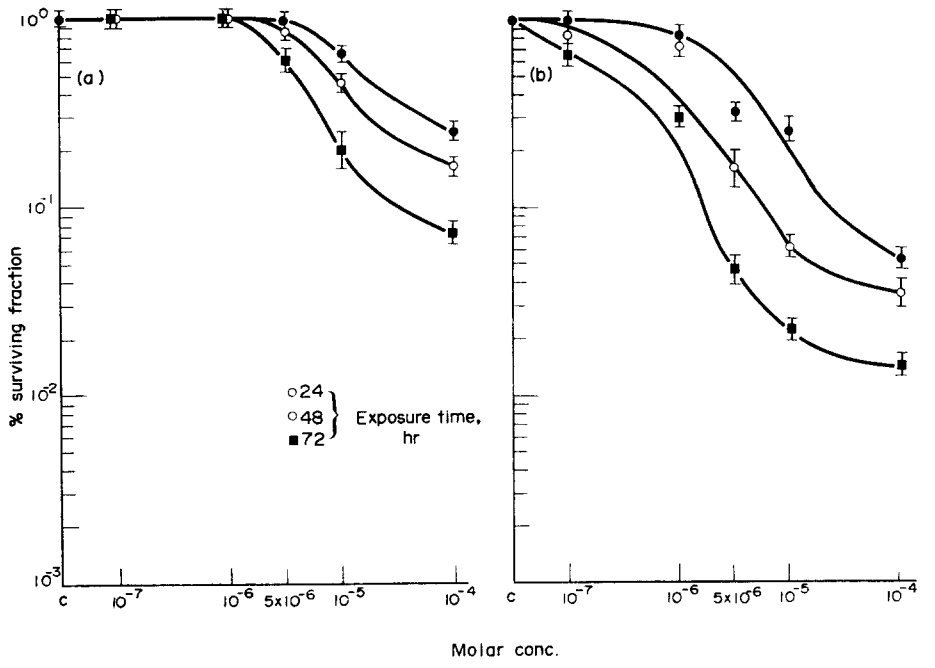

Fig. 4(a). Effect of 24, 48 and 72 hr exposures to increasing concentrations of methotrexate on of L5178Y cells (methotrexate resistant). Percentage surviving fraction plotted against drug concentration in moles.

Fig. 4(b). Effect of 24, 48 and 72 hr exposures to increasing concentrations of pyrimethamine on L5178Y cells (methotrexate resistant). Percentage of surviving fraction plotted against drug concentration in moles.

compared. Figure 4(a) shows the effects of 24, 48 and 72 hr exposures to methotrexate on the resistant cells. The shape of the survival curve differs significantly from that seen with the sensitive cell line. Regardless of the duration of exposure there is no significant reduction in cell viability until a concentration of drug greater than 10^{-6} molar is employed. Once this threshold concentration is exceeded progressive reduction in cell viability is seen, the shape of the curves being notably less steep than with the sensitive cells. The longest exposures show the largest reduction in cell viability. Figure 4(b) shows the effects of 24, 48 and 72 hr exposures to pyrimethamine on the methotrexate resistant cells. With progressively longer exposures to the drug, there is a progressively steeper reduction in cell viability with a plateau effect being noted at the highest drug concentrations.

Figure 5 illustrates the effects of pyrimethamine on the transport of methotrexate into the sensitive L5178Y cells. Pyrimethamine at a

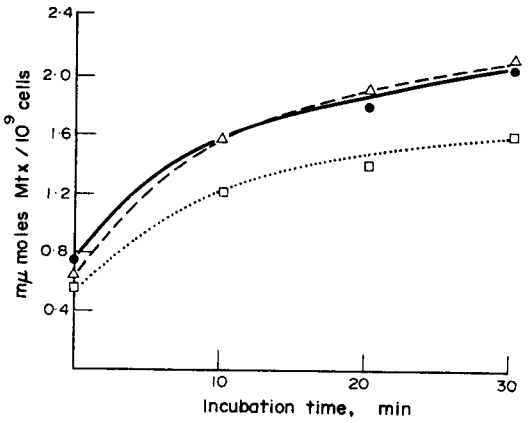

Fig. 5. Uptake of ^3H Methotrexate by L5178Y cells in the presence and absence of pyrimethamine.

● —— ● *MTX* ($4 \times 10^{-6} M$)
△ —— △ *MTX* ,, *+pyrimethamine* ($4 \times 10^{-6} M$)
□ —.— □ *MTX* ,, *+pyrimethamine* ($4 \times 10^{-5} M$)

Each point represents the mean of duplicate determinations.

concentration equimolar to that of methotrexate (4×10^{-6} molar) was without effect on the uptake of methotrexate. The addition of pyrimethamine at a concentration 10 times in excess of methotrexate i.e. (4×10^{-5} molar) inhibited the uptake of the latter drug to some extent (20% inhibition after 30 min).

DISCUSSION

The shapes of the dose ,response–survival curves seen with methotrexate and pyrimethamine against the methotrexate sensitive cell lines are similar to those which have been reported by others using the *in vivo* spleen colony assay as well as *in vitro* tests [14, 15, 16]. These survival curves are produced by drugs that are thought to act during only one phase of the cell cycle; in the case of methotrexate during the S phase [16]. During a particular time exposure to the drug only those cells which pass through a vulnerable phase of the cell cycle will be killed, the remaining cells by virtue of being in an invulnerable part of the cell cycle will constitute the resistant "trail" or plateau that is seen. As their resistance depends primarily on their growth kinetics, increasing concentrations of drug over a limited time period will not be effective in further reducing the cell viability. Extending the duration of drug exposure will allow most or all of the cells that are in an insensitive part of the cell cycle to then pass through a sensitive phase and consequently be killed. This is presumably the basis for the greatly increased reduction in cell viability that is seen with exposure to methotrexate longer than 24 hr. The shape of the survival curve produced by pyrimethamine is similar to that of methotrexate, though on a molar basis pyrimethamine is less effective in killing the sensitive cells.

When the effects of the two agents on the resistant cell line were studied different results were noted. With methotrexate there can now be seen a wide shoulder in the cell viability curve. Reduction in cell viability does not occur until a critical threshold concentration of drug is reached. Extending the duration of exposure to the drug from 24 to 72 hr does not significantly alter the shape of the curve but does result in a moderate increase in the fractional cell kill. This result is consistent with the known property of these resistant cells, namely that they can grow continuously in 10^{-6} molar methotrexate. A different situation is seen with pyrimethamine. In contrast with its effects on the sensitive cells a moderate shoulder is seen in the survival curve. However, compared with methotrexate, it can be seen that the pyrimethamine is significantly more effective on a molar basis in reducing the viability of the resistant cells. Again, the longest drug exposures produce the greatest reduction in cell viability.

The question arises as to what is the basis for the different effects noted between methotrexate and pyrimethamine on the resistant cells. It has been previously established [13] that the basis for the methotrexate resistance in this line of L5178Y cells is due to defective transport of the drug and not to differences in sensitivity or amounts of the dihydrofolate reductase. Utilization by pyrimethamine of a

different transport mechanism could provide a possible explanation for the observed effect. This hypothesis was tested in the experiment where the effect of pyrimethamine on the uptake of methotrexate by L5178Y cells was studied (Fig. 5). At equimolar concentrations, pyrimethamine appeared to be without effect on the uptake of methotrexate. A ten-fold excess of pyrimethamine showed a slight inhibition of methotrexate uptake. Two possibilities might account for these findings. Either methotrexate and pyrimethamine are transported into the cells through different sites and do not compete with one another, or alternatively pyrimethamine has a far lower affinity for a common transport site than methotrexate, and does not compete effectively. Since pyrimethamine has almost equal activity against the viability of methotrexate-sensitive cells and cells resistant to methotrexate due to depleted drug transport, it is suggested that the former possibility is the more likely. The slight inhibition of methotrexate transport by the higher concentration of pyrimethamine may possibly result from competition for the binding sites on the intracellular dihydrofolate reductase.

CONCLUSION

Data has been presented which indicate that pyrimethamine acts as a potent folic acid antagonist against L5178Y cells. Against the sensitive subline of cells it was less effective on a molar basis than methotrexate itself. However, a different situation was seen when both drugs were tested against the methotrexate resistant subline, where pyrimethamine proved to be superior to methotrexate and produced a different shaped survival curve. Against the resistant cells, a wide shoulder was seen with methotrexate. This presumably reflects the fact that with these cells a high extracellular concentration of the drug is required to produce sufficient intracellular drug concentration to cause lethal injury [13]. This type of resistance can be overcome by high drug concentration, but not by prolonged exposure to a lower concentration.

When pyrimethamine was tested against the resistant cells the wide shoulder seen with methotrexate was not apparent. Pyrimethamine showed nearly equivalent effects against both sensitive and resistant sublines. Although direct evidence is lacking, we would suggest that this effect of pyrimethamine may be due to more efficient transport of pyrimethamine into the resistant cells.

These transport properties of pyrimethamine suggest a number of clinical applications. The drug's ability to penetrate the CNS has been mentioned previously [8]. Clinical counterparts to the defective transport based resistance in our experimental system [17] might be susceptible to treatment with pyrimethamine. As the effects of both methotrexate and the diamino-pyrimidine class of drugs can be readily reversed by reduced folate, then conceivably the "kill and rescue" chemotherapeutic strategy could be applied employing combinations of different classes of anti-folate drugs.

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Immunosuppression Mediated by Ehrlich Ascites Fluid

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Abstract—*One intraperitoneal injection of cell-free Ehrlich ascites fluid, which did not induce the tumour, inhibited very strongly, and for a long time, the formation of antibody-producing cells (PFC's) in the spleens of recipient mice. The same procedure did not alter cellular immunological reactivity of these splenocytes (assayed by graft-vs-host reaction). At the time when the strongest suppression of PFC formation was registered, cellularity of the spleens increased. Several interpretations of these data are given, the most attractive one being that immunosuppressive agent(s) present in the ascitic fluid of Ehrlich tumour selectively eliminated or inhibited function of the population of B lymphocytes.*

INTRODUCTION

IN MICE bearing Ehrlich ascites tumour the formation of humoral antibodies was found to be depressed [1], and rejection of allogeneic and xenogeneic skin grafts delayed [2]. Moreover, when the skin from animals with Ehrlich tumour was transplanted onto normal allogeneic recipients, it was tolerated longer than the skin from non-tumorous donors [3]. Some data indicate that an active factor which causes immunosuppression is present in the cell-free ascitic fluid of this tumor. Thus, daily injections of the fluid selectively inhibited secondary immune response to allografts of mouse skin, but did not influence the primary response [4].

We have investigated immunological functions of normal mice injected with cell-free ascitic fluid from Ehrlich tumour, which by itself could not induce the malignancy, and will describe the observed modifications in the number of antibody forming cells, and the graft-vs-host reactivity of spleen cells of mice injected with that material.

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*On leave from pharmaceutical factory "Pliva", Zagreb.

MATERIAL AND METHODS

Animals

Male and female mice, 2–4 months old, of four inbred strains: A, CBA, C57BL and B10LP, as well as two hybrid lines: (C57BL × A)F_1—thereafter named LAF_1, and (CBA × C57BL)F_1—named CLF_1, were used. They were kept, no more than 5 to a cage, on a standard diet with food and water given *ad libitum*.

Tumours

Ehrlich ascites tumour has been maintained in our Laboratory for several years in CBA, A and LAF_1 mice by serial intraperitoneal transplantations. After injection of 0·5 ml of terminal ascites, without counting the number of malignant cells, the tumour develops in all recipient animals, and they die in 11–13 days. In terminal stages of the disease CBA mice had approximately 4–6 ml of ascites in the abdominal cavity, and LAF_1 mice had 5–8 ml.

Reticulosarcoma used in these experiments developed spontaneoulsy and was discovered incidentally in a C57BL mice of our breeding colony, in the year 1971. Since then, it has been maintained in the same strain by intraperitoneal transplantations. This tumour is strain-specific

and grows only in C57BL mice, or in F_1 hybrids of this strain. In LAF_1 hosts injected with 10×10^6 malignant cells the tumour causes the development of 3–5 ml of ascites, and the recipients die in 25–30 days.

Preparation and administration of ascitic fluid

Ascites were collected from tumorous animals under aseptic precautions, a day or two before death. Pooled fluid from 5 to 10 mice was then centrifugated for 10 min at 800 *g*, the supernatant was carefully aspirated, divided in 2 ml samples in sterile tubes, frozen at −18°C, and rapidly thawn just before use. Only one injection, firstly 0·5 ml and later 0·3 ml, of Ehrlich ascitic fluid (EAF) or reticulosarcoma ascitic fluid (RAF), obtained and preserved in such way, was given to experimental animals intraperitoneally. This quantity of EAF, introduced into young adult mice did not induce the Ehrlich tumour during the observation period of 8 months, neither did the malignancy develop within 6 months in animals injected with 0·1 ml of EAF one day after birth.

Immunological tests

To assess the humoral immunological reactivity, the total number of antibody forming cells (PFC's) in the spleen was determined 4 days after imunization with 0·5 ml of 10% suspension of sheep red blood cells (SRBC). For this purpose, the technique of Jerne *et al.* [5] was modified for use on microscopic slides, as follows. Two ml of 1% agar (Indubiose A 37^R)† were poured onto a microscopic slide to serve as bottom layer. An upper layer was made of 0·5 ml of 0·5% agar incorporating $2·5 \times 10^5$ cells and 0·05 ml of 20% SRBC suspension, thoroughly mixed. After 1hr incubation in humid chamber at 37°C, the slides are arrayed on plastic plates, upper side downwards, facing a shallow trough in which guinea-pig complement (diluted 1 : 10) was instilled. Following 2 hr incubation at 37°C, clearly visible plaques could be counted by unaided eye.

Cellular immunological reactivity was tested by measuring the graft-vs-host (GVH) activity following Nouza's [6] modification of Simonsen's test. C57BL splenocytes (4×10^6) were injected into 2–3 day-old CLF_1 hybrid recipients. Seven days later the host mice were killed, their spleens weighed, and the indices of splenomegaly calculated.

Experimental design

Experimental mice were treated with EAF or RAF at several intervals before injection of SRBC, or 1 day after it. The number of PFC's was always determined 4 days after SRBC administration. Each experimental group, consisting of 4 to 10 animals, included also two or three control mice injected with Hank's solution instead of ascites fluid. The PFC counts in experimental mice were then correlated with counts in the controls and presented as percentage of control value.

The splenocytes used for GVH assay were harvested from mice injected with EAF 7, 10, 14, 18, 21 or 28 days before. Here again each experimental group was matched by its own control, i.e. mice injected with Hank's solution instead of EAF.

†Produced by l'Industrie Biologique Française, Gennevilliers, France.

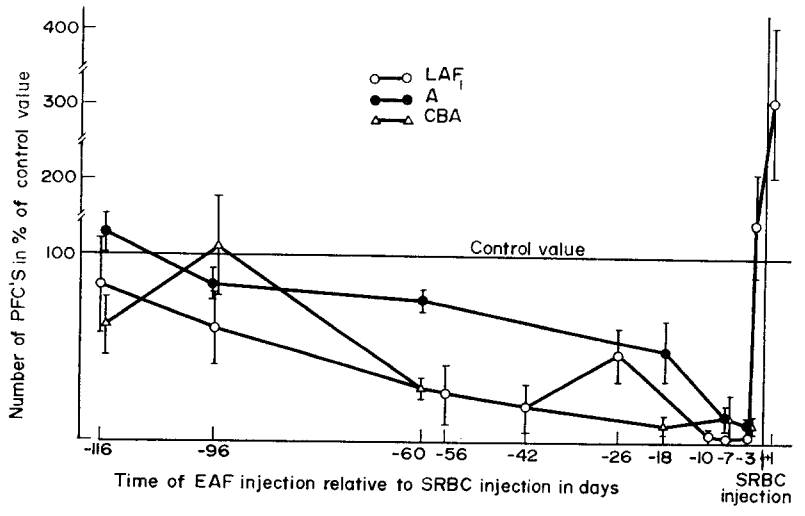

Fig. 1. *The total number of PFC's per spleen in LAF_1, A and CBA mice injected with EAF from donors of the same strain. Each point represents mean ± S.E. of 4–10 individual results.*

Statistics

Differences between experimental and control groups were statistically evaluated by Student's *t*-test, or by Mann–Whitney U-test [7].

RESULTS

One intraperitoneal injection of 0·3 or 0·5 ml of EAF from LAF_1 mice into normal LAF_1 recipients caused dramatic change in their reactivity to sheep erythrocytes (Fig. 1). If the animals received the ascitic fluid one day *after* antigen, the number of PFC's increased about three times normal. However, if EAF was administered 3 or more days *before* antigen, the development of antibody-forming cells was substantially checked. The strongest inhibition was registered in animals injected with EAF 3–10 days prior to SRBC, thus assayed for the number of PFC's 7–14 days after injection of EAF. At that time their spleens contained no more PFC's than 3–4% of control values. As more time elapsed after injection of EAF, the reactivity to sheep erythocytes gradually recovered, but normal immunological response was only registered in mice treated with EAF 4 months before injection of antigen.

Very similar effect on the formation of PFC's was observed in A and CBA mice treated with EAF from A or CBA donors.

Ehrlich fluid harvested from one mouse strain suppressed the immunological reactivity in other strains as well (Table 1). Only A mice seemed to be slightly more resistant to the influence of EAF than the other 5 strains tested. In A strain, as well as in LAF_1 mice, EAF from "syngeneic" donors (A or LAF_1, respectively) induced somewhat stronger suppression than EAF from "allogenic" donors. In CBA mice, however, this was not the case.

The effect of injection of reticulosarcoma ascitic fluid was also tested. Half a ml of RAF caused only slight decrease in the reactivity to SRBC in C57BL, CBA and CLF_1 animals, did not change the response of A and B10LP mice, and even stimulated the formation of PFC's in LAF_1 mice (Table 1). Thus, in two host strains of this reticulosarcoma (C57BL and LAF_1), the results diverged, like in other 4 strains tested. We concluded, therefore, that ascitic fluid from reticulosarcoma does not contain such potent immunosuppressive agent(s), as the fluid of Ehrlich ascites tumour.

The weights and cellularity of the spleens were measured in LAF_1 mice at various intervals after EAF injection (Table 2). In comparison to control values, both of these parameters increased and were significantly higher in animals treated with EAF, throughout the whole period when the humoral immunological reactivity was mostly depressed.

The results of GVH assay, in which splenocytes of parental mice, treated or not with EAF, were injected into hybrid recipients, are presented in the Table 3. In two repeated experiments, donors of spleen cells received ascitic fluid from two different harvests, and their GVH activity was assayed six times between the 7th and the 28th day after administration of EAF. The results in experimental and in control groups were the same, in general, and different splenomegaly indices were only registered in 2 of the 12 experimental groups. In both cases the administration of EAF did not inhibit, but stimulated the GVH activity of the spleen cells.

DISCUSSION

The results clearly show that ascitic fluid from Ehrlich tumour contains agent(s) which induce strong and long-lasting suppression of humoral immunological reactivity in mice but do not influence cellular immunity. Because of this discrepancy and as the number of spleen cells increased simultaneously with the greatest suppression of plaque formation, this effect can hardly be attributed to a nonspecific toxic effect of EAF on splenocytes. More probably, there occurred a selective inhibition, or elimination, of some cell type involved in production of humoral antibodies. If so, EAF might have affected either B or T cells.

Loring and Schlesinger observed that theta positive (T) lymphocytes disappear in thymuses and lymph nodes of RIII mice with growing Ehrlich ascites tumour [8]. This could explain suppressive effect of EAF on humoral immunological reactivity in our experimental mice, which involves cooperation of T and B cells [9], but does not fit well with the data of GVH assay. As T cells are prerequisite for normal GVH reactivity, and as it is questionable whether T–B cooperation takes place in GVH reaction [10, 11], better explanation for both suppressed humoral and normal cellular immunological reactivity would be that EAF influenced the population of B lymphocytes. In support to this interpretation it may be noticed that synthesis of DNA in bone marrow cells cultured *in vitro* decreased if cell-free ascitic fluid from Ehrlich tumour was added to the culture medium [12]. In our experimental mice, cells which proliferate in response to SRBC, and synthetized new DNA, were antibody-producing cells, i.e., B lymphocytes. In

Table 1. *The total number of PFC's per spleen in various strains of mice* 11 *days after injection of EAF from* LAF_1, *A and CBA donors, or RAF from* LAF_1 *mice. Results are expressed as percentage of control value* (100%). *In parentheses, number of mice*

Material	Donor strain	Recipient strain					
		LAF_1	A	CBA	C57BL	B10LP	CLF_1
EAF	LAF_1	2·8 ± 1·3 (8)	24·7 ± 9·4 (6)	15·6 ± 1·5 (5)	5·5 ± 1·5 (4)	11·9 ± 1·4 (4)	5·6 ± 4·9 (4)
	A	12·5 ± 11·5 (3)	13·8 ± 5·5 (6)	4·2 ± 0·3 (4)	12·0 ± 6·1 (5)	3·6 ± 1·8 (4)	8·6 ± 6·8 (3)
	CBA	5·6 ± 3·9 (6)	28·6 ± 12·4 (4)	14·3 ± 11·6 (8)	N.T.*	3·0 ± 2·9 (4)	3·6 ± 1·7 (5)
RAF	LAF_1	308·0 ± 68·7 (7)	125·5 ± 35·5 (5)	58·4 ± 8·8 (5)	56·0 ± 15·0 (7)	113·0 ± 21·2 (5)	48·9 ± 9·2 (5)

*N.T. = not tested.

Table 2. Weight and cellularity of the spleens of LAF_1 mice injected with EAF from LAF_1 donors. In parentheses number of mice

	Control value	Days after injection of EAF						
		3	7	11	14	30	60	100
Spleen weight (mg)	118·5 ± 7·7 (10)	128·5 ± 9·7 (4)	130·7 ± 5·8 (4)	171·9 ± 10·1† (13)	184·7 ± 29·4* (4)	161·6 ± 12·8† (7)	191·6 ± 26·0* (6)	116·8 ± 16·2 (5)
Number of cells ($\times 10^6$)	126·7 ± 28·3 (7)	155·0 ± 24·2 (4)	116·2 ± 14·0 (4)	183·4 ± 15·1† (13)	210·0 ± 40·1 (4)	223·7 ± 29·8* (7)	176·3 ± 26·0 (6)	116·2 ± 22·1 (6)

Statistical difference to the control value: *$P < 0{\cdot}05$.
†$P < 0{\cdot}01$.

Table 3. GVH activity of 4×10^6 splenocytes from C57BL mice injected or not with EAF from LAF_1 or CBA donors. Results represent splenomegaly indices in CLF_1 recipients. In parentheses, number of receipient mice

Donors injected with	Days after injection of EAF					
	7	10	14	18	21	28
EAF from LAF_1 mice	2·00 ± 0·08† (9)	2·16 ± 0·07* (13)	2·32 ± 0·29 (9)	2·48 ± 0·25 (5)	2·08 ± 0·20 (13)	1·74 ± 0·15 (6)
Hank's solution	1·62 ± 0·09 (9)	1·93 ± 0·09 (11)	2·56 ± 0·29 (6)	2·10 ± 0·16 (9)	2·09 ± 0·18 (13)	1·86 ± 0·24 (5)
EAF from CBA mice	2·51 ± 0·18 (3)	2·10 ± 0·40 (3)	2·04 ± 0·16 (7)	N.T.‡	2·01 ± 0·16 (8)	N.T.‡
Hank's solution	2·76 ± 0·13 (4)	1·99 ± 0·33 (4)	2·34 ± 0·10 (9)	N.T.‡	1·98 ± 0·21 (8)	N.T.‡

Statistical difference to the control value: *$P = 0{\cdot}032$.
†$P = 0{\cdot}020$.
‡N.T. = not tested.

C3H mice a syngeneic mammary tumour which grows in ascitic form was even found to produce a peculiar gama globulin destroying antibody-forming cells [13].

Another possible explanation of the observed effect on the PFC's would be the interference of EAF with function of macrophages. From the ascitic fluid of Ehrlich's tumour a dialysable factor was isolated which inhibited formation of pseudopodia and adhesion to the glass of L cells in tissue culture [14]. Perhaps the same factor is operating also *in vivo*, in mice injected with EAF, by preventing the contact of macrophages and antigen (SRBC) with consecutively inhibited formation of humoral antibodies.

Futhermore, depressed reactivity against sheep erythrocytes may be the consequence of a process of antigen competition. In the spleens of mice with Ehrlich ascites tumour, enhanced formation of germinal centers was registered and interpreted as indicating an immunological response of the host to tumour antigens [15]. However, antigenicity of Ehrlich tumour is relatively feeble [16], so it is hard to believe that these antigens could have competed with more stronger antigens in sheep erythrocytes. Development of new germinal centers may partly explain augmentation of the number of splenocytes in mice injected with EAF, but another factor which probably contributed more to this increase of the cellularity was the proliferation of immunologically inactive cells, presumably haematopoietic cells from the red pulp. It was already shown that Ehrlich tumour [15], as well as other tumours, can stimulate haematopoiesis in the spleen [17].

Normal GVH reactivity of spleen cells in our experimental mice injected with EAF disagree with the observation that the rejection of allogeneic skin grafts is retarded in mice bearing Ehrlich ascites tumour or treated with cell-free ascitic fluid [2, 4]. This discrepancy may be caused by differences in the test used and by different administration schedules of effective agent(s). In GVH assay the cells to be tested were removed from the animals inoculated with EAF and their reactivity was evaluated in normal recipients. The rejection of skin grafts, on the other hand, was determined in mice with growing tumour, or in those treated with daily injections of EAF, thus in animals under the continuous influence of the tumour or ascitic fluid. Our pilot experiments with skin grafting in mice after one injection of EAF showed no changes in the rejection time in comparison to controls. Perhaps, to inhibit cellular immunological reactivity, longer influence or greater quantities of active factor(s) are required, than to inhibit humoral immunological response.

Injection of the ascitic fluid from reticulosarcoma-bearing mice caused very heterogenous results. In any case, if in RAF similar immunosuppressive agent(s) are present like in EAF, they are less potent, or less concentrated, than in ascites fluid formed during the growth of Ehrlich tumour. One speculation can be given at this point: reticulosarcoma is a strain specific tumour, while Ehrlich tumour grow more or less unrestricted in all strains of mice. This is feasible, perhaps, just because of strong immunosuppression related with this tumour.

On the ground of results presented we can discuss the possible mechanism of EAF influence on the immunological reactivity and can only guess about the causative agent(s). Very strong suppressive effect, visible early after inoculation of EAF, long duration of this effect, and the increase in the weights and cellularity in the spleens, hints at viruses as causative agents. Infection of mice with oncogenic [18, 19] and non-oncogenic [20] viruses may lead to very similar changes in the response of recipient splenocytes to SRBC. Since the ascitic fluid used in our experiments cannot induce the malignancy, immunosuppression observed could be ascribed to the action of non-oncogenic viruses. At this moment, however, this is only a speculative statement.

Acknowledgement—We acknowledge the constructive discussions with Dr. M. Boranic, and greatly appreciate the technical assistance of Mrs. Lj. Bošković and Mrs. J. Miholjević.

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Effect of Neocarzinostatin on V-79 Chinese Hamster Cells *in vitro**

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Abstract—*Chinese hamster cells V-79 were treated with neocarzinostatin. The survival curve was made up of two components, the first with D_0 of 0·025 μg/ml and the second with D_0 of 0·05 μg/ml. When a correction for cell multiplicity is made, the first component was an exponential dose relation curve. The dose fractionation for 1-hr intervals and 6-hr intervals did not increase the survival. This illustrates that recovery of the Elkind type did not exist for neocarzinostatin.*

INTRODUCTION

RADIATION–drug interaction is of considerable interest because evidence of a clear interaction between drug and radiation may show the way to enhance the possibility of curing certain cancers. Elkind and Sakamoto [1] suggested that interaction between radiation and drugs was unlikely when the drug gave an exponential dose response curve [1, 2], e.g., the survival curve of Chinese hamster cells treated with nitrogen mustard is exponential and this system does not show the drug–radiation interaction.

Neocarzinostatin is an anti-tumour drug recently developed in Japan. It is an acidic protein with a molecular weight of about 9000 [3], whose chemical characteristics have been described in detail by Maeda and Ishida [4]. Neocarzinostatin has been found to have a strong anti-tumour activity against several tumours, such as Ehrlich ascites tumour [5], murine ascitic leukemia SN–6 [6] and L1210 murine leukaemia [7]. The drug has also been tried clinically. Its mode of anti-tumour action is only partially understood. The present paper describes the dose–response relationship using V–79 Chinese hamster cells and to examine whether there is recovery from sub-lethal damage from this drug.

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MATERIAL AND METHODS

As the experimental system used is essentially the same as described by Elkind *et al.* [8, 9], it will only be presented briefly.

Cells and their cultivation

A number of subclones of Chinese hamster cells designated originally V-79-1 by Elkind have been used. The doubling time of these cells when grown on glass surfaces is 10–11 hr. They have a fibroblast-like morphology, and produce colonies of about 2 mm in diameter in 9–10 days. Colony-forming ability was used as a measure of the cells' survival *in vitro*.

Using a modified Eagle's medium [10] with the addition of 15% foetal calf serum (EM-15), cells plated in 6 cm. Petri dishes were incubated overnight in a CO_2 incubator before an experiment was started. This procedure was used to help insure that the distribution of cells throughout their growth cycle (i.e. the age-density distribution) was close to that resulting from asynchronous, log-phase growth and hence fairly reproducible from experiment to experiment. During this overnight growth period, initially single cells formed micro-colonies, on average about three cells; consequently the experiments began with populations of micro-colonies rather than single cells.

Following a given treatment sequence, cells were incubated long enough for a maximum yield of colonies. They were then stained with methylene blue and counted. Results are expressed as the fraction of cells surviving a given treatment relative to the number in the initial suspension plated, which were able to form colonies. (The percentage of colony formers in the starting suspension is designated the plating efficiency, P.E.) In the results, $\bar{N}$ = the average multiplicity at the start of the experiment and was determined, in each case, from measurements of the increase in the number of colonies obtained when the cells in microcolonies were plated after trypsinization. Beyond surviving fraction of 0·5, the effect of multiplicity is simply to shift survival upward by $\bar{N}$ [11].

Neocarzinostatin treatment

The stock solution of neocarzinostatin was prepared at a concentration of 0·5 mg/ml in saline and kept frozen between experiments. For use, stock solution was diluted in Eagle's medium with 15% foetal calf serum to 10 times higher than the final concentrations required. They were kept on ice, and neocarzinostatin treatment was made by adding 0·5 ml aliquots of an appropriate drug solution to 4·5 ml of medium at 37°C for 20 min. In preliminary experiments, colony forming ability of Chinese hamster cells incubated with neocarzinostatin was statistically similar whether the treatment was for 20 min, 30 min, 1 or 2 hr. A treatment time of 20 min was used throughout the rest of the experiments. At the end of the treatment, neocarzinostatin containing medium was removed with a pipette, the cells were rinsed once with buffer and supplied with fresh medium at 37°C and incubated to observe the eventual colony formation.

RESULTS

Figure 1 shows the survival curve of the subclone V-79-B1M to single dose treatments with neocarzinostatin. The curve has two exponential components from a surviving fraction of 0·5 and this exponential to a surviving fraction of about 0·01. D_0 of the first exponential portion is 0·025 μg and that of the second exponential portion is 0·05 μg. However, experiments started with micro-colonies on average 2·7 cells and the adjustment of this curve to that for single cells [11] seems to make it exponentially throughout. Therefore, the curve may result from single hit to kill inactivation in which case a cell may survive after any dose without having any sub-lethal damage relative to the end point.

If neocarzinostatin does not cause sub-lethal damage in Chinese hamster cells, the Elkind type recovery [8] may not be detected. Therefore, the evident existence of Elkind type recovery in neocarzinostatin damage on cells was studied. For this purpose, split dose experiments were performed for doses corresponding to the first exponential curve. The results are

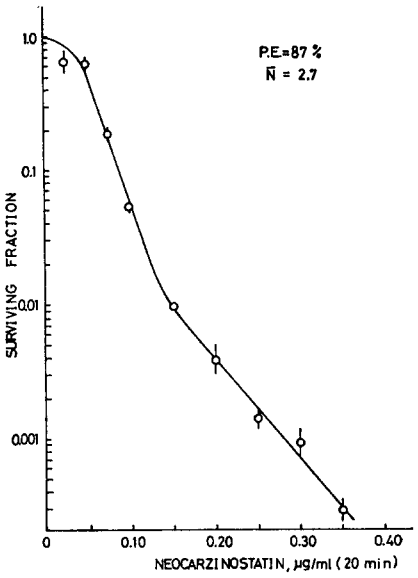

Fig. 1. The effect of treatment with neocarzinostatin on V-79 B1M Chinese hamster cells. P.E. = plating efficiency; and $\bar{N}$ is the multiplicity at the start of the experiment (standard errors are shown where the means of several points are plotted).

shown in Fig. 2 and 3. Though the points for survival for single dose treated cells in Fig. 2 are scattered compared with Fig. 1, the extrapolation numbers $\bar{N}$ and D_0 are almost the same as in Fig. 1. The survival of cells which were given a first dose of 0·05 μg/ml neocarzinostatin, followed after one hour by a graded dose of neocarzinostatin, is shown in Fig. 2. The cells were kept in 37°C CO_2 incubator during the two doses. These responses are also scattered, the Elkind type recovery seems not to be demonstrated in this figure.

In another experiment, the interval between the first and second doses was 6 hr. The result is shown in Fig. 3. Although the points do not fall on the regression, Elkind type recovery was not demonstrated in this experiment. Hence, it is strongly suggested that cells surviving neocarzinostatin treatment have not sustained sub-lethal damage, as there was no evidence of a repair type response.

DISCUSSION

The effect of neocarzinostatin on Chinese hamster cells is similar to that of nitrogen mustard [2]. The response to nitrogen mustard has also shown an exponential survival curve. However, neocarzinostatin-dose–response curve bends at the surviving fraction of around 0·01. This may suggest that the cells surviving after neocarzinostatin treatment of more than 0·15 μg/ml may be more resistant than the population as a whole. In other words, Chinese hamster cells V-79-B1M used in these experiments may consist of two populations with different sensitivities to neocarzinostatin. Another possibility is that the drug was too concentrated to kill cells proportionally to the dose level when it was 0·15 μg/ml, some of the drug put into medium might be wasted, that is, to kill a cell requires a given dose and at this dose level cells die proportionally depending on doses given, but, if the dose exceeds the level, some of the drug may not contribute to the cell killing. The bending of survival curve is also seen in survival curves after bleomycin treatment [12], the dose response curve of bleomycin on mouse L cells shows the upward-concave and is followed by exponential portion from a surviving fraction of 0·2. Terasima *et al.* [12] considered the reason for bending of survival curve to be the induction of resistance by the antibiotic, however we have no evidence of this phenomenon in our experiment. Further investigations will be needed to find out if resistance to neocarzinostatin occurs.

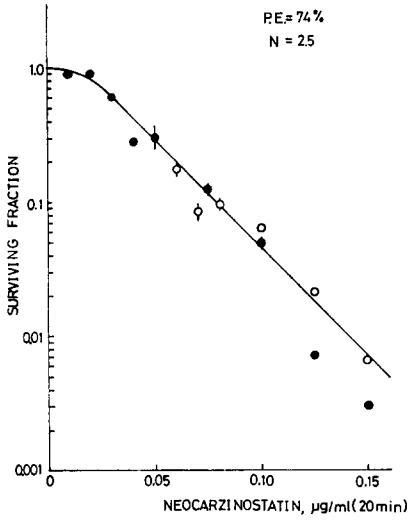

Fig. 2. Survival curves of Chinese hamster cells V-79-B1M after single and double treatments with neocarzinostatin.

●: *Survival of cells treated with the drug at various concentrations for 20 min.*

o: *Survival of the cells which were treated first with the drug at 0·05 μg/ml for 20 min and followed by a second treatment for 20 min with the drug of various concentrations 1 hr after the first treatment.*

The surviving fraction was the ratio of plating efficiency of a treated cell over that of the non-treated control. The survival curves were always constructed by plotting survival against total dose of the drug used.

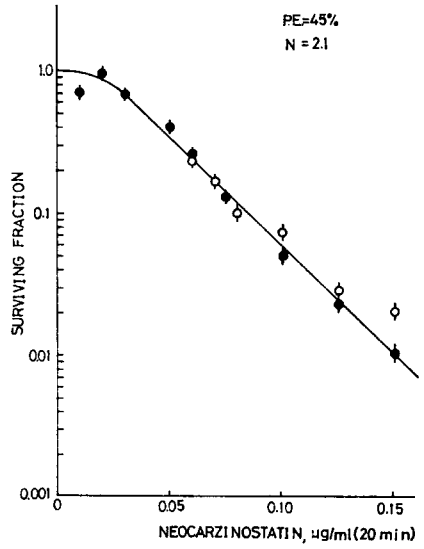

Fig. 3. Survival curves of V-79-B1M Chinese hamster cells after single and double treatments with neocarzinostatin.

●: *Survival of the cells treated with the drug of various concentrations for 20 min.*

o: *Survival of the cells which were treated first with a constant dose (0·05 μg/ml) of the drug then by various additional doses 6 hr after the first treatment.*

The surviving fraction was calculated by the same method as in Fig. 2.

In previous experiments, we did not detect the interaction between nitrogen mustard and X-rays [2]. Nitrogen mustard caused an exponential survival curve and no recovery, suggesting it acts on cells by a single hit to kill the mechanism, without causing sub-lethal damage. Neocarzinostatin shows the same shape of the survival curve as in nitrogen mustard. Therefore, the possibility of a neocarzinostatin and radiation interaction does not seem likely.

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Sinus Histiocytosis with Massive Lymphadenopathy—Properties of Cultured Histiocytes

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Abstract—*This paper describes the properties of cultured histiocytes derived from lymph node material of a patient with sinus histiocytosis. Before culture the cytoplasm of the histiocytes contained numerous leucocytes, residual bodies and large spherical membrane-bound inclusions of moderate electron density. After prolonged culture, these features disappeared from the cytoplasm. However, when buffy coat cells from a normal individual were brought in contact with the cultured histiocytes, they were phagocytozed and the residual bodies and the large spherical electron dense bodies then reappeared in the cytoplasm of the histiocytes.*

INTRODUCTION

SINUS histiocytosis is a benign disease of man which has only recently been recognized as a definite entity [1, 2]. The clinical course of the disease is prolonged, and the affected individuals suffer from fever, massive lymphadenopathy, especially of the cervical region, and leukocytosis. Pathologically, the disease is characterized by a massive enlargement of the lymph nodes associated with a proliferation of histiocytes in the distended medullary and subcapsular sinuses. The histiocytes contain in their cytoplasm a variable number of apparently phagocytosed cells amongst which lymphocytes are most frequent. The aetiology of the disease is not known.

In this report we describe the properties of the cultured histiocytes from a young patient presenting the typical clinical and pathological picture of sinus histiocytosis.

MATERIAL AND METHODS

Case report

The patient was a 14-year-old Caucasian male who presented with a generalized lymphadenopathy of at least 3 years duration. Recent cervical node biopsy showed the typical histological features of sinus histiocytosis (Fig. 1) as described by Rosai and Dorfman [2, 3] and this tissue was used for the studies described in this paper.

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Cell culture

A tissue biopsy from an enlarged cervical lymph node was collected under sterile conditions. A cell suspension was prepared by pressing the tissue through a sterile stainless steel mesh. The cells were then suspended in Ham's F-10 medium and washed once. The pellet of cells was divided into equal parts; one of these was suspended in Ham's medium supplemented with 10% tryptose broth and 20% foetal bovine serum. The other was suspended in RPMI-1640 medium containing 20% foetal bovine serum. Streptomycin (100 μg/ml) and penicillin (100 i.u./ml) were added to both cultures. Both sets of cultures were maintained in glass vessels at 36·8°C in a gassing incubator containing 5% CO_2 in air; the medium was changed every five days. Samples of the cells which grew in suspension were taken first at four weeks and again after ten weeks, stained with Leishman and Giemsa and incubated for acid phosphatase activity (4).

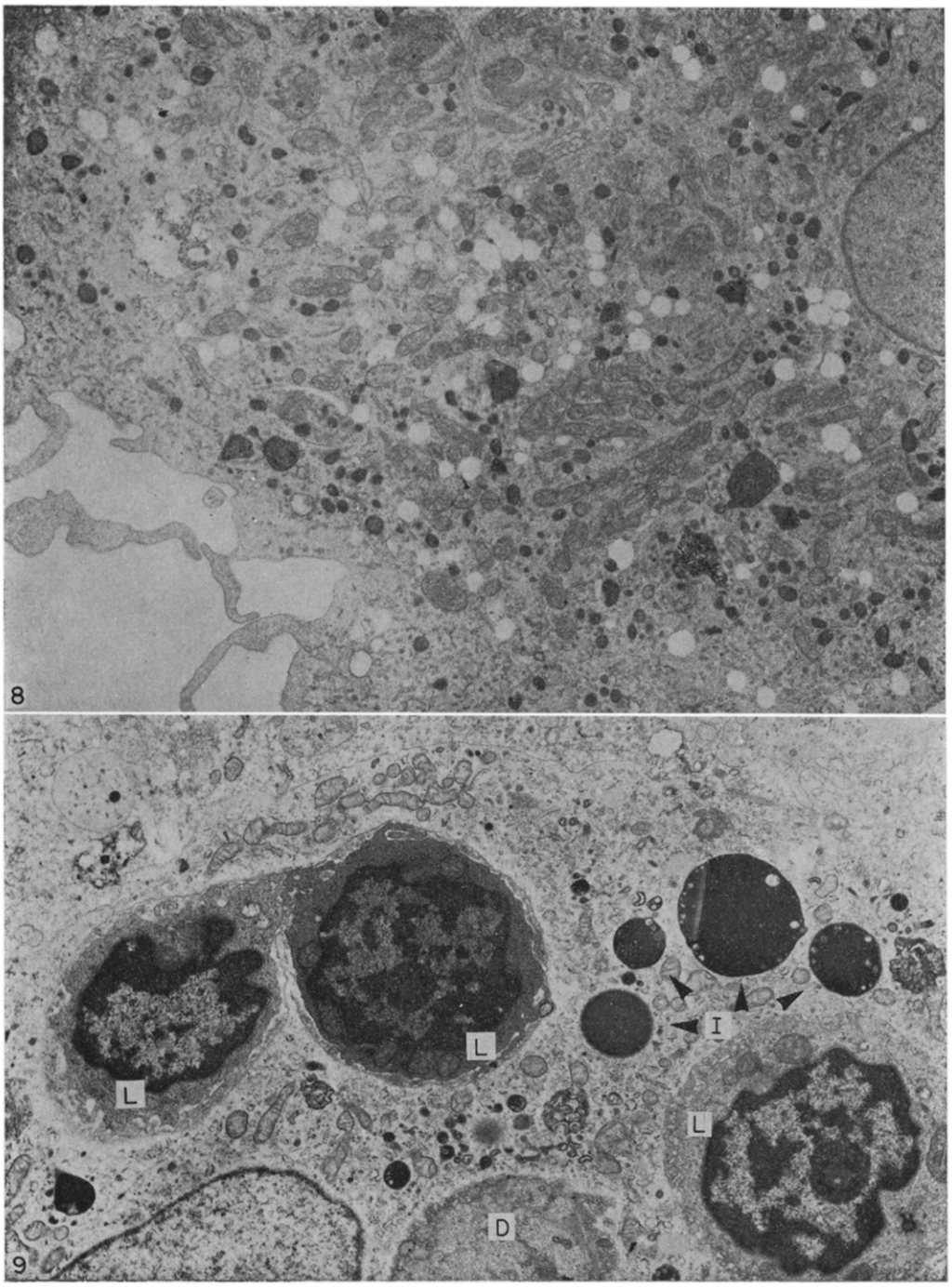
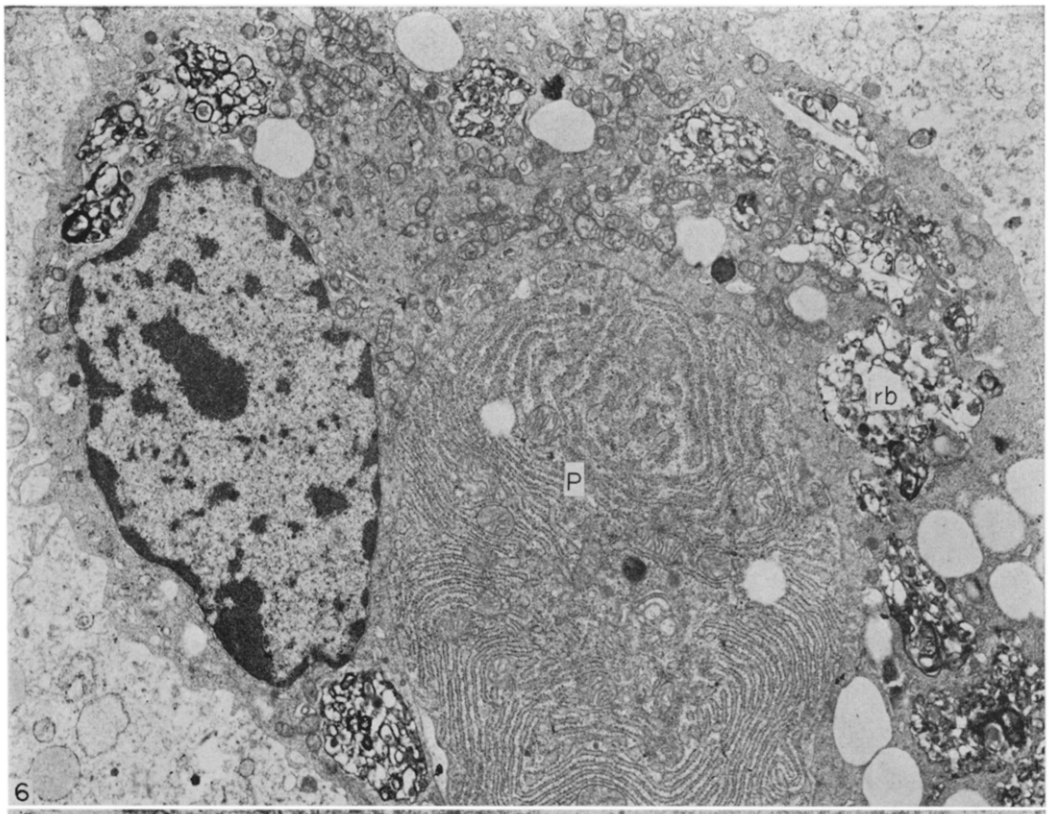


Fig. 8. *Histiocyte cultured for* 10 *weeks. The cytoplasm of this cell contains numerous lysosomal granules. However, no phagocytosed cells are seen and residual bodies are markedly fewer and smaller in size than in the histiocytes of the biopsy specimen. No round membrane-bound structures like that illustrated in Fig.* 7 (*I*) *are present.* (× 6000).

Fig. 9. *Cultured histiocyte after incubation with normal buffy coat cells for* 15 *hr. The cytoplasm contains three intact lymphocytes* (*L*), *a partially digested cell* (*D*) *and four rounded membrane-bound inclusions* (*I*). (× 6000).



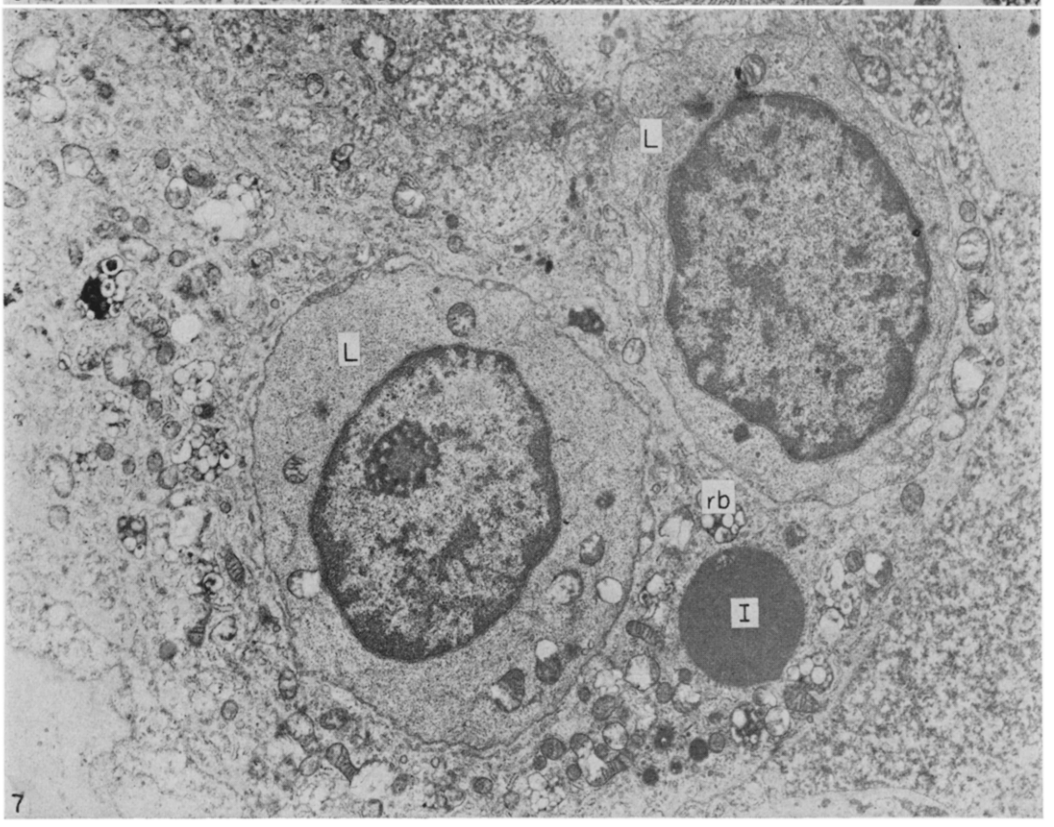


Fig. 6. *Lymph node biopsy. The cytoplasm of this phagocytic cell contains a plasma cell (P), numerous prominent osmiophilic residual bodies (e.g. rb) of variable size and shape, and numerous scattered vacuoles and mitochondria. The eccentric nucleus contains moderate peripheral chromatin condensation and a possible nucleolus.* × 12,500.

Fig. 7. *Lymph node biopsy. The cytoplasm of this histiocyte contains two intact lymphocytes (L) and a rounded membrane-bound inclusion (I). A few residual bodies (e.g. rb) are seen together with scattered strands of rough endoplasmic reticulum and abundant mitochondria. The mitochondrial ballooning is probably the result of sub-optimal fixation. No histiocyte nucleus is seen in this thin section.* (× 12,500)

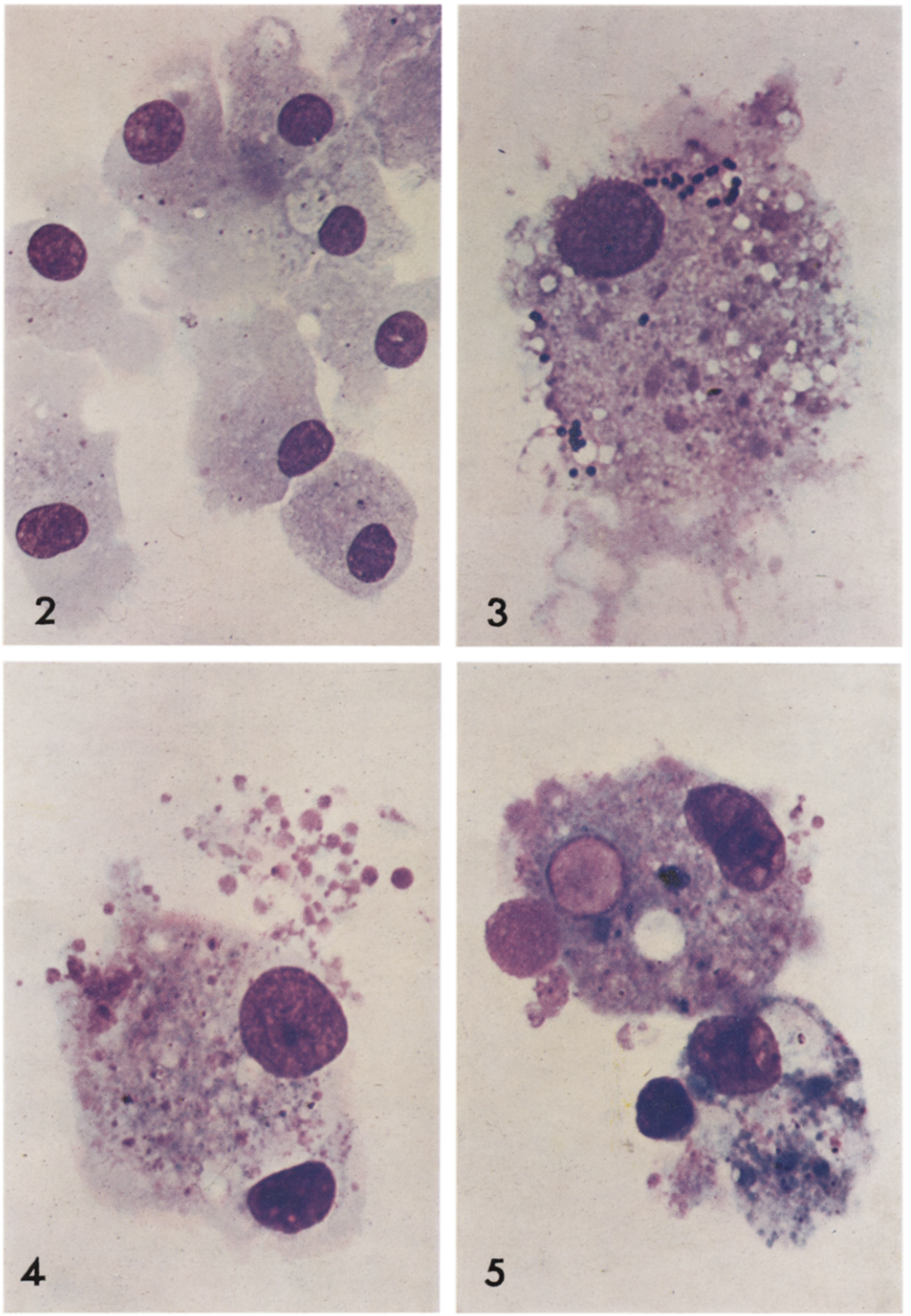


Fig. 2. *Leishman-stained spread of histiocytes cultured for* 4 *weeks. All cells have abundant cytoplasm and an eccentric nucleus.*

Fig. 3. *Geimsa-stained spread of cultured histiocytes exposed to* Streptococcus faecalis. *The cytoplasm of the cells appears highly vacuolated and contains numerous cocci.*

Fig. 4. *Leishman-stained smear of histiocytes cultured for four weeks and exposed to normal buffy coat cells for* 15 *hr. The cytoplasm is vacuolated and contains several platelets. A number of platelets are also seen extra-cellularly. Lymphocyte present in the lower part of the field may be about to be ingested by the histiocyte.*

Fig. 5. *Leishman-stained spread of histiocytes cultured for ten weeks and incubated with buffy coat cells of a normal individual for* 30 *hr. Both histiocytes appeared vacuolated. The upper cell contains a partially digested mononuclear cell* (↑).

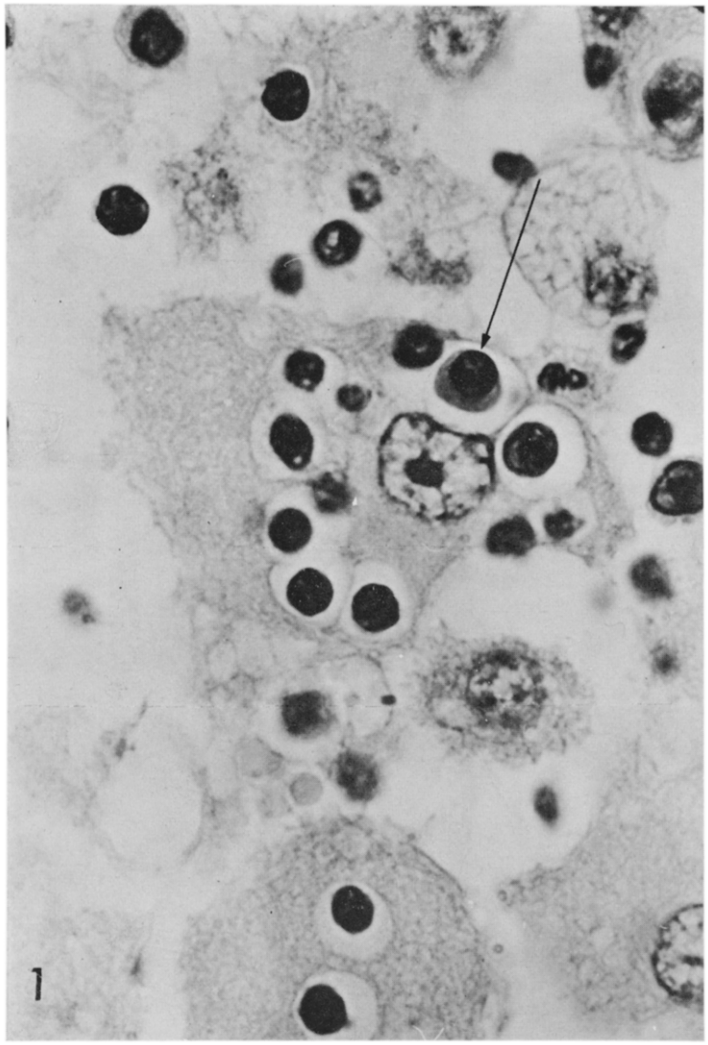


Fig. 1. *Paraffin section of the lymph node stained with H & E. The large histiocytes containing several lymphocytes and a probable plasma cell* (↑).

(to face p. 730)

Tests for phagocytic activity of cultured cells

Histiocytes were taken from the culture flasks and placed in three tubes at 37°C; two of these contained normal human buffy coat cells, while the third tube contained a suspension of of *Streptococcus faecalis*. After incubation with the Streptococcal suspension for 40 min, the histiocytes were spread on a glass slide, fixed, and stained with Giemsa. The two tubes containing histiocytes and buffy coat cells were incubated for 15 and 30 hr respectively before fixation. Some were then stained with Giemsa, others for acid phosphatase activity.

These experiments were carried out after four weeks culture and repeated after ten weeks.

Electron microscopy technique

Part of the biopsied material was passed through a stainless steel mesh to produce small clumps of tissue which were fixed for 1 hr at room temperature in 3% glutaraldehyde in 0·1M cacodylate–HCl buffer (pH 7·4). Subsequent processing for electron microscopy was carried out as previously described [5]. Cells cultured for 10 weeks were fixed both before and after incubation with buffy coat cells, and processed in the same way as the biopsy material.

RESULTS

Cell cultures

A mixed cell population grew out of the cell suspension prepared from the lymph node biopsy. In both the Ham's and RPMI cultures, fibroblastic cells were observed growing on the glass surface. Between the fibroblasts, refractile rounded cells of variable size and irregular outline could be seen. Similar refractile cells were also observed floating free in the culture fluid: these cells had a few coarse cytoplasmic processes of variable size.

Staining reactions

The cells grown in suspension closely resembled the histiocytes seen within the sinuses of the original lymph node. The majority were mononuclear (Fig. 2) but some contained more than one nucleus.

Although examination of the histiocytes before culture showed many lymphocytes within their cytoplasm, after the periods of four and ten weeks in culture, the cytoplasm was empty. Either this population was made up only of cells which had replicated *in vitro*, or there had been digestion and absorption of the intracytoplasmic cells. Indeed, both events may have taken place. Although dividing histiocytes are rarely seen *in vivo* in this disease, an occasional mitosis was observed among the cultured histiocytes. The slow rate of cell division explains why the histiocytes were always rather sparse in the cultures.

Histiocytes from the original biopsy contained strong coarse acid phosphatase positivity. The cultured histiocytes were also strongly acid phosphatase positive but this was of a more finely particulate nature; after incubation with buffy coat cells the acid phosphatase positively became more coarsely distributed and resembled that in the original biopsy.

Phagocytic activity of the cultured histiocytes

After incubation with *Streptococcus faecalis* for 40 min the cultured histiocytes contained many of the organisms in their cytoplasm (Fig. 3). The histiocytes incubated for 15 hr with buffy coat cells were found to contain many leucocytes and platelets (Fig. 4). Most of the phagocytosed cells appeared intact, suggesting that living cells had been ingested. This contrasted with the appearance seen after 30 hr incubation when the cytoplasm of the histiocytic cells was highly vacuolated and contained leucocytes in different stages of digestion (Fig. 5). These observations correlated well with the cytological picture of the original lymph node biopsy and indicate that, even after prolonged culture, the histiocytes are capable of phagocytosing leucocytes from a normal individual.

ULTRASTRUCTURE

Before culture

The histiocytes were very large and often contained eccentric nuclei with slight to moderate peripheral chromatin condensation, and one or more well-developed nucleoli.

The abundant cytoplasm contained numerous prominent osmiophilic inclusions of variable size and outline (Fig. 6); these probably represent residual bodies. The cytoplasm of many of the histiocytes contained leucocytes which, on many occasions, could be recognized as lymphocytes, and less frequently as plasma cells (Figs. 6 and 7). The cytoplasm also contained large spherical membrane-bound structures of moderate electron density (Fig. 7). These inclusions were usually of homogenous content but sometimes contained ill-defined rounded structures of variable electron density. The cytoplasm also contained abundant mito-

chondria and scattered short strands of rough endoplasmic reticulum.

After ten weeks culture

The cultured histiocytes broadly resembled those examined before culture. However, although numerous dense lysosomal granules were present, the number of dense osmiophilic residual bodies was markedly reduced, and only occasional such structures were observed. Furthermore, the spherical membrane-bound inclusions were rare in the cultured cells (Fig. 8).

Histiocytes incubated with normal buffy coat cells

The histiocytes which were incubated for 30 hr with buffy coat cells from a normal individual contained intact as well as partially digested leucocytes and platelets (Fig. 9). In addition, the cytoplasm of these cells contained numerous dense osmiophilic residual bodies, and the number of such inclusions appeared to resemble that seen in the histiocytes before culture. These cells also contained more spherical inclusions than the cultured histiocytes which had not been incubated with buffy coat cells.

DISCUSSION

Sinus histiocytosis is an apparently benign disease of extreme rarity. Since the original description of the condition as a separate entity by Rosai and Dorfman in 1969 [3], the same authors have published another paper summarizing studies of 30 further cases [2], over half of which have occurred in negro children. Our report deals with some of the characteristics of these bizarre histiocytes which were derived from a local case of the disease in a Caucasian patient. Even after culture, they retain the property of phagocytosing other cells and micro-organisms, in the absence of specific stimulation. The ability to engulf apparently living cells, not derived from the patient, suggests that the abnormality is not related to an impaired immune response. This impression is strengthened by the number of different cell types (plasma cells, neutrophils and erythrocytes as well as lymphocytes) that were recognized as having been phagocytosed in the original lymph node. The cause of this alteration in the histiocytes is not known. The finding of an occasional histiocyte in mitosis indicates that at least some of the cultured cells emerged *in vitro*.

Electron microscopy of the resting histiocytes in culture showed that many of the cytoplasmic vacuoles and osmiophilic residual bodies disappear (Fig. 8). However, when the cultured histiocytes were brought in contact with buffy coat cells derived from a normal individual, the vacuolization of their cytoplasm and the number of residual bodies increased, with the result that the cells resembled the histiocytes seen in the original biopsy. Although lysosomal granules persisted in large numbers throughout the culture period, most of the larger rounded spherical inclusions disappeared in culture, but re-appeared after the addition of buffy coat cells to the cultured histiocytes. Therefore it may be concluded that both the osmiophilic residual bodies and the large round spherical inclusions are closely associated with active phagocytosis and digestion.

So far, the culture studies have provided no clues about the aetiology of this condition. One would expect that if a micro-organism or microbial product were involved, such an organism might have been detected *in vitro*. Also if a specific antibody were involved in the pathogenesis of the disease its effect should have subsided in culture. Although no overt micro-organism could be detected in the histiocytes after prolonged *in vitro* culture, the possibility remains that a latent virus may be involved. A cellular integrated genome or part of a genome of such a virus could have induced the initial cellular changes manifested by an uncontrolled phagocytic activity.

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Combination Chemotherapy: The Interaction of Methotrexate and 5-Fluorouracil

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Abstract—*The combination of methotrexate and 5-fluorouracil was found to have less than additive effects against tumour cells growing* in vitro *and* in vivo. *Under some circumstances these drugs were mutually antagonistic, and the molecular basis for these observations has been defined. Methotrexate treatment caused an accumulation of cellular deoxyuridylic acid and this reduced the effectiveness of 5-fluorouracil. Conversely 5-fluorouracil reduced the blocking of purine biosynthesis caused by methotrexate. The implications of these findings are discussed in relation to the differential reversal of drug toxicity caused by thymidine and purines.*

INTRODUCTION

DRUG COMBINATIONS which include both methotrexate (MTX†) and 5-fluorouracil (5-FU) are used to treat patients with a variety of malignant tumours [1, 2, 3]. Combination drug schedules are numerous, and all have been derived empirically, frequently with little consideration of the possible biochemical consequences of administering drug mixtures. Greater knowledge of the mechanism of action of cytotoxic drugs should lead to a more rational approach to multiple drug therapy.

Factors which must be considered in attempting to understand the basis of interaction of drugs used in combination are the clinical pharmacology of the component drugs, the biochemical relationships of their respective target sites, and the temporal relationships of drug administration and metabolism. When two drugs acting on a common biochemical pathway are used together, the resulting disturbance of the control of that pathway makes the prediction of probable effects particularly difficult. MTX and 5-fluoro-deoxyuridylate (the active metabolite of 5-FU) form a sequential blockade [4, 5]. We have measured the extent to which these drugs interact *in vivo* and *in vitro* and have detected biochemical changes which may account for the results observed.

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†The abbreviations used are:

AdR	2′-deoxyadenosine
AICAR	5-amino-4-imidazolecarboxamide ribotide
$CH_2FH_4N^{5,10}$	methylene tetrahydrofolate
$CHFH_4N^{5,10}$	methenyl tetrahydrofolate
dUMP	deoxyuridylate
dUTP	deoxyuridine triphosphate
5FdUMP	5 fluorodeoxyuridylate
FH_2	dihydrofolate
FH_4	tetrahydrofolate
5FU	5 fluorouracil
GAR	glycinamide ribotide
10-HCO FH_4	10 formyl tetrahydrofolate
MTX	methotrexate
PCA	perchloric acid
TdR	thymidine
UdR	uridine

MATERIAL AND METHODS

Reagent chemicals were obtained from Hopkin and Williams Ltd., or from British Drug Houses, Ltd., AnalaR grades being used when available. Nucleosides and nucleotides were purchased from Boehringer, and tritium-labelled deoxyuridine (24Ci/mM) from the Radiochemical Centre, Amersham. Fischer's

medium for leukaemic cells of mice was supplied by Grand Island Biological Co., MTX and folinic acid (5-formyltetrahydrofolic acid) were purchased from Lederle, 5-methyltetrahydrofolic acid from Sigma Chemical Co., and 5-FU from Roche Products. 5, 10-Methylene tetrahydrofolic acid was prepared according to Dunlap *et al.* [6].

Cell culture

The line of L5178Y cells used in the *in vitro* culture experiments has been maintained in suspension culture for many years, and details of the culture conditions have been reported [7]. Cells were subcultured twice weekly and had a mean doubling time of 12 hr. The sensitivity of the cells to MTX and 5-FU used singly and in combination was assessed by growth experiments with drugs added to suspension cultures which had been established 24 hours previously at a cell concentration of 2×10^4 ml. Visual cell counts were performed daily, in duplicate, using a modified Fuchs–Rosenthal haemocytometer. The interaction of the two drugs was investigated by constructing both dose and response based isobolograms. using concentrations of the drugs which resulted in a 50% reduction in cell number, relative to untreated controls, 48 hr after drug addition. In some experiments MTX was added to the cultures 6 hr before 5-FU, and *vice versa*, to investigate whether the sequence of drug administration influenced the cytotoxicity.

In vivo *experiments*

The L1210 tumour was carried in ascites form in BDF1 mice by inoculation with about 10^6 cells. Treatment was started two days later, and comprised five successive daily intraperitoneal doses of either MTX or 5-FU, singly or in combination. The outcome of treatment was assessed by prolongation of survival of the treated animals.

Deoxyuridylate assays

Two methods were used to investigate the disturbance of deoxyuridine metabolism caused by methotrexate treatment.

(*a*) *Radioisotope incorporation.* ^{3}H-deoxyuridine (24Ci/mMol, 6μCi) was added to bottles containing 1×10^7 cells growing in 50 ml suspension cultures; simultaneously the cultures were treated with MTX 10^{-6} M, MTX 10^{-6} M plus folinic acid 4×10^{-5} M, or with an equal volume of physiological saline. Two hours later, the cells were washed twice in ice-cold phosphate buffered saline and collected by centrifugation. One millilitre of 60% methanol was added to the precipitate which was stored at −20°C overnight. The resultant suspension was centrifuged and the supernatant evaporated to dryness in a rotary evaporator at 35°C. Fifty microlitres of a 0·1 M solution of carrier was added and the distribution of label between various components was estimated following descending chromatography on Whatman No. 1 paper in two solvent systems: (*a*) isobutyric acid, 1N NH_4OH, 0·1M EDTA tetrasodium (50; 30; 1), (*b*) 0·1 M sodium phosphate buffer pH 6·8, NH_4SO_4, *n*-propanol (1,000 ml; 600 mg; 20). EDTA was then added to a final concentration of 0·001 M. Following their identification by examination in u.v. light, the spots were cut out and the radioactivity counted in liquid scintillant (toluene—1925 ml, dioxan—1925 ml, methanol—1150 ml, naphthalene—400 g, butyl —PBD 35 g). The precipitate, following the methanol extraction, was washed twice with cold 0·5 M PCA, and then hydrolysed at 85° in 0·5 M PCA for 20 min. Aliquots of this supernatant were counted following addition of scintillant.

(*b*) *Enzymic determination of dUMP.* dUMP was assayed directly by using it as the limiting substrate for thymidylate synthetase (EC. 2.1.1.b). A preparation of thymidylate synthetase obtained from a MTX-resistant strain of *L. casei* was purified as far as the Sephadex G. 100 stage [6]. Extracts of L5178Y cells were prepared as follows: 1·5 litres of cell culture in mid-logarithmic phase (3×10^5 cells/ml) were centrifuged, and the cell-pellet washed in phosphate buffered saline. Cells were resuspended in 2 ml 0·05 M tris HCl buffer pH 7·3 (containing 0·01 M mercaptoethanol) and disrupted by a 30-sec exposure on a MSE ultrasonicator (20 kHz). Extracts were deproteinized by placing in a boiling water bath for 10 min then centrifuged at 3,000 *g* for 10 min. The supernatant was used for the subsequent assay. Assays were performed in a Cary model 16K spectrophotometer as follows: To a cuvette were added 0·25 ml of 5,10 methylene-tetrahydrofolate solution (2×10^{-3} M), 0·3 ml 0·5 M tris. HCl buffer pH 7·3, and extract and water to 2·9 ml. Following equilibration, 0·1 ml of thymidylate synthetase solution (0·1 i.u./ml) was added. The reaction was allowed to go to completion at 30°C (about 10 min) and the overall optical density change at 340 nm was measured using a Varian G-2500 recorder set to give full scale deflection for an optical density change of 0·02. The optical density change after correction for the enzyme blank,

was proportional to the dUMP content of the extract for levels up to 200 nm/sample. As little as 0·5 nm can be measured. The standard curve showed an optical density change of 0·039 for 20 nm of dUMP.

RESULTS

Figure 1 shows the effect of different concentrations of MTX and 5FU on the growth of L5178Y cells in suspension culture. The dose-response curve of MTX is of the "threshold" type usually found for this drug, whereas that for 5-FU is of the exponential shape expected for an inhibitor of a rate-limiting enzyme. The ID_{50} concentrations, as here defined, were 6×10^{-8} M for MTX and 2×10^{-7} M for 5-FU. Drug interactions are conveniently demonstrated by constructing isobolograms [8]—diagrams showing the amount of one drug required to produce a stated effect (in this case, a 50% reduction in cell number 48 hr after drug addition—ID_{50}) in the presence of a range of concentrations of the other drug. The amounts of either drug are expressed as fractions of that concentration which alone produces the stated effect. These fractions can be expressed in one of two ways. The fraction can refer to a proportion of the ID_{50} dose, and we will call such diagrams Dose Isobolograms. Alternatively, the fraction can refer to that dose which gives a stated proportion of the ID_{50} response, and we will call such diagrams Respose Isobolograms. Thus, 0·5 on the MTX axis of a response isobologram means that concentration of MTX which gives a 25% reduction in cell number compared to the control 48 hr after drug addition (i.e., half the ID_{50} response). This dose is not necessarily the same as half the ID_{50} dose. Figure 2 shows dose and response isobolograms and, in each graph, the plot refers to combinations of the two drugs which resulted in 50% reduction in cell number 48 hr after drug addition. The broken line in the figures shows the form the plot would take if the two drugs were additive. From Fig. 2 it is clear that the combination of MTX and 5-FU produces less than additive effects. The sequence of drug administration did not influence the outcome of drug interaction, as can be seen in Fig. 2(a).

Figure 3 summarizes the effect of MTX and

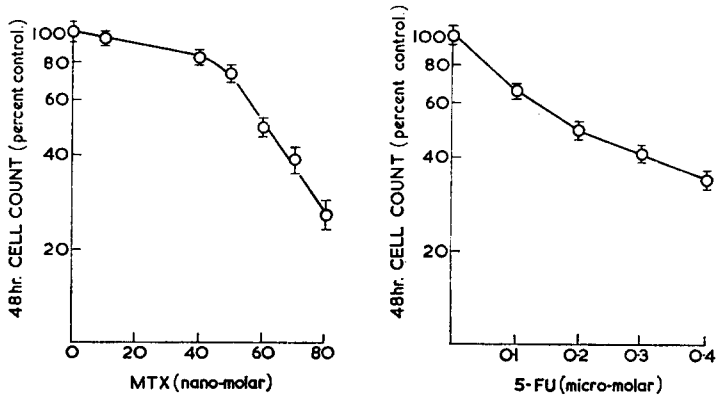

Fig. 1. MTX and 5-FU dose-response curves for L5178Y cells. Cells were grown in suspension culture as described in the methods section.

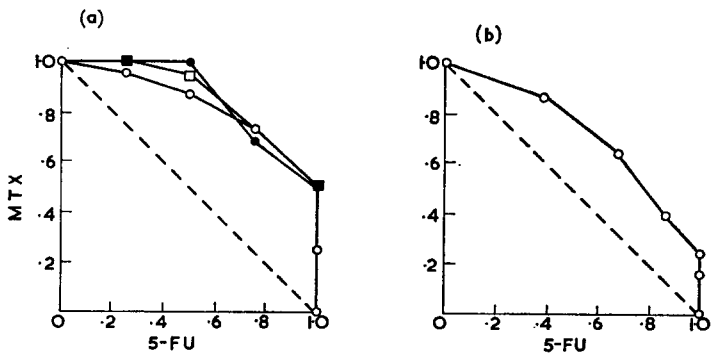

Fig. 2. Isobolograms for combinations of MTX and 5-FU. (a) Dose isobologram: —○— MTX and 5-FU added simultaneously —□— MTX added 6 hr before 5-FU —●— 5-FU added 6 hr before MTX. (b) Response isobologram MTX and 5-FU added simultaneously. The broken line in both figures shows the form the plot would take if the two drugs exerted additive effects. Experimental details are given in the Methods section, and interpretation of the plots is discussed in the text.

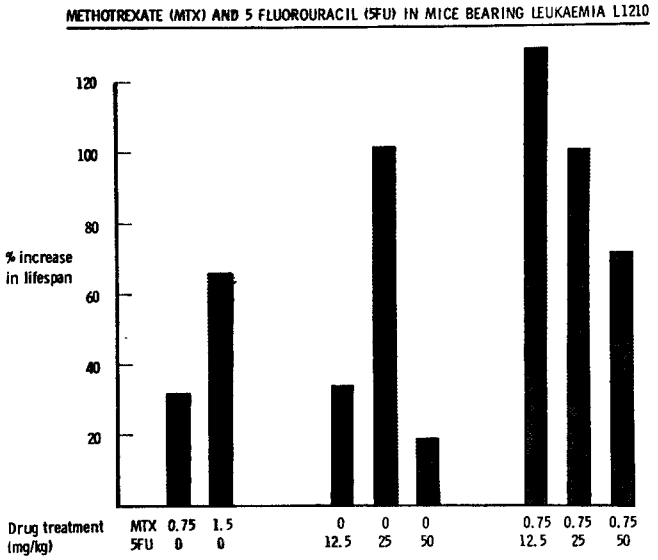

Fig. 3. The effects of combinations of MTX and 5-FU.

5-FU given daily intraperitoneally for five days to groups of five mice bearing the L1210 leukaemia in ascites form. The results show that the exact doses of the combination schedule determines whether the effects of treatment are the same or superior to treatment with only one of the drugs used in the combination. For instance, the combination of MTX 0·75 mg/kg with 5-FU 25 mg/kg does not produce any increase in survival on that achieved using 5-FU 25 mg/kg alone. However, the same dose of MTX in combination with 12·5 mg/kg of 5-FU does result in better than additive effects.

Figure 4 shows the sites of action of MTX and 5-FU on the thymidylate and purine biosyn-

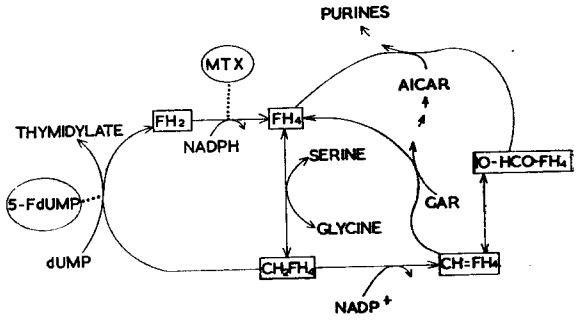

Fig. 4

thetic pathways. MTX is a tight-binding competitive inhibitor of dihydrofolate reductase [9, 10] and thus disturbs the methylation of deoxyuridine monophosphate (dUMP) by depleting the pool of 5,10 methylene tetrahydrofolate—the one-carbon group donor in this reaction. 5-FU, following its activation to 5-fluorodeoxyuridine monophosphate (5F-dUMP), is an inhibitor of thymidylate synthetase, competing with deoxyuridine monophosphate (dUMP) [11].

Table 1 shows the distribution, as determined chromatographically, of titium-labelled deoxyuridine 2 hr after the addition of MTX to cultures of L5178 cells. This treatment strikingly reduces the uptake of labelled UdR into

Table 1. ^{3}H-UdR uptake by L5178Y cells. Distribution of label 2 hr after addition of ^{3}H-UdR to 1×10^7 logarithmically growing cells simultaneously treated with MTX $10^{-6}M$, alone and folinic acid $4 \times 10^{-5}M$ and MTX $10^{-6}M$ together

Identity	Control (counts/min)	MTX (counts/min)	MTX+ Folinic acid (counts/min)
DNA	10285	714	3907
UdR	2130	4837	3757
dUMP	207	1100	306
dUTP	87	146	106
TMP	119	77	180
TTP	280	116	325
TdR	30	23	20

DNA. This inhibition is overcome to a great degree if folinic acid is added with the MTX. There is also an altered distribution of label among nucleotides in cells treated with MTX. The most obvious effect of methotrexate is to cause an accumulation of label in deoxyuridine monophosphate (dUMP). This build-up is not seen in cells rescued with folinic acid. There is a slight accumulation of radioactivity in deoxyuridine triphosphate (dUTP), and a reduction in the radioactivity in thymidine triphosphate (TTP) in MTX-treated cells. These effects are reduced in the presence of folinic acid.

Since results obtained from isotope incorporation studies may not necessarily be related directly to the total cellular content of a given

metabolite, it was desirable to confirm the changes in dUMP level by an independent method. The enzymic assay for dUMP showed that untreated logarithmically growing L5178Y cells contain 27 ± 5 nm dUMP/10^9 cells. Four hours after addition of MTX to the culture medium at a concentration of 5×10^{-7} M, the dUMP pool was increased to 164 nm/10^9 cells, and 9 hr after drug addition it had reached 212 nmole. (Values are means from three cultures). Thus MTX treatment caused a progressive increase in dUMP pools which reached 8× control values 9 hr after drug addition. These findings confirm the results of labelled UdR incorporation studies.

The thymidylate synthetase reaction causes oxidation of tetrahydrofolate cofactor to dihydrofolate (Fig. 4). Methotrexate, by blocking the regeneration of tetrahydrofolate, causes a depletion of cellular tetrahydrofolate pools [12] and thus a depression of *de novo* purine biosynthesis. Since 5-FdUMP inhibits thymidylate synthetase, and might therefore be expected to minimize the loss of cellular tetrahydrofolate which occurs in the presence of MTX, we have studied the effects of 5-FU on the antipurine activity of MTX. Figure 5(a) shows that thymidine alone does not rescue L5178Y cells from the effects of MTX.

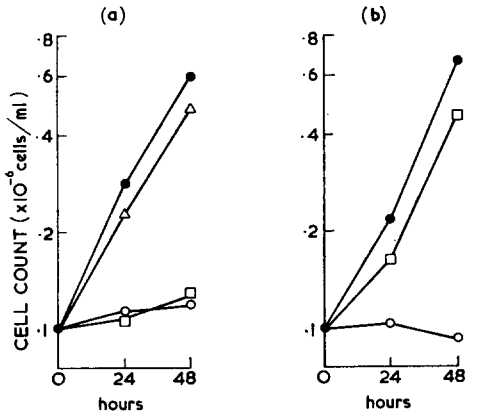

Fig. 5. Reversal of MTX and 5-FU toxicity by nucleosides. (a) MTX —●— control —○— MTX, $10^{-6}M$ —□— MTX, $10^{-6}M$ + TdR, $2 \times 10^{-5}M$ —△— MTX, $10^{-6}M$ + TdR, $2 \times 10^{-5}M$ + AdR, $2 \times 10^{-5}M$. (b) 5-FU —●— control —○— 5-FU, $1 \times 10^{-6}M$ —□— 5-FU, $1 \times 10^{-6}M$ + TdR, $2 \times 10^{-5}M$

However, thymidine and deoxyadenosine together will reverse the toxicity of MTX. In contrast, only thymidine is required to rescue cells from the effects of 5-FU (Fig. 5b). In the presence of excess thymidine, the toxicity of MTX may be attributed solely to its antipurine activity. The results of an L5178Y growth experiment are shown in Table 2. In the presence of thymidine, addition of 5-FU to cultures antagonises the toxic effects of MTX. These findings indicate that 5-FU protects cells from the antipurine effects of MTX.

Table 2. Interactions of MTX and 5-FU in the presence of nucleosides during in vitro *growth of L5178Y cells*

Culture	Drugs added	Nucleosides added	Cell count after 48 hr (× 10^6/ml)	Per cent control
1	—	—	0·685	(100)
2	MTX	—	0·048	7
3	MTX + 5-FU	—	0.055	8
4	MTX	TdR	0.075	11
5	MTX	TdR + AdR	0·640	93
6	MTX + 5-FU	TdR	0·295	43
7	MTX 5-FU (after 4 hr)	TdR	0·126	18
8	MTX F-FU (after 9 hr)	TdR	0·091	13

All cultures were initiated at $0{\cdot}1 \times 10^6$ cells/ml. Unless otherwise indicated drugs and nucleosides were added at zero time. MTX and 5-FU were used at 10^{-6}M; TdR and AdR were used at 2×10^{-5}M. Values quoted are means of triplicate cultures.

DISCUSSION

The belief that multiple drugs are always more effective than single drugs in the treatment of human malignant disease has arisen from the demonstration that certain multiple drug regimens have proved superior to single drugs in the treatment of a few human tumours [13, 14]. Recently, there have been a number of reports showing that combination chemotherapy in the treatment of some tumours may be less effective than treatment with one drug alone [15, 16]. Thus, it is clear that pure empiricism in the concoction of multiple drug regimens can have an uncertain outcome. A greater knowledge of the mechanisms of action and interaction of drugs used in cancer treatment will lead to more rational design of multiple drug therapy schedules, but until it is possible to individualise chemotherapy based on a knowledge of tumour cell sensitivity, it is likely that treatment for cancer patients will remain largely empirical.

The sites of action of MTX and 5-FU have been known for many years [9, 10, 11]. Each drug has been shown to have useful antitumour effects in a number of human malignant diseases, and recently there have been reports of combination chemotherapy regimens which include both drugs [1, 2, 3].

The *in vitro* and *in vivo* data shown in Figs. 2 and 3 indicate that MTX and 5-FU may have less than additive effects in certain circumstances. Dose additivity would only be expected of drugs which act at the same site, so it is important also to consider the degree of response additivity. Previous studies of the interactions of anti-tumour drugs have expressed results on a dose basis [8]. It should be noted that a "fractional inhibitory concentration", calculated on a response basis, will differ from the value calculated on a dose basis, unless the dose–response relationship for the system is linear. Figure 2(b) shows that the effects of MTX and 5-FU are infra-additive. 5F-dUMP inhibits thymidylate synthetase by competing for the dUMP binding site, whilst MTX reduces thymidylate synthetase activity by depleting the 5,10-methylene tetrahydrofolate pool. Thus, from considerations of enzyme kinetics it may be predicted that these drugs will have less than additive effects. In this particular case, the prediction of the outcome of combination treatment with MTX and 5-FU is made even more complicated by 5-FU having to be metabolized before it is an inhibitor of thymidylate synthesis. In addition to kinetic grounds for non-additivity of this drug combination, our studies suggest molecular bases for actual drug antagonism. Firstly, MTX treatment causes a build-up of dUMP (Table 1 and text). This has two possible consequences which may influence the cytoxicity of 5-FU. (*a*) The increased pool of dUMP will competitively reduce the inhibition of thymidylate synthetase caused by a given concentration of 5F-dUMP. For example, if the Km of dUMP for thymidylate synthetase is $6{\cdot}3 \times 10^{-6}$ M [17], the Ki for 5F-dUMP is $3{\cdot}1 \times 10^{-9}$ M [17], and the cellular enzyme concentration is 2×10^{-7} M when the cellular dUMP concentration is 27 nm/10^9 cells, a 5F-dUMP concentration of $1{\cdot}25 \times 10^{-7}$ M will result in 44% inhibition of thymidylate synthetase, as calculated from the Strauss–Goldstein equation [18]. An eight-fold elevation of this dUMP level, as found in the present study, will reduce the inhibition to 21%. Thus although 5F-dUMP is a tight binding inhibitor, the competitive effect of the elevated dUMP can be considerable. (*b*) Increased pools of dUMP in cells may have feed-back effects on enzymes which activate 5-FU. For example, it seems possible that high concentrations of dUMP may inhibit UdR kinase. We have previously suggested that this mechanism may cause a redirection of UdR towards deoxycytidine via UTP aminohydrolase [19]. Activation of this pathway may be responsible for the raised deoxycytidine triphosphate pools which have been observed in cells treated with MTX. The raised dUMP pool in MTX-treated cells explains why MTX may, in certain situations, antagonize the effects of 5-FU. Of course 5-FdUMP alone, by inhibiting thymidylate synthetase, might itself be expected to cause an accumulation of dUMP; how much greater this effect will be when MTX is also present cannot be predicted without more detailed knowledge of the control of dUMP production and utilization.

Data shown in Table 2 indicate that 5-FU antagonizes the antipurine effect of MTX. An explanation of the purine effects of 5-FU in combination with MTX is suggested by considering Fig. 4. The antipurine effects of MTX are caused by depleting cells of the reduced folate compounds which are needed for *de novo* nucleotide biosynthesis. In the presence of 5F-dUMP, the utilisation of 5,10-methylene-tetrahydrofolate for thymidine biosynthesis will be inhibited. Consequently, the accumulation of most of the cellular folate, as dihydrofolate, caused by MTX [12] will be prevented and purine biosynthesis will be maintained. In the presence of excess thymidine the toxicity of MTX is a consequence of its antipurine effect and thus under these circumstances 5-FU will antagonize the cytotoxic effect of MTX.

The studies reported here indicate that, under certain conditions, MTX may antagonize 5-FU and also that 5-FU may antagonize MTX. The combination is in most situations less than additive due to the sequential sites of action of these drugs. These experiments have concentrated principally on cellular aspects of the drug interactions, and it is recognized that in the greater complexity of the whole animal situation over-riding pharmacological considerations might still make this combination a useful one: nevertheless recognition of the possible causes of antagonisms of MTX and 5-FU indicate that caution must be exercised when using these drugs together to treat human malignant disease.

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The Cure and Concomitant Immunization of Mice Bearing Ehrlich Ascites Tumors by Treatment with an Antibody–Alkylating Agent Complex*

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Abstract—*BALB/c mice challenged with* 2×10^6 *Ehrlich ascites cells (EAC) and beginning the next day treated for* 4 *consecutive days with a heterologous (rabbit) anti-EAC antibody complexed with chlorambucil, are still alive after* 150 *days of observation and apparently tumor-free. On the other hand, all the controls developed fatal tumors with a mean survival time of* 17·6 *days. The treated mice were able to withstand a second challenge with the same lethal tumor dose which was given* 14 *days after the first challenge, this time without any follow-up treatment. All the corresponding controls died after a mean survival time of* 18·3 *days. Spleen cells* (6×10^7) *from treated mice adoptively transferred some degree of protection into normal mice challenged with* 5×10^4 *EAC, as expressed by the median survival time* (27 *vs* 23 *days*).

INTRODUCTION

IN THE absence of a specific action of antitumor drugs, one way to achieve specificity is to reach the tumor cells selectively. Carriers such as hormones and antibodies with specific affinity for the tumor cells have been suggested for this purpose [1]. Encouraging results have been obtained with hormones conjugated with nitrogen mustard [2] and with tumor specific antibodies or anti-fibrin antibodies carrying radioiodine [3, 4], chlorambucil [5, 6] or diphtheria toxin [7].

The beneficial therapeutic effects that were observed were considered to be due to the toxic action of the above mentioned substances on the tumor cells, the antibody serving merely as a vehicle for directing the drug to the target cells. Assuming that one cell in a million of transplanted tumor cells survives the cytotoxic action of the drug, which is more than likely, and knowing that two live tumor cells are sufficient to induce fatal tumors [8] as is the case with Ehrlich ascites cells (EAC), cures can be obtained in animals injected with millions of cells, only when the defense mechanism of the host can be promoted.

In order to verify whether an immune response is mounted by animals transplanted with EAC and treated with anti-EAC antibodies complexed with chlorambucil, we studied the resistance of these mice to a new challenge with the same tumor line and the possibility of conferring protection to untreated mice by adoptive and passive immunization.

MATERIAL AND METHODS

The EAC were kept by serial intraperitoneal (i.p.) passages using random-bred Swiss mice. The experimental animals were BALB/c male mice, weighing 20 ± 2 g at the beginning of the experiments. The EAC used in the experiments were obtained one week after i.p. passage.

The anti-EAC sera were prepared in rabbits as described by Lindner [9]. The γ-globulin

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fraction was isolated by three successive precipitations with $(NH_4)_2SO_4$ at 33·3% saturation. The dialyzed γ-globulin fraction was exhaustively absorbed with homogenates of liver, spleen, kidney and lung obtained from normal BALB/c mice. When tested by Ouchterlony's double diffusion the γ-globulin fraction before absorption gave two precipitin lines with the tissue homogenates tested as well as with the EAC homogenate. Following absorption no precipitin arcs were obtained with either. The cytotoxic titre of the absorbed γ-globulin was 1:256 (the concentration of the undiluted γ-globulin was 5 mg protein/ml). For some experiments the absorbed γ-globulin fraction was purified using EAC treated with glutaraldehyde as immunoadsorbent [10]. Chlorambucil (Sigma) was bound to the different antibody preparations as well as to a γ-globulin fraction isolated from normal rabbit serum, using the method described by Israels and Linford [11].

The cytotoxic activity of the rabbit antibody preparations and that of mouse sera was determined according to Kato *et al.* [12] by the trypan blue exclusion method.

Spleen cells and sera were obtained from normal mice and mice that had been injected 16 days previously with EAC and then treated with the complex γ-globulin anti-EAC-chlorambucil complex as described in Table 1. Spleen cell suspensions were prepared as described by Grohsman and Nowotny [13] and the adoptive immunization was performed by injecting 6×10^7 cells/mouse intravenously 3 hr after the intraperitoneal injection of 5×10^4 EAC. For passive immunization 0·25 ml serum were injected intravenously as above.

Protein estimations were made by ultraviolet spectrophotometry at 280 nm.

RESULTS AND DISCUSSION

From Table 1 it can be seen that all the mice treated with γ-globulin isolated from anti-EAC sera prepared in rabbits and complexed with chlorambucil, or antibodies specifically purified using EAC treated with glutaraldehyde as an immuno-adsorbent and complexed with chlorambucil, are still alive after 150 days of observation and apparently tumor-free, while all the controls developed fatal tumors with a mean survival time of 17·6 days. A small percentage of the mice treated with γ-globulin isolated from normal rabbit serum complexed with chlorambucil remained alive as well for a long period of time, but later all of them developed solid tumors and a haemorrhagic peritoneal effusion, and died about 70 days after tumor inoculation. One possible interpretation of the partial and transient protection conferred by the normal γ-globulin–chlorambucil complex is that malignant cells have a more pronounced ability to take up proteins from the medium than normal cells [14]. Antibodies to lactate dehydrogenase for example have been shown to be taken up and accumulate by some mouse and rabbit malignant cells but not by normal cells [15, 16]. On the other hand, one has to take into account that hydrophobic proteins, as γ-globulins, are known to slow down the hydrolysis of alkylating agents [17].

When the treated mice were rechallenged with EAC 14 days after the first inoculation of tumor cells, they were able to withstand the lethal tumor dose and were still alive after an additional 136 days of observation while all the controls died after a mean survival time of 18·3 days.

In order to answer the question whether the resistance is humoral or cellular in nature, adoptive immunization with spleen cells from "resistant" mice and passive immunization with the corresponding sera have been attempted. As can be seen in Table 2, some degree of protection as expressed by the median survival time has been achieved with spleen cells of "resistant" mice (27 vs 23 days). The finding is in line with the data reported by Warnatz *et al.* [18] and Sakashita [19] who showed that the transfer of lymphocytes from actively immunized mice is able to delay or suppress the growth of EAC. Since we did not have an adequate quantity of serum from "resistant" mice we have used only 5 mice to check the effect of passive immunization on the growth of EAC. Under the conditions employed not only was there no prolongation of the median survival time of the mice treated with "resistant" sera, but it proved to be shorter (21 vs 23 days). The difference however is not statistically significant due to the small number of animals used in the experiment. It is worthwhile to mention that the cytotoxicity of the "resistant" sera as tested *in vitro* was nil. Although we can venture a guess that the shorter median survival time was due to enhancement, no conclusion can be drawn at present on the basis of these data.

Since the adoptive immunization experiments showed that spleen cells from "resistant" mice are able to induce some degree of protection in normal mice and given the importance assigned to cell-mediated immunity (CMI) in the rejection of tumors one may assume that antibodies complexed with an alkylating agent can influence the balance between the humoral

Table 1. *Effect of treatment on survival and resistance to a new challenge*

Group No.	Description of the group	No. of mice*	Description of the treatment	Quantities per injection†	Interval between challenge and treatment (hr)	Interval between first and second challenge	Mean survival time ± SD	survivors total‡	*P* value§
1	Effect of treatment on survival	10	γ-globulin-anti-EAC--chlorambucil	2 mg γ-globulin 100 μg chlorambucil‖	24	—	—	10/10 (150)	0·01
2	Effect of treatment on survival	10	Specifically purified anti-EAC antibody-chlorambucil	20 μg antibody-56 μg chlorambucil	24	—	—	10/10 (150)	0·01
3	Effect of treatment on survival	10	γ-globulin of NRS-chlorambucil	2 mg γ-globulin 100 μg chlorambucil	24	—	22·0 ± 1·6 for 7 mice 70·0 ± 10 for 3 mice	—	—
4	Effect of treatment on survival	10	γ-globulin-anti-EAC- and separately chlorambucil**	2 mg γ-globulin and 100 μg chlorambucil	24	—	20·0 ± 1·4	—	—
5	Control for groups 1-4	10	—	—	—	—	17·6 ± 1·9	—	—
6	Resistance to a second challenge	10	γ-globulin-anti-EAC-chlorambucil	2 mg γ-globulin 100 μg chlorambucil	24	14 days ¶	—	10/10 (150)	0·01
7	Control for group 6	10	—	—	—	—	18·3 ± 2·7	—	—

*All the mice were inoculated intraperitoneally with 2×10^6 viable EAC.

†Intraperitoneal injections once daily for 4 days.

‡Figures in parentheses denote days of observation.

§Calculated according to Fisher's test.

‖The amount of chlorambucil added to the protein fraction.

¶No treatment was given after the second challenge.

**Within 5 minutes following the intraperitoneal injection of γ-globulin-anti-EAC, chlorambucil was injected by the same route.

Table 2. Adoptive transfer of tumor resistance

Group No.	No. of mice	Intravenous injection of	Survivors/total after 30 days of observation	Median survival time of the mice which died due to tumors (days)	Mean survival time ± SD
1	10	6×10^7 "immune" spleen cells	2/10	27·0	26·7 ± 2·05
2	10	6×10^7 normal spleen cells	1/10	23·0	23·0 ± 1·05
3	5	0·25 ml "immune" serum	0/5	21·0	20·4 ± 0·9
4	5	0·25 ml normal serum	0/5	23·0	22·8 ± 1·3
5	10	Controls	0/10	23·0	23·0 ± 0·95

response, which may lead to enhancing antibodies and CMI in favour of the latter. One possible mode of action is as follows; antigens of tumor cells which are released from live and/or damaged cells combine with the anti-tumor antibodies carrying the cytotoxic drug and are chemically modified by alkylation. The anti-tumor antibody can serve as an immunological carrier. The modified antigens can then act and produce an enhanced cell-mediated immune response with concomitant suppression of antibody formation as is the case with the acetoacetylated flagellin described by Parish [20].

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Prophylactic X-Irradiation of the Lung in Canine Tumours with Particular Reference to Osteosarcoma

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Abstract—*Following intravenous injection of canine osteosarcoma cells into immunosuppressed puppies one lung was X-irradiated. One lung only was also prophylactically X-irradiated* (1200 *rad*) *in* 2 *dogs with spontaneous primary melanomas in which the primary tumour had been ablated. In all these dogs less tumour grew on the X-irradiated side.*

In 20 *dogs with spontaneous osteosarcoma, differences between X-irradiated and non-irradiated lungs were nil or small. In* 2 *other dogs with spontaneous osteosarcomas and* 2 *with carcinomas, where the primary tumour was not completely destroyed, thoracic irradiation on one side only resulted in more tumour developing in the X-irradiated lung than in the non-irradiated lung.*

INTRODUCTION

OSTEOSARCOMAS and other malignant tumours, both in man and dog, metastasize to the lungs but at the time of initial examination less than 10% show radiographic evidence of this spread. The problem of how to kill these malignant cells in the lung before they become apparent radiographically or clinically is a major one in cancer research. At some hospitals human patients with osteosarcoma are receiving prophylactic lung X-irradiation and at others chemotherapy or immunotherapy.

We have carried out experiments on X-irradiation of one or both lungs in 21 dogs injected intravenously with a canine osteosarcoma cell-line [1].

In 27 other dogs with spontaneous malignant tumours, including 22 osteosarcomas, one lung only was X-irradiated. In one further case of osteosarcoma both lungs were X-irradiated.

TRANSPLANTED TUMOURS

Methods

New-born Beagle or mongrel puppies were immunosuppressed with antilymphocyte serum [1] using a dose of 3 ml per kg of body wt followed by 1·5 ml/kg of body wt 3 times weekly. Within 14 days of birth they were injected with canine osteosarcoma cells (O.S.2) grown in tissue culture, between 5×10^6–10^8 cells being injected slowly into the right or left jugular vein in a well dispersed suspension. Twenty-four hours later one or both sides of the thorax were X-irradiated with 600 rad (mid-plane dose) from a 230 kV radiotherapy machine. No correction for lung tissue was made. In 10 dogs further doses of 300 rad were given at weekly intervals to total doses of 900 rad (4 dogs), 1200 rad (3 dogs), 1500 rad (2 dogs) and 1800 rad (1 dog). In 2 dogs 600 rad X-irradiation was given to the left thorax 2 days after birth and osteosarcoma cells were injected intravenously 14 days later.

Euthanasia was carried out 16–35 days post tumour cell injection at which time radiographic signs of lung tumours were usually present. The left and right sides of the lungs were examined macroscopically, weighed and subjected to histological examination, together with lungs from control animals in the same litter which had received cells, but no irradiation.

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It was not usually possible to count the individual tumour nodules in these animals because of their large number.

Results

The weights of the left and right lungs in X-irradiated and control dogs are given in Table 1. The lower weight of the X-irradiated side is due to less tumour tissue as the reduced growth of the lung on this side is only minimal.

Table 1. Weight of lungs in immunosuppressed puppies injected i.v. 2–5 weeks previously with canine osteosarcama cells. In some of the puppies one or both lungs were X-irradiated

Dog No.	Dose X-irrad. (R)	Lung irradiated	Wt left lung (g)	Wt right lung (g)
1	600	R	28·0	17·0
2	600	R	37·2	20·8
3	600	R	22·0	12·0
4	600	R	11·8	11·0
5	900	L	15·3	23·6
6	900	L	14·2	53·0
7	900	Both	9·7	7·1
8	900	Both	9·9	8·1
9	1200	L	12·8	42·6
10	1200	Both	9·1	16·4
11	1200	Both	4·7	7·7
12	1500	Both	5·2	6·5
13	1500	Both	7·3	7·8
14	1800	L	14·0	40·5
15	Nil	Neither	33·0	29·5
16	Nil	Neither	27·0	28·8
17	Nil	Neither	10·9	15·7
18	Nil	Neither	71·0	49·0
19	Nil	Neither	25·9	37·8
20	600 before inj.	L	10·6	10·0
21	600 before inj.	L	12·2	9·1

Where one lung only was irradiated considerably less tumour tissue was found on this side compared with the control side in 7 out of 8 dogs. In the eighth dog tumour tissue grew poorly on both sides. In 6 dogs where both lungs were irradiated the weight of tumour tissue was much less than in the four control dogs, in the fifth control dog tumour grew poorly on both sides.

Anatomically similar areas of lung, from both right and left sides, were examined histologically to confirm the gross post-mortem appearance and weight differences. After only 600 rad, histological examination showed marked differences in the amount of tumour tissue present.

In the 2 puppies given X-irradiation to the left lung and injected intravenously 14 days later with osteosarcoma cells slightly more tumour was present in the X-irradiated than in the untreated lobes in each dog (Dogs 20, 21). The distribution of the tumours differed and many more tumours were present in the left apical lobe when compared with the right apical lobe (Fig. 1).

SPONTANEOUS TUMOURS

Methods

Osteosarcoma. Twenty-three dogs with spontaneous osteosarcomas were examined. The breeds of dog were: Retriever (6), Alsatian (5), Great Dane (3), St. Bernard (2), Bull Mastiff (2), Boxer (2), Pointer (1), Dalmatian (1), and Greyhound (1). The primary sites of osteosarcoma were: distal radius (12), proximal humerus (4), proximal tibia and fibula (2), distal humerus (1), distal tibia (3), scapula (1). Fourteen tumours were on the left side of the body and nine on the right. Biopsy was made in 11 dogs and post-morten examination performed on the 21 dogs now dead. Radiography of the chest showed no metastases when therapy was started.

In one dog the affected limb was amputated before radiotherapy was begun and, in 4 other dogs, X-irradiation up to 1500 rad was given as one dose followed by amputation a few days later.

In the other 17 dogs the primary tumour was X-irradiated using a 15 MeV Mullard linear accelerator giving 120–130 rad/min and with 100% penetration at 3 cm in tissue. Under Halothane anaesthesia 1000 rad midplane dose was given at weekly intervals to a total dose of 3,500 rad in one dog 4000 rad in 14 dogs and 5000 rad in 2 dogs. A few days after amputation or at the time of the first irradiation of the primary tumour, 600 rad midplane dose with no correction for lung tissue was given to the right thorax in 22 dogs and this dose was repeated one week later. In one of these dogs a further dose of 300 rad was given after a one week interval and one other dog received irradiation on both sides of the chest to a total dose of 1200 rad. The anatomy of the canine chest makes it more difficult to irradiate than the human chest and appropriate packing with tissue equivalent material was carried out before irradiation.

Following treatment of the primary tumour and X-irradiation of the right chest, dogs were examined clinically and radiographically at frequent intervals. When pain recurred at the primary site or other clinical illness developed euthanasia was performed. On post-mortem examination the lungs were inflated, radio-

graphed, fixed in formalin, sliced thinly and every grossly visible metastatic tumour nodule measured.

Melanoma. In a Springer Spaniel with a malignant melanoma of the left cheek the tumour was excised surgically, together with the left mandibular lymph node in which metastatic tumour was present, and both operation sites X-irradiated with 1000 rad at weekly intervals to a total of 3000 rad. The right thorax was X-irradiated (2 × 600 rad) as described for the osteosarcomas.

In a long haired Dachshund with a malignant melanoma of the right lip the tumour recurred after surgery. Further surgical excision was made together with the tumorous right submandibular lymph node. Radiotherapy of the operation sites and right thorax was then performed as in the case of the Springer Spaniel.

Adenocarcinoma. A poorly differentiated adenocarcinoma was excised from the right cheek of a Retriever and the operation site given 3000 rad (weekly intervals of 1000 rad). Because of tumour recurrence a further 1000 rad was given 8 weeks later. The left thorax received 2 × 600 rad with one week's interval.

Squamous cell carcinoma. In a crossbred dog with a squamous cell carcinoma of the right tonsil, the affected tonsil and the right submandibular lymph node, containing metastatic tumour, were excised and both sites given 3 × 1000 rad. The right thorax was given 2 × 600 rad with one week's interval. At the time of X-irradiation of the thorax in the above 4 dogs there were no radiographic signs of metastasis.

RESULTS

Osteosarcomas

Amputated. At periods, varying between 4–11 months post-amputation euthanasia was performed in 5 dogs. The number and volume of metastases are shown in Table 2. Dogs 1, 2 and 3 had approximately equal numbers of metastases and tumour volume on each side. In Dog 4 only 2 ml of tumour tissue was found on the irradiated side compared with a tumour volume of 31 ml on the non-irradiated side. In Dog 5 one metastasis of large volume occurred on the X-irradiated side, while on the non-irradiated side six very much smaller metastases were present. One Alsatian is still alive 46 weeks after amputation.

Table 2. Lung metastases in spontaneous osteosarcoma of dogs in which the tumour bearing limb was amputated and the right lung X-irradiated

Dog No.	Right lung (X-irradiated) No.	Right lung (X-irradiated) Vol. (ml)	Left lung (non-irradiated) No.	Left lung (non-irradiated) Vol. (ml)	Survival time in weeks
1	1	1·0	0	0	49
2	5	10·1	4	6·0	13
3	309	82	255	74	13
4	3	2	8	31	21
5	1	15·7	6	4·5	18

X-irradiated and left in situ. Within 2 days of the first dose of X-irradiation to the primary tumour pain was considerably decreased and lameness improved. Except in two very advanced cases the tumour showed some slow regression and in particular a reduction in periosteal reaction was seen radiographically. Using a dose up to 5000 rad, given in 1000 rad weekly doses, there was loss of hair but no skin ulceration, pruritis or secondary infection. Pain-free survival time varied between 1 month and 1 year with an average of 4 months. Of 17 dogs treated euthanasia was performed in 14 because of recurrence of pain at the primary site and in one dog because of metastases in the lungs. One dog died with tumour in the myocardium and 1 dog is still alive. In the 16 dogs now dead no metastases were found in 5 animals, one of which was the animal surviving for a year.

In the one dog where X-irradiation was given to both lungs 9 metastases with a volume of 6·4 ml were found in the right lung and 4 metastases with a volume of 3·6 ml in the left lung. The number of metastases and volume of tumour for the remaining 10 dogs are shown in Table 3.

Table 3. Lung metastases in spontaneous osteosarcoma of dogs in which the primary tumour and the right lung were X-irradiated

Dog	Right lung (X-irradiated) No.	Right lung (X-irradiated) Vol.	Left lung (non-irradiated) No.	Left lung (non-irradiated) Vol.	Survival time in weeks
1	1	2·1	1	0·1	17
2	3	14·8	0	0	26
3	4	1·1	4	0·9	13
4	8	1·0	4	0·3	12
5	18	22·7	15	9	13
6	41	46·8	42	35·8	22
7	144	7·3	165	6·4	12
8	13	9·6	2	2	30
9	16	32·0	7	9·2	20
10	200	9·2	67	5·6	9

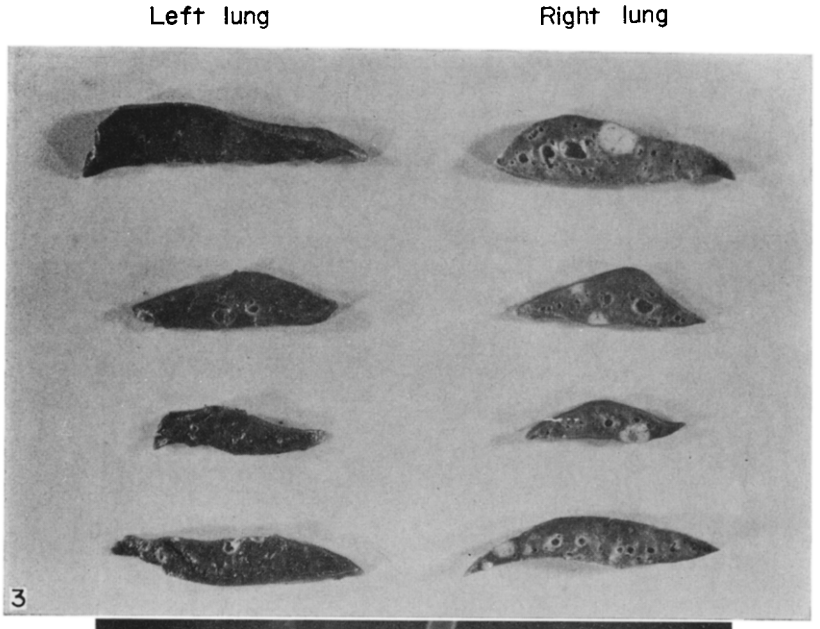

Fig. 3. Metastases in the left lung from a spontaneous tonsillar carcinoma. This left side was X-irradiated at a time when no metastases were radiographically visible. No metastases were found in the right (non-irradiated) lung.

Fig. 4. Metastases from a spontaneous osteosarcoma. Three times as many small metastases occurred on the right (X-irradiated) side than the left (non-irradiated) side.

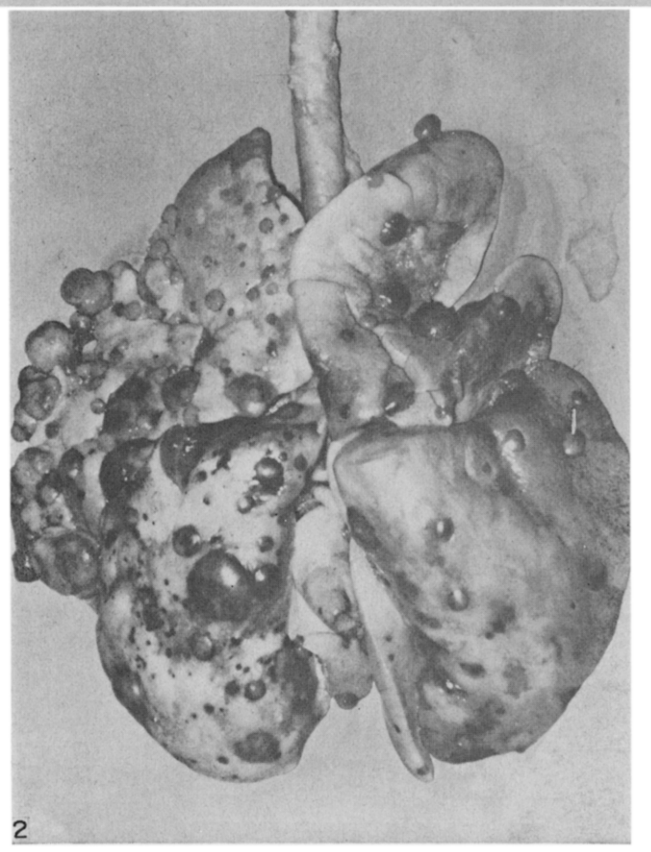

Fig. 1. *Osteosarcoma growing more in the left apical lobe than the right apical. The left lungs were X-irradiated* 24 *hours before the i.v. injection of cells.*

Fig. 2. *Metastases from spontaneous melanoma. The right lobes were prophylactically X-irradiated and there is less tumour on this side.*

(to face p. 750

In 7 dogs there were more metastases on the right (X-irradiated) side than on the normal side and, in all 10 animals, the volume of tumour was greater on the X-irradiated side. While for most dogs the figures for the two sides are not significantly different, in one dog (No. 9) more than three times the volume of tumour occurred on the X-irradiated side than the normal side and in another dog (No. 10) three times as many small metastases were present on the irradiated side (Fig. 4).

Table 4 shows the number and volume of lung metastases in 10 dogs receiving no X-irradiation. The number of metastases in right and left sides were very similar but large differences in tumour volume occurred in 3 dogs (Nos. 3, 4, 7).

Table 4. No. and volume of lung metastases found at 10 routine post-mortem examinations of dogs with various primary neoplasms which were untreated or removed surgically

Dog. No.	Diagnosis	Left lung No.	Left lung Vol. (ml)	Right lung No.	Right lung Vol. (ml)
1	Osteosarcoma	1	1·0	0	0
2	Mastocytoma, skin	2	4·4	1	0·8
3	Fibrosarcoma of bone	2	37·0	2	6·6
4	Mammary carcinoma	5	13·3	6	284·0
5	Mammary carcinoma	5	2·7	8	9·4
6	Carcinomatosis	16	27·0	16	15·7
7	Bile duct carcinoma	21	2·4	19	17·2
8	Mammary carcinoma	30	5·6	40	8·0
9	Pancreatic carcinoma	68	1·2	54	2·7
10	Mammary carcinoma	78	5·5	95	6·8

Melanoma. Springer Spaniel. Twenty weeks after the first X-irradiation of the primary tumour and the thorax, there was radiographic evidence of metastases in the lungs and euthanasia was performed. There was no recurrence of the primary tumour and no histological evidence of this tumour could be found at the primary site or at the site of the submaxillary lymph node. On post-mortem examination of the lungs there was appreciably less tumour on the X-irradiated side. In the right (irradiated) lobes 57 metastases with a total volume of 46 ml were found compared with 72 metastases with a total volume of 187 ml on the non-irradiated left side (Fig. 2).

Dachshund. Four months after radiotherapy there was recurrence of the primary tumour and euthanasia was carried out. In the right (X-irradiated) lung 24 metastases with a total volume of 3·7 ml were found. In the left, non-irradiated, lung there were 51 metastases with a total volume of 14·9 ml. Multiple metastatic nodules were also found in the myocardium and in the lymph nodes draining the primary tumour.

Adenocarcinoma. Sixteen weeks after the first irradiation euthanasia was performed because of lung metastases. At this time the tumour at the primary site measured 2 × 2 cm and there was metastatic tumour in the right submandibular lymph node.

More tumour was present in the left, irradiated, lobes than in the untreated lobes, the number of metastases being 1484 (approx.) on the left side and 720 (approx.) on the right. The total tumour volume on the left side was 79·6 ml and on the right 25·6 ml.

Squamous cell carcinoma. Twenty weeks after the first irradiation euthanasia was performed because of pain and dysphagia due to primary tumour recurrence, and, at this time, tumour was also present in the regional lymph nodes.

Metastatic tumours to the lungs occurred solely on the right, irradiated, side. There were 56 grossly visible tumours in the right lobes with a diameter of 0·1–0·8 cms and a total volume of 2·14 ml (Fig. 3). Following histological examination of the right lobes many more smaller tumours were found. A detailed macro- and microscopic examination of the left lobes failed to reveal any evidence of tumours. In addition to the tumours in the right lobes other areas of lung showed perivascular and interstitial fibrosis with pseudoepithelialisation of the alveolar walls and desquamation of the septal cells into the alveolar lumen. This interstitital pneumonia was confined to the right lobes, the left, non-irradiated, lobes appearing normal.

Discussion

The results of X-irradiation of one lung following the intravenous injection of osteo-

sarcoma cells into immunosuppressed animals clearly show a reduction in the amount of tumour growing in the irradiated lung as compared with the normal lung.

Some spontaneous melanomas in the dog are radiosensitive. Using a total dose of 5000 rad given in 3 or 4 fractions only, Silver [2] found a one-year survival rate of 45% in 26 canine melanomas. In the two cases described here, tumour cells, not radiographically apparent, were almost certainly present in the lungs at the commencement of therapy. The subsequent result of very much less tumour tissue being found on the X-irradiated side in both dogs is similar to the results obtained with puppies injected with osteosarcoma cells.

In the 5 dogs now dead in which the osteosarcoma was ablated, and, in 8 of 10 dogs where the primary osteosarcoma was irradiated but left *in situ*, the distribution of tumours on right and left sides was not significantly different following 1200 rad X-irradiation when compared with untreated dogs. In one dog, however, there was over three times the volume of tumour and, in a further, dog three times as many metastases occurred on the irradiated side compared with the normal side. The difference in volume of tumour may be coincidental but a ratio of 3:1 in the number of metastases between the two sides has not been seen in untreated dogs (Table 4).

The results of lung irradiation in the dog with adenocarcinoma again showed more tumour on the irradiated side but the most dramatic result occurred in the dog with the metastasising tonsillar carcinoma. The finding of 56 gross and multiple microscopic metastases on the irradiated side when none could be found on the normal side leaves no doubt that X-irradiation was harmful in this case. With the primary tumour still present and growing in this dog it appears probable that metastatic cells lodged in the lungs at some time post-irradiation. The marked pneumonitis found in the right lobes of this dog was not a feature of the irradiated lungs of other dogs receiving 1200 rad and post-irradiation infection may have contributed to the pathological changes found. In other dogs where the thorax was irradiated, thorough and extensive examination of sections of right and left lung lobes under the light microscope showed little or no differences in appearance.

Possible reasons why the tumour cells became established in the irradiated lung include damage to small blood vessels, or impairment of local immunity following destruction of perivascular and peribronchiolar lymphoid follicles. Further investigations to elucidate this problem are being conducted.

In the normal lung circulating tumour cells must have passed through the normal tissue or been destroyed there. Apart from the draining lymph nodes and the irradiated lung no other metastases were found.

The effects of X-irradiation on normal dog lungs have been studied by several workers. Leroy *et al.* [3] showed that 44 hours after 850 rad absorbed X-irradiation many granular pneumocytes were damaged and that there was some focal oedema. Michaelson and Schreiner [4] gave 1700–2100 rad over a 4-day period and found that during the first year after irradiation the animals experienced acute respiratory difficulty as a result of pulmonary fibrosis and alveolar capillary block. Most cases exposed to 2100 rad died during this period.

In our study, the thoracic dose of 600 rad given twice with a one-week interval was chosen as a dose which might well be expected to produce differences in tumour growth but which would not cause severe pneumonitis. In man, 13 fractionated doses to the lungs of 150 rad over a period of $2\frac{1}{2}$ weeks to a total dose of 1950 rad have been given. [5] Because of the problems associated with repeated general anaesthesia we have not tried these smaller daily fractionated doses but 2 doses of 600 rad is equivalent in biological terms to slightly more than 14×150 rad (relevant to normal connective tissue).

Although the number of clinical cases treated is small and the fractionation of the X-irradiation dose could be improved two main conclusions can be drawn:

when the primary tumour is completely excised or otherwise killed, prophylactic X-irradiation of the lungs can delay the growth of a radiosensitive tumour. A combination of this approach combined with chemotherapy or immunotherapy is worthy of further study;

when the primary tumour is not completely excised or otherwise killed prophylactic X-irradiation of the lung can sometimes increase the number of metastases.

Acknowledgements—We are grateful to Professor J. S. Mitchell for his encouragement and for permission to use the linear accelerator.

We wish to thank Professor H. A. Van Peperzeel for valuable advice and Dr. D. W. van Bekkum for facilities for some of the experimental work. The dogs were anaesthetized by Dr. W. McDonall, Miss K. Clarke, Miss M. Massey and Mr. A. Eales. We thank Mr. J. Green and Mr. T. Carter for technical help and Mr. F. K. Bampton for help with the manuscript.

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Proliferation Behaviour of P and Q Cells in a Rat Rhabdomyosarcoma after Irradiation as Determined by DNA Measurements

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Abstract—*DNA distributions of R–1 tumour cells from a transplantable rat rhabdomyosarcoma grown* in vitro *and* in vivo *have been obtained at different time intervals after irradiation with* 2000 *rad of* ^{137}Cs *gamma-rays.*

From the changes observed in the DNA histograms, the following conclusions can be drawn:

1. *Changes in the fractions of cells in* G_1, *S and* $G_2 + M$ *phases are similar for R–1 cells cultured* in vitro *and R–1 cells irradiated in the tumour.*
2. *Non-proliferating cells (Q cells) have a DNA content equal to that of* G_1 *and S phase cells.*
3. *Up to* 20 *hr after irradiation no recruitment of Q cells could be observed.*
4. *R–1 tumour cells can quite easily be distinguished from normal cells present in the tumour due to differences in DNA content.*

INTRODUCTION

THE RESPONSES of cells in solid tumours to various types of treatments are known to depend on their proliferative behaviour. Many tumours contain cells in proliferative cycle (P cells) as well as resting or quiescent cells (Q cells). Significant differences have been observed in the efficiency with radiation or cytotoxic agents which induce cell reproductive death in proliferating and in non-proliferating cells. Treatments of solid tumours by radiotherapy or chemotherapy may induce proliferating cells to become synchronized in their cycle and may cause quiescent cells to start proliferating. It is evident that the efficacy of individual doses of radiation or of cytotoxic drugs in fractionated or protracted treatments must depend on changes induced by previous doses.

With the advent of ultrarapid cytophotometers, such as the flow microfluorometer [1] and the impulscytophotometer [2], DNA distributions can be obtained. This can be done by quantitative measurements of fluorescent light emission from cells stained with fluorescent dyes (e.g., acriflavine or ethidium bromide) which stain the double-stranded DNA. A distribution can be obtained from a large number of cells, usually several tens of thousand, within a few minutes. Changes in the DNA distributions as a result of various treatments can be analyzed. Such techniques have been used in studies of the effects of various agents on the cell cycle [3], of phase progression in the two-dose response of Ehrlich ascites tumour cells [4], and for comparison with cell-volume changes [5].

Almost all R–1 cells *in vitro* are in the proliferative stage (P cells), while, in the solid tumour, the number of proliferating cells and non-proliferating or quiescent cells (Q cells) is equal. In this report, a comparison between the proliferative behaviour of R–1 cells growing *in vitro* (P cells) and R–1 cells growing in the

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solid tumour (P and Q cells) after an acute single dose of 2000 rad of ^{137}Cs gamma-rays will be made by means of DNA measurements with the impulscytophotometer ICP 11*.

MATERIAL AND METHODS

The origin and growth characteristics of the tumour, a rhabdomyosarcoma transplantable in the inbred strain of WAG/Rij rats, have been described previously [6]. This tumour, designated R–1, was obtained by a special selection procedure. It consists of cells which, after excision of the tumour and application of a cell dispersion technique [7], can be cultured directly *in vitro* [8] with a population doubling time of 17 ± 2 hr. From experiments in which semi-continuous labelling was applied, it was concluded that, at a volume of 0·3 cm^3, the fraction of P cells varied between 0·35 and 0·5 depending on their location in the tumour [9]. This indicated that about one half of the cells are Q cells. It was shown by Barendsen *et al.* [10] that the cell dispersion technique applied to prepare a cell suspension from the tumour did not preferentially select P or Q cells. The tumours to be investigated had volumes between 0·7 and 1·0 cm^3 and a tumour doubling time of 4 days; the cell cycle time of the proliferating cells is about 20 hr [11].

With the impulscytophotometer ICP11 described by Dittrich and Göhde [2], DNA distributions of R–1 cells growing *in vitro* and of R–1 tumour cells obtained by the cell dispersion technique have been determined. After fixation of single cells in pure alcohol at -30°C, a temperature at which cells can be stored for several months, cells are washed with physiological salt, incubated with RNAse for half an hour, and stained with the fluorescent dye ethidium bromide. The cells are directed in single file fashion through a relatively large measuring area parallel to the optical axis of the microscope objective and are intensely illuminated by light from a high pressure mercury tube. Cells with appropriate staining emit a fluorescent light signal which is proportional to the amount of the stained compounds; i.e., the double-stranded DNA. The light signal is amplified by a photoelectrical device and classified in a multichannel analyzer which is interphased with an *X–Y* recorder to obtain a hard copy.

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Tumours in rats receiving a total-body exposure, and R–1 cells *in vitro*, were irradiated with a dose of 2000 rad of ^{137}Cs gamma-rays at a dose rate of 122 rad/min.

RESULTS AND DISCUSSION

In Fig. 1, curve A represents the DNA distribution of *in vitro* cultured unirradiated R–1 cells. The first peak represents cells in G_1 phase. The second peak is illustrative of cells in G_2+M phase which contain nearly twice the amount of DNA as do G_1 cells. Cells in S phase are represented between these two peaks. Curve B of Fig. 1 represents the

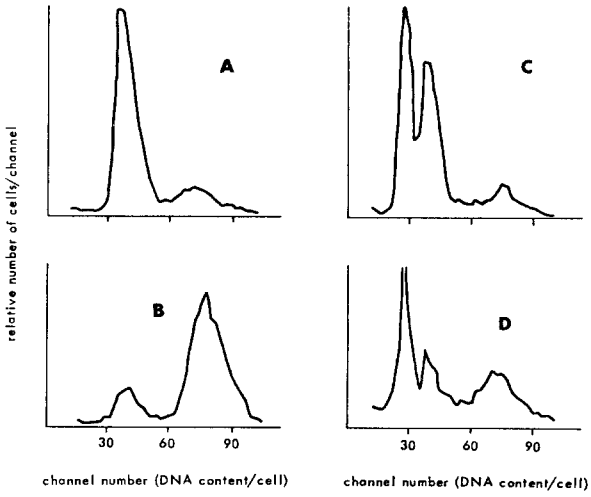

Fig. 1. Distributions of DNA content per cell of R–1 cells. Numbers of cells counted per channel are plotted on an arbitrary scale as a function of the channel number which is proportional to the DNA content per cell. At least 20,000 *cells are measured in each sample. A: R–1 cells* in vitro; *control cells. B: R–1 cells* 16 *hr after a dose of* 2000 *rad of* ^{137}*Cs gamma-rays. C: cells from R–1 tumours; control tumour. D: cells from R–1 tumours* 16·5 *hr after a dose of* 2000 *rad of* ^{137}*Cs gamma-rays.*

DNA distribution of R–1 cells 16 hr after irradiation with an acute single dose of 2000 rad of ^{137}Cs gamma-rays. From a comparison of these curves, it can be concluded that the majority of the irradiated R–1 cells are accumulated into the G_2+M phase.

DNA distributions have also been obtained at other time intervals after a dose of 2000 rad. From these distributions, the percentage of cells in G_1, S, and G_2+M phases can be derived as a function of time after irradiation and these are presented in Fig. 2A. From the sharp decrease in the number of G_1 and the increase of S cells in the first hours after irradiation, it is evident that this is due to a

radiation-induced inhibition of division leading to an accumulation of cells in the G_2+M phase. A maximum in the number of G_2+M cells is reached at about 16 hr after irradiation. The percentage of cells in G_2+M phase is about 78%. For lower doses, a lower percentage has been observed at a shorter time interval after irradiation (unpublished results).

Curve C in Fig. 1 represents the DNA histogram of unirradiated rhabdomyosarcoma cells growing in the solid tumour. Curve D represents the DNA distribution of tumour cells 16·5 hr after irradiation *in situ* with a dose of 2000 rad of ^{137}Cs gamma-rays.

The first peak of curves C and D is due to normal diploid cells in the tumour. These cells cannot be completely removed by the trypsinization technique employed to obtain a monocellular cell suspension. The second and third peaks are comparable with the peaks at the same channel numbers of Figs. 1A and 1B. Similar DNA distributions have been obtained by Raju *et al.* [12] who studied the effect of radiation doses on the mouse KHT tumour by flow microfluorometry instrumentation.

From these two curves, it is clear that, although more cells are found in the G_2+M phase in the irradiated tumour as compared with the control tumour, a considerable fraction of cells is present in G_1 and S phases. This is in contrast to the findings obtained with cultured R–1 cells as demonstrated in Fig. 1, curve B.

The percentages of cells in G_1, S, and G_2+M phases as a function of time after a dose of 2000 rad of gamma-rays to tumours *in situ* are shown in Fig. 2B. The same pattern is found for the R–1 tumour cells as compared with the *in vitro* cultured R–1 cells. It indicates that P cells in the tumour are affected in a similar way as R–1 cells cultured *in vitro*. The number of G_2+M cells also reaches a maximum, and the number of S and G_1 cells a minimum, at 16–20 hr after irradiation. The fraction of tumour cells in G_2+M phases is 0·48, and the fractions of G_1 and S cells are 0·28 and 0·24, respectively. These values differ from the values found for the cultured R–1 cells, which were 0·78, 0·14 and 0·08, respectively. They indicate that a population of cells with a DNA content of cells in G_1 and S phases, which is present at the time of irradiation, will not progress into the G_2+M phases after irradiation. This population of cells must be composed of non-cycling cells, Q cells, in a resting phase of the cell cycle. This population is still present in this phase 16–20 hr after irradiation. It indicates that in that period recruitment of Q cells could not be observed.

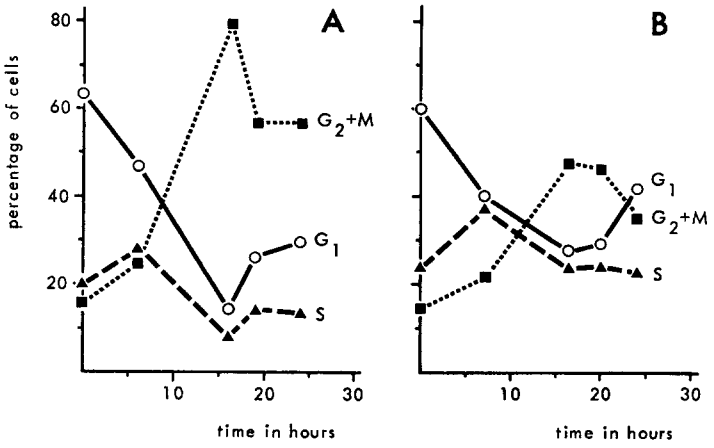

Fig. 2. Percentages of cells in G_1, S, and G_2+M phase as a function of time after a dose of 2000 *rad of* ^{137}*Cs gamma-rays. A: R–1 cells* in vitro. *B: cells from R–1 tumours.*

From the changes observed in the DNA histograms, the following conclusions can be drawn:

1. Changes in the fractions of cells in G_1, S and G_2+M phases are similar for R–1 cells cultured *in vitro* and R–1 cells irradiated in the tumour.

2. Non-proliferative cells (Q cells) have a DNA content equal to that of G_1 and S phase cells.

3. Up to 20 hr after irradiation no recruitment of Q cells could be observed.

4. With this cytofluorographic method, tumour cells can quite easily be distinguished

from normal cells present in the tumour due to the differences in DNA content.

It can be concluded that comparison of DNA histograms can provide a useful tool for investigating the influence of various types of agents on the proliferative behaviour of cells in tumours and in culture.

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Characterization and Partial Purification of Normal and Tumor Associated Transplantation Antigens of Rauscher Leukemia Cells

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Abstract—*Partially purified hydrosoluble extracts were obtained by isoelectrofocusing from the membranes of RC_{19} tumor cells carried in ascites form in Balb/c mice. The biological activity was assayed* in vitro *by the capacity of the partially purified antigen to absorb cytotoxic activity from allogeneic antisera specific for the FMR tumor associated antigen (TAA) as well as for antisera specific for normal H–2 transplantation antigens. The partially purified material contained both TAA and normal H–2 antigen. No separation of normal and tumor antigens was observed at this stage of purification by the methods used. Separation on acrylamide gel revealed two protein bands the identities of which are currently being investigated. The results and their implications are discussed in terms of the physicochemical technique used and the nature of the cell surface antigen.*

INTRODUCTION

ALTHOUGH tumor biologists have documented the presence of both normal and tumor specific transplantation antigens on the surface of experimental tumor cells, little is known of their characteristics. The antigenic changes which have been found in virtually every experimental tumor system studied, as well as in many tumors studied in man, can be divided into two main groups. The first are quantitative changes in the density of normal histocompatibility antigens on malignant cells [1, 2, 3]. These changes have been found by antibody adsorption studies as well as by light and electron microscope studies using antibodies tagged with appropriate markers. The other major change in antigenicity has been qualitative, that is to say, antigenic determinants have been found on tumor cells which are not present on cells of the non-tumor bearing isogeneic host; in most cases the host is capable of responding immunologically to these antigens [4, 5, 6].

The relationship between the antigenic changes observed in both chemically and virally induced tumours and the malignant transformation is poorly understood and indeed is a central question in tumor immunology to-day.

Comparative results indicate that histocompatibility antigens are found in a higher concentration on surface membranes of a cell than are tumor antigens [1, 3]; the latter are, however, present and immunologically active in the isogenous host. We have demonstrated that the transplantable ascitic murine leukemia induced in Balb/c mice by Rauscher virus carries an extractable and immunologically active cellular antigen. Pre-immunization with the hydrosoluble fraction obtained from the ascites tumor cells can inhibit the induction of the leukemia by cell-free virus and can facilitate the growth of the ascitic graft of leukemia

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cells [7]. We have partially purified the antigenic fractions of the extract by the method of electrofocusing which we have previously used to partially purify the soluble H–2 antigens of C_3H mice [8]. Moreover, we have used the method of electrofocusing to compare the isoelectric pH, charge, and mobility of the tumor specific and normal transplantation antigens of our extract.

MATERIAL AND METHODS

Mice

Young adult male and female Balb/c mice were used.

Tumor

RC_{19} is a murine leukemia induced by the Rauscher virus in Balb/c mice and carried in ascites form in Balb/c mice.

Normal cells

The spleen cells of non-tumor-bearing Balb/c mice were used as a source for normal cellular antigens.

Preparation of the membrane fraction

The method of preparing the membrane fraction has been described in detail previously [9, 10]. The ascites cells from RC_{19} bearing Balb/c were submitted to lysis by successively hypotonic solutions of NaCl (0·07 M; 0·035 M) and finally distilled water. After each incubation of 30 min the material was centrifuged for 10 min at 800 ***g*** at room temperature and the supernatant is saved. The three supernatant fractions are pooled and centrifuged at 75,000 ***g*** for 90 min. The precipitate obtained represents the membrane fraction. The normal spleen cell membranes were prepared using the following modifications of the technique above: only two exposures to hypotonic solution were used and both incubations were carried out in 0·13 M NaCl.

Solubilization of the membrane fraction

For both the normal and tumor cell membrane fractions, solubilization was carried out by the autodigestion method previously described [11, 12]. The pellet obtained from the rapid centrifugation of the cell lysate was suspended in a solution of 0·05 M Tris-HCl at pH 7·7 at 37°C for 3 hr. The suspension was then centrifuged for 1 hr at 105,000 ***g***, and the supernatant was used as the hydrosoluble fraction.

Electrofocusing of the hydrosoluble fractions

The electrofocusing technique used was exactly that described for the purification of the H–2 antigens of the C_3H mouse [8, 13]. The carrier ampholyte was selected to give a pH gradient between pH 3 and pH 10. Electrofocusing was performed for 48 hr with a potential of 600V at 10°C. Absorbance of each 2 ml fraction collected was determined at 280 hm.

Electrophoresis of biologically active fractions

The biologically active fractions of both tumor and normal cell extracts recovered from electrofocusing were submitted to electrophoresis on acrylamide gel at pH 9·4 in 10 M urea for 1 hr at 100V, 5 mA per tube at 20°C.

Determination of the biological activity

The biological activities of both the hydrosoluble extracts and the fractions obtained after electrofocusing were determined *in vitro* by inhibition of cytotoxic activity of reference sera. In the case of the tumor fractions, the activity was assayed by the ability to absorb the cytotoxic activity of an allogeneic antiserum directed gainst the Friend–Moloney–Rauscher (FMR) common antigens, as well as another allogeneic serum directed against the $H–2^d$ antigens. In the case of the normal splenic fractions, the activity was assayed by the ability to absorb the cytotoxic activity of anti $H–2^d$ antiserum.

The anti-FMR serum was prepared in $C_{57}Bl/6$ ($H–2^b$) mice by injections of ascitic cells from RC_{19} tumor-bearing Balb/c mice. The resulting serum was absorbed with normal Balb/c spleen cells until no cytotoxic activity against the normal cells could be found. The anti $H–2^d$ serum was prepared in $C_{57}Bl/6$ mice by the injection of DBA/2 spleen cells.

The biologic activity was determined as the absorptive capacity of each fraction after dialysis of each eluate against 0·9% NaCl buffered with Na_2HPO_4 to pH 7·8 at 4°C for 48 hr. Serial two-fold dilutions of 0·05 ml of each fraction were incubated for 30 min at room temperature with 0·05 ml antiserum, giving a final dilution of 50% cytotoxic activity, and 0·1 ml of a suspension containing 10^5 target cells; then 0·1 ml of rabbit complement was added, and after 45 min incubation at 37°C the residual cytotoxic activity was tested in the presence of trypan blue; the target cells are RC_{19} tumor cells and normal lymph node cells of Balb/c mice.

RESULTS

Purification by electrofocusing of the hydrosoluble fractions of cell lysates

Purification of the hydrosoluble fraction of both normal and malignant cell lysates by the methods of electrofocusing has been carried out on different samples. The results are reproducible. Figures 1 and 2 are representative

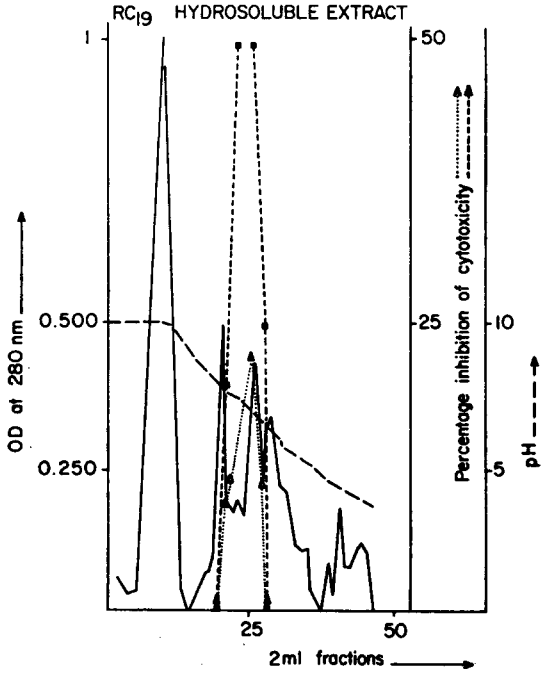

Fig. 1. Isoelectric focusing between pH 3 and 10 of the hydrosoluble fraction obtained by autodigestion from murine RC_{19} (Balb/c) tumor cell membranes ——— absorbancy at 280 nm; — — — pH; ■- - - - - - - ■ inhibition of cytotoxicity of anti FMR antiserum; △..... △ inhibition of cytotoxicity of anti H−2^d antiserum.

elution profiles of soluble normal and RC_{19} cell antigens, respectively, obtained from electrofocusing (absorption at 280 hm). The relationship between the total fraction eluted and the biologically active fraction indicates a purification* greater than 3–4-fold (see Figs. 1 and 2), concerning the soluble material.

Biological activity

The electrofocusing of the normal cell extract yielded absorptive activity for anti H–2^d serum only in two or three fractions eluated between pH 6·3 and pH 7·4.

The electrofocusing eluates of tumor cell extract, when tested against the same anti

*Extract quantification unknown because an undetermined amount of protein was lost by precipitation on the column.

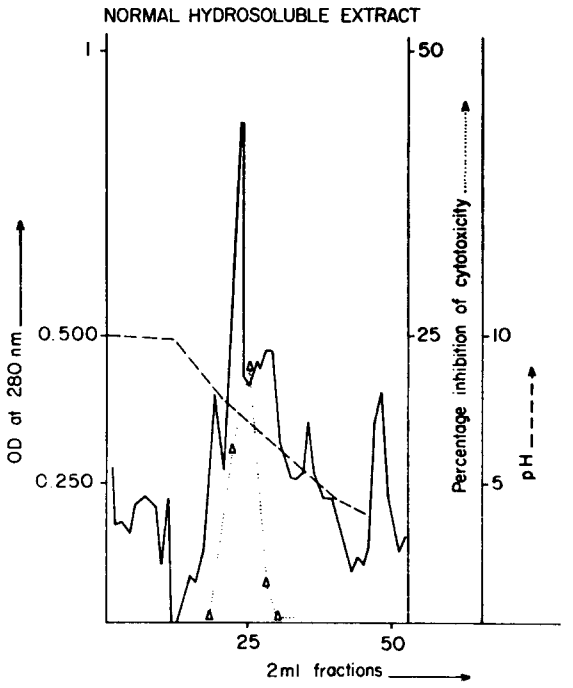

Fig. 2. Isoelectric focusing of the hydrosoluble fraction obtained by autodigestion from normal spleen cell membranes of Balb/c mice (for details, see Fig. 1).

H–2^d alloantiserum, showed a pattern very similar to that of the normal cell extracts. Moreover, when the electrofocusing eluates of the tumor cell extracts were tested for absorptive activity against the anti FMR alloantiserum, the activity was again found in the same fractions as the absorptive activity of the H–2 histocompatibility antigens.

Acrylamide gel electrophoresis of biologically active fractions obtained by electrofocusing

Active fractions of electrofocusing eluate obtained from both tumor and normal cells were pooled and submitted to analytical electrophoresis on acrylamide gel. Concerning the tumor fractions, two protein bands were obtained. In the normal fractions two protein bands were also obtained in which the second band seemed to be heterogenous (Fig. 3). In both cases, they have not, as yet, been characterized.

DISCUSSION

The solubilized extracts of murine tumor cells and normal murine spleen cells carrying identical H–2 antigens were characterized and partially purified by electrofocusing. All the biological activity was eluated in a narrow pH range and yielded products purified greater

than 3–4 fold in terms of protein concentration. The elution patterns of the normal and tumor cells were similar. Moreover, the biological activity was eluted in the same pH range for both extracts. Each extract retained its characteristic absorption capacity after purification. The normal cell extract absorbed the anti H–2 cytotoxic antisera and the tumor cell extract absorbed the cytotoxic activity of both

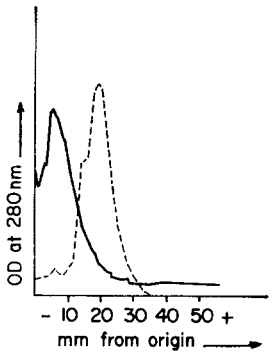

Fig. 3. Electrophoresis scanning: ——— normal fractions; - - - - - - - - tumoral fractions.

anti H–2 and anti FMR sera. By the technique of electrofocusing, we were not able to separate the tumor antigens from the normal histocompatability antigenic determinants. The possibility exists that quantitative separation was missed because of the antisera we used or because of the limited sensitivity of the cytotoxic assay. We have not pursued this particular possibility to date. The inability to separate the biological activity by a method which depends on charge alone may be explicable in terms of the nature of the cell surface antigens.

If the H–2 specificities are a part of a molecular complex while other antigenic determinants, such as tumor-associated antigens, are another part of the complex, one could expect that separation is possible with further physicochemical manipulation. If, however, the hypothesis of Boyse [14] is valid, and if the antigenic specificity is based on a supramolecular geometric arrangement on the cell surface, further purification would ultimately destroy antigenicity. In the latter case, purification seems possible only with specific immunoabsorbants. We have some evidence that the biologically active fraction of our tumor cell eluates is not homogeneous. We have found two distinct protein bands after electrophoresis in acrylamide gel. We have also observed two protein bands in the normal fractions. In both the cases of the RC_{19} extract and the normal extract, all the bands observed migrated differently. The cause for this different electrophoretical behaviour is under investigation.

The attempts at solubilization, separation and purification of normal and tumor-associated antigens, which have interested our laboratory and many others, have been motivated by the possibilities for specific immune manipulation by the modification of antigenic determinants. While certain immunological effects have been demonstrated by the modification of whole cells, the determinants responsible for the altered immunogenicity understandably remain obscure [15, 16, 17, 18]. Modification of cell-free extracts [19] have likewise been shown to modulate host response, but again little knowledge has been gained with reference to the criteria necessary for specific immunogenicity.

By contrast, well defined modification of a well defined molecule has recently demonstrated some of the factors involved in the production of either immunity or tolerance in both humoral and cell mediated immune responses [20, 21].

"Well defined" is the key phrase and it is our objective to pursue the definition of the antigenic determinants in order to approach systematically the manipulation of the immune response.

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Enzyme Patterns of Glucose Catabolism in Hormone-Dependent and -Independent Mammary Tumours of GR Mice*

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Abstract—*Mammary tumours were induced in ovariectomized GR female mice according to the method of van Nie, by feeding estrone in the drinking water and giving weekly injections of progesterone pellets. The tumour incidence was* 90% *after* 14 *weeks of treatment. More than* 90% *of the induced tumours proved to be hormone dependent, and remained so for the next few serial transplantations after which they changed into hormone independent tumours.*

The determination of hexokinase, phosphofructokinase, pyruvate kinase, and lactate dehydrogenase activity of hormone dependent tumours showed a consistent increase as compared to virgin, though not to lactating mammary gland. However, only hexokinase activity was further increased by the transition to autonomy. In agreement with this, lactate accumulation was significantly higher in autonomous compared to hormone dependent tumours.

*The activity of glucose-*6*-phosphate dehydrogenase was found to be significantly lower in autonomous tumours as compared to hormone dependent as well as to virgin mammary gland. No increase in activity could be induced in autonomous tumour tissue by administering estrone.*

INTRODUCTION

THE UNDERSTANDING of hormone influence on tumour induction, growth and metabolism is important in the elucidation of the pathogenesis of human breast cancer. Based on the success rate of endocrine ablation as a treatment for breast cancer, the frequency of hormone responsive breast cancers is about 30% [1].

In a review, Dao [2] summarizes experiments illustrating that hormonal imbalance causing hyperfunction of the pituitary gland may lead to neoplasia in the target organ of experimental animals. In mammary tumourogenesis, it has been shown that implantation of pituitaries into mice free of mammary tumour agent increases the incidence of tumours [3]. In mouse strains carrying the MTV, such as the GR strain, hormones also have a considerable influence on tumourogenesis by shortening the induction time as compared to spontaneous tumourogenesis [4]. Mammary tumours in rats are generally hormone-dependent, whether they are induced by hormones or by chemical carcinogens. In mice, pituitary and steroid hormones have a profound effect on the growth of the precancerous nodules in the mammary gland, but seem to be without effect in later stages of tumour development [5]. However, hormone dependent mammary tumours have been described in the BR6 strain [6] and in the GR strain [7].

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Abbreviations used: ALD—aldolase; EDTA—ethylendiaminetetraacetate di-sodium salt; GPDH—glycerin-1-phosphate dehydrogenase; GPI—glucose-phosphate isomerase, G6PDH—glucose-6-phosphate dehydrogenase; HD—hormone dependent; HI—hormone independent; HK—hexokinase; HR—hormone responsive; LDH—lactate dehydrogenase; MTV—mammary tumour virus; PFK—phosphofructokinase; PK—pyruvate kinase; TPI—triosephosphate isomerase.

The following are our observations of tumour induction and hormone dependence as well as measurements of the activity of key enzymes in carbohydrate metabolism. Hormone dependent and independent mammary tumours of the GR mouse are compared.

MATERIAL AND METHODS

Induction of tumours

Ten to twelve-week old female mice of the GR/A strain [8] were ovariectomized. The following day, treatment with progesterone and estrone was started. Progesterone was administered in pellets and introduced subcutaneously on the back of the mouse with an injection needle equipped with a stiletto. The dose was 3 pellets (5–10 mg progesterone) per animal per week. Estrone was dissolved in ethanol (2 mg/ml). This solution was added to the drinking water to give a final concentration of 0·5 μg/ml. The animals were checked once a week by palpation of the mammary glands for tumours. When the largest tumour of an animal had grown to about 15 mm in diameter, the animal was killed, and the tumour transplanted in the following manner:

Transplantation of tumours

For serial transplantation, tumour tissue was minced with scissors to a degree allowing the tissue to pass a 1·2 mm needle. In a few cases, the tissue was diluted with a few drops of phosphate buffered saline. 0·1 ml of the minced tissue was inoculated subcutaneously in the right flank of an animal. For testing of hormone dependence, tumour tissue was inoculated into 2 castrated female or male mice which received no hormonal treatment. Two castrated animals treated with progesterone and estrone, as above, served as controls. The animals were checked every week over 2 months. If no tumour appeared in the animals that did not receive hormones, but grew progressively in one or both of the treated animals, the tumour tested was considered hormone dependent (HD). If the tumour, however, grew in the non-treated animals, as well and the time for tumour appearance was equal to that of the treated group, it was considered hormone independent (HI). Tumours that were transplantable to non-treated castrated animals but appeared more than one week later than in the hormone treated animals, were called hormone responsive (HR).

Hormone-dependent tumours were passaged every 4–6 weeks, hormone independent tumours every 2–4 weeks, reaching a weight of about ½–1g.

Tissue preparation and enzyme assay

Mammary glands Nos. 4 and 5 of GR mice and tumours of these glands were used for enzyme studies. Furthermore, mammary glands Nos. 4 and 5 of virgin and lactating C3H mice were included in the studies.

The animals were killed by cervical dislocation. The tumour (or mammary gland) was removed and placed in ice-cold sucrose 0·25 M. A part of the tumour was used for transplantation and histology. The remaining part was cut with scissors and homogenized in 0·25 M sucrose for 1 min × 2–3. The 10–20% (w/v) homogenate was centrifuged at 500 × *g* for 10 min. The supernatant was further centrifuged at 100·000 × *g* for 60 min and this supernatant was used for enzyme assay. Through all procedures samples were kept at about 4°C.

Enzyme activities were determined spectrophotometrically at 340 nm in 1 cm cuvettes by the amount of NADH oxidized or NADP reduced at about 25°C using the principle of Kubowitz and Ott [9]. HK, PFK and LDH were determined by modifications of previous methods [10]. Methods for PK and G6PDH were modified by Guminska *et al.* [11] and Daehnfeldt *et al.* [12]. The reaction mixtures are given in Table 1. The supernatant was added to give a ΔE/min between 0·020 and 0·060 except in the case of G6PDH where the interval chosen was between 0·015 and 0·030. Zero order kinetics were ascertained by direct proportionality between enzyme amount and ΔE/min determined at least at 2 enzyme concentrations.

Lactate accumulation

Tissue for metabolic studies was cut with razor blades into slices less than 1 mm thick. About 50 mg of tissue was incubated in Erlenmeyer flasks containing 4 ml of Krebs–Ringer-phosphate buffer [13] with the addition of Tris 30 mM and glucose 10 mM. The flasks were either gassed with 100% oxygen or 100% nitrogen for 5 min and incubated at 37°C in a shaking water bath. At times 0, 1 and 2 hr samples were taken for lactate determination. After the experiment the dry weight of the tissue was determined. The lactate concentration was determined according to the method of Horn and Bruns [14].

Chemicals: All enzymes, substrates and cofactors were obtained from Boehringer, Mannheim, except ATP and Tris which were purchased from Sigma, St. Louis, U.S.A. The remaining chemicals were analytical grade. Progesterone and estrone were generously granted by Leo Pharmaceuticals, Ballerup, Denmark.

Table 1. *Enzymatic methods*

Assay mixture (final concentrations)	Final volume ml
Hexokinase (HK): Tris buffer, about 70 mM (pH 7·7), Supernatant 25–100 µl, $MgSO_4$ 10 mM, NADP 0·4 mM, G6PDH 6·6 µl/ml, ATP 5 mM, glucose 0·33 mM	1·0
Phosphofructokinase (PKF): Tris buffer, about 70 mM (pH 8·0), Supernatant 25–100 µl, ATP 2·5 mM, $MgCl_2$ 10 mM, EDTA 2 mM, ALD 0·2 mg/ml, TPI 0·04 mg/ml, GDPH 0·2 mg/ml, NADH 0·38 mM, fructose-6-phosphate 1·0 mM	1·0
Pyruvate kinase (PK): Tris buffer 75 mM (pH 7·5), KCl 76 mM, $MgSO_4$ 10 mM, ADP 4·5 mM, NADH 0·34 mM, Supernatant (diluted) 200 µl, LDH 0·015 mg/ml, Phosphoenolpyruvate 4·5 mM	2·2
Lactate dehydrogenase (LDH): Tris buffer 92 mM (pH 7·5), NADH 0·25 mM, Supernatant (diluted) 50 µl, sodium pyruvate 1·66 mM	3·0
Glucose-6-phosphate dehydrogenase (G6PDH): Tris buffer 332 mM, NADP 0·33 mM, $MgCl_2$ 0·033 mM, supernatant 25–100 µl, Glucose-6-phosphate 0·07 mM	3·0

Statistical analysis was carried out according to the Wilcoxon rank test [15], if not otherwise stated. When determinations were made simultaneously, tests for paired observations were applied.

RESULTS

Tumour induction

The incidence of mammary tumours in female castrated GR mice was recorded after 14 weeks of treatment with estrone and progesterone. Of 174 animals 156 (90%) had palpable tumours. Some of the animals were killed before 14 weeks because of the size of the tumour. In 123 animals, the induction time (i.e., the time from initiation of treatment until tumour was palpable) was recorded and found to be distributed as shown in Fig. 1 with a median value of 10 weeks.

Usually the animals had more than one mammary tumour. Table 2 shows that the largest tumour was localized to mammary gland No. 5 in about ⅓ of the tumour-bearing animals, and in decreasing frequency in mammary gland Nos. 4 to 1. There was no significant difference between the induction time of mammary tumours of different localization.

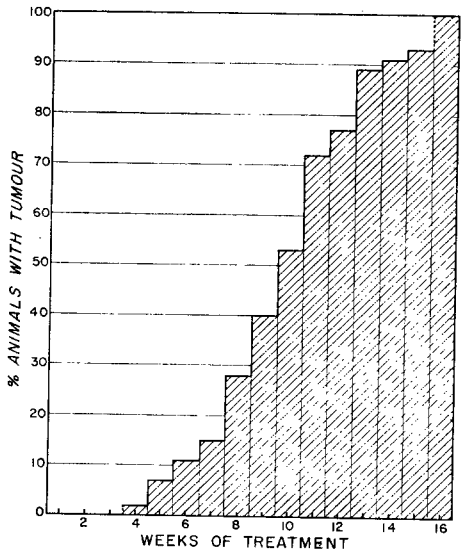

Fig. 1. *Distribution of induction time of mammary tumours induced in castrated female GR mice by treatment with estrone and progesterone. Ordinate:*

$$\frac{\textit{No. of animals with tumour} \times 100}{\textit{Total No. of animals with tumour after 16 weeks}}$$

Hormone dependence

Table 3 shows the hormone dependence of the tumours, primary as well as transplanted. HI tumours were not tested in the following passage. Only 6 out of 68 primary tumours were

HI. Even after 2 passages, the frequency was only 5 out of 28. Of 18 HR tumours tested, 5 were HD in the following passage, but HR again later on. This probably reflects the inaccuracy of the test rather than a reversal of the sequence: HD to HR to HI.

Table 4 shows that hormone treatment for less than 14 weeks resulted in 43 HD tumours out of 53 tumours, whereas the corresponding figures for treatment exceeding 14 weeks were 9 out of 15. This indicates that the risk for inducing autonomous tumours increases with the duration of treatment. However, the difference is not statistically significant by the chi-square test, with Yates correction for small numbers ($\chi^2 = 1{\cdot}85$, $P > 0{\cdot}10$).

Table 2. Localization and induction time of mammary tumours induced in castrated female GR mice by treatment with estrone and progesterone for 14 weeks. (Only largest tumour from each animal)

	Mammary gland No.*				
	1	2	3	4	5
No. of animals	3	15	25	27	38
Induction time (weeks)	9·7	11·8	10·4	9·4	9·7
Standard deviation	1·2	1·5	2·8	3·3	2·0

*Mammary glands Nos. 1–3 are thoracic, Nos. 4–5 abdominal.

Table 3. Development of autonomy in relation to number of transplantation passages

Transplantation passage	Number of tumours tested	Hormone dependence*		
		HD	HR	HI
Primary	68	51	11	6
1. passage	47	35	11	1
2. passage	28	18	5	5
3. passage	8	3	3	2
4. passage	3	2	1	0
5. passage	2	0	1	1
6. passage	1	0	1	0

*HD: hormone-dependent.
HR: hormone-responsive.
HI: hormone-independent.

Enzyme activity

Glycolytic enzyme activities (HK, PFK, PK, LDH) were found to be higher in HD tumours than in mammary gland of virgin mice (Fig. 2). The difference was statistically significant for all enzymes except PFK. The mean G6PDH activity was 18% higher in the tumours, a difference which, however, was not statistically significant.

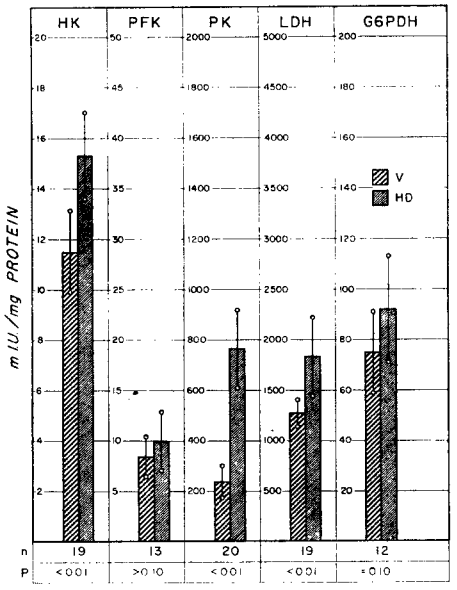

Fig. 2. Enzyme pattern of hormone dependent mammary tumours (HD) compared to mammary glands of virgin mice (V). Ordinate: enzyme activity (mU/mg protein). **n** *= number of animals. The vertical bars designate* $\pm 2e$. P *values were calculated according to the Wilcoxon rank test for paired observations.*

The activities of HK, PFK, PK, and LDH of HI tumours also were observed to increase (Fig. 3). However, in contrast to the HD tumours, the G6PDH activity declined compared to virgin mammary gland. All the differences were statistically significant.

A comparison of HD and HI tumours (Fig. 4) showed a higher mean activity of the glycolytic enzymes in the HI tumour. However, this difference was only statistically significant in

Table 4. Development of autonomy in relation to duration of hormone treatment

Duration of hormone treatment (weeks)	5	6	7	8	9	10	11	12	13	14	15	16	17	18	Total number of tumours
Dependent	1				1	9	6	6	9	11	2	5	2		52
Responsive						2			2	3	2	1			10
Independent							1		1	1	2	1			6

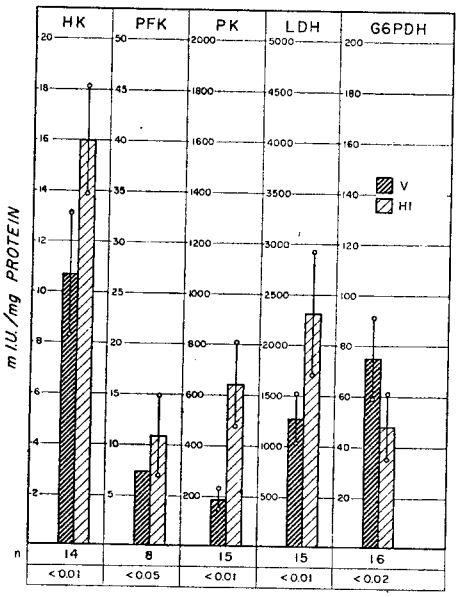

Fig. 3. Enzyme pattern of hormone independent mammary tumours (HI) compared to mammary glands of virgin mice (V). Ordinate: enzyme activity (mU/mg protein). n = number of animals. The vertical bars designate ±2e. P values were calculated according to Wilcoxon rank test for paired observations.

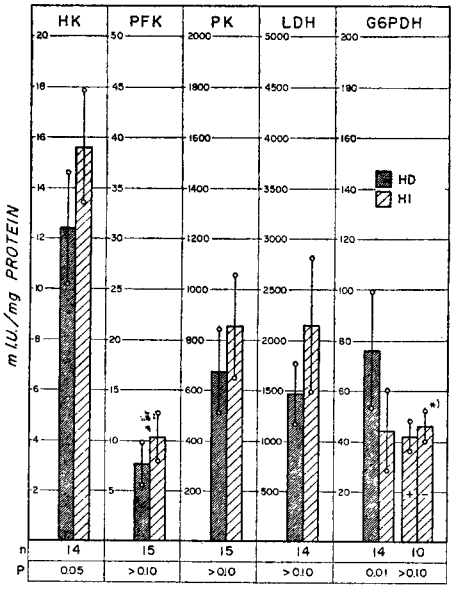

*Fig. 4. Enzyme pattern of hormone independent mammary tumours (HI) compared to hormone dependent mammary tumours (HD). *The G6PDH activity was determined in hormone independent tumours in hosts treated with estrone and progesterone (+) as well as not treated (−). Ordinate: enzyme activity (mU/mg protein). n = number of animals. The vertical bars designate ±2e. P values were calculated according to the Wilcoxon rank test for paired observations.*

the case of HK. The activity of G6PDH was lower in the HI tumours than in the HD tumours with a P value of 0·01 (Fig. 4). As the HI tumours were not exposed to hormones, an additional experiment was carried out. A group of mice were transplanted with a HI tumour. Half the animals were treated with hormones, the other half were not. However, tumour assays for G6PDH activity revealed no difference between the groups.

Except for HK the enzyme activity of lactating GR mouse mammary gland was found to be much higher than that of the mammary gland of virgin GR mice (Fig. 5), and the values were at a level above tumour values. For comparison, enzyme activity of virgin and lactating female C3H mice were determined. It was found that activities not only of PFK and LDH but also of HK were higher in lactating than in virgin glands in this mouse strain.

Corresponding to the increased enzyme activity of HI tumours as compared to HD tumours, a higher accumulation of lactate was found in the HI tumours in all of 5 experiments

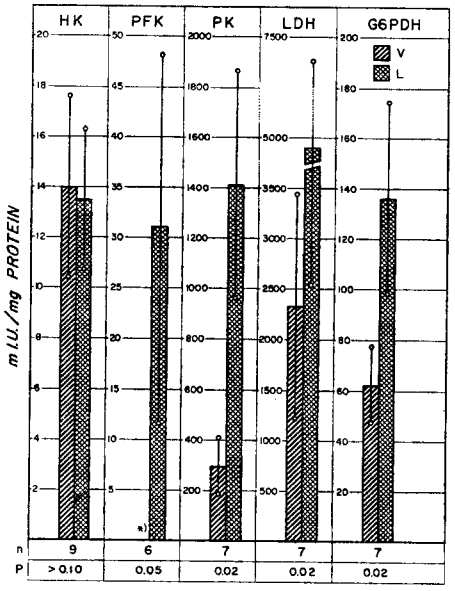

Fig. 5. Enzyme pattern of lactating (L) compared to virgin (V) mammary gland. Ordinate: enzyme activity (mU/mg protein). n = number of animals. The vertical bars designate ±2e. P values were calculated according to the Wilcoxon rank test for paired observations.

*Below the sensitivity of the method.

Table 5. The activity of HK, PFK and LDH of mammary glands of virgin and lactating female C3H mice

		Enzyme activity* HK	PFK	LDH
Virgins	$\bar{x}$	3·4	6·2	2292
	s	1·4	2·4	943
	N	4·0	4·0	4
Lactating females	$\bar{x}$	8·4	19·0	3939
	s	0·9	2·2	328
	N	4·0	4·0	4

*mU/mg protein.

(Table 6). Under anaerobic conditions the increase was more than 50% and statistically significant.

Table 6. Anaerobic and aerobic lactate accumulation of hormone dependent and hormone independent mammary tumours of GR mice

	L^{N_2}*			L^{O_2}†		
	N	$\bar{x}$	*s*	*N*	$\bar{x}$	*s*
Hormone dependent	4	464	115	5	305	90
Hormone independent	4	745	109	5	426	72

*nmol lactate/mg d.w. per hr under anaerobic conditions.

†nmol lactate/mg d.w. per hr under aerobic conditions. Tissue slices were incubated in Krebs–Ringer–phosphate buffer, with Tris 30 mM and glucose 10 mM.

DISCUSSION

Generally, mammary tumours can be induced in mice by estrogens only in the presence of mammary tumour virus (MTV) [16]. In the GR strain of mice, which carries the MTV, mammary tumours have been induced in several ways [4]: (1) ovariectomy + estrone treatment, (2) pituitary grafting, (3) forced breeding.

In intact virgin mice of the GR strain, mammary tumours will arise "spontaneously" in a high percentage. The result of the above mentioned methods is first of all a considerable decrease of the induction time from about 14 to 3 months in the case of forced breeding. A similar short induction time can be obtained by castration and treatment with estrone and progesterone (van Nie, personal communication).

In the present investigation, mammary tumours were induced in 90% of the animals within 14 weeks. Only 6 out of 68 tumours were hormone-independent, showing that this model is satisfactory for production of hormone-dependent mammary tumours in mice. This incidence of hormone-dependent tumours is similar to that of tumours induced by forced breeding, whereas only 1 of 10 spontaneous mammary tumours, 1 of 5 tumours induced by pituitary grafting, and 1 of 9 tumours induced by ovariectomy + estrone, were hormone dependent after transplantation [4].

It is assumed that the hormone-independent tumours with a longer induction time are induced by prolactin, either by stimulatory effect of estrone on pituitary prolactin production or by increased prolactin production from a pituitary graft due to lack of hypothalamic inhibition. The hormone dependent tumours with short induction time are supposed to be induced and maintained by a direct effect of estrone and progesterone on the mammary glands [5, 16]. However, our preliminary results with the anti-prolactin compound CB 154 (Sandoz) have indicated that the growth of HD tumours with short induction time also is sustained by prolactin [17].

Enzyme activities of the mammary gland are subjected to considerable variations depending on the hormonal state of the animal.

During pregnancy the activity of HK, PFK, LDH and G6PDH is low in the mammary gland of C3H and BR6 mice [18, 19, 20] whereas the pregnant rat mammary gland has a higher activity of HK compared to that of virgins [21].

In the lactating mammary gland of the GR mouse, we found no change in HK, but the specific activity of PFK, PK and LDH was much higher than in the inactive gland. These results differ in various ways from those reported by other authors. Thus a lower specific activity of HK, PFK and LDH has been found in the lactating, compared to virgin, mammary gland of C3H and BR6 mice [18, 19, 20]. However, in our hands, the activity of HK, PFK and LDH was found higher in lactating compared to virgin mammary gland of C3H mice. In agreement with this, the specific activity of HK was increased several fold in lactating mammary gland of Sprague–Dawley rats [21].

The high activity of G6PDH found in the lactating mammary gland of the GR mouse is in agreement with results obtained with other mouse strains [18, 19, 20] and with rats [21].

Increased glycolytic enzyme activity is found in human malignant tumours of the mammary gland [22, 23]. In the rat mammary tumours, the activity of GPI and G6PDH has been found higher than in normal, inactive mammary gland [24].

In the mouse mammary tumours, Kopelovich *et al.* [19] found increased specific activity of PFK and decreased specific activity of HK and G6PDH as compared to normal mammary gland from virgins. There was no difference in LDH activity. In the present work, all glycolytic enzymes studied showed an increased activity during tumour development, but no significant increase of G6PDH activity could be demonstrated.

A comparison between hormone dependent and hormone independent mammary tumours in rats has shown that LDH activity was significantly higher in the latter, whereas G6PDH

was about the same [25]. In the present investigation, the average glycolytic enzyme activity of the HI tumour was higher than that of HD tumours, but the difference was only statistically significant in the case of HK. However, the anaerobic lactate accumulation of HI tumours was significantly larger than that of HD tumours. The G6PDH activity which was only insignificantly increased in the HD tumours, was significantly decreased in HI tumours. In BR6 mice, the G6PDH activity was decreased already at the hormone dependent step and remained decreased in the hormone independent tumours [20].

In the hormone dependent tumours, enzyme activities are influenced by the hormonal environment. In rat mammary tumours, ovariectomy generally results in a decrease in enzyme activity and treatment with estrogens in an increase[22]. The continuous increase in glycolytic enzyme activity during mammary tumourogenesis in GR mice seems to be related to growth rate rather than to direct hormonal influence, since the independent tumours which were growing on castrated animals had a growth rate about twice that of the HD tumours. The changes in G6PDH activity seemed to be correlated to hormone dependence in the sense that when tumour growth responded to hormones, the activity of the enzyme also responded, and when the tumour became hormone independent, G6PDH activity not only decreased, but also failed to respond to hormone treatment. This may be taken as a sign of molecular dedifferentiation.

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Letter to the Editor

Effect of Bleomycin on the Sheep Erythrocyte-Induced Rossette Formation and on the Allograft Survival in Mice

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BLEOMYCIN has a considerable carcinostatic activity in laboratory animals and in man, but has only little myelosuppressive effect in clinical experiences [1, 2] and little or no immuno-suppressive effect *in vivo* [3, 4], though it exerts an inhibiting effect on lymphoblast transformation [3], and an *in vitro* suppressive action was observed by the Jerne plaque technique [1]. It was concluded that either this drug might be inactivated by the lymphoid tissues, or excreted in large amounts by the kidneys [3]. These controversial results led us to investigate the effect of the bleomycin on the sheep-erythrocyte (SRBC) induced rosette formation and on the allograft survival in mice.

Male Balb/c and C57BLP10 mice were used. The drug was administered intraperitoneally. Two dosage schedules were applied: (1) 2 doses of 1/5 LD_{10} (2) 1/25 LD_{10} once a day for a week. Each animal was injected i.p. with 3×10^8 SRBC. The animals were killed on the 4th and 8th day after immunization. The technique for rosette formation described by McConnell *et al.* [5] was applied. The total of the rosette-forming cells (RFC) was calculated by the formula:

$$TRFC = RFC\% \times \text{number of spleen cells}$$

Haemolysin and haemagglutinin titrations were made on Takátsy's microplates. Skin grafting was carried out in a strong allograft system (Balb/c—CBA). The technique was reported previously [6]. 1/5 LD_{10} of bleomycin was given i.p. in 6 doses.

The dose of $2 \times 1/5$ LD_{10} given i.p. caused no death (Balb/c mice). In these experiments (Table 1) higher spleen weights were found after immunization in the controls. This showed a further increase on the 8th day. Immunization produced a three-fold increase in the number of TRFC in the control mice, whereas a slight decrease was found in the treated group. Thus more than 5 times more RFC were obtained in the control group on the 8th day after immunization than in the treated

Table 1. *Effect of* $2 \times 1/5$ LD_{10} *bleomycin on the immune-rosette formation of spleen cells in BALB/c mice*

Treatment	Untreated control		Bleomycin	
Days after immunization	4	8	4	8
Spleen weight (mg)	194 ± 12	275 ± 22	70 ± 9	117 ± 15
Number of spleen cells ($\times 10^8$)	3·3 ± 0·2	4·8 ± 0·1	2·7 ± 0·1	1·7 ± 0·2
Rosettes (1000 spleen cells)	11 ± 2	23 ± 3	9 ± 1	11 ± 1
TRFC ($\times 10^6$)	3·7 ± 0·4	11·0 ± 0·8	2·4 ± 0·1	1·9 ± 0·2

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Table 2. Effect of 7 × 1/25 LD_{10} bleomycin on the immune-rosette formation of spleen cells in BALB/c mice

Treatment	Untreated control		Bleomycin	
Days after immunization	4	8	4	8
Spleen weight (mg)	205 ± 11	295 ± 10	150 ± 7	116 ± 6
Number of spleen cells ($\times 10^8$)	3·2 ± 0·1	5·1 ± 0·2	1·3 ± 0·1	1·2 ± 0·1
Rosettes (1000 spleen cells)	15 ± 2	29 ± 2	9 ± 1	6 ± 0·4
TRFC ($\times 10^5$)	48 ± 3	148 ± 5	12 ± 1	8·2 ± 1

one. The difference is highly significant. Similar results were obtained with the lower dosage (Table 2) lower spleen weights were observed on the 4th day and there was no further increase in the treated group on the 8th day after immunization. More than 18 times more RFC were observed in the control group on the 8th day than in the treated one. C57BLP10 mice: the results were similar to those obtained in Balb/c mice.

Neither 2 × 1/5 LD_{10} nor 7 × 1/25 LD_{10} bleomycin caused any significant difference in circulating haemolysin and haemagglutinin antibody titres on the 4th and 8th day after immunization.

Skin graft survival: there was no significant difference between the treated and control mice.

Our observations suggest that bleomycin does have some immunosuppressive affect. Despite the lack of graft protection, it definitely interferes with the immune rosette formation, which is a distinct sign of impairment in the production of RFC. The controversial data obtained suggest that this immunosuppressive effect must be short or reversible.

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Letter to the Editor

Cell Killing Effectiveness of an Alkylating Steroid (Leo 1031)*

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A STEROID with a bis-chloro-aethylamino-phenyl–butyric acid group in the side chain was prepared by AB Leo, Helsingborg, Sweden, (pregna-1, 4-diene-3, 20-dione, 11β, 17α, 21-trihydroxy-, 21-4-*p*-/bis-(2-chloro-aethyl)-amino/-phenyl-, butyrate (EORTC 1502, NSC 134087)) [1]. Its toxicity and effectiveness against experimental tumours have been described [2]. This report describes the effectiveness of this compound, after subcutaneous injection in the mouse, on the survival of resting and rapidly proliferating haemopoietic stem cells and of L1210 leukaemia cells, as well as on a transplantable osteosarcoma. The drug was administered subcutaneously, suspended in 0·2% carboxymethylcellulose in a volume of 0·01 ml/g of body weight. The method of determining stem cell survival by the spleen colony technique was described earlier, [3] as well as the technique of measuring osteosarcoma volume reduction after a single dose of the drug in an amount equal to the LD10 [4]. L1210 leukaemic cell survival was determined by the method of Skipper *et al.* [5]: L1210 cell suspensions were diluted to contain the following average cell numbers per 0·5 ml: 10^5, 10^4, 10^3, 10^2, 10, 3 and 1, and 0·5 ml quantities of each of these dilutions were injected i.v. in 10 (DBA × C57BL)F_1 hybrid mice. In addition, a number of similar groups of mice received 10^5 cells i.v. and, 24 hr later, different doses of Leo 1031 s.c. The mice were observed daily and the median survival time (MST) was noted for each group. Reduction of cell survival was estimated by comparison of the MST for each treated group with the graph relating effective cell number to MST. The results are presented in Fig. 1 for the stem cells and leukaemic cells. The D_0 values (reciproke of the slope) of the survival curves are 516 mg/kg for resting stem cells, 125 mg/kg for rapidly-proliferating stem cells and 22·2 mg/kg for L1210 cells.

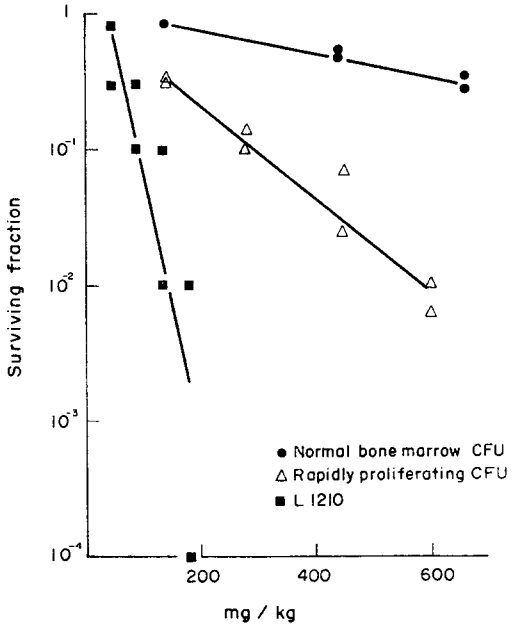

Fig. 1. Dose effect curves for the survival of resting and rapidly-proliferating spleen colony forming haemopoietic stem cells (CFU) and L1210 leukaemia cells after subcutaneous administration of Leo 1031 *to mice.*

It is interesting to note that the ratio of effectiveness against rapidly-proliferating cells compared with resting cells, 4·13, is higher than that observed for any other alkylating agent of 19 tested in a similar way [4]. The slope of the curve describing the killing of L1210 cells is

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Table 1. L1210 Leukemia cell killing effectiveness of different alkylating agents

Drug	Relative killing effectiveness compared to normal stem cells (ratio of slopes of survival curves)	Extrapolated cell survival after LD_{10} dose	
		CFU	L1210
BCNU	8·0	0·07	10^{-9}
CCNU	9·2	0·03	10^{-15}
MCCNU	18·8	0·09	10^{-19}
Cyclophosphamide	5·8	0·03	10^{-5}
DTIC	3·4	0·003	10^{-10}
TEM	1·5	0·001	10^{-3}
LEO 1031	23·3	0·84	10^{-2}

even steeper, and the ratio of effectiveness compared to resting stem cells is 23·3. Again this value is much higher than for other alkylating agents (see Table 1, second column). However, this high ratio does not imply that Leo 1031 is very effective against L1210; rather it is a consequence of its low toxicity for normal stem cells. At the LD10, only 16% of these cells are killed; much less than for other alkylating agents, and the killing of L1210 cells is not impressive after this dose. (Table 1, columns three and four). The agent apparently has very little toxicity for the bone marrow. This is in agreement with the clinical data[6].

In a screening study on a transplantable osteosarcoma it was found that the LD10 administered subcutaneously caused a volume reduction by a factor of 0·22. This result is of the same order of magnitude as observed with nitrosoureas and cyclophosphamide and its congeners. Further studies with this agent seem indicated.

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Letter to the Editor

Ovariectomy and Postpartum Growth of Rat Mammary Tumors*

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WHILE the stimulatory effect of pregnancy on the growth of rat mammary tumors induced by 7,12-dimethylbenz[α]anthracene (DMBA) seems to be well established [1], the ability of the subsequent lactation to maintain such growth is highly variable. When rats are allowed to nurse their full litters, some tumors regress, some grow rapidly, while others maintain a constant size [1]. Litter removal at birth is followed by regression of all tumors [2, 3], indicating that nursing is necessary for tumor growth. However, ovariectomy of lactating tumor-bearing dams abolishes tumor growth [2]. Because progesterone seems to be the principle hormone secreted by the rat ovary during lactation, it may be concluded that progesterone is necessary for postpartum tumor growth. In the present study, ovariectomized tumor-bearing dams were allowed to nurse litters of increasing numbers, in order to determine whether the increased prolactin secretion seen when large litters are nursed [4] could maintain tumor growth in the absence of ovarian progesterone.

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Virgin female Sprague–Dawley rats were stomach-tubed at 50 days of age and fed 20 mg DMBA in sesame oil. Eighteen days after DMBA feeding, females were placed with males and allowed to breed. Two groups, one intact and one ovariectomized on day 2 postpartum, nursed 6 pups for 21 days. Two other groups were ovariectomized on day 2 postpartum, and nursed 9 and 12 pups, respectively, for 21 days. Tumors were detected by palpation, and measured weekly with calipers. Tumors were considered to have regressed significantly if their size at the end of the 21 day postpartum period was equal to one-half the maximum size attained at parturition.

All rats developed palpable mammary tumors during their pregnancy, and all tumors grew until parturition. Some grew more rapidly than others. The majority of the tumors could be classified as either adenocarcinomas or fibroadenomas. Some tumors seemed to be composed of equal portions of fibrous and epithelial elements and were classified as "mixed".

In intact dams nursing 6 pups, 53·8% of all tumors were static or growing (Table 1). In ovariectomized rats nursing 6 pups, a significant decrease was seen in the number of tumors which were static or growing. The difference between the number of growing tumors in ovariectomized rats nursing 6, 9 or 12 pups was not statistically significant (chi-square). However, the number of growing tumors in ovarectomized lactators nursing 9 or 12 pups was also significantly different from intact controls

Table 1. *Effect of litter size on postpartum tumor growth in ovariectomized rats*

Group	No. rats	No. tumors static of growing/no. of tumors (%) AC*	FA†	Mixed
Intact, 6 in litter	9	6/9 (66·9)	1/3 (33·3)	0/1 (0)
			7/13 (53·8)‡§‖	
Ovex., 6 in litter	6	2/7 (28·6)	0/0 (0)	0/2 (0)
			2/9 (22·2)‡	
Ovex., 9 in litter	8	2/7 (28·6)	0/3 (0)	0/4 (0)
			2/14 (14·3)§	
Ovex., 12 in litter	7	1/3 (33·3)	0/4 (0)	0/2 (0)
			1/9 (11·1)‖	

(chi-square). In all rats, significant regression was seen in all tumors which were not static or growing.

The present experiment lends further support to our previous conclusion that prolactin has little direct effect on postpartum growth of rat mammary tumors. Prolactin secretion is greater when larger litters are nursed [4]. If prolactin had any direct effect on postpartum tumor growth, then increased growth should have been seen when ovariectomized dams nursed larger litters. Such was not the case, and we must still conclude that ovarian progesterone is necessary for postpartum growth of DMBA-induced rat mammary tumors, even in the presence of increased endogenous prolactin secretion.

References to Table

*AC = Adenocarcinomas.
†FA = Fibroadenomas.
‡Significantly different at the 5% level (Chi-square).
§Significantly different at the 1% level (Chi-square).
‖Significantly different at the 1% level (Chi-square).

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Letter to the Editor

Radiation Responses of Cells in Tumours and Cells in Culture. Have Anomalous Differences been Demonstrated?

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IN THEIR recent paper entitled "Studies on the radiobiology of a rat sarcoma treated *in situ* and assayed *in vitro*", Dawson and co-workers [1] conclude that a significant and unexplained difference exists between the radiosensitivities of cells of the Visking sarcoma irradiated *in situ* and *in vitro* respectively. This conclusion is of importance for experimental and clinical radiotherapy, because of the implication that responses of tumours cannot be interpreted on the basis of cellular radiosensitivity with respect to clonogenic capacity derived from *in vitro* studies. It appears of interest, therefore, to evaluate the evidence presented by Dawson *et al.*, and to relate this evidence to data published for other tumours.

It should be first noted that Dawson *et al.* are quite correct in stating that various authors have pointed out the considerable spread, observed among survival parameters of mammalian cells, with D_0 values ranging from about 50 to 200 rad, and D_Q values ranging from 1 to 100 (leaving aside a few less well documented and extreme values). However, contrary to the assertion by Dawson *et al.*, it must be doubted that informed investigators have ever made "a common assumption that all cells of mammalian origin have very similar radiosensitivities" and this assumption is certainly not accepted at present. If the authors make this statement they should quote literature relevant to this point.

In relation to this first point, it is of interest to note also that the D_0 and D_Q values derived from the Visking sarcoma cells, irradiated *in vitro*, ranging from 136 to 180 rad and 67 to 165 rad respectively, are well within the range of values generally derived for mammalian cells. Moreover, the shapes of these curves are sufficiently documented by four points at regular dose intervals down to the region of a few percentage of survival. By contrast, however, the survival points for tumour cells irradiated *in situ*, and subsequently assayed by the plating technique, are not taken at regular dose intervals, and the lowest surviving fraction is equal to about 0·07. These data are evidently inadequate to derive a D_0 and a D_Q. It is commonly accepted that, in the region between 100% and 10% survival, the curve is in general not exponential and consequently the D_0 must be derived from at least two experimental data points below 10% survival. Furthermore, the data presented are insufficient to establish whether the survival curve has a complex shape and is composed of the two parts frequently observed for tumours containing proportions of oxygenated and severely hypoxic cells [2–4]. Before arriving at important conclusions about cellular sensitivity, it would have been appropriate to obtain data at larger doses and to carry out experiments with tumours made anoxic by killing the animal before irradiation, in order to assess the oxygenation status of these tumours.

With respect to the data obtained from experiments on growth delay of tumours after irradiation, it is of interest to note that for other tumours, a major cause for the small degree of volume response has been shown to be due to an increase in the rate of cell production, due to a shortening of the cell cycle [2, 5–7]. It can be concluded that, especially for tumours which grow with volume doubling times in excess

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of 24 hr, growth delay does not provide a direct measure of cell survival. In addition, it is evident that for the few survival data, derived from assessments of growth delay, a similar criticism with respect to the range of fractions of surviving cells must be made, as for the data derived for tumours irradiated *in situ* and assayed *in vitro*.

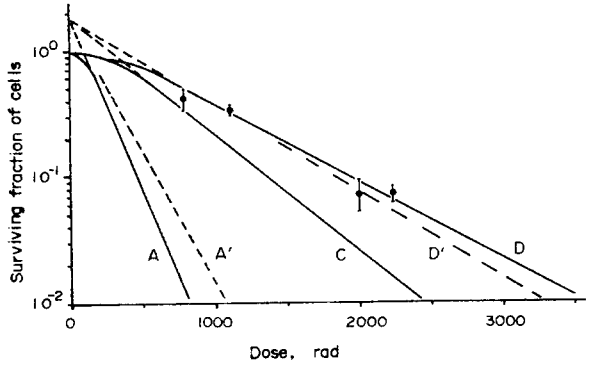

Fig. 1. *Comparison of survival curves of Visking sarcoma cells irradiated* in situ *and assayed* in vitro.

Curve A: *Cells irradiated* in vitro *and assayed* in vitro *in well oxygenated conditions.*

Curve C: *Curve derived for anoxic cells by assuming an OER of 3 for cells of curve A.*

Curve D and points: *Cells irradiated* in vivo *and assayed* in vitro.

Curve D': *Curve consistent with points and having the same extra-polation number as curves A and C.*

Curve A': *Curve derived for oxygenated cells, assuming an OER of 3 for cells of curve D'.*

A third point to be noted concerning the data presented by Dawson *et al.* is concerned with their evaluation of the possible influence of anoxia of cells in the tumours. In Fig. 1 I have replotted curves A, C and D from Fig. 7 of Dawson *et al.*, together with the data points of their Fig. 5. Their curve C was derived from curve A by assuming an OER = 3. Curve D' has been added with a D_0 = 620 rad and the same extrapolation number as curves A and C. This curve D' is not inconsistent with the experimental data. If an OER of 3 is applied to curve D', curve A' with a D_0 of 207 rad is obtained. This value is very close to the range of D_0 values mentioned earlier. Curve A applies to cells which were presumably all proliferating (P-cells) but the tumour may also contain non-proliferating cells (Q-cells). These Q-cells may differ from P-cells in radiosensitivity. It appears, therefore, that the evidence is not sufficient to conclude that: "the survival of cells irradiated in a tumour *in situ* is greater than could be accounted for in terms of survival of cells irradiated in culture, if anoxia of the tumour cells was the only factor involved." Cells in culture should be studied in the non-proliferative stage as well. The presence of Q-cells may also influence the effectiveness of a treatment with hydroxyurea and consequently an interpretation of these data of the fraction of Q-cells must be known.

It may be concluded that, although it would be imprudent to state that responses of tumours can be completely accounted for on the basis of cell survival curves obtained from *in vitro* studies and cell proliferation kinetics, the suggestion of an anomaly for the Visking sarcoma appears insufficiently documented.

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Reply to Dr. Barendsen's Letter

DR. BARENDSEN has raised a number of criticisms of our paper [1]. Whilst this is an important subject for those interested in the behaviour of irradiated tumours and one that is receiving increasing attention, in the interests of brevity I will only try to answer his specific points.

"Informed investigations have never made 'a common assumption that all cells of mammalian origin have a similar radiosensitivity' ". Of course this is true for "informed investigators", but over the years since Puck's original work I have frequently heard it stated or implied by those using radiation who are not actively engaged in research and it has on occasion appeared in print (see for example the chapter on "Radiation response and recovery: radiobiological principles and their relation to clinical practice" in [2]).

He states that our *in vivo–in vitro* and the *in vivo* results were not taken to low enough survival values to determine the curve parameters accurately or to see if the curves had a complex shape. Our reservations on these data and the assumptions involved in their interpretation were discussed in the paper. Unless the anoxic fraction was relatively large it would not be possible to demonstrate it in our experimental system—in Powers and Tolmach's original survival curve [3] they had to go down past the 4th decade to demonstrate an inflexion and a hypoxic component. However, even at these relatively high survival levels, the difference between the two irradiation situations has to be explained. If our data were such that we were still working on a broad shoulder and had not reached the exponential parts of the curves, the implications for radiotherapy are still very important as Hornsey [4] has emphasised.

It was an omission that no direct experiments were made on tumours rendered anoxic *in situ*, but this does not invalidate our conclusions as, even assuming a maximum OER of three and that all the tumour cells were anoxic, which is very improbable, the results of the two situations are not compatible. This is shown in Barendsen's figure, where curve A′ with these assumptions is similar, but still does not fit the experimental data for curve A. Using a similar experimental technique and direct measurements on hypoxic tumours, McNally [5] found qualitative differences in the amount of damage expressed *in situ* and *in vitro*.

When we come to the question of P and Q cells, I do not think this explains any discrepancies but rather poses the problem. If there are P and Q cells in a tumour then their relative proportions and their respective radiosensitivities *in vivo* and *in vitro* (there is still discussion on this) will have to be known as well as other factors before the fate or an irradiated tumour can be predicted. Durand and Sutherland [6] have discussed this briefly and also suggested that one of the differences between the *in vivo* and *in vitro* radiosensitivity is cell-to-cell contact, although it could be argued that this is a special feature of their ingenious experimental model.

Nowhere do we state that the responses of tumours cannot be interpreted on the basis of cellular radiosensitivities; rather we stress that the response of a tumour cannot necessarily be predicted from *in vitro* data and that laboratory screening based on this approach should be employed with extreme care. In this relatively simple animal system we believe we have indicated a discrepancy between the tumour cells irradiated in culture and the tumour cells irradiated *in situ*. Hermens [7] has illustrated how in the human situation the techniques for collecting the necessary information are not available in the majority of cases—even assuming there are no unknown factors. When the complexities of fractionation are added, it is difficult at the present time to see how the fate of an irradiated tumour can be predicted other than from the criteria of "clinical experience".

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Announcement

The organization is now under way of the 6th L. H. Gray Conference, on

"The Initial Shapes of Cell Survival Curves: Clinical and Theoretical Implications".

It will take place at Bedford College, Regent's Park, London, 16–21 September, 1974.

It is hoped that the meeting will be of interest to radiotherapists as well as to radiobiologists.

The organizers have prepared a list of topics which they envisage as the subject matter, and they plan to arrange the programme to some extent in accordance with the availability of relevant results. The list may be obtained from Dr. Tikvah Alper, Medical Research Council, Experimental Radiopathology Unit, Hammersmith Hospital, London, W12 0HS, England.

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Nuclear and Nucleolar Ultrastructural Lesions Induced by 1-Nitro-9-Aminoacridine (C-283) in Human Ovarian Carcinoma Cells*

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Abstract—*The effect of 1-nitro-9-aminoacridine derivative (C-283) on the ultrastructure of tumour cells obtained from peritoneal and pleural cavity exudates of patients with ovarian carcinoma was studied. C-283 induces condensation and margination of the chromatin, segregation of the nucleolar components and the appearance of helical ribosomal forms in the cytoplasm of carcinoma cells.*

It is here suggested that the observed lesions are the morphological manifestation of RNA biosynthesis disturbances. The clinical effect of the drug resulted in the absence of carcinoma cells in effusion fluid 24 hr after a single injection of C-283.

INTRODUCTION

RECENTLY published clinical observations show an inhibitory effect of C-283† on the formation of effusion in pleural and peritoneal cavities of patients with ovarian carcinomas [1, 2, 3].

The results of postmortem examinations suggest that this suppressive effect does not result from obliterative changes in body cavities, but rather from direct action of C-283 on carcinoma cells present in the exudate [1].

Biochemical studies on the mechanism of C-283 action performed *in vitro* and *in vivo* show a strong inhibitory effect of this compound on RNA biosynthesis [4, 5].

In rat hepatocytes this compound induces characteristic nuclear ultrastructural changes which are considered to be the morphological expression of RNA synthesis alteration [6, 7].

The main purpose of the present work is to examine the influence of C-283 on the ultrastructure of carcinoma cells obtained from peritoneal and pleural cavity effusions of patients with ovarian carcinomas.

MATERIAL AND METHODS

Five patients with ovarian carcinomas showing pleural exudates were treated with C-283 given by single intracavitary injection in a dose of 10 mg. One case of peritoneal cavity exudate was also examined. In this case 25 mg of C-283 was injected intraperitoneally.

In each case samples of exudate taken immediately before injection of C-283 were processed for an ulstrastructural examination of untreated carcinoma cells.

For studies of the effect of C-283 the samples of exudate were taken 3, 6 and 24 hr after the drug injection. The aspirated exudate was centrifuged in a refrigerated centrifuge (4°C) for 10 min at 1800 rev/min. The supernatant was discarded after which the pellet was fixed with cacodylate-buffered 2·5% glutaraldehyde

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†1-nitro-9/3′-dimethylpropyloamine/acridine-2HCl synthetized at the Department of Drug Chemistry and Technology, Technical University, Gdańsk, Poland.

followed by postfixation with veronal acetate-buffered 1% osmium tetroxide. After dehydration in ethanol, the pellets were embedded in Epon 812 mixture.

Ultrathin sections were stained with uranyl acetate and lead citrate and examined under a Tesla BS 513 electron microscope.

RESULTS

The pellets obtained from exudates of untreated patients are composed of various types of cells. In addition to the carcinoma cells, numerous macrophages and blood cells, including erythrocytes, are present. Despite some differences in individual patients, the recognition of cancer cells is relatively easy because of their large size and ultrastructural features. The surface of the usually round cancer cell is slightly undulated, forming short microvilli and protrusions. The cytoplasm contains all typical organelles with rather scanty, rough endoplasmic reticulum. In some cases, large aggregations of glycogen granules are present.

The cancer cell nuclei are large and irregular in shape due to the prominent indentations of the cytoplasm into the nucleus. The chromatin is always dispersed, forming small spots throughout the entire nucleus. A thin layer of condensed chromatin is always visible along the nuclear envelope. Between chromatin spots loose conglomerations of interchromatinic granules are present (Fig. 1, arrows). The nucleolus-associated chromatin is usually dispersed, adhering to the nucleolar body in small clumps (Figs. 1 and 2). As a rule the nucleoli are large, of round or oval shape, and are composed of characteristic nucleolar components with a clearly visible nucleolonema (Fig. 2). Three nucleolar components—fibrillar (f), granular (g) and fibrillar centers (fc)—can be distinguished.

Three hours after injection of C-283 dramatic changes in the ultrastructure of carcinoma cells occur. The most evident changes are noted in the nuclei and nucleoli. These consist in the margination of chromatin which becomes more condensed and electron-dense. The condensed clumps of chromatin are frequently visible near the nucleoli (Fig. 3, arrows). At this time the nucleoli of all examined cancer cells show more or less advanced steps of segregation (Fig. 3). The fibrillar component (f) forms round, condensed clumps with adhering fibrillar centers (fc). The granular component (g) is condensed, also forming an irregular mass. In some cases the granular component of segregated nucleoli completely disappears (Fig. 4). In such nucleoli only two components, fibrillar and fibrillar centers, are visible. The nucleolus-associated chromatin shows characteristic changes, for example, the formation of spotty, hypercontrasted clumpings (Fig. 4, arrows).

Six hours after introduction of C-283, the ultrastructural changes are more pronounced. They consist in more evident margination and condensation of the chromatin which forms dense spots and clumps (Fig. 5). At this time the nucleoplasm becomes more transparent than in untreated cells. The interchromatinic granules form characteristic large clusters. The nucleolar material is very dense, which makes the differentiation of the segregated components difficult. In some nucleoli a clear central area is present. The nucleoli are frequently present in close proximity to deep invaginations of the cytoplasm (Fig. 5c).

In all examined cases, after treatment with C-283, a very characteristic arrangement of the ribosomes in cell cytoplasm can be noted. The ribosomes are arranged in the form of elongated helical structures scattered throughout the entire cytoplasm (Fig. 6 and 7, arrows). They occur together with free and membrane-bounded, typically arranged, ribosomes.

Similar changes in cell nucleus and nucleolus as well as the appearance of helical polyribosomes are observed not only in carcinoma cells, but also in macrophages present in the samples. Figure 8 shows a fragment of a macrophage 3 hours after injection of C-283. In the nucleus the margination and condensation of the chromatin and segregation of nucleolar material are visible. The helical ribosomal structures are also present in the cytoplasm of this cell (Fig. 8, arrow). We were unable to find any cancer cells in any of the samples taken 24 hr after the injection of C-283 into the body cavity.

DISCUSSION

Our results show that the aminoacridine derivative C-283 induces specific changes in the ultrastructural pattern of the nucleus and nucleolus of human ovarian carcinoma cells present in the exudate. These changes consist mainly in segregation of the nucleolar components as well as condensation and margination of the chromatin. Identical changes caused by C-283 were observed by one of us [6] in normal and regenerating rat liver.

Similarly, some other acridine derivatives, such as acridine orange [8], proflavin [8, 9, 10] and quinacrine [11], induce these characteristic nucleolar changes. Furthermore, the phenomenon of nucleolar segregation was observed in

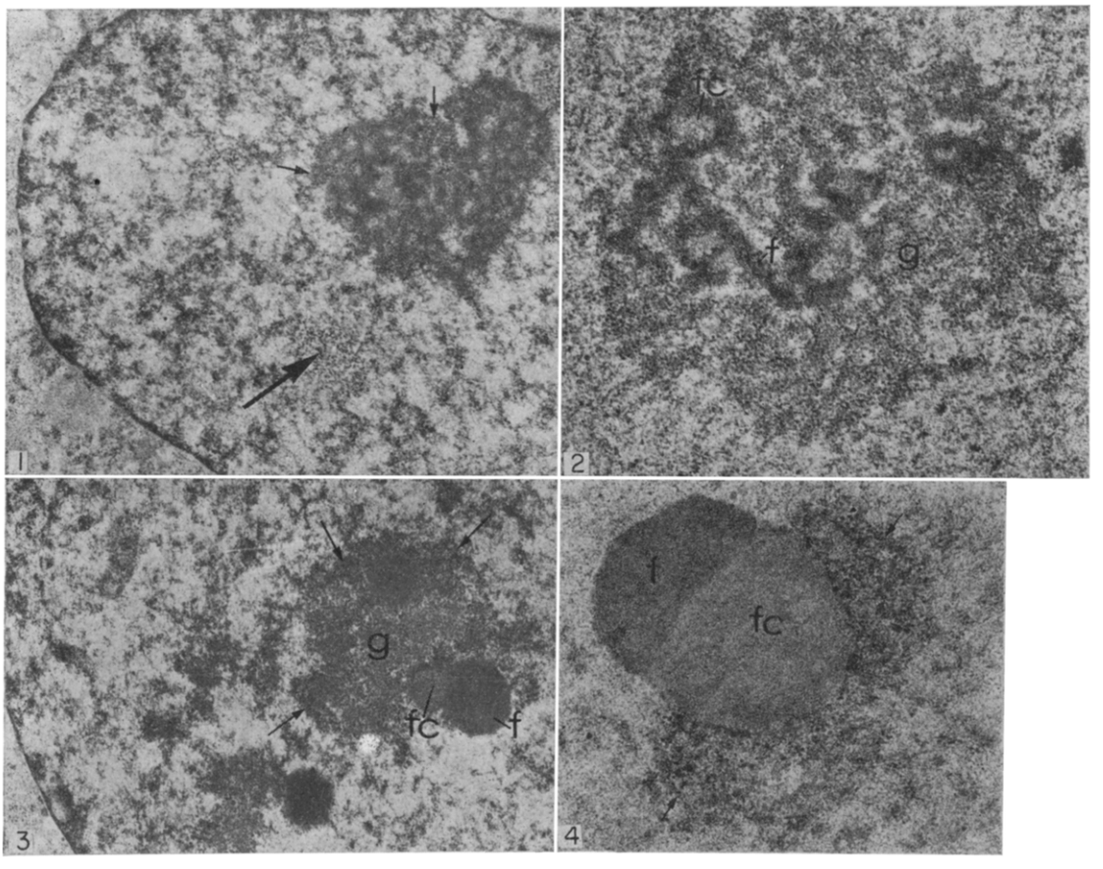

Fig. 1. *Nucleus of an untreated cancer cell obtained from the pleural cavity. Chromatin is scattered throughout the nucleoplasm and forms a thin condensed layer near the nuclear envelope. Nucleolus consists of a highly visible nucleolonema. Short arrows—nucleolus-associated chromatin; long arrow—interchromatinic granules.* × 15600.

Fig. 2. *Nucleolus of an untreated carcinoma cell obtained from the pleural cavity. The nucleolus is rich in granular component (g). The fibrillar component (f) surrounds the fibrillar centers (fc).* × 24000.

Fig. 3. *Fragment of a cancer cell nucleus* 3 *hr after intrapleural injection of C-283. The chromatin spots are a little larger as compared to Fig.* 1. *The nucleolus shows distinctly visible features of segregation. f—fibrillar component; fc—fibrillar center; g—granular component; arrows—nucleolus associated chromatin.* × 15600.

Fig. 4. *Nucleolus of a cancer cell from the pleural cavity obtained* 3 *hr after C-283 injection. Only two nucleolar components distinctly segregated are visible; the fibrillar component (f) and the fibrillar center (fc). The nucleolus associated chromatin (arrows) shows many small hypercontrasted spots.* × 24000.

(to face p. 786)

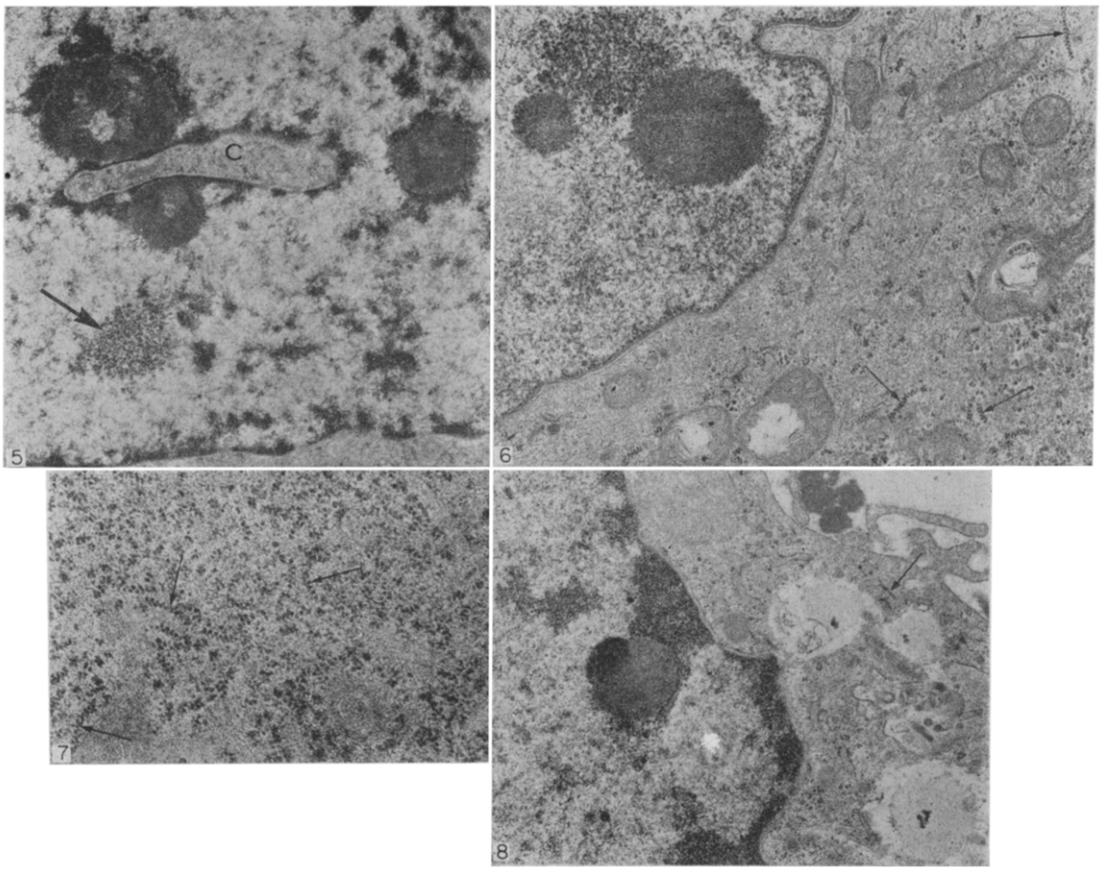

Fig. 5. *Fragment of a cancer cell nucleus* 6 *hr after intrapleural injection of C-283. Distinct margination of chromatin at the nuclear envelope and nucleoli is visible. The nucleoplasma appears lighter. The interchromatinic granules (arrow) form a condensed conglomeration. The nucleolar components are segregated. c—a deep invagination of cell cytoplasm into the nucleus.* × 15600.

Fig. 6. *Fragment of a cancer cell from the peritoneal cavity taken* 3 *hr after C-283 injection. The nucleolus shows distinct features of segregation. In the cytoplasm numerous helical ribosomal forms (arrows) are noticeable.* × 24000.

Fig. 7. *Helical ribosomal forms (arrows) in a cancer cell obtained from the pleural cavity* 3 *hr after C-283 injection.* × 43000.

Fig. 8. *Fragment of a macrophage from the peritoneal cavity taken* 3 *hr after C-283 injection. Segregation of the nucleolus as well as helical ribosomal forms (arrow) are distinctly visible.* × 15600.

mammalian cells exposed to a variety of non-acridine compounds such as actinomycin D [12, 13, 14, 15, 16, 17], aflatoxin [18, 19], anthramycin [20], daunomycin and ethidium bromide [9], mitomycin C [21] and azaserin [22]. A typical segregation of the nucleolar components was recently described in monkey kidney cells infected with Coxsackie-virus A9 [23]. Some physical agents are also capable of inducing this phenomenon, e.g., ultraviolet rays [24] and X-rays (unpublished data).

It is generally believed that nucleolar segregation is a morphological expression of nucleolar disfunction resulting from disturbances in the DNA-dependent RNA synthesis [25].

As was already mentioned, the compound C-283 strongly inhibits the synthesis of RNA [5]. C-283, similar to other acridine compounds, forms complexes with DNA molecules [26]. It seems very probable that this compound's mechanism of action is similar to that of other substances which are able to form complexes with DNA. According to Goldblatt *et al.* [16], the formation of such complexes leads to the coiling of the chromatin strands concomitant with the segregation of the nucleolar components. Another structural manifestation of the formation of complexes between DNA and interacting compounds is condensation and margination of the chromatin [9, 17], which was also observed in our material after C-283 treatments.

In the cytoplasm of carcinoma cells treated with C-283 we have noted the presence of unusual ribosomal helical forms. Similar helical forms were already observed in different plant [27, 28, 29] and animal cells [30, 31, 32, 33]. They are considered to be a type of polysomes but their significance is not known. The appearance simultaneously in our material of the helical ribosomal forms with the nucleolar segregation suggest that these may be a manifestation of disturbances in the ribosomal RNA biosynthesis.

In our study the nucleolar segregation and the appearance of helical arrangements of ribosomes is observed not only in carcinoma cells but also occasionally in other types of cells, namely, in macrophages. This observation suggests that the action of C-283 is not exclusively directed to proliferating cells, although the final effect of the drug is more evident and easily perceptible in the latter. Clinical expression of the cytotoxic action of C-283 is the suppression of the exudate production in body cavities. In our experiments the rapid action of the drug was manifested by the lack of carcinoma cells in effusion samples taken 24 hr after C-283 injection.

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Growth Curve Modification of Measurable Tumors by 75 mg/m^2 of CCNU every 3 Weeks

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Abstract—*Thirty patients with measurable tumors of known spontaneous doubling time were treated by CCNU 75 mg/m^2 every 3 weeks. Hematopoietic toxicity was minimal with this original schedule. Efficacy has been judged on therapeutic growth curves. Complete response occurred in 3 cases (10%), partial response over 50% in 2 (7%), partial response between 50 and 25% in 3 (10%), static response in 12 (40% with a median duration of 4 months), significant slowing down in 3 (10%) and total failure in 7 (23%). In 9 cases resistance, as judged by the resuming of the previous growth rate despite the treatment, occurred after a median duration of 1·5 months.*

The analysis of the data shows that: (a) this model allows the detection and measure of weak responses that are overlooked by the usual trials though they may be of great benefit to the patients in terms of survival gain; (b) in all responders the response is seen within one week after the beginning of the treatment; (c) maximal response has been always obtained before the time of the third dose; (d) maximal response and onset of total resistance follow in close succession (1 month); (e) there is no correlation between spontaneous growth rate and the degree of response. These unexpected findings show that resistance plays a more important role with this drug than non-proliferating cells as a limiting factor.

These findings are discussed in view of the strategy of chemotherapy.

APART from other advantages, the study of volume variations induced by chemotherapeutic agents in measurable tumors of known spontaneous doubling time makes it possible to assess the true effect of a given drug with greater accuracy [1–4]. This is why the EORTC decided to create a group using this evaluation model. The aim of this study is to demonstrate the possibilities of this model by measuring the effects of 75 mg/m^2 of CCNU every three weeks on various measurable tumors. The reason for choosing this dose rather than the traditional 130 mg/m^2 every six weeks will be explained in the discussion. This paper also discusses certain effects on tumor growth often overlooked by standard therapeutic screening methods, the time taken for response to appear, the relationship between the degree of response and spontaneous growth rate, and determination of the clinical resistance coefficient.

Accepted 6 August 1973.
*Chairman.
†Secretary of the group.

MATERIAL AND METHODS

Patient selection

Only patients with known histology were included in this study. In most cases, the measurable tumor was situated in the lung (28 cases). Of these, 10 were primary bronchogenic carcinomas (8 squamous cell, 2 adenocarcinomas) and 18 were pulmonary metastases of various origins: breast (4), larynx, head and

neck (3), uterus (2), bladder (2), salivary glands (2), colon (1), kidney (1), oesophagus (1), uterine leiomyosarcoma (1), osteosarcoma (1). In addition, 1 case of a palpable metastatic lymph node due to a primary liposarcoma of the pharynx and 1 case of subcutaneous metastases of a malignant melanoma were included. This brought the total number of cases to 30.

To be included in this study, measurable tumors had to meet the following requirements: round or slightly elliptical shape, all contours visible, identical conditions of measurement on all films for any given tumor. The diameter taken into account was the mean of the vertical and horizontal diameters. To calculate the spontaneous doubling time using Collins graphic method [5], at least three measurements were required. A minimal spontaneous progression period (SPP) was defined as a function of the doubling time (DT). Values for the SPP were determined according to a straight line which was a logarithmic function of the DT (since the DT forms a log normal distribution [6, 7]), and on the basis of a spontaneous period of progression of 60 days for a doubling time of 60 days (Fig. 1). This was chosen because

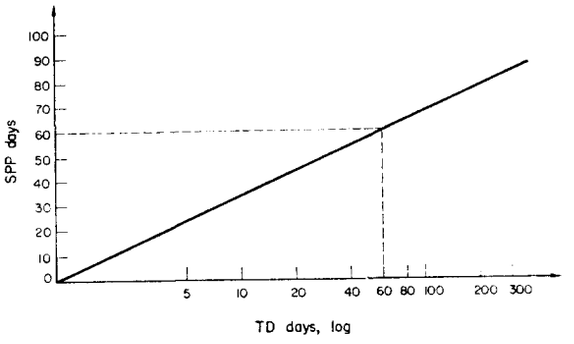

Fig. 1. Required spontaneous progression period (SPP) as a function of doubling-time (DT). (SPP = 34 log TD).

60 days corresponds to an overall mean doubling time, as established in 530 cases by Charbit *et al.* [8], and because in practice both the duration of the investigations needed to determine the extent of the disease and/or the duration of the retrospective observation, as shown by previous X-ray films, are compatible with this mean value. This means for example that in order to accept a DT of 200 days, the SPP had to be of at least 78 days, whereas a DT of 10 days could be measured with the same accuracy in a period of 34 days.

The following cases were excluded from this study: patients in poor general health, in the terminal phase of disease with a life expectancy of less than 2 months; patients with abnormal blood counts (WBC $\leqq$ 3,000/mm^3, platelets $\leqq$ 120,000/mm^3); patients with disturbed renal function (creatinine clearance $\leqq$ 60 ml/mn and/or blood creatinine level > 1·2 mg/100 ml). We also excluded cases in which surgery or radiotherapy were indicated. If the patient had been previously treated (surgery, radiotherapy, chemotherapy), a therapy-free interval of at least one month was required.

Of the 30 patients, 20 were male and 10 were female. Their ages ranged from 22 to 76 years and the doubling times of their tumors from 9 to 240 days (Table 1).

Treatment prior to this study was as follows: none = 8 cases; surgery only = 8 cases; radiotherapy only = 2 cases; chemotherapy only = 3 cases, surgery + radiotherapy = 3 cases; surgery + chemotherapy = 1 case; surgery + radiotherapy + chemotherapy = 5 cases.

Therapeutic schedule

CCNU was supplied in capsules of 40 and 100 mg containing lactose and magnesium stearate as bulk and was stored at −10°C until use to prevent decomposition. The drug was administered orally in a standard of 75 mg/m^2 every 3 weeks. When required, the calculated dose was obtained by emptying and weighing part of the capsule content. Each dose was administered at the same time under medical supervision and one vial of 10 mg of metoclopramide was injected intramuscularly to delay the onset of nausea and vomiting. Other symptomatic therapy, particularly antiemetics, was authorized. Administration of corticosteroids was also authorized, but only for short periods. Before starting therapy, the following parameters were recorded: blood count, including platelets and differential white count, alkaline phosphatases, SGOT and SGPT, blood urea and creatinine, intradermal reaction to tuberculin (10 units i.p. 48, Institut Pasteur). These investigations were completed by chest X-rays (face-on and lateral). During the treatment, before administering each dose, these investigations were repeated except for the intradermal tuberculin test which was repeated 4 and 8 weeks after the start of therapy.

Plotting the growth curve and evaluation of results

Response was assessed as follows: (1) No response: growth rate unchanged; (2) Slowing down: progression continues but the growth rate becomes significantly slower than the spontaneous growth rate. Three administrations were required before a change of treatment could be considered. (3) Static response or plateau: treatment is continued as long as the

Table 1. Patients treated by CCNU (75 mg/m² every 3 weeks)

Cell type	Case No	Age (Years)	Sex	DT (Days)
I. Primary bronchogenic carcinomas:				
Squamous cell	1	68	M	27
	2	51	M	37
	3	66	M	40
	4	56	M	48
	5	54	M	53
	6	70	M	67
	7	68	M	86
	8	63	M	110
Adenocarcinoma	9	60	M	55
	10	73	F	135
II. Pulmonary metastases:				
Breast	11	51	F	38
	12	50	F	78
	13	51	F	102
	14	72	F	52
Larynx	15	62	M	60
	16	64	M	114
	17	44	M	157
Uterus	18	67	F	98
	19	42	F	100
Bladder	20	58	M	20
	21	46	M	24
Salivary Glands	22	76	F	57
	23	67	M	240
Colon	24	76	M	105
Kidney	25	73	M	20
Oesophagus	26	63	M	22
Uterine leiomyosarcoma	27	59	F	30
Osteosarcoma	28	22	M	22
III. Metastatic lymph node of a liposarcoma	29	69	M	47
Subcutaneous metastase of a melanoma	30	60	F	9

plateau is maintained; (4) Regression: the slope of the curve and the lowest point reached are recorded, with two subgroups (partial response > 50%: regression of more than 50% in the product of two diameters, and partial response < 50%); (5) complete response: disappearance of all measurable tumors; (6) resistance: is defined as the point at which the growth curve under treatment once again becomes parallel to the spontaneous growth curve after an initially favorable effect of the drug. In this situation treatment is changed.

Moreover, survival gain was calculated according to a method previously described [9]. These results were compared to the usually accepted criteria for evaluable, but non-measurable tumors.

RESULTS

1. *Duration of treatment*

The treatment with CCNU has been discontinued for the following reasons:

Initial failure	7 (F)
Acquired resistance after response	9 (R)
Recurrent toxicity	4 (HT)
Death during weak response	5 (D)

and 5 patients are under treatment at present time (T). Table 2 indicates the number of courses of CCNU per patient.

Table 2. Number of doses of CCNU

Number of doses of CCNU	1	2	3	4	5	6	8	9	13
Number of patients	1	2	10	4	4	3	3	2	1

2. *Tolerance and side-effects*

No cases of kidney or liver damage were recorded in this series. From a gastrointestinal standpoint, nausea and vomiting were almost

constant and began within a few hours of administration. The antiemetics used (intramuscular metoclopramide) delayed their onset. This nauseous state was transient and disappeared after 24 48 hr. It is extremely important to ensure that the capsules have not been vomited. It is noteworthy, on the other hand, that in one case of cerebral metastases with intracranial hypertension inducing repeated vomiting, administration of CCNU was particularly effective and overcame neurologic symptoms, including vomiting (case No. 12).

The hematopoietic toxicity will be thoroughly discussed in another paper comparing two different schedules of CCNU. We will only indicate here that 24 patients (80%) tolerated the present schedule without any adverse hematologic effect, whereas in 4 patients we had to delay the therapy for 3 to 6 weeks on one occasion, and, in 2 others, we had to discontinue it for a long-lasting thrombocytopenia.

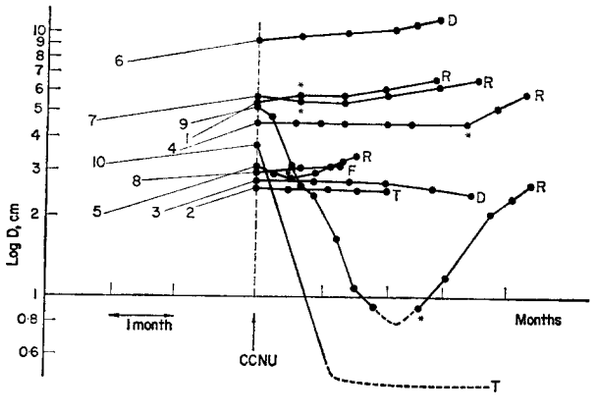

Fig. 2. Growth curves of primary bronchogenic carcinomas—Cases 1–10 (the arrow indicates the onset of CCNU)

* = *resistance*
R = *acquired resistance at the point**
D = *death*
F = *failure*
HT = *hematologic toxicity*
T = *still under treatment*

3. *Efficacy*

Figures 2, 3, and 4 and Table 3 summarize the results obtained. Out of 30 cases, 3 complete responses were obtained (10%), 2 of which were in cases of primary adenocarcinoma of the lung. This is a noteworthy result. The third patient was a case of breast cancer with pulmonary and cerebral metastases attended by signs of intracranial hypertension which disappeared under the influence of treatment. Five partial responses were recorded: 2 (7%) of more than 50% in the product of two diameters (liposarcoma: 1, melanoma: 1) and 3 (10%) of less than 50% but more than 25% (squamous cell bronchogenic carcinoma: 2, oesophagus: 1). The most commonly encountered response was a plateau: 12 cases (40%) including half the cases of bronchogenic squamous cell carcinoma.

Thus some therapeutic benefit was observed in 20 patients (67%). 3 cases (10%) showed only a slowing down or lengthening of the doubling time with maintained progression (squamous cell bronchogenic carcinoma: 1, uterus: 1, osteosarcoma: 1). In 7 cases (23%) there was no change in the spontaneous growth rate: bronchogenic squamous cell carcinoma: 1, breast: 1, uterus: 1, bladder: 2, kidney: 1, leiomyosarcoma: 1.

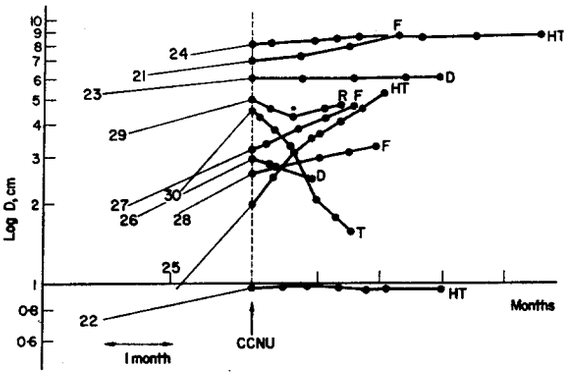

Fig. 4. Growth curves of metastatic tumors (Cases 21–30).

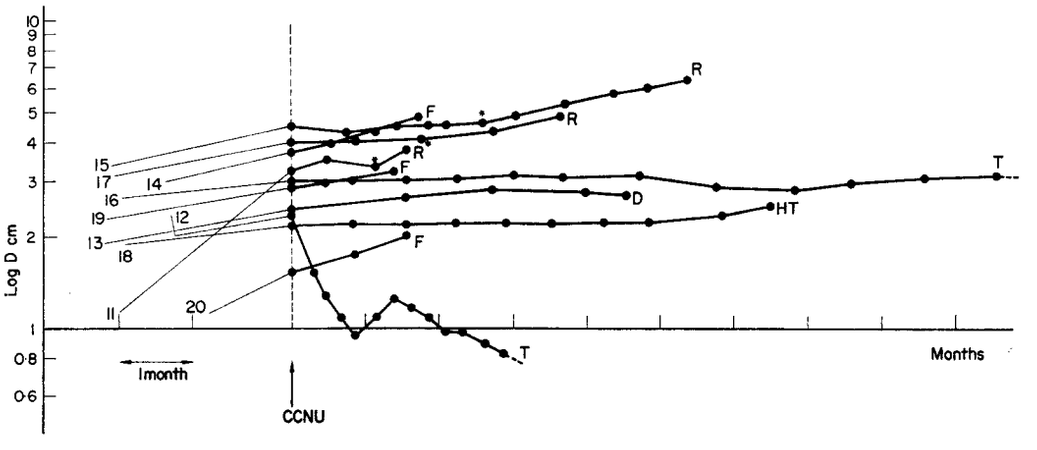

Fig. 3. Growth curves of metastatic tumors (Cases 11–20).

Table 3. Responses classified by cell type (CCNU 75 mg/m² every 3 weeks)

	CR*	PR† > 50%	PR‡ < 50%	SR§	SD‖	NR¶
Squamous cell			2	4	1	1
Adenocarcinoma	2					
Breast	1			1	1	1
Larynx				3		
Uterus				1		1
Bladder						2
Salivary glands				2		
Colon				1		
Kidney						1
Leiomyosarcoma						1
Osteosarcoma					1	
Oesophagus			1			
Liposarcoma pharyngeal		1				
Cutaneous melanoma		1				

*CR: complete responses, defined as disappearance of all measurable tumors.
†PR > 50%: Partial response with regression of more than 50% of the product of the longest and widest perpendicular diameter.
‡PR < 50%: Partial response with regression of less than 50% of the product of the two diameters.
§SR: static response = plateau, or no change.
‖SD: slowing-down, progression but lengthening of the doubling-time.
¶NR: no change of the growth rate.

Table 4. Duration of responses in months (CCNU 75 mg/m² every 3 weeks)

Response	Cell type	Interval between onset of therapy and maximum response	Duration of response	Survival gain
CR	Adenocarcinoma	1·5	2·5	13
	Adenocarcinoma	1	7†	23†
	Breast	1	3†	12†
PR > 50%	Liposarcoma	1	1·5	2
	Melanoma	1·5†	1·5†	3
PR < 50%	Squamous cell	3	3	3·5
	Squamous cell	0·5	1	1
	Oesophagus	0·7	0·7	1
SR	Squamous cell	—	1·5	1·5
	Squamous cell	—	1·5	1·5
	Squamous cell	—	3·5	3·5
	Squamous cell	—	2	2
	Larynx	—	3	3
	Larynx	—	2	2
	Larynx	—	12†	12†
	Breast	—	4·5	4·5
	Uterus	—	5	5
	Saliv. glands	—	3	3
	Saliv. glands	—	4	4
	Colon	—	4	4
SD	Squamous cell	—	1	1
	Uterus	—	1·5	1
	Osteosarcoma	—	1·5	0·5

*Table 5 Comparison with the duration of response according to international trials**

Response	Cell type	Duration of response in months	
		Measurable tumors	International trials
CR	Adenocarcinoma	4	2
	Adenocarcinoma	8†	6·5†
	Breast	4†	2·5†
PR > 50%	Liposarcoma	2·5	0·5
	Melanoma	3†	1†

* Defined as the duration between reduction of 50% and progression of 25% in the product of two diameters.

4. *Duration of response*

Table 4 compares response duration according to the criteria defined above for measurable tumors. The values recorded are compared to those obtained in protocols of international trials on "evaluable" tumors which are not, strictly speaking, measurable (Table 5).

In 9 cases, we were able to evaluate the time taken for resistance to appear and the percentage of resistant cells (Table 6).

Table 6. *Appearance of resistance to CCNU* (9 *cases*)

Case No.	Delay of appearance of resistance in months	Extrapolated % of resistant cells
1	1·5	70
4	3·5	7
5	0·5	52
7	1·5	53
9	2·5	0·006
11	1	24
15	2·5	39
17	2	51
29	1	85
Median	1·5	51

DISCUSSION

1. *Toxicity*

Comparative toxicity of CCNU in the usual dose of 130 mg/m^2 every 6 weeks and in the dose used in this trial is being assayed in a separate study using the EORTC lung group and our personal work [10]. We simply wish to stress here that the number of doses administered, the total duration of treatment before permanent discontinuation because of the drug's toxicity, and the total dose administered are all markedly increased with the therapeutic schedule used in this study.

In this respect it should be pointed out that the aim of the trials which established the dose of 130 mg/m^2 every 6 weeks [11] was to determine the maximum tolerable dose. These trials then showed that for this maximum dose the mean safety interval between two doses was 6 weeks. Clinical application of the results of these toxicity trials is based on two unproven assumptions: (a) the maximum dose is the optimal dose; (b) a dose of $2D$ administered at intervals of $2T$ is as effective or more effective in controlling tumor growth than a dose of D given at intervals of T. In fact, only a randomized study involving a large number of measurable tumors of known spontaneous doubling time can provide answers to these questions. We would like to point out however, that, according to our theoretical results [12, 13], the increase in the number of courses per doubling time is a fundamental parameter in any eradication strategy. If one takes into account both the mean doubling time of human tumors (between 50 and 100 days) [8] and the rapid increase in repopulation rate after effective therapy [14, 15], it is evident that the compromise which should be sought in each case is trifactorial: maximal efficacy, minimal toxicity, shortest optimal interval between two successive doses. To be precise, this means that the term maximal efficacy is not only dose-dependent but also time-dependent. In this respect as well, tumors of known doubling time are an invaluable tool for clinical investigation.

2. *Comparison of data derived from standard evaluation methods of measurable tumors*

1. *Marked responses.* Complete response occurred in 3 patients (10%) and partial response of more than 50% in 2 patients, that is 5 responders out of 30 patients, or 17%. For bronchogenic carcinomas, the figures are 2 out of 10 which is close to other reported series of treatment of bronchogenic carcinoma with CCNU 130 mg/m^2 every 6 weeks (Carter [16]: 21% of regression, Takita [17]: 4% of complete response and 14% of partial response > 50%).

2. *Weak responses.* As can be seen in Tables 5 and 6 and Figs. 2, 3 and 4, 6 different types of responses may be distinguished from the analysis of our results. The overall response rates of 67% in this series is much higher than that of international trials using CCNU. This is due to the possibility given by the growth curves to detect weak responses (partial response < 50%, no change and slowing-down).

Interpretation of these results according to international nomenclature shows 15 cases of no change (50%) and 10 failures (33%). It is noteworthy that in the 15 cases of no change we recorded 3 responses of less than 50% and 12 plateaus which were maintained for 4 months (mean value). This means that in these cases prolonged equilibrium between tumor growth and cell-killing therapy was obtained. This effect is far from negligible, and all the more so since it is obtained without major toxicity. The association of several non cross-resistant drugs capable of inducing a plateau would be of considerable efficacy. Lastly, in 3 cases classified as failures there was in fact a slowing down of growth rate. The remarks made about plateaus are equally applicable here. It should be mentioned that multiplication of the spontaneous doubling time by 3 or

4 completely changes life expectancy, even when tumor growth continues under treatment. The above mentioned data illustrates that in addition to commonly accepted efficacy criteria, accurate evaluation of a drug requires a separate study using a measurable tumor model which permits more detailed analysis of response rates.

3. *Time taken for response to appear.* Examination of the curves reproduced in this paper confirms a finding which we believe to be of prime importance and which we have observed in all our earlier investigations, namely the fact that in practically all cases destined to respond, whether the response be a regression, a plateau or a slowing down of tumor growth, the response is initiated after the first dose and is recorded within a week. There is no such thing as initial resistance or inertia overcome by a cumulative drug-effect, but an immediate response. This is consistent with all available data on cell kinetics, but the usual response criteria were not able to demonstrate this fact.

4. *Response duration.* The usual criteria do not provide sufficient details concerning response duration since they postulate that response begins only after regression has attained 50%, and ceases only after progression has reached 25% with regard to the lowest point recorded. This evaluation method has two drawbacks: firstly, it may, as mentioned earlier, overlook weaker, but nevertheless significant responses, and secondly, it may lead to futile continuation of therapy once progression has begun but before it reaches the 25% required for it to be taken into account. In tumors of long doubling time, this time-wasting period may reach several months.

The model studied here permits accurate evaluation of the time required to obtain maximal response, irrespective of the degree of the latter. This is valuable data for comparing drugs of different action mechanisms and for calculating, as we shall do below, a possible correlation between this time-lapse and spontaneous growth rate. In the 7 cases for which regression was recorded, the mean time-lapse required to obtain a maximal response was 1·3 months. In other words, maximal response occurred after the second dose but before the third. We are of the opinion that this finding is of considerable importance in planning an eradication strategy.

We would now like to draw attention to another fact brought to light by examination of growth curves, namely the fact that although the depth of a volumetric response provides valuable data on cell kinetics, there is very little parallelism between the former and the benefit obtained by the patient. This is particularly well illustrated by the comparison of curves 9 and 16. Survival gain (12 months) was identical in both cases. One of these showed a complete response which was short-lived (1 month) while the other presented a plateau, i.e. a very weak response according to standard criteria, but which was much more prolonged.

This example shows the importance of strict evaluation criteria. Indeed, a drug which could induce a high percentage of lasting plateaus would be preferable by far to one which could induce the same number of complete but short-lived responses, even though most screening investigations performed during the past few years tend to show the contrary.

3. *Acquired clinical resistance*

We have already stressed the idea of acquired clinical resistance as it appears on growth curves [1, 4, 9]. Once a tumor reaches a certain point, its spontaneous growth rate reappears despite continuation of treatment. In our experience, continuation of therapy beyond this point has always been useless. We are thus able to determine the onset of resistance at a very early phase. This occurred in 9 of our cases within a mean time-lapse of 2 months, which is very short, extreme values being 0·5 and 3·5 months. In view of what has been said earlier concerning the time required to obtain a maximal response, it is apparent that maximal response and resistance follow in close succession. This is what we have observed in all trials involving a single agent [1, 18] contrary to data obtained with an association of five non cross-resistant drugs [3, 18]. This is a strong argument in favour of associating cytostatics.

4. *Spontaneous growth rate in cases of regression and in cases of no response*

One has only to examine the growth curves to remark that there is no correlation between spontaneous growth rate and degree of response. This clearly shows that the proliferation coefficient is not the only important parameter in the efficacy of a given treatment and that the percentage of cells resistant to this therapy from the outset is of considerable consequence. Slowly growing tumors with a low resistance fraction respond far better than rapidly growing tumors with a high resistance fraction, at least to CCNU. In an earlier investigation we studied the mathematical relationships between these two parameters [2, 12]. Again this points out the necessity of associating cytostatics without cross-resistance.

CONCLUSION

The study of responses of 30 measurable tumors of various types to 75 mg/m^2 of CCNU administered every 3 weeks confirmed the insufficiency of generally accepted efficacy criteria for "evaluable", but not strictly measurable tumors. This insufficiency lies in the fact that these criteria overlook responses classified as weak but which are, nevertheless, very important in that they reflect therapeutic activity. Moreover, these criteria provide only very approximate data concerning the initiation of response, the time required to obtain a maximal response and the onset of resistance. With regard to all these parameters, the model using measurable tumors of known spontaneous growth rate gives far better results.

In view of the findings described above, we feel that clinical screening of any new drug should include, in addition to the cases usually used, a group of measurable tumors of known spontaneous growth rate.

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Comparative Toxicity on Leukocytes and Platelets of Two Regimens of CCNU: The Relationship Between Maximum Dose and Optimum Dose

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Abstract—*Two different schedules of administration of CCNU have been retrospectively compared from the stand point of toxicity: regime A included 18 patients treated with 130 mg/m² every 6 weeks and regime B included 34 patients treated with 75 mg/m² every 3 weeks, without any difference in age, sex and previous therapies. Regime B induced a thrombopenia (< 100,000 platelets per ml) and a leukopenia (< 3000 per ml) in 8 patients out of 34, instead of 15 out of 18 for regime A ($P < 0.001$). Moreover, in those patients with hematopoietic toxicity the mean duration of treatment until the course inducing the toxicity was 105 days for regime B against 48 days for regime A ($P < 0.05$), so that patients with regime B could receive 606 mg of CCNU before toxicity against 320 mg in group A (mean values).*

These data, together with conclusions of previous theoretical work on the relationship between doubling times and eradicating schedules, show that there is no necessary relationship between optimal and maximal tolerated dose and that the former should be determined, in each clinical situation, through appropriate trials.

CCNU and related drugs possess delayed onset hematologic toxicity which has been accurately investigated by comparing the effects of a single administration of increasing doses in successive patient groups [1, 2].

These investigations led to the conclusion that the maximum tolerable dose was around 130 mg/m² every 6 weeks, and numerous studies using this dose/schedule relationship have already been published. We ourselves have participated in studies using this regimen [3, 4, 5]. Moreover, we have initiated, for reasons to be discussed below, protocols using 75 mg/m² every 3 weeks. The aim of this study is to make a retrospective comparison of the toxicity observed in these two non-randomized patient groups. In performing this study we took into account earlier personal investigations which showed that, tolerance permitting, courses should be as frequent as possible [6]. We also attempted to determine whether the maximum tolerable dose and the optimum dose were equivalent.

Accepted 6 August 1973.

MATERIAL AND METHODS

1. Group A (130 mg/m² every 6 weeks) included 18 patients: 12 cases of glioblastoma treated after surgery, 4 cases of bronchogenic squamous cell carcinoma, and 2 cases of metastatic breast cancer. Group B (75 mg/m² every 3 weeks) included 34 patients: 16 cases of primary bronchogenic carcinoma (squamous cell: 10, adenocarcinomas: 5, oat cells: 1) and 18 cases of pulmonary metastases of various origins (breast: 4, larynx: 3, uterus: 2, bladder:

2, salivary glands: 2, colon: 1, kidney: 1, soft tissue sarcoma: 2, osteosarcoma: 1).

2. In all cases CCNU was administered orally half an hour after an intramuscular injection of 10 mg of metoclopramide to suppress vomiting or delay its onset by several hours. The drug was administered only if the platelet count was above 100,000/mm^3 and the white blood cell count above 3,000/mm^3. If this was not the case, administration was postponed until these values were attained. Platelet and white blood cell counts were performed every week. Patients with a life expectancy of less than two months or those with an unsatisfactory hematologic status were not included in either of the groups. Permanent discontinuation of therapy was decided after unquestionable progression of the disease.

3. In view of the fact that the two groups were not randomized, we made sure, retrospectively, that there was no significant difference between the A and B groups for the following parameters: age (57·8 years and 58·8 years respectively, $t = 0{\cdot}25$); sex (8 females, 10 males—11 females, 23 males respectively, $\chi^2 = 0{\cdot}74$); previous therapy (4 patients out of 18 had received chemotherapy in group A, and 10 out of 34 in group B, $\chi^2 = 0{\cdot}30$).

4. Table 1 shows the number of courses of CCNU for each patient and for each group. Treatment was discontinued because of progression in 13 group A patients and in 21 group B patients, even though there were no signs of toxicity.

RESULTS

It should be remembered that we defined hematologic toxicity as a white blood cell count of less than 3,000/mm^3 or a platelet count of less than 100,000/mm^3 at the anticipated time of administration as required by the protocol.

1. *Number of patients who showed signs of hematologic toxicity at once* (less than 100,000 platelets and/or less than 3,000 leukocytes)

In group A, 15 patients out of 18 exhibited hematologic toxicity as against 8 out of 34 in group B ($\chi^2 = 22$, $P < 0{\cdot}001$). Table 2 shows the details of these disturbances. It is noteworthy that leukocytes were affected in only 1 group B patient as against 9 group A patients.

Table 1. *Total number of courses per patient and per treatment group*

Number of doses	Group A 130 mg/m^2 every 6 weeks	Group B 75 mg/m^2 every 3 weeks
1	0	0
2	6	2
3	6	9
4	2	4
5	1	4
6	1	5
7	2	2
8	0	4
9	0	2
10	0	1
13	0	1

2. *Mean duration† of treatment before the course which induced the signs of toxicity*

In group A, the mean for 15 patients was 48·7 days (±26·7) as against 105·5 days (±61) for the 8 group B patients (t: 2·51, $P < 0{\cdot}05$).

3. *Total dose of CCNU administered before the course which induced the first signs of toxicity*

The mean was 320 mg for group A (±154 mg) as against 606 mg for group B (±312 mg, $t = 2{\cdot}44$, $P < 0{\cdot}05$).

The study of the ratio between total dose and total administration time showed that the dose per unit time was identical in both groups (6·7 mg daily in group A as against 6·3 mg daily in group B), the only difference being that group B patients were able to take the drug almost twice as long before showing signs of toxicity.

†All the mean values are given with one standard-deviation.

Table 2. *Incidence of hematologic disturbances in the two groups* ($P < 0{\cdot}001$)*

Group	Thrombocytopenia only Pts	(%)	Leucopenia only Pts	(%)	Both Pts	(%)	Neither Pts	(%)
A 130 mg/m^2	6	33·3	3	16·7	6	33·3	3	16·7
B 75 mg/m^2	7	20·5	0		1	3	26	76·5

*Pts: Absolute number of patients.

*Table 3. Summary of the data for the first hematological toxicity**

	Group A 130 mg/m² every 6 weeks	Group B 75 mg/m² every 3 weeks	Statistical significance†
Mean duration of treatment inducing the first hematologic toxicity	48·7 days (±26·7)	105·5 days (±61)	$P<0.05$
Total dose of CCNU administered before the first hematologic toxicity	320 mg (±154)	606 mg (±312)	$P<0.05$
Time-lapse between toxicity nadir and return to normal values	19 days (±8·5)	16·4 days (±6·2)	N.S.
Toxicity nadir for platelets	50,000/mm³ (±27,000)	57,000/mm³ (±22,000)	N.S.

*Mean values and one standard-deviation.
†N.S. = not significant.

4. *Time-lapse between toxicity nadir and return to normal values*

This comparison is only valid for platelets since only one group B patient exhibited leukopenia. Mean values were 19 days (±8·5) for group A and 16·4 days (±6·2) for group B. This difference is not significant.

5. *Comparison of toxicity nadir*

They are the same in both groups with a mean platelet count of 50,000/mm³ (±27,000) for group A and 57,000/mm³ (±22,000) for group B.

The mean value of leukopenia in group A was 2250 WBC/mm³ and in group B the one and only patient to exhibit leukopenia had 2700 WBC/mm³.

6. *Time-lapse between initiation of therapy and return to normal values*

Mean values were 67 days (±28) for group A and 120 days (±59) for group B ($P<0.05$) which only reflects the findings exposed in 2., that is the difference in treatment duration toxicity. Table 3 summarizes the main data for the first hematologic toxicity.

7. *Tolerance to therapy*

With regard to tolerance to therapy administered after the first toxic disturbance, we would simply like to point out that in both groups the probability of inducing toxic manifestations by resuming treatment with CCNU is 8 out of 10. The retrospective conclusion which may be drawn from these findings is that after the first drop in blood count, doses should usually be reduced by 1/3 if the drug is to be continued.

8. *Progression*

Lastly, it should be mentioned that 1 patient in each group showed progression due to discontinuation of therapy because of toxicity. In group A the patient died 12 weeks after the last course with a platelet count of 50,000/mm³. This patient had received no prior therapy. In group B the patient died 9 weeks after the last course with a platelet count of 40,000/mm³. This patient had received prior cytostatic therapy for 18 months with cyclophosphamide, fluorouracil and methotrexate.

DISCUSSION

1. It should be stressed once again that the above results were obtained by a non-randomized retrospective study and that, consequently, their value is purely indicative. Nevertheless, a number of facts are noteworthy:

(*a*) The degree and duration of toxic disturbances are the same with both schedules, as is the risk of recurrence when treatment is resumed after recovery from a first hematologic toxicity.

(*b*) However, the incidence of toxic manifestations and the toxic-free therapeutic interval vary considerably in the two groups. A dose of 75 mg/m² of CCNU every 21 days, i.e., 3·6 mg/m² per day induced toxicity in 8 patients out of 34 as against 15 patients out of 18 with 130 mg/m² every 42 days, i.e. 3 mg/m² per day. Moreover, patients who exhibited toxic manifestations received therapy for twice as long in the 75 mg/m² per 3 weeks group as in the 130 mg/m² per 6 weeks group before the onset of these manifestations. In other words, the former group was given a total dose twice that of the latter group. The findings confirm the results obtained with single doses by Hansen [1], who observed one case of thrombocytopenia out of 6 and no cases of leukopenia in patients given 75 mg/m² as against 11 cases of thrombocytopenia and 7 cases of leukopenia in 15 patients given 130 mg/m².

2. In the light of these findings an important problem arises. Despite the disadvantages of increased toxicity and a lower total dose, is it advantageous to administer the maximum tolerable dose every 6 weeks rather than resort to 3-weekly administrations of a little over half this dose? This question may be answered in two ways:

(*a*) From a practical standpoint, this question cannot be clearly answered as no randomized study is yet available. However, we can mention two studies [7, 8] which indicate that the 75 mg/m^2 every 3 weeks regimen of CCNU is effective within the limits of efficacy usually attributed to this cytostatic drug. Personally, we feel that because of the possibility of continuing therapy for twice as long, it is possible to maintain the initial response for a considerably longer period of time.

(*b*) From a theoretical standpoint, we have shown that in planning an eradication strategy using any drug which is partially or wholly cycle-dependent, the number of courses per doubling time should be as high as possible [6].

3. To what extent can these results be generalized? This is impossible to determine at the present time since the toxicity of nitrosoureas is of a particularly delayed type. But it is obvious that the optimum dose is that which, for equal antitumor activity, enables therapeutic pressure to be sustained the longest. In terms of kinetic considerations (that is number of courses per doubling time), as well as in terms of chronic toxicity, it appears that this optimum dose may be very different from the maximum tolerated dose as usually determined in phase I trials [9].

The most important conclusion we would like to draw from the above results is that the optimum dose should be determined for each type of carcinoma through appropriate trials including measurable tumors of known doubling time and taking into account not only the acute toxicity, but also the time during which an acceptable toxicity allows the therapeutic pressure to be maintained.

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Biological Properties of a C-Type Leuko-Virus Isolated from Mouse Myeloma Cells*

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Abstract—*The properties of a C-type virus from mouse myeloma cells (MMCV) are described. The virus was isolated in 3T3 cell cultures, but the infected cells did not transform nor did they produce abnormal proteins. MMCV acted as a helper in the rescue of murine sarcoma viral (MSV) genome from transformed non-producing mouse and hamster cells. The pseudotype virus formed (MSV–MMCV), transformed mouse and rat cells in culture, and induced rhabdomyosarcoma when inoculated into neonatal BALB/c-mice. However, newborn mice inoculated with MMCV did not develop any tumours. MMCV was found to share the group specific antigen of the murine leukaemia sarcoma viruses, but contained a type specific viral antigen which was not neutralized by anti-MSV-H serum.*

Mouse cells chronically infected with MMCV were resistant to superinfection and transformation by different pseudotypes of MSV (MSV–H, MSV–FLV, MSV–FLV and MSV–MMCV).

INTRODUCTION

We recently reported the *in vitro* isolation of a C-type virus from BALB/c mouse myeloma (P-3) cells [1]. Subsequently, we studied the relationship of the A- and C-type particles which are frequently associated with the myeloma cells [2]. These studies suggested that intracisternal A-type particles are not infectious and that they are probably not the precursors of the mouse myeloma C-type virus (MMCV). Although no evidence is yet available as to whether this MMCV is involved in the development of the mouse myeloma, the studies reported in this paper did not demonstrate any leukaemogenic activity attributable to this virus, but showed that it can act as a helper in the rescue of the defective murine sarcoma viral (MSV) genome. Additional biological properties of this mouse myeloma C-type virus are outlined in the present paper.

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MATERIAL AND METHODS

Cells and culture media

P-3 cell line. These cells were a cloned plasma cell line originated by Dr. K. Horibata and M. Cohn from an oil-induced myeloma of BALB/c mice. The cells were kindly provided by Dr. C. Milstein who grew them in Dulbecco's modified Eagle's medium (DMM) supplemented with 10% horse serum.

3T3 cell lines. These cell lines were derived from mouse embryos of the BALB/c strain and the NIH strain.

HT-1 cell line. A hamster tumour cell line which carries the defective MSV genome [3 and 4].

D-243 cell line. MSV transformed 3T3 cells (of NIH strain). These cells, which carry the defective MSV genome (S^+L^- cells), were kindly provided by Dr. R. Bassin [5].

NRK cell line. These normal rat kidney cells were derived from Fisher rats and were kindly provided by Dr. R. Gilden.

XC cell line. This rat cell line carries the defective Rous sarcoma virus (RSV) [6].

Following the infection of the 3T3 and NRK cells with virus, culture media containing only 5% FBS was used.

Animals. Newborn litters of BALB/c and the NIH strain of mice were used for viral inoculation, while rabbits were used for the production of antiviral sera.

Rescue of the murine sarcoma viral genome from non-producer cells by the mouse myeloma cell virus (MMCV)

The mouse myeloma ecll virus (MMCV), pseudotype of the MSV genome, was obtained in the following ways: (*a*) after fusion of HT-1 or D-243 cells with the mouse myeloma cells, (*b*) after co-cultivation of the mouse myeloma cells with 3T3 cells together with either HT-1 or D-243 cells, (*c*) after fusion of MMCV infected 3T3 cells with HT-1 cells. The technique used for the assay of the MSV pseudotypes was similar to the one described by Huebner *et al.* [3].

For comparative studies the Rauscher (RLV) and Friend (FLV) leukaemia virus pseudotypes of the MSV genome were prepared by fusion of virus-infected BALB/3T3 cells with HT-1 cells. All cell fusions were done with β-propiolactone-inactivated Sendai virus according to the procedure described by Enders *et al.* [7]. The fused cells were seeded separately in flasks containing DMM + 10% FBS. After three days of culture the cells together with the fluid were frozen and thawed three times, spun at low speed, and the supernatant passed through a 0·45 μ millipore filter.

Induction of focus formation in vitro. 3T3 cells of both NIH and BALB/c strain as well as NRK cells were used for assay of viral transformation. Sixty-mm plastic plates were seeded with 3×10^5 cells per plate, and on the following day the cells were treated with 100 μg DEAE-D per plate for 30 min. Following this treatment, the cells were washed twice, and then separately infected with the different pseudotypes of the MSV. After incubation for 1 hr, 5 ml of DMM + 5% calf serum was added to each plate. For the quantitative assay of viral transformation BALB-3T3 cells were used. The infected plates were checked twice weekly and were kept for up to three weeks. Colonies of BALB-3T3 cells which were transformed by the MSV pseudotype of the mouse myeloma cell virus were picked up from the infected cultures which were overlayed with medium containing 0·5% agar on the second day after infection. These transformed cells were then propagated separately and assayed for viral production.

Neutralization of focus-formation. Virus neutralization tests were carried out using the focus reduction assay. The antiserum to the C-type virus of the mouse myeloma was prepared in rabbits from virus which was concentrated by ultracentrifugation of P-3 culture medium Antiserum to MSV–H (Harvey strain) was also prepared in rabbits. 10^3 focus forming units (FFU) of MSV–MMCV and 10^3 FFU of MSV–H were incubated for 45 min. with equal volumes of two-fold dilutions (1:2 to 1:32) of anti-MMCV and MSV–H rabbit serum. The sera had been earlier inactivated by heating at 56°C for 30 min.

Resistance inducing factors (RIF) tests. This test was based on the original observation of Rubin who was the first to show that avian leukaemia infected chick cells were resistant to superinfection and transformation by Rous sarcoma virus [8]. BALB-3T3 cells which were chronically infected with MMCV and separately with RLV and FLV were seeded in 60 mm plates (3×10^5 cells/plate). On the following day the plates were treated with 100 μg/plate of diethyl-aminoethyldextran (DEAE-D) for 30 min, washed twice, and then infected separately with the following pseudotypes of the MSV: MSV–H, MSV–RLV, MSV–FLV and MSV–MMCV.

Mixed culture cytopathogenicity test. This test which can be used to detect the presence of murine leukaemia viruses is based on the observation that when the XC cells are placed in contact with cells infected with murine leukaemia viruses, syncitium formation occurs [9]. The test was performed with BALB-3T3 cells which were infected separately with MMCV, RLV and FLV. These cells were brought in contact with XC cells in order to assess whether the size of the syncytium formation reflects the poorer rate of the MMCV replication when compared to the other murine leukaemia viruses.

Antigen for immunodiffusion. The group specific (GS) soluble antigen of the murine leukaemia and sarcoma viruses was prepared in the following way. MSV–H released into the media was concentrated by spinning the culture fluid at 30,000 ***g*** for two hours. The pellet which contained the virus was then suspended in Hank's buffer. To this virus suspension, three parts of ether were added. After continuous

agitation for 2 hrs at room temperature, the mixture was centrifuged for 15 min at 1,000 *g* in order to eliminate the ether. The ether-disrupted virus suspension was then spun at 100,000 *g* for 2 hr, the pellet discarded and the supernatant used as the GS antigen. For the production of anti GS antibodies rabbits were inoculated with GS antigen preparation three times, at two week intervals, and were bled seven days after the last injections.

Immunodiffusion. Immunodiffusion reactions were conducted by using 1% agar in barbitol buffer (pH 8·6). The wells were prepared by an LKB punch. The group specific (GS) antigen which was prepared from MSV–H was placed in the centre well. The antisera used were obtained from rabbits which were inoculated with MMCV, MSV–H and RLV. These different antiviral sera were used in the double diffusion test.

Complement fixation tests. Complement fixation tests (CFT) for the assay of murine leukaemia in cell culture [10] were performed by the micro-procedure technique with 1·8 units of guinea pig complement. The MMCV antigen was prepared from the supernatant of homogenized 20% P-3 cell suspension. Antiserum to the MSV–H derived GS antigen of the murine leukaemia sarcoma was prepared in rabbits.

Animal inoculation

I. Newborn BALB/c and NIH mice were inoculated intraperitoneally (I/p) and subcutaneously (s/c) with two preparations of the myeloma cell virus: (*a*) Concentrated MMCV released from cultured P-3 cells, (*b*) Concentrated MMCV released from infected BALB-3T3 cells.

In both cases the virus was concentrated in the following way. First, the culture fluid was spun at 500 *g* for 20 min at 4°C. The supernatant was collected and spun at 30,000 *g* for 2 hr at +4°C. The pellet was suspended in 1/100 of the original volume culture medium.

II. Neonate BALB/c mice were inoculated intramuscularly (I/M) and subcutaneously (s/c) with approximately 10^2 FFU of MSV–MMCV per animal. Samples of tumour tissues which developed in the inoculated mice were fixed in 3% glutaraldehyde or absolute methanol, for histological examination.

RESULTS

Rescue of the murine sarcoma virus (MSV) genome from non-producing (N-P) cells by the mouse myeloma cell virus (MMCV)

The MMCV acted as a helper virus and rescued the defective MSV genome after cell fusion or co-cultivation of the mouse myeloma cells with the transformed N-P mouse (D-243) and hamster (HT-1) cells. This new MSV–MMCV pseudotype transformed not only mouse cells of both BALB/c and NIH strains, but also rat (NRK) cells. Several transformed foci were picked up from the infected 3T3 cultures which had been overlayed with medium containing agar. Those transformed cells were propagated separately and were found to release an infectious virus which in turn transformed both mouse and rat cells. In infected 3T3 BALB/c cells two types of transformed foci could be seen; one with highly refractile spindle-shaped and rounded cells growing in different directions (Fig. 1) and the other with mainly elongated spindle-shaped cells growing parallel to each other. In the 3T3 FL cells, derived from NIH strain, the foci consisted of rounded and spindle-shaped cells intermingled with morphologically normal cells. Transformed foci in NRK cells consisted mainly of refractile rounded and spindle-shaped cells (Fig. 2). After an initial increase in the number of the transformed NRK cells there was a gradual decrease in the size of the foci. Eventually, the transformed RNK cells disappeared and no infectious MSV–MMCV could be recovered from the culture fluid. This was in sharp contrast to NRK cells which were infected and transformed by MSV–H. The foci of MSV–H transformed NRK cells increased rapidly in size (Fig. 3) and infectious MSV–H could be recovered from the culture fluid. Culture fluid from MSV–MMCV transformed BALB/c 3T3 cells contained a low titre of transforming virus, usually not higher than 10^2 FFU per ml. However, following centrifugation of the culture fluid at 30,000 *g* for 2 hr there was between 10 to 50-fold increase in the FFU per ml of MSV–MMCV.

Concentrated MSV–MMCV preparations which were stored for three months at −70°C in medium deficient in serum, lost their capacity to transform 3T3 cells. However, the ability of the virus pseudotype to transform cells *in vitro* could be restored simply by spinning the virus preparation again at 30,000 *g* for 2 hr.

Neutralization tests

Antiserum which was prepared in rabbits following the repeated injection of MMCV (released from P-3 cells) neutralized the infectivity of the MSV–MMCV pseudotype, after 1 hr of incubation, but failed to neutralize the infectivity of the MSV–H. However, rabbit anti-MSV–H serum neutralized the infectivity of the MSV–H, but not that of the MSV–

MMCV (Table 1). These tests indicate that the MMCV contain a type specific envelope antigen which differs from MSV–H.

RIF tests

The results of the RIF tests are summarized in Table 2. They indicate that BALB/c-3T3 cells which were chronically infected by MMCV or RLV or FLV were completely resistant to infection by MSV–H, MSV–RLV, MSV–FLV and MSV–MMCV. BALB-3T3 cells which were infected with RLV or FLV were fully resistant to infection and transformation by the MSV pseudotypes within two weeks after the initial infection. However, BALB-3T3 cells which were infected by MMCV had to be cultured for two months before they became completely resistant to infection and transformation by the different pseudotypes of MSV.

Table 1. *Neutralization tests*

Viruses	Antisera to MSV–H	Antisera to MMCV
MSV–H	–	+
MSV–MMCV	+	–

Each of the above MSV pseudotypes was incubated separately for one hour with the two rabbit antisera.

+ induction of foci.

– no foci.

Mixed culture cytopathogenicity test

In BALB/c-3T3 cells, which were infected with RLV or FLV, large plaques of fused giant XC cells were easily detected throughout the culture, whereas in 3T3 cells, infected by MMCV, no large plaques of XC cells could be detected. The MMCV infected cultures contained only small multinucleated (2–4 nuclei) cells; such cells could be found in the control cultures which were overlayed with XC cells.

Detection of murine leukaemia sarcoma GS antigens

The results of the immunodiffusion tests are presented in Fig. 4. The rabbits anti-MMCV serum (Well 1) formed a line of precipitation with the GS antigen prepared from MSV–H. Likewise, the rabbits anti-MSV–H (Well 2) and anti-RLV (Well 3) formed similar lines o precipitation with the GS antigen. Figure 4 illustrates that the three reactions are identical and therefore indicate that the MMCV shares the GS antigen with other murine leukaemia and sarcoma viruses. The complement fixation tests confirmed this observation by the reaction of the MMCV derived antigens with the rabbit antisera which contained murine leukaemia–sarcoma group specific antibodies.

Animal inoculations

The newborn BALB/c and NIH mice which were inoculated with the MMCV developed normally for over six months following the viral injections. On the other hand, three day old BALB/c mice which were inoculated each with approximately 10^2 FFU of the mouse myeloma pseudotype of the murine sarcoma virus (MSV–MMCV) were seen to develop very large tumours at or near the site of the inoculation, two weeks after the viral injection. Histologically, this tumour was found to be a rhabdomyosarcoma. Some of the mice which had these very large tumours were killed by their mother during the following week while others

Table 2. *Resistant inducing factor (RIF) tests*

BALB/c cell cultures	Induction of cellular transformation by the different MSV pseudotypes			
	MSV–H	MSV–RLV	MSV–FLV	MSV–MMCV
3T3	+	+	+	+
3T3–RLV	–	–	–	–
3T3–FLV	–	–	–	–
3T3–MMCV	–	–	–	–

+ = induction of foci of cellular transformation.

– = no cellular transformation.

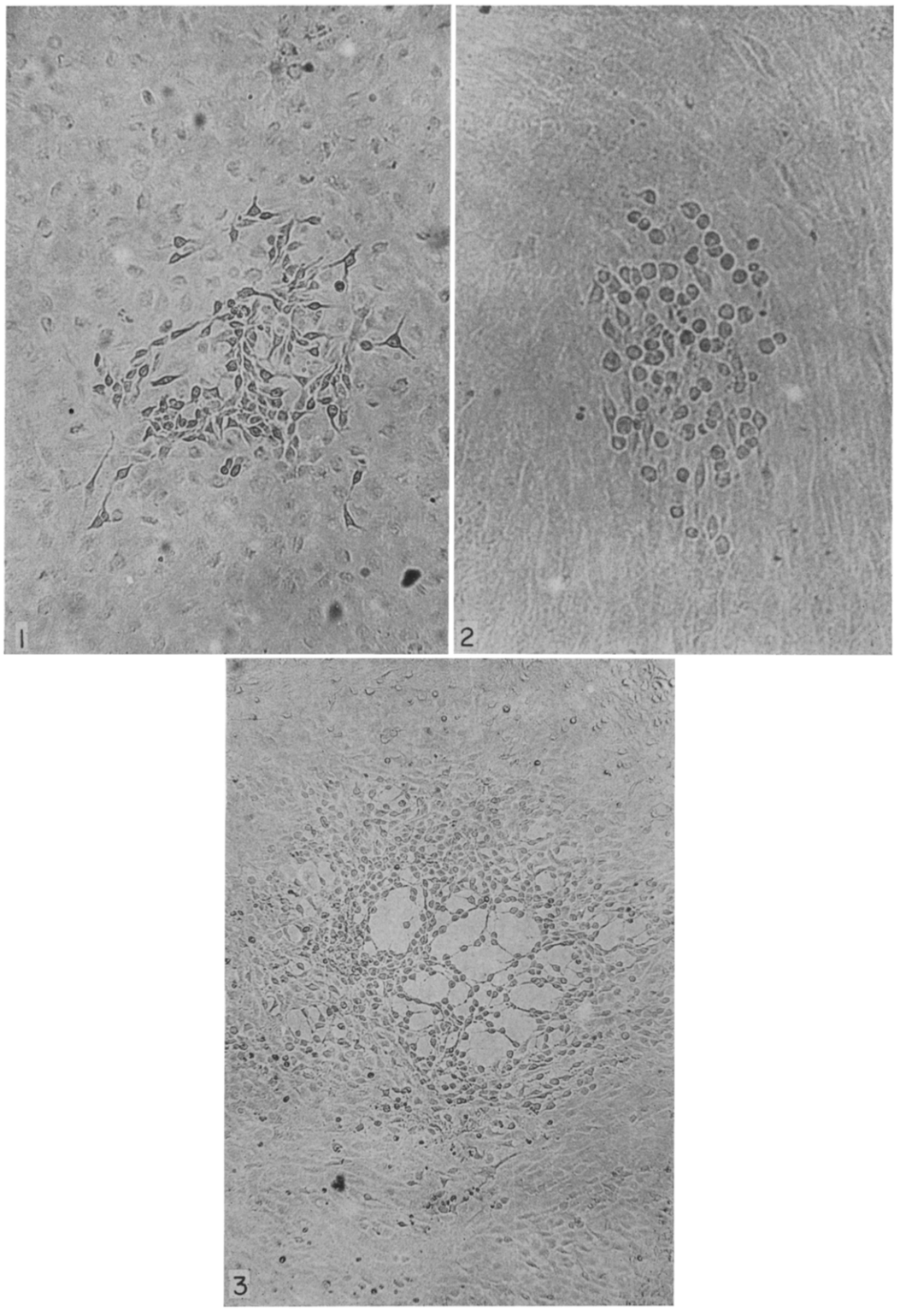

Fig. 1. *Balb-3T3 cells infected with MSV–MMCV. A typical focus of cellular transformation is demonstrated.*

Fig. 2. *Rat (NRK) cells one week after the infection by MSV–MMCV. A small focus of cellular transformation is shown.*

Fig. 3. *NRK cells one week after the infection by MSV–H showing a very large focus of transformation with numerous transformed cells.*

(to face p. 806)

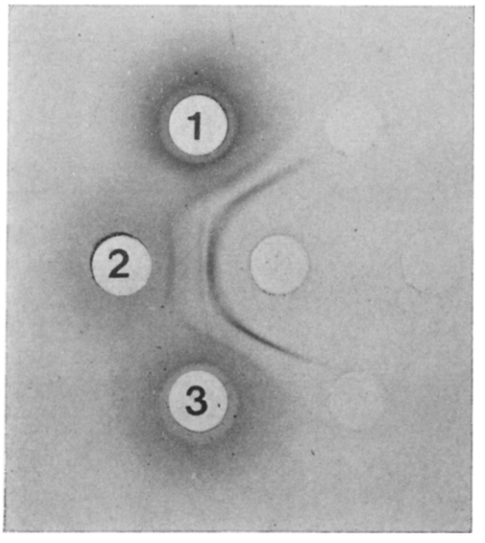

Fig. 4. *Micro-Ouchterlony plate. Central well: GS antigen of the murine leukaemia–sarcoma virus derived from MSV–H.*

Well 1 *: contains rabbit anti–MMCV serum.*

Well 2 *: contains rabbit anti–MSV–H serum.*

Well 3 *: contains rabbit anti–RLV serum*

were sacrificed and bled. Blood smears were prepared from the inoculated animals and were found to be free from leukaemic cells.

DISCUSSION

The P-3 mouse myeloma cells contain intracisternal A-type particles and release C-type virus from the cell membrane. In spite of the fact that the initial C-type virus isolation was done by co-cultivation of P-3 cells with normal mouse fibroblasts, the 3T3 cells which became infected and released C-type virus did not contain any intracisternal A-type particles [2]. On the other hand, normal mouse embryo fibroblasts (MEF) of BALB/c strain which contained intracisternal A-type particles were as susceptible as BALB/c-3T3 cells to infection and transformation by MSV–H. After infection of the MEF cells which contained A-type particles with MSV–H there was no increase in the number of intracisternal A-type particles. Those observations suggested that the C-type virus of mouse myeloma is probably not related to the intracisternal A-type particles.

The studies of the MMCV indicate that in spite of the fact that this virus does not induce leukaemia in inoculated mice it does share several basic properties with the leukaemogenic C-type viruses. It acted as a helper virus and rescued the defective MSV genome from non-producing (N-P) mouse (D-243) and hamster (HT1) cells. The N-P mouse D-243 cells carry the group specific (GS) antigen of the murine leukaemia and sarcoma viruses, while the hamster HT-1 cells lack the information coding for this antigen. Therefore, for the rescue of the MSV genome from the hamster cells, the MMCV presumably had to furnish more of its genetic information in the formation of its MSV pseudotype. The pseudotype virus formed transformed both BALB-3T3(B) and NIH-3T3(N) cells and therefore has dual NB tropism [12]. The MSV–MMCV also transformed rat cells, but the transformed rat cells did not release the virus and after prolonged incubation the transformed cells disappeared from the culture. This was in sharp contrast to MSV–H infected rat cells where the entire culture became rapidly transformed and infectious MSV–H was released into the medium.

The bondage between the MMCV and the MSV genome is probably not very strong. When the MSV–MMCV pseudotype was stored in serum free medium at −70°C, it lost its ability to transform 3T3 cells. However, the ability of the MSV–MMCV to transform 3T3 cells *in vitro* could be restored following the ultracentrifugation of this virus suspension at 30,000 *g* for 2 hr. Under this condition physical encapsidation of the MSV genome by the MMCV probably took place. Such an event was earlier reported in the formation of the feline leukaemia pseudotype of the MSV genome [11]. The RIF test revealed that the MMCV infected BALB-3T3 cells were resistant to infection and transformation not only to MSV–MMCV pseudotype, but also to MSV–H, MSV–RLV and MSV–FLV. Gross leukaemia virus being a N-tropic virus [12] was not used in the RIF test, which was carried in BALB/c-3T3 cells. This together with the absence of G antigen on the surface of plasma cells, rules out any relationship between those two C-type viruses.

In vitro MMCV has a very poor rate of replication. Infected cells became resistant to challenge by the MSV pseudotypes only after prolonged culture and repeated passage. Likewise the absence of XC plaques in the MMCV infected cells when compared to the very much larger XC plaques produced in RLV and FLV infected cultures could be attributed to the poor rate of MMCV replication and release.

The immunodiffusion and complement fixation tests provide evidence indicating that the MMCV shares a common group specific antigen with other viruses (MSV–H and RLV) of the murine leukaemia–sarcoma group. The neutralization tests, on the other hand, reveal that the MMCV has a different envelope type of specific antigen from the MSV–H.

Newborn mice, inoculated with MMCV, developed normally, while BALB/c mice of similar age, inoculated with the MSV–MMCV pseudotype, developed large tumours which were diagnosed as rhabdomyosarcoma at or near the site of inoculation.

Herberman and Aoki [13] who recently studied the immune and natural antibodies to syngenic murine plasma cell tumours presented strong evidence for the close association of a virus with these tumours. They described the presence of naturally occurring antibodies in mice with myelomas which reacted with an antigen common to all plasma cell tumours. They have found that the envelope of a C-type virus which was released by the plasma cells contained this antigen. This antigen was distinct from that of Gross leukaemia virus and unrelated to the intracisternal A-type particles. Earlier, Laspinats [14] found transplantation antigens common to several plasma cell tumours and also suggested that its presence may be consistent with the viral aetiology of the disease. The failure to induce myeloma in inoculated mice by MMCV within six months

indicates that if this virus is involved in the development of the myeloma, either a longer incubation period is needed or that it needs an unspecific initiating carcinogenic factor such as mineral oils or plastic. These chemical agents can induce this malignancy in the BALB/c strain of mice possibly because of the latent presence of the MMCV. A similar situation may exist with the C3H strain where it was also observed that following inoculation of mice with similar chemical agents myeloma developed and the protein producing cells contained a C-type virus [15]. Further experiments that will attempt to verify the role of the isolated C-type virus in the development of this malignancy are under way.

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Synergistic Cytotoxic Effect of Macrophages and Normal Mouse Serum on Neuraminidase-Treated Murine Leukaemia Cells*

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Abstract—*The results of this study indicate that the cultured leukaemia L1210 cells pre-treated with* Vibrio cholerae *neuraminidase became susceptible to synergistic cytotoxic effect of peritoneal macrophage like (PML) cells in the presence of serum (NMS) obtained from normal DBA/2J mice. But such cytotoxic effect was not apparent when untreated or leukemic cells treated with heat-denatured neuraminidase were used. Sera alone were not cytotoxic* per se. *Pretreatment of normal PML cells with VCN had no effect on the growth of untreated L1210 cells in the presence of NMS. Pre-treatment of L1210 cells with a variety of other enzymes with known substrates or other reagents did not render the tumour cells susceptible to the synergistic cytotoxic effect of normal PML cells and NMS. The observed cytotoxicity was related to the ratio of normal PML cells to VCN-treated L1210 cells. The NMS factor(s) required for inducing normal PML cells-mediated damage on VCN-treated L1210 cells could be substituted by NMS from another mouse strain i.e. BALB/c. Heating of the NMS at 58°C for 30 min did not abolish its property to induce PML cell-mediated cytotoxicity towards VCN-treated target cells. The NMS factor(s) could be selectively absorbed onto VCN-treated L1210 cells.*

INTRODUCTION

RECENT studies with a variety of non-specific and syngeneic tumor–host systems have demonstrated that pre-treatment of tumor cells with enzyme neuraminidase from either *Vibrio cholerae* (VCN) or *Clostridium perfringens* can alter their *in vivo* transplantability to various extents [1, 2, 3, 4, 5]. Treatment of murine leukaemia L1210 cells with VCN can completely destroy their transplantability for strain-specific (compatible) DBA/2 mice and the recipients of the enzyme-treated cells develop specific immunity against large challenge doses of untreated L1210 cells [6, 7, 8].

We have reported earlier that normal sera from some mouse strains possess the property of damaging VCN-treated, but not untreated, L1210 cells; this effect could, however, be observed only at low serum dilutions [9]. VCN-treated TA3 (mouse mammary adenocarcinoma) cells have also been reported to be destroyed by normal guinea pig serum or allogeneic C3 H mouse serum while untreated TA3 cells were unaffected [10].

In the present communication evidence is presented which indicates that VCN-treated

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Abbreviations:

VCN: *Vibrio cholerae* neuraminidase
PML Peritoneal macrophage-like cells
NMS: Normal mouse serum
GM: Fischers medium supplemented with 10 per cent heat inactivated horse serum
GPS: Guinea pig serum

L1210 cells can be destroyed under *in vitro* conditions by the synergistic cytotoxic effect of normal (non-immune) peritoneal macrophage-like cells* (PML) from the compatible mouse strain DBA/2J in the presence of normal DBA/2J mouse serum at a dilution which is not cytotoxic *per se*.

MATERIAL AND METHODS

Mice

Inbred strain DBA/2J (SPF) male (G. L. Bomholtgard, Denmark) mice, weighing 20–25 g, were used for the preparation of PML cells and the collection of sera.

Media

Growth medium (GM) used consisted of Fischer's medium (Grand Island Biological) supplemented with 10% of heat-inactivated horse serum, penicillin (100 units/ml) and streptomycin (0·1 mg/ml). Tissue culture medium 199 (TC199) was obtained from the Microbiological Associates.

Preparations of sheets of PML cells

Peritoneal exudate cells were induced in normal DBA/2J mice by intraperitoneal injections of 1 ml of a 3% starch solution. The exudate cells were harvested 3 days later by injecting 3 ml of GM into the peritoneal cavity and withdrawing the suspension with a syringe. The peritoneal exudate cell suspensions from 3 to 5 mice were pooled, washed 3 times in TC199, and suspended in GM at a final concentration of 10×10^6 viable cells per ml. The suspension was then pipetted onto Falcon plastic Petri dishes (60 × 15 mm) in 1 ml amounts to which 7 ml of GM were added and the cultures were incubated in a humid incubator at 37°C with an atmosphere of 5% CO_2. After 2 hr incubation the cultures were washed, until all the non-adhering or loosely adhering cells were removed and 8 ml of fresh GM were added to each culture and the plates were reincubated. After a further incubation of 3 hr the cultures were again washed and then used in cytotoxicity tests. Differential counts carried out in 1% crystal violet solution indicated 85–90% of the adhering cell population as macrophages. To facilitate examination under the phase contrast microscope sheets of PML cells were also prepared on sterile glass cover slips (24 × 16 mm) placed in tissue culture dishes (35 × 10 mm). After incubation with target cells, the cover slips were removed and either fixed and stained or examined under phase contrast microscope. The cultures were checked for mycoplasma contamination and only mycoplasma free cultures were used.

Leukaemia L1210 cells

Lymphocytic leukaemia L1210, which arose in DBA/2 mouse following skin paintings with methylcholanthrene (11), is a strain-specific leukaemia as it grows only in DBA/2 or related hybrid hosts and a single cell is capable of producing fatal leukaemia in DBA/2 hosts [12]. L1210 cells grown in suspension culture were obtained from Prof. D. W. van Bekkum of the Radiobiological Institute, Rijswijk, Netherlands. The cell line was maintained as an unagitated suspension culture in GM. The cells were cultured after every 24 hr in fresh GM and routinely checked for mycoplasma contamination. The cells retain their characteristic lymphoblast morphology and do not attach to glass. This cell line originally developed from the tumor maintained in DBA/2 C57 BL mice, possessed oncogenic potential for DBA/2J male or female mice (Average killing time after i.p. injection of 100 cells was 10–14 days).

Sera

Blood from mice was drawn by cardiac puncture and allowed to clot at room temperature. Fresh sera from 2 to 5 mice were pooled and checked for sterility before used in cytotoxicity tests. Guinea pig serum (GPS) was obtained from outbred animals and a single pool preabsorbed with VCN-treated cells, in order to remove its cytotoxicity *per se*, was used as a complement source. The absorption procedure did not affect the hemolytic complement activity of the serum.

Enzymes and reagents

Neuraminidase (Behringwerke, West Germany) prepared from the filtrate of *Vibrio cholerae* (VCN) was obtained in vials containing 500 U/ml as indicated by the manufacturer; VCN was diluted in 0·05 M sodium acetate buffer (pH 5·5) containing 0·9% sodium chloride and 1 mg/ml calcium chloride. When desired the enzyme preparation was inactivated by heating at 100°C for 10 min. Trypsin and pronase were Difco products. Ribonuclease (Bovine pancrease), hyaluronidase, phospholipase D, were obtained from General Biochemicals, Chagrin Falls, USA. Protamine sulphate (Roche) was obtained as a 2%

*The adhering cells used in cytotoxicity test contained 85–90 per cent macrophages as judged by morphologic criteria. Since completely pure macrophage preparations were difficult to obtain we have referred to the adhering cell population as peritoneal macrophage-like (PML) cells throughout the text.

solution. The desired concentrations of these enzymes and reagents were prepared in normal saline and pH adjusted in each case at 7·2.

Treatment of L1210 cells

2×10^5 cultured L1210 cells suspended in 0·5 ml of GM were mixed with VCN solution or desired reagent and the mixture was incubated at 37°C for 30 min with occasional shakings. After incubation the cells were washed thoroughly with TC 199 and were then suspended in GM to a final concentration of 2×10^5 viable cells/0·1 ml. Control cells were subjected to the same procedure but without reagents or enzymes.

Treatment of sheets of DBA/2J PML cells with VCN

The culture medium was removed from the sheets of PML cells and the monolayer was covered with 1 ml of 25 U or 50 U of VCN. After incubation at 37°C for 30 min the fluid was removed and the sheets were washed thoroughly with GM before use in cytotoxicity tests.

Cytotoxicity test

Appropriate numbers of untreated or treated cultured L1210 cells (usually 2×10^5) in 0·1 ml volumes of GM were added onto washed sheets of DBA/2J PML cells and additional GM was added to make the total volume to 8 ml. NMS was added at a final dilution of 1:50. The plates were replaced in the incubator and after 48 hr the growth of L1210 cells in the cultures was determined. Controls included L1210 cells cultured alone and sheets of PML cells incubated alone. All tests were carried out in quadruplicates. In order to assess the growth of L1210 cells the medium containing L1210 cells was poured off, the PML sheets themselves remaining firmly attached. The PML sheets were thoroughly washed with GM containing 0·1% trypsin and the wash was added to the collected cells. The collected cells were centrifuged at $100 \times g$ for 5 min and suspended in 5 ml of GM. The number of viable cells in the suspension was determined by 2 procedures:

Trypan blue exclusion test. A quantity of 0·2 ml of the test cell suspension was removed and mixed with an equal volume of 0·5% trypan blue in TC 199 and the number of viable cells were counted by hemocytometer. Due to marked difference in the size and morphology between cultured L1210 cells and the PML cells no difficulties were encountered in distinguishing L1210 cells from some detached PML cells in the suspensions. Cytotoxicity was quantified by determining the difference in the percentage of the viable cells remaining after 48 hours incubation as compared to the controls.

Colony formation on agarose. The ability of cultured L1210 cells to form colonies (13) was investigated by seeding appropriate number of cells in nutrient medium consisting of Eagle's basal medium (Difco), 10% heat inactivated horse serum and 0·375% agarose. Macroscopic colonies were counted after incubation for 14 days at 37°C.

Absorption of NMS

Washed cell suspensions of L1210 cells or HeLa cells (untreated or VCN treated) were suspended in phosphate buffered saline (pH 7.2) to a concentration of 3×10^4 cells per 0·5 ml. One half millilitre of this suspension was then mixed with an equal amount of the undiluted test serum and the mixture was incubated at 37°C for 1 hr followed by 24 hr incubation at 4°C. The supernatant was then centrifuged twice at $300 \times g$ for 30 min and then used in the cytotoxicity tests. In some instances the procedure of absorption was repeated 4 times using fresh cell preparations following each absorption.

RESULTS

From the data summarized in Table 1 it is apparent that normal DBA/2J PML cell-exerted a marked cytotoxic effect on VCNs treated cultured L1210 cells in the simultaneous presence of DBA/2J NMS after 48 hr of incubation. Phase contrast microscopy indicated that the destruction of VCN-treated cultured L1210 cells was a consequence of a non-phagocytic mechanism. Under an identical set of conditions no cytotoxic effect could be observed when either untreated L1210 cells or L1210 cells pre-incubated with inactivated preparations of VCN were used. Neither PML cells nor NMS alone at the test dilution i.e. 1:50 could exert a cytotoxic effect on VCN-treated L1210 cells. The data on the effect of treating sheets of normal DBA/2J PML cells with VCN on their subsequent interaction with untreated or VCN-treated L1210 cells is presented in Table 2. It can be noted that sheets of normal DBA/2J PML cells pretreated with 25 U or 50 U of VCN for 30 min had no cytotoxic effect on untreated cultured L1210 cells. Higher doses of the enzyme were not tolerated well by the cells and therefore could not be used. It may be mentioned that the synergistic cytotoxic effect

Table 1. In vitro *cytotoxicity towards VCN-treated cultured leukaemia L1210 cells mediated by sheets of normal (non-immune) DBA/2J PML cells in the simultaneous presence of DBA/2J NMS*

Reaction mixture*			Cytotoxicity on L1210 cells	
L1210 cells (2×10^5) (Treatment; 30 min)	Non-immune sheet of PML cells	DBA/2J NMS (Final dilution) 1:50	Viable cells remaining after 48 hr ($\times 10^4$)†	Colonies on agarose, 10^5 cells seeded ($\times 10^2$)
No	+		176 (97·7)‡	572
No		+	181 (100)	597
No	+	+	173 (96·1)	549
VCN (250 U/ml)	+		176 (97·7)	574
VCN (250 U/ml)		+	171 (95·0)	560
VCN (250 U/ml)	+	+	16 (8·8)	12
Inactivated VCN (250 U/ml)	+	+	173 (96·7)	569

*2×10^5 viable untreated or treated L1210 cells + sheet of PML cells (10×10^6 peritoneal exudate cells). NMS when added was at a final dilution of 1:50.
†Figures average of quadruplicate experiments.
‡Figures in parentheses = percentage of viable cells remaining as compared to numbers of viable cells in control cultures which attained an average figure of 180×10^4 cells after 48 hr.

Table 2. *Effect of treating sheets of normal (non-immune) DBA/2J PML cells with VCN on their subsequent interaction with either untreated or VCN-treated cultured leukaemia L1210 cells in the simultaneous presence of DBA/2J NMS at final dilution of* 1:50

	Synergistic cytotoxicity* on			
	Untreated L1210 cells		VCN-treated L1210 cells	
Non-immune (PML) sheets (Treatment) 30 min	Viable cells remaining after 48 hr ($\times 10^4$)†	Colonies on agarose/ 10^3 cells seeded	Viable cells remaining after 48 hr ($\times 10^4$)	Colonies on agarose/ 10^3 cells seeded
None	129 (> 100)	> 250	14 (10·9)	24
VCN (25U/ml)	126 (98·4)	> 250	10 (7·8)	21
VCN (50 U/ml)	121 (94·5)	> 250	13 (10·1)	19

*2×10^5 viable untreated L1210 cells or treated with VCN (250 U/ml for 30 min) + sheet of PML cells (10×10^6 peritoneal exudate cells) + NMS at a final dilution of 1:50.
†Figures average of quadruplicate experiments.
‡Figures in parentheses = percentage of viable cells compared with the numbers in control cultures of L1210 which attained an average figure of 128×10^4 cells after 48 hr.

of PML cells and NMS was slightly less on L1210 cells pretreated with 25 or 50 U of VCN for 30 min than observed on those pretreated with 250 U of VCN. Furthermore there was no increase in cytotoxicity when VCN-treated L1210 cells were included in the reaction mixture.

It is clear from the results presented in Table 3 that pretreatment of cultured L1210 cells with a variety of other enzymes such as ribonuclease, hyaluronidase, pronase, trypsin, phospholipase D or with reagents such as protamine sulphate, did not render the treated L1210 cells susceptible to the PML cell mediated cytotoxicity in the presence of NMS.

The relative cytotoxicity of 10×10^6 normal DBA/2J PML cells on varying numbers (i.e. 10^5, 2×10^5 or 10^6) of VCN-treated L1210 cells was examined in the simultaneous presence of NMS. Significantly less cytotoxicity was observed in cultures containing 10^6 VCN-treated L1210 cells as compared to that in cultures containing either 10^5 or 2×10^5 VCN-treated L1210 cells.

To investigate whether the DBA/2J NMS factor(s) required for the DBA/2J PML cell

Table 3. Failure of certain other enzymes and reagents to render cultured leukaemia L1210 cells susceptible to the cytotoxic effect mediated by sheets of normal (non-immune) DBA/2J PML cells and DBA/2J NMS at a final dilution 1:50

L1210 cells (2×10^5) (Treatment)	Concentration (Incubation at 37°C/30 min)	Synergistic cytotoxicity* Viable cells remaining after 48 hr ($\times 10^4$)†	Synergistic cytotoxicity* Colonies on agarose/10^3 cells seeded
Buffer		156 (> 100)‡	> 250
VCN	250 U/ml	18 (11·6)	28
Ribonuclease	1·0 mg/ml	150 (97·4)	> 250
Protamine sulphate	2·0 mg/ml	152 (98·7)	> 250
Hyaluronidase	500 U/ml	152 (98·7)	> 250
Phospholipase D	0·5 mg/ml	155 (> 100)	> 250
Trypsin	2·0 mg/ml	148 (96·1)	> 250
Pronase	1·0 mg/ml	152 (98·7)	> 250

*2×10^5 viable untreated L1210 cells or treated with various reagents + sheet of PML cells (10×10^6 peritoneal exudate cells) + NMS at a final dilution of 1:50.
†Figures average of quadruplicate experiments.
‡Figures in parentheses represent the percentage of viable cells remaining compared with the numbers in control cultures of L1210 cells which attained an average figure of 154×10^4 cells after 48 hr.

mediated cytotoxicity on VCN-treated L1210 cells could be provided by another mouse strain, NMS from BALB/c mouse strain was tested. This serum showed no cytotoxicity *per se* towards VCN-treated L1210 cells even when used in undiluted form. From the results in Table 4, it is evident that there was no detectable difference in the normal DBA/2J PML cell mediated cytotoxicity when DBA/2J NMS was substituted by BALB/c NMS.

As can be seen from the results in Table 5, cytotoxicity mediated by normal DBA/2J PML cells was not abolished in the presence of heat-inactivated NMS and, furthermore, the addition of fresh guinea pig serum (pre-absorbed to remove its own cytotoxicity *per se* towards VCN-treated L1210 cells) as a source of complement did not augment the cytotoxic effect towards VCN-treated L1210 cells. To determine the specificity of the DBA/2J NMS factor(s), L1210

Table 4. Substitution of the DBA/2J NMS factor(s) required for inducing the cytotoxic effect mediated by sheets of normal (non-immune) DBA/2J PML cells on VCN-treated cultured leukaemia L1210 cells by sera from mouse-strain BALB/c§

NMS (Donor and Final dilution)	Synergistic cytotoxicity* on VCN-treated L1210 cells: Viable cells remaining after 48 hr ($\times 10^4$)†	Synergistic cytotoxicity* on VCN-treated L1210 cells: Colonies on agarose/10^3 cells seeded
DBA/2J (1:50)	19 (9·5)‡	18
BALB/c (1:5)	14 (7·0)	26
BALB/c (1:50)	21 (10·5)	16

*2×10^5 viable L1210 cells treated with VCN (250 U/ml for 30 min) + sheet of PML cells (10×10^6 peritoneal exudate cells). Reaction mixture supplemented with desired concentration of NMS.
*Figures average of quadruplicate experiments.
‡Figures in parentheses = percentage of viable cells remaining compared with the number of cells in control cultures which attained an average figure of 200×10^4 cells after 48 hr.
§Serum from this mouse strain did not show cytotoxicity towards VCN-treated L1210 cells *per se* even when used at a final dilution of 1:2. The DBA/2J mouse showed a marked direct cytotoxicity towards VCN-treated L1210 cells at low dilutions.

cells or HeLa cells (untreated or VCN-treated) were used to absorb the factor(s). The results indicate that the cytotoxicity towards VCN-treated L1210 cells in the presence of DBA/2J NMS pre-absorbed with VCN-treated L1210 cells was not stronger than observed in controls. DBA/2J NMS subjected to four consecutive absorptions with untreated L1210 cells could, however, contribute to induce normal PML cell mediated cytotoxicity towards VCN-treated L1210 cells, although the extent of cytotoxicity was much reduced. Normal DBA/2J PML cell mediated cytotoxicity on VCN-treated L1210 cells in presence of NMS preabsorbed with untreated or VCN-treated HeLa cells was in the same range as observed in the presence of unabsorbed NMS. It would appear therefore that the DBA/2J NMS factor(s) participating in the PML cell mediated cytotoxicity towards VCN-treated L1210 cells could be selectively absorbed onto VCN-treated L1210 cells.

Table 5. Induction of normal (non-immune) DBA/2J PML cell-mediated cytotoxicity towards VCN-treated cultured L1210 cells in presence of heated DBA/2J NMS

NMS; DBA/2 (Treatment)	Synergistic cytotoxicity* towards VCN-treated L1210 cells	
	Viable cells remaining after 48 hr ($\times 10^4$)†	Colonies on agarose/ 10^3 cells seeded
None	19 (10·0)‡	18
Heated (58°C/30 min)	17 (8·9)	18
None; +10% GPS§	16 (8·4)	23

*2×10^5 viable L1210 cells treated with VCN (250 U/ml for 30 min) + sheet of PML cells (10×10^6 peritoneal exudate cells) + NMS at final dilution of 1:50.
†Figures average of quadruplicate experiments.
‡Figures in parentheses = percentage of viable cells remaining compared with the number of cells in control cultures which attained an average figure of 190×10^4 cells during 48 hr.
§GPS used as a source of complement was preabsorbed to remove its own cytotoxicity *per se*.

Table 6. Attempts to absorb the DBA/2J NMS factor(s) which participate in the cytotoxicity towards VCN-treated cultured leukaemia L1210 cells in presence of normal (non-immune) DBA/2J PML cells

DBA/2J NMS (Absorption with)	Synergistic cytotoxicity* on Untreated L1210 cells	Synergistic cytotoxicity* on VCN-treated L1210 cells
	Viable cells remaining after 48 hr ($\times 10^4$)†	Colonies on agarose/ 10^3 cells seeded
Untreated (Normal) L1210 cells (4 times)	22 (11·0)‡	29
	24 (12·0)	36
VCN-treated L1210 cells (250 U/ml/30 min) (1 time)	180 (90·0)	> 200
Untreated HeLa cells (4 times)	19 (9·5)	21
VCN-treated HeLa cells (250 U/ml/30 min) (4 times)	17 (8·5)	29

*$2 \cdot 10^5$ viable L1210 cells treated with VCN (250 U/ml/30 min) + sheet of PML cells (10×10^6 peritoneal exudate cells) + untreated or treated NMS at a final dilution of 1:50.
†Figures average of quadruplicate experiments.
‡Figures in parentheses = percentage of viable cells remaining compared with the number of cells in control cultures which attained an average figure of 200×10^4 cells after 48 hr.

DISCUSSION

The results of the present study have demonstrated that under *in vitro* conditions a marked non-phagocytic cytotoxic effect could be exerted by non-immune PML cells from DBA/2J mice on VCN-treated L1210 cells (presumably syngeneic) in the simultaneous presence of DBA/2J NMS. The dilutions of NMS used in cytotoxicity tests could not exert any cytotoxic effect on VCN-treated L1210 cells *per se* even in the presence of 10% GPS. A prerequisite for the normal DBA/2J PML cell-mediated cytotoxicity towards VCN-treated L1210 cells, apparently was the presence of naturally occurring mouse serum factor(s) in the reaction mixture. The observation that the heated DBA/2J NMS did not loose its capacity to evoke normal DBA/2J PML cell mediated cytotoxicity on VCN-treated L1210 cells does not completely rule out the dependence of the observed cytotoxicity on complement since macrophages are known to possess themselves several components of complement system [14]; DBA/2J mice are known to lack C5 components [15]. It was possible to deplete the normal DBA/2J mouse serum factor(s) participating in the normal DBA/2J PML cell cytotoxicity on VCN-treated L1210 cells by a single absorption with VCN-treated L1210 cells which shows the high affinity of the factor(s) for determinant(s) expressed on VCN-treated L1210 cells; untreated L1210 cells could absorb only a small fraction of the activity after 4 consecutive absorptions. Normal mouse serum from strain BALB/c which did not possess cytotoxic activity for VCN-treated L1210 cells *per se* even when undiluted could be substituted in place of DBA/2J NMS in the cytotoxicity tests. Studies are in progress to investigate whether or not sera from mice strains other than DBA/2J and BALB/c could render normal PML cells cytotoxic for VCN treated L1210 cells. It is not clear whether a relationship exists between the NMS factor(s) which participate in the normal DBA/2J PML cell mediated cytotoxicity towards VCN-treated L1210 cells and the NMS factor(s) in undiluted sera which is capable of exerting a direct cytotoxic effect *per se* on VCN-treated tumor cells [9, 10].

The data presented in this paper do not permit to decide about the nature of the antigenic determinant(s) towards which the NMS factor(s) is directed and the mechanism by which VCN-treated L1210 cells are killed by the synergistic action of normal DBA/2J PML cells and NMS. However, the following possibilities may account for the observations: The development of histocompatible differences between L1210 cells and the DBA/2 host either due to antigenic mutations in the leukaemia cell line during its transplantation-history or within the inbred DBA/2 mouse colonies obtained from various sources has been reported [16, 17] and it has been suggested that minor histocompatible differences may not be revealed by transplantation tests. It can be argued that VCN-treatment enhances the expression of those antigenic determinant(s) on L1210 cells which are not carried out by DBA/2J PML cells. If such is the case then the observed cytotoxicity on VCN-treated L1210 cells can be interpreted as an expression of allogeneic inhibition [18, 19] ensuing in the presence of a naturally occurring antibody-like factor(s) in mouse serum directed against VCN-expressed antigenic determinant(s) carried by leukaemia cells. Such a mode of cytotoxicity will then be analogous to that described by MacLennan *et al.* [20] who reported a factor in normal human serum with the properties of an antibody which could render target cells (Chang liver cells) susceptible to lysis by non-immune human lymphoid cells.

Another possible explanation of the cytotoxicity observed in the present system will be that naturally occurring serum factor(s) arm the normal macrophages which become activated on coming in contact with the target cell antigens (tumor specific transplantation antigens or histocompatible determinant(s) not shared by normal DBA/2J PML cells) expressed as a result of VCN treatment resulting in target cell death. Evans and Alexander [21] have reported that normal DBA/2 peritoneal macrophages acquire the capacity to kill syngeneic L5178 lymphoma cells following incubation either with immune spleen cells or the supernatant from a culture of immune spleen cells and antigen. The arming of peritoneal macrophages by the immune serum was, however, not achieved.

Whatever the nature of the antigenic determinant(s) may be towards which the DBA/2J NMS factor(s) are directed, it is clear that VCN treatment leads to their effective expression. Since pretreatment of normal DBA/2J PML cells did not show any cytotoxicity towards untreated L1210 cells in the presence of NMS, it can be concluded that the effect of VCN is directed towards the tumor cells. The observation that L1210 cells preincubated with protamine sulphate, which is known to reduce the cell surface charge failed to render the L1210 cells sensitive to the synergistic cytotoxic effect

of DBA/2J PML cells and NMS, rules out the argument that the susceptibility of VCN-treated L1210 cells is due to their reduced cell surface charge. Treatment of L1210 cells with a number of other enzymes (e.g., ribonuclease, hyaluronidase, phospholipase D) including proteolytic enzymes i.e., trypsin and pronase which are known to uncover antigenic sites on cell surfaces [22] failed to modify the sensitivity of L1210 cells towards the cytotoxic activity of normal DBA/2J PML cells and NMS. It can be inferred, therefore, that the effective expression of antigenic determinant(s) on VCN-treated L1210 cells is related to the enzymatic removal of cell surface sialic acid. Such a suggestion has already been made by several investigators from certain *in vivo* studies [1, 2, 5]. The increased expression of tumor cell antigenic determinant(s) can be interpreted as either to unmasking due to removal of cell surface sialic acid or due to stearic changes in the configuration of the antigenic determinant(s) involved [1]. There is already *in vitro* evidence also which suggests that VCN-treatment of tumor cells results in increased expression of tumor associated antigens [23, 24, 25]. However, the situation in case of the *in vitro* interactions between mastocytoma (P815) cells and immune spleen cells appears to be different since VCN-treatment of target cells had no effect on immune cytolysis of target cells [26]. Evans [27] has reported increased phagocytosis of VCN-treated L5178Y lymphoma cells by syngeneic DBA/2 peritoneal macrophages in the presence of specific antiserum. The *in vitro* cytotoxicity towards VCN-treated L1210 cells observed in the present system was, however, of non-phagocytic nature.

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A Factor Reducing Protein Synthesis from Ehrlich Ascites Cells

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Abstract—*Ehrlich ascites cells show a decreasing rate of incorporation of labeled amino acids into the cold acid insoluble material when they are incubated in HBSS. This decreasing rate of incorporation is partly due to a dilution of the labeled amino acid by unlabeled amino acids escaping from the cells. It is demonstrated that also the net rate of protein synthesis is reduced under these conditions and this reduction occurs on a step following aminoacylation of transfer-RNA. When the medium is changed after 3–5 hr of incubation, the cells regain their original high rate of amino acid incorporation and the medium harvested after incubation with ascites cells reduces the rate of protein synthesis in freshly harvested cells. It is hypothesized that ascites cells under the incubation conditions release a factor into the medium which reversibly inhibits protein synthesis. The possible role of this factor in regulating the transformation of growing ascites cells into non-growing cells is discussed.*

INTRODUCTION

FOLLOWING an exponential phase of cell-multiplication ascites tumours reach a steady state between cell production and cell death (plateau phase). Bichel has demonstrated that this plateau phase in the growth of ascites tumours is not due to a lack of substrate resulting from exhaustion of the host [1–3] and he proposed a factor involved in the growth regulation of ascites tumours. A similar mechanism was discussed by Sylvén and Holmberg [4–7]. These authors isolated from ascitic fluids a protein which had an inhibitory effect on the growth rate of various cells lines in tissue culture. Though Waller *et al.* [8] could not reproduce these experiments there seems to be some good evidence for the existence of such a factor. The contradictory results of Waller *et al.* [8] may well be based on an insufficient biological test system. It is desirable, therefore, to set up a test system which allows objective evaluation of the effects of the postulated factors on the molecular level, e.g., by measurement of cellular syntheses. Ascites cells in the exponential growth phase *in vivo* stop growing upon transfer to a balanced salt solution *in vitro*. The rate of incorporation of radioactive thymidine into the cold acid insoluble material is drastically reduced in this system within the first 30 min of incubation (95%) and cinematographic time lapse studies of these cells reveal that no cell goes into mitosis under these conditions (unpublished results). Starting from the assumption that this artificially induced growth inhibition could serve as an *in vitro* model for the investigation of the growth regulation in the plateau phase of ascites tumours, we tried to establish an inhibition of cellular syntheses by factors contained in the medium of these non-growing cell cultures.

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MATERIAL AND METHODS

Ascites cells

Specific pathogen free NMRI mice (30 g) were bought from the Süddeutsche Versuchstierfarm (Tuttlingen, W. Germany) and fed with a basal diet (Fa. Altromin, Lage, W. Germany) and tap water *ad libitum*. Ascites tumour was produced by i.p. injections of 5×10^4 Ehrlich ascites cells of the Gϕ [9] strain per animal under sterile conditions. The ascitic fluid was aspirated seven days after inoculation and the ascites cells were washed free from serum by sedimentation and resuspension in

HBSS [10], pH of 7·4. Finally the cells were resuspended in the same volume of HBSS. This suspension was used for estimation of cell numbers and following dilution with the same medium for incubation experiments.

Preparation of supernatants from preincubated cells

Cell suspensions as described above were incubated at 37° in Erlenmeyer flasks under gentle shaking in a Gyrotory Shaker (New Brunswick Scientific Instruments, New Brunswick, N.J., USA, model G 25) for 5 hr. The cells were then sedimented by centrifugation (4°, 5000 × *g*, 20 min) and the cell free supernatant was stored in 1 ml aliquots at −20°. No loss of activity with respect to inhibition of incorporation of labeled amino acids into freshly harvested ascites cells was detectable within 5–6 weeks.

Radioisotopes

Amino acid-^{3}H (G) mixture was bought from Fa. NEN-Chemicals (Dreieichenhain, W. Germany). Other radiochemicals used in this work were products of the Radiochemical Center (Amersham, U.K.).

Radioscans and liquid scintillation countings

Radiochromatography was performed as described in a previous paper [11]. Liquid scintillation countings were carried out either in homogeneous solution (Dpm-analyses) containing 1 ml of a solubilizer (Protosol, NEN-Chemicals, Dreieichenhain, W. Germany) per 10 ml scintillation fluid or by means of a filter technique [12] modified by Süß *et al.* [13]. The scintillation fluid contained 0·01% *p*-bis [2-(5-phenyl-oxazolyl)] benzene (POPOP) and 0·4% 2,5-diphenyloxazole (PPO) in toluene (Toluene p.a., Fa. Merck, Darmstadt, W. Germany). The countings were performed in a Tri Carb Liquid Scintillation Spectrometer (Fa. Packard Instruments, Frankfurt, W. Germany, model 3380 provided with an analyzer for absolute activity countings, model 544).

RESULTS

Reduction of the rate of incorporation of labeled amino acids into the cold acid insoluble material of ascites cells when incubated in HBSS

From Fig. 1 it is seen that the labeled amino acid is mixed with the functional precursor pool of ascites cells *in vitro* and utilized for protein synthesis. Following the high initial rate of incorporation, a plateau phase is reached and the data of Fig. 2 indicate that this plateau is

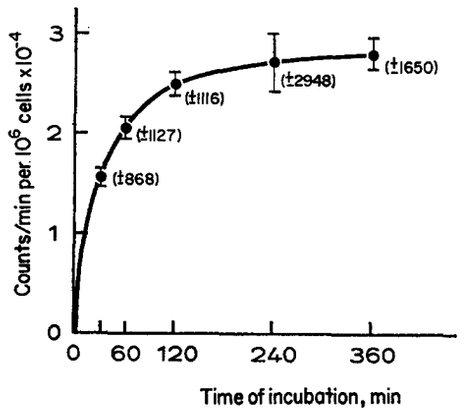

Fig. 1. Incorporation of L-*lysine-*^{14}*C (U) into the cold TCA insoluble material of ascites tumour cells* in vitro. *The incubation mixture contained* 6 × 10^7 *cells,* 10 *μCi of* L-*lysine-*^{14}*C (U)* (269 *mCi/mmole*) *in* 5 *ml of HBSS. Incubation was performed in an Erlenmeyer flask,* 25 *ml of volume, under gentle shaking at* 37°. *At the time intervals, indicated in the graph,* 5 × 50 *μl aliquots were taken and analyzed for TCA insoluble radioactivity. The graph presents mean values of the five aliquots taken at the same time and corrected for* 10^6 *ascites cells.*

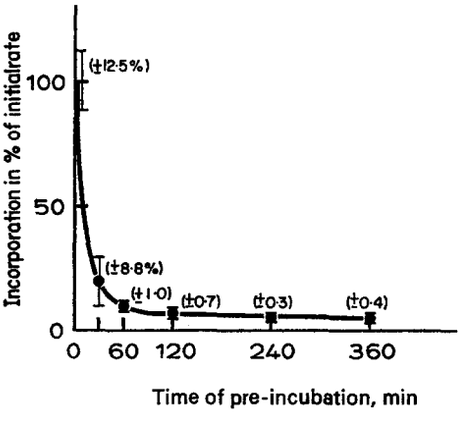

Fig. 2. Effect of the time of pre-incubation on the initial rate of incorporation of L-*lysine-*^{14}*C (U) into the cold TCA insoluble material of ascites tumour cells* in vitro. *Each of six incubation mixtures contained* 86·5 × 10^6 *ascites cells in a total volume of* 5 *ml of HBSS. The time of preincubation was as indicated in the graph. Following the preincubation time* 10 *μCi of* L-*lysine-*^{14}*C (U)* (269 *mCi/mmole*) *were added and the incubation continued at* 37° *for another hour.* 5 × 50 *μl aliquots were then taken and analysed for TCA insoluble radioactivity. The rates of incorporation are expressed as percentage of the initial rate.*

the result of decreasing rates of incorporation of the labeled amino acid into the high molecular material of the cells. The high initial rate of incorporation of the radioactive amino acid at the beginning of the incubation is due to the high specific radioactivity of the added amino acid in the incubation mixture and to a high rate of protein synthesis in freshly harvested

ascites cells. The reduction of the rate of incorporation (Fig. 2) could be explained by a dilution of the radioactive precursor by amino acids escaping from the cells [14, 15] or by an inhibition-factor of protein synthesis accumulating in the time course of incubation. These mechanisms would require that the original high rate of amino acid incorporation should be restored after renewal of the incubation medium. Renewal of the medium should remove either the amino acids which could dilute the exogenously added amino acids or the postulated

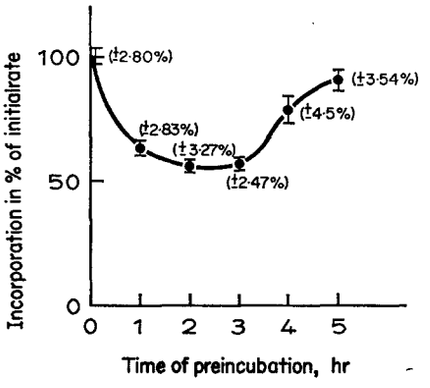

Fig. 3. Recovery of the initial rate of incorporation of radioactively labeled amino acids into the cold acid insoluble material of ascites cells after renewal of the medium. $4{\cdot}2 \times 10^8$ *ascites cells were incubated in a total volume of* 10 *ml of HBSS at* 37° *in a Gyrotory Shaker under gentle shaking over a period of* 5 *hr. The cell number proved to be constant* ±5% *within this incubation period. At time intervals as indicated in the graph* 1 *ml aliquots were taken from the original mixture. The cells contained in these* 1 *ml aliquots were centrifuged, the supernatant was discarded and the cells were resuspended in HBSS to give a total volume of* 1 *ml.* 5 *μCi of* H^3*-amino acid mixture were added and the incubation continued. After* 30 *min* 5×50 *μl aliquots were taken and analyzed for TCA insoluble radioactivity. The rates of incorporation are expressed in percentage of the initial rate.*

inhibition factor. The experiment described in the legend to Fig. 3 proves that the reduction in incorporation of labeled amino acids into protein was 50–90% reversible, depending on the preincubation time, through renewal of the medium prior to addition of the labeled amino acids (Fig. 3).

Reduction of the net rate of protein synthesis of ascites cells when incubated in HBSS

In order to rule out the possibility of a dilution of the radioactive label through the catabolism of unlabeled cellular proteins under the conditions of incubation, the rate of incorporation of label into the direct precursor pool for protein synthesis (charged transfer–RNA) was measured. Charging of transfer–RNA with labeled amino acids was indeed reduced by a factor of 0·4 under our experimental conditions (Table 1). However, protein synthesis measured at the same time showed a further 10-fold reduction in the incorporation of labeled amino acids, indicating that in addition to a 60% reduction in the labeling of proteins through dilution of the isotope, there was a sharp drop in labeling due to a block following aminoacyl–transfer–RNA formation.

Table 1. Effect of preincubation on the rate of incorporation of various radioactively labeled amino acids into the cold acid insoluble material of ascites cells

A. Hot acid soluble fraction:

Amino acid	Dpm × 10^{-3} Not pre-incubated	Dpm × 10^{-3} Pre-incubated	%
L-Alanine	9·6	3·6	31·3
Glycine	42·0	19·0	47·4
L-Leucine	79·4	21·6	27·2
L-Lysine	160·6	81·0	50·4
L-Valine	57·9	12·4	21·4
Sum	349·5	137·9	39·5

B. Hot acid insoluble fraction:

Amino acid	Dpm × 10^{-3} Not pre-incubated	Dpm × 10^{-3} Pre-incubated	%
L-Alanine	46·6	2·1	4·5
Glycine	174·3	6·2	3·6
L-Leucine	2434·0	141·2	5·8
L-Lysine	1139·1	16·1	1·4
L-Valine	1283·7	14·4	1·1
Sum	5077·6	180·0	3·6

10^8 freshly harvested ascites tumour cells were incubated in 5 ml of HBSS containing 2 μCi of one of the following amino acids in a Gyrotory Shaker at 37° for 1 hr: (L-alanine-^{14}C (U) 75 mCi/mmole, glycine-^{14}C (U) 50 mCi/mmole, L-leucine-^{14}C (U) 150 mCi/mmole, L-lysine-^{14}C (U) 150 mCi/mmole, L-valine-^{14}C (U) 75 mCi/mmole). Pre-incubation was performed under the incubation conditions for 2 hr. Following the precipitation of the cold acid insoluble material by addition of 1 ml of cold TCA (30%) and extraction of the sediment with alcohol (20°) and alcohol/ether (2:1, 40°), the sediment was treated for 30 min with 2 ml of hot TCA (5%, 90°). The hot TCA insoluble sediment was dissolved in 1 ml of Protosol. Aliquots of the TCA insoluble and of the TCA soluble fraction were mixed with 1 ml of Protosol and 10 ml of scintillation fluid and submitted to absolute activity analysis. Activities of the total fractions were calculated from the values of the aliquots.

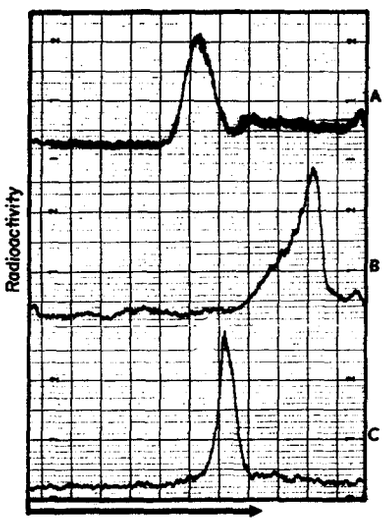

Fig. 4. Radioscans of paper chromatograms of the hot TCA soluble fraction and ribonuclease digests of ascites cells which were incubated with L-*lysine-*^{14}C *(U). Aliquots containing about* 10,000 *Dpm were used in ascending chromatography with phenol saturated by a solution which contained* 6·3% *sodium-citrate and* 3·7% *potassium-dihydrogen-phosphate as solvent.*

A. L-*lysine-*^{14}C *(U)* (150 *mCi/mmole*)

B. Hot TCA soluble fraction of ascites cells pre-incubated for 2 *hr and then incubated with* L-*lysine-*^{14}C *(U). For details see preparation of the hot TCA soluble fraction in the legend to Table* 1.

C. Ribonuclease digest of cold TCA insoluble material of ascites cells pre-incubated for 2 *hr and then incubated with* L-*lysine-*^{14}C *(U) for another hour. The cold TCA precipitate was washed by resuspension in alcohol (room temperature) and alcohol/ether* (2:1) *and then dried in a desiccator. Finally it was incubated for* 1 *hr at room temperature with* 3 *ml of acetate buffer* (0·1 *M; pH* 5) *containing* 1 *mg of panc atic ribonuclease (EC* 2.7.7.16) *(Fa. Boehringer, Mannheim, W. Germany,* 40 *U/mg). The digestion was stopped by addition of cold TCA* (0·5 *ml,* 15%) *and the supernatant used for chromatography.*

The radioactivity found in the hot acid soluble fraction is originated from labeled amino acids bound to transfer–RNA

The assumption that the radioactivity found in the hot acid soluble fraction is actually due to amino acid charged transfer–RNAs becomes evident by the result of the experiment shown in Fig. 4. Following the incubation of ascites cells with radioactive amino acids, the transfer–RNA is hydrolyzed by the hot TCA (5%) into oligonucleotides, but the amino-acyl binding to the terminal residue of transfer–RNA is relatively stable at lower pH values [16]. The R_f- value of the resulting mixture of compounds (amino-acid-ApC...) is different from that of the free amino acid, proving that no relevant amount of radioactively labeled amino acid has been hydrolyzed from proteins or from amino-acylated transfer–RNA under these conditions and that no contamination of this fraction by free amino acids is detectable. In Fig. 4, it is also demonstrated that the radioactively labeled compound in the hot acid hydrolysate consists of an amino acid originally bound to a terminal residue of RNA and not of a small protein or a peptide because pancreatic ribonuclease treatment of the high molecular weight material of ascites cells, following the procedure described above, results in a homogeneous compound with a characteristic R_f-value. According to the mode of action of pancreatic ribonuclease, one has to assume for this compound the structure of amino-acyl-adenosine [17]. Similar experiments (not shown) were performed with the amino acids listed in Table 1.

Effect of supernatants of pre-incubated ascites cells on the rate of protein synthesis of freshly harvested ascites cells

Cell-free medium harvested after incubation with ascites cells markedly inhibited incorporation of labeled amino acids into protein of freshly harvested ascites cells (Table 2). This effect can not be due to a simple dilution of isotope through the release of unlabeled amino acids into the medium, since the medium harvested after incubation with ascites cells does not reduce the incorporation of ^{3}H-lysine into the immediate precursor pool (charged amino-acyl-transfer–RNA) for protein synthesis (Table 3). At the same time, the rate of incorporation of ^{3}H-lysine into protein was significantly lowered

Table 2. Effect of supernatants of preincubated ascites tumour cells on the rate of incorporation of amino acids into the cold acid insoluble material of freshly harvested ascites cells

Experiment	Counts/min $\times 10^{-3}$ controls	Counts/min $\times 10^{-3}$ experiments	% of controls	P ≦
1	27·4 ± 2·7	6·1 ± 0·7	23	0·001
2	27·2 ± 4·1	9·2 ± 0·7	34	0·001
3	31·1 ± 3·6	8·9 ± 0·9	29	0·001
4	28·5 ± 3·2	5·6 ± 1·6	20	0·001
5	34·2 ± 1·2	7·1 ± 2·2	21	0·001
6	24·2 ± 2·7	8·1 ± 0·3	33	0·001

Freshly harvested ascites cells (~ 5×10^6 cells) were incubated for 1 hr at 37° with 5 μCi of an amino acid-^{3}H mixture in 130 μl of HBSS (controls) and in 80 μl of HBSS and 50 μl of supernatant of preincubated cells. Following the incubation, 5×50 μl aliquots were taken and the radioactivity was detected as described in the legend to Fig. 1. Six separate experiments were carried out. The mean values (corrected for 10^6 cells) of the experiments and standard deviations are given in the Table. Student's t-test for unpaired values was used for the calculation of P-levels.

by medium pre-incubated with ascites cells, leading us to postulate that such medium contains a factor which is able to reduce the rate of net protein synthesis in freshly harvested cells at a step following aminoacyl-transfer–RNA formation. Dialysis shows the molecular weight of the active substance to be greater than 500 daltons (Table 4).

Table 3. Effect of supernatants of pre-incubated ascites tumour cells on the incorporation of DL-lysine-4,5-^3H into the hot TCA soluble (A) and into the hot TCA insoluble (B) fraction of freshly harvested ascites cells

(A) Hot TCA soluble fraction

Sample	Dpm × 10^{-4} Controls	Dpm × 10^{-4} Plus supernatant
1	2·74	2·30
2	3·40	3·55
3	3·88	3·12
4	3·22	3·63
5	3·07	2·84
6	3·20	3·55
$\bar{X} \pm$ S.D.	3·25 ± 0·13	3·27 ± 0·33

Student's *t*-test: $t = 0{\cdot}1496$; no significant difference

(B) Hot TCA insoluble fraction

Sample	Dpm × 10^{-4} controls	Dpm × 10^{-4} plus supernatant
1	4·92	3·15
2	3·73	3·02
3	4·06	2·46
4	4·01	2·56
5	4·29	2·44
6	3·55	3·22
$\bar{X} \pm$ S.D.	4·11 ± 0·15	2·82 ± 0·36 68% of control

Student's *t*-test: $t = 5{\cdot}2971$; $P \leqq 0{\cdot}01$

Freshly harvested ascites cells ($\sim 5 \times 10^6$) were incubated for 1 hr at 37°C with 5 μCi of DL-lysine-4, 5-^{3}H (12·8 Ci/mmole) without supernatants of pre-incubated cells (controls) and with supernatants of preincubated cells as described in the legend to Table 2. Following the incubation the high molecular weight material was precipitated and fractioned into the hot TCA soluble and into the hot TCA insoluble material as described in the legend to Table 1. Aliquots of the fractions dissolved in Protosol were submitted to Dpm analysis. Total radioactivity was calculated from these values and corrected for 10^6 cells. The mean values and standard deviations are given in the Table. Student's *t*-test for unpaired values was used for the calculation of *P*-levels.

Table 4. Effect of dialyzed supernatants on the incorporation of labeled amino acids into the cold acid insoluble material of freshly harvested ascites cells

Experiments	Counts/min × 10^{-3} controls	Counts/min × 10^{-3} experiments	% of controls
1	6·3	3·7	54
2	7·2	3·6	50
3	6·9	4·2	61
$\bar{X} \pm$ S.D.	6·8 ± 0·5	3·8 ± 0·3	55

67 ml of the crude medium (HBSS) of preincubated ascites cells were filtered through an Amicon-membrane (UMO5) by means of an ultrafiltration apparatus (Amicon N.V., Oosterhout, Netherlands, Model TCF 10) and washed by 750 ml of 1 mM phosphate buffer of pH 7·5 to remove molecules with a molecular weight lower than 500. Aliquots of the remaining retentate (70 ml) were tested for inhibition of incorporation of amino acids into freshly harvested ascites cells according to the method described in the legend to Table 2. Incubation mixtures contained $6{\cdot}1 \times 10^6$ cells, 2·5 μCi amino acid-^{3}H mixture and 50 μl of the retentate in a final volume of 150 μl (HBSS). Controls contained 50 μl of 1 mM phosphate buffer pH 7·5 instead of retentate. Mean values and standard deviations are corrected for 10^6 cells.

DISCUSSION

It was the aim of this work to find out whether the decreasing rates of incorporation of labeled amino acids into the high molecular weight material of ascites cells incubated in a balanced salt solution *in vitro* are caused by simple dilution of the radioactively labeled amino acids or by an inhibition of the rate of net protein synthesis. It could be demonstrated that these decreasing rates of incorporation may partly be due to a dilution of the radioactively labeled precursor pool, but that there must occur also an additional inhibition of the net protein synthesis because the incorporation of radioactive amino acids into the functional precursor pool is less reduced in the time course of incubation than the incorporation into the protein. This inhibition of the rate of net protein synthesis can also be produced by aliquots of medium pre-incubated with ascites cells. We therefore hypothesize that this medium contains a factor which is able to inhibit also the net protein synthesis of freshly harvested cells at a step following aminoacyl–transfer–RNA formation. The search for this factor which is under investigation indicates its protein nature. Predictions for the biological significance of this factor are only speculative until now; however, we are following the working hypothesis that ascites tumour cells under special incubation conditions release a factor or factors into the medium which reduce protein synthesis reversibly and

that these *in vitro* conditions may reflect the situation of ascites tumours in the plateau phase.

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Influence of Genetic Strain on the Induction of Lung Cancer in Hamsters by Alpha Radiation*

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Abstract—*The response was studied of four inbred strains of Syrian hamsters to multiple intratracheal instillations of the alpha emitting radionuclide polonium-210 adsorbed onto hematite particles. Marked differences occurred among strains in their tolerance to the instillations. There also appeared to be significant differences in the tumor induction times, but the frequency of lung tumors was similar in the four strains—varying between* 33% and 50%. *The influence of genetic strain might well be more marked with chemical carcinogens in which mechanisms of activation or detoxification become important factors which come under genetic control.*

INTRODUCTION

SYRIAN golden hamsters have been widely used as test animals in carcinogenesis studies [1, 2]. These investigations have employed random bred animals, although all laboratory hamsters are in reality derived from three animals originally brought to Israel from Syria in 1931 [3]. Recently, studies with inbred hamster lines have indicated an influence of genetic strain on susceptibility to polynuclear hydrocarbons following subcutaneous and gastric administration [4, 5] and on the response to adenovirus type 12 [6].

Syrian hamsters have received increasing use in the past several years in studies of experimental respiratory carcinogenesis [7]. The occurrence of spontaneous lung tumors in hamsters is rare, and the incidence of infectious and other pulmonary complications is also low [8, 9]. It would therefore be important to know whether an inbred line might be a better model for such studies on respiratory carcinogenesis. In addition, the occurrence of significant differences in response among strains would allow us to study factors which control sensitivity to carcinogens.

We have investigated the influence of genetic strain on the response to repeated intratracheal instillations and on the susceptibility to lung tumor induction by polonium-210 (^{210}Po, a naturally occurring radionuclide which emits a 5·3 MeV alpha particle) in four inbred hamster lines. ^{210}Po was chosen as a carcinogen because its activity does not depend on chemical activation or degradation in the animals, and it has been found reproducibly to yield a high incidence of lung tumors in random bred hamsters [10, 11].

MATERIAL AND METHODS

Male and female hamsters in about equal proportions from four inbred lines were kindly supplied by Dr. Freddy Homburger of the Bio-Research Institute, Cambridge, Massachusetts. These lines were identified as BIO 2·4 (originally LSH), BIO 4·22, BIO 15·16 and BIO 87·20, and are now available from TELACO, Bar Harbor, Maine 04609, U.S.A. At the time of this investigation, brother–sister matings had been carried out for 28–30, 39–41, 17–18 and 18–20 generations respectively in these lines. Further details as to the origin and character-

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istics of these BIO hamsters have been reported [5]. The animals were housed 3–4 to a cage according to sex in an environment controlled room, and were fed Purina chow and water *ad libitum.*

Intratracheal instillations were begun when the hamsters reached 10 weeks of age. Each animal received 15 weekly instillations of 0·2μ Ci ^{210}Po absorbed on 1·5 mg of hematite particles and suspended in 0·1 ml of isotonic saline. Hamsters were lightly anesthetized with sodium brevitol i.p. prior to each instillation. Details concerning the preparation and intratracheal instillation of ^{210}Po suspensions have been described [10, 11]. The surviving animals were weighed either weekly or biweekly from the 4th through the 30th week after the beginning of treatment.

The hamsters were sacrificed when moribund, the inflated lungs and trachea removed together, fixed in alcohol–formalin fixative and embedded in paraffin blocks. Sections 8 μ thick were cut and stained with hematoxylin and eosin for pathological study. Sections were obtained from each lobe of the lungs, as well as at several levels in the trachea.

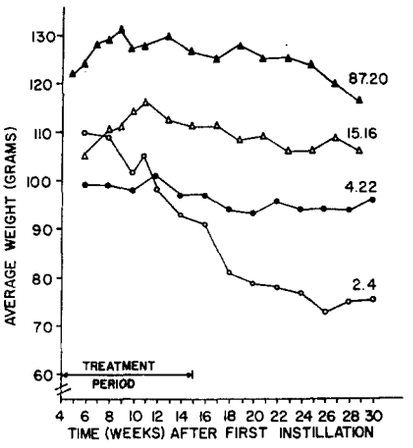

Fig. 1. *Average weight of hamsters of* 4 *genetic strains during and after a course of* 15 *weekly intratracheal instillations of* 0·2μ *Ci* ^{210}*Po on* 1·5 *mg hematite particles suspended in* 0·1 *ml isotonic saline.*

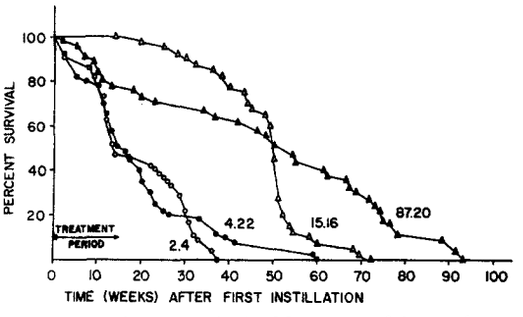

Fig. 2. *Cumulative survival of hamsters of* 4 *genetic strains during and after the course of* 15 *weekly intratracheal instillations of* 0·2μ *Ci* ^{210}*Po.*

RESULTS

The changes in the average weight of the hamsters in the four groups during and after the course of intratracheal instillations are shown in Fig. 1. The initial weights are similar to those reported by Homburger *et al.* [5] for comparably aged animals. In each group the average weight of the males was slightly lower than that of the females. The 87·20 and 15·16 strains continued to gain weight during the treatment period, while that of the 4·22 hamsters remained constant. The 2·4 strain, however, showed a rapid decline in weight during the treatment period which continued until 5 weeks after the last instillation.

The cumulative survival for each strain vs time after the first instillation is shown in Fig. 2. The lung tumor incidence data are tabulated in Table 1, and data indicating the time of death for each animal and the presence or absence of lung tumors at autopsy are presented in Fig. 3. Only one tracheal tumor was found among these animals: a carcinosarcoma occurring in an 87·20 hamster which died 77 weeks after the first instillations. There were no sex related differences in tumor incidence or induction time.

With the exception of the single carcinosarcoma of the trachea, the induced tumors all involved peripheral lung parenchyma. We categorize these as "combined" tumors [10, 11]

Table 1. *Lung tumor incidence in hamsters receiving* 15 *weekly intratracheal instillations of* 0·2μ *Ci polonium-*210

Strain	No. of animals autopsied*	No. of animals dead with no lung tumor		No. of animals dead with malignant lung tumors	Tumor incidence	
		5–15 wks	15–92 wks		% of all animals	% of animals surviving 15 wk treatment period
2·4	39	18	11	10	26%	48%
4·22	32	11	13	8	25%	38%
15·16	33	0	22	11	33%	33%
87·20	40	8	16	16	40%	50%

*Does not include hamsters which died before fifth week of treatment.

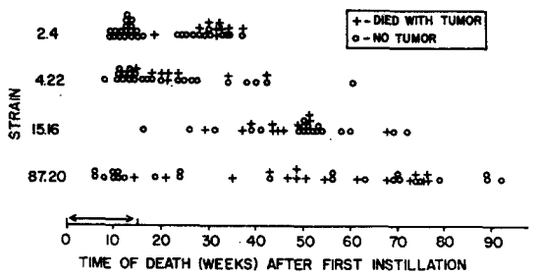

Fig. 3. Time of death of individual hamsters from each of the 4 groups. Crosses or zeros indicate whether or not lung tumors were present at the time of death.

because both adenomatous and epidermoid features are found in different fields of the same tumor (Fig. 4). Only three of the total tumors showed epidermoid characteristics alone; two of these appeared to be bronchial in origin. Categorization of these lesions as tumors was based on their replacement of supporting lung tissue. As in our previous report [11], invasion of bronchi, blood vessels and pleura was seen in instances of extensive involvement. There were no notable differences in the histopathologic detail of these tumors among the four experimental groups, though, on the whole, tumors in the 87·20 group appeared more extensive.

DISCUSSION

These results indicate that significant differences do exist in the response of gentically inbred strains of Syrian hamsters to intratracheally administered ^{210}Po. These differences appear to involve the tolerance of the animals to multiple intratracheal instillations, and the carcinogenic response to alpha radiation. As can be seen in Fig. 2, over 50% of animals of strains 2·4 and 4·22 died during the treatment period, whereas no deaths occurred amongst the 15·16 group. In parallel experiments, about 20% of random bred hamsters died during the treatment period [10], comparable to the results with the 87·20 strain in these experiments. The more resistant strains actually gained weight during the treatment period (Fig. 1); this gain was similar to that observed in untreated control animals (F. Homburger, personal communication).

The reasons for the marked differences in the mortality experience after treatment are not entirely clear. It is possible that the 2·4 and 4·22 strains were more sensitive to the toxic (non-carcinogenic) effects of radiation on lung tissue, although pathological evidence to support this hypothesis was not present. The degree of inflammatory change, fibrosis and necrosis appeared grossly similar in animals of similar ages. Interestingly, the 87·20 strain is normally an unusually long-lived hamster; its longevity under standard conditions is 670 days, as compared with 532 days for the 2·4 and 481 days for the 4·22 strain [5].

The differences in the frequency of lung tumors among the four strains were small (Table 1), although the overall tumor incidence was slightly higher among the 87·20 animals. In this regard, Homburger *et al.* [5] found that bronchiolar adenocarcinomas developed in 10% of 87·20 hamsters following subcutaneous administration of benz[a]anthracene, whereas no lung tumors developed in untreated controls nor in similarly treated hamsters of several other strains including 4·22 and 15·16. In general in their study, Homburger *et al.* [85] found that 87·20 hamsters were more susceptible to the induction of tumors by subcutaneous and gastric administration of polynuclear hydrocarbons than were the other strains.

Although the frequency of lung tumors was similar, a difference in tumor induction time was apparent among the four strains in the present study. As can be seen in Fig. 3, lung tumors were found in eight of the seventeen 87·20 animals which died between weeks 51 and 80, whereas only one of nine 15·16 hamsters which died after week 51 had a tumor. Tumors found in 15·16 animals were grouped primarily between weeks 35 and 51 (Fig. 3); only one tumor occurred before week 35. Tumors in the 4·22 hamsters tended to group before week 25, however, and tumors occurred in only two of the nine hamsters which died between weeks 25 and 60. Both within and among all strains, late occurring tumors did not appear on the whole to be more extensive or advanced histologically than earlier tumors, although tumors in 87·20 hamsters dying at all times appeared more extensive than those in the other strains. These findings suggest that the induction time effect is real, and that it cannot be explained simply on the basis that tumors were recognized earlier in those strains in which the animals died sooner from other causes. Furthermore, they suggest that the tumor incidence in the 2·4 and 4·22 strains would not necessarily have been greater had the animals survived longer.

The results indicate that genetic strain does have some influence on the response to multiple intratracheal instillations of ^{210}Po. Radiation, however, deposits its energy at random in cells and thus acts directly to initiate the carcinogenic process. The influence of genetic strain might well be more marked with chemical carcinogens in which mechanisms of activation or detoxification become important factors which may come under genetic control.

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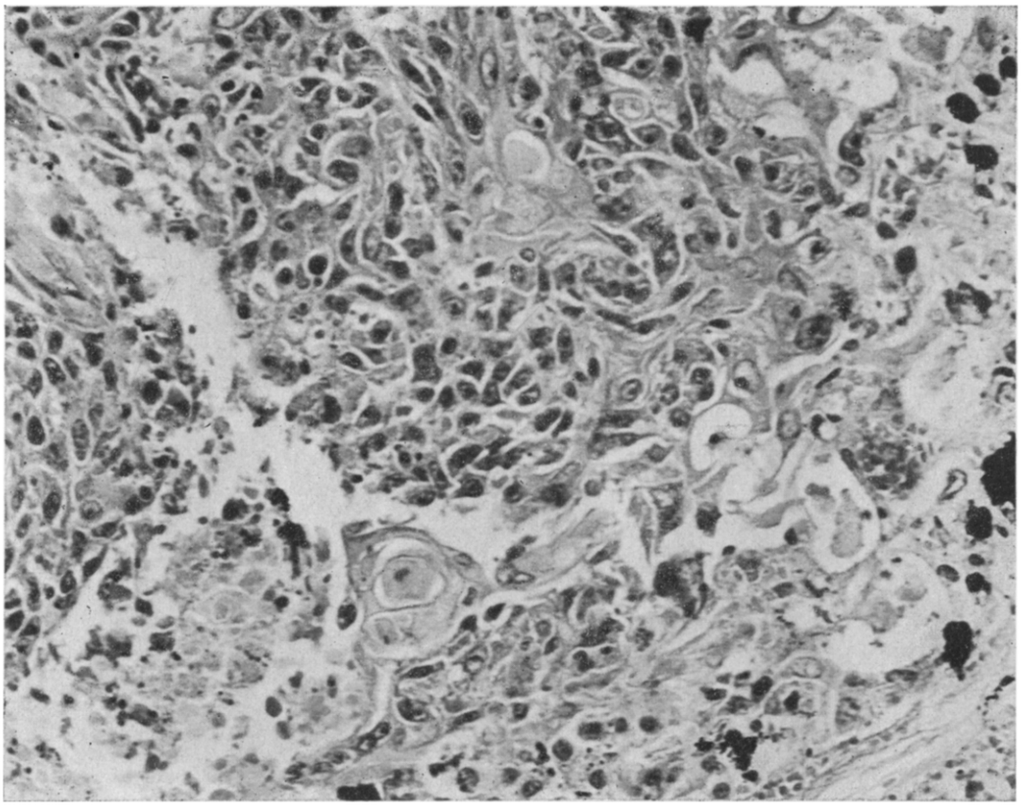

Fig. 4. *Peripheral lung field from* 15·16 *hamster which died* 45 *weeks after first instillation with an extensive tumor. This field shows a poorly differentiated epidermoid pattern* × 400, *H & E*).

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Survival of Clonogenic Cells of a Murine Epithelioma Treated with Neocarzinostatin*

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Abstract—*Neocarzinostatin is a polypeptide anti-tumour substance produced by Streptomyces carzinostatics. Neocarzinostatin has demonstrated a strong anti-tumour activity towards several experimental tumours and has also been tried clinically. However, there are no experimental data for the effect on squamous carcinomas. Therefore, we studied the effect of neocarzinostatin on a murine squamous carcinoma. The survival curve was a two component curve and* D_0 *values were* 1·1 *mg/kg in the first part and* 8·5 *mg/kg in the second part. On the other hand, neocarzinostatin was quite toxic for the WHT/Ht mice used in the experiment; e.g. LD*50 (30) *was* 2·4 ± 0·9 *mg/kg. This dose level corresponds to the dose required to obtain a surviving fraction of* 0·1 *of tumour cells treated with the drug.*

INTRODUCTION

NEOCARZINOSTATIN is an acidic protein with a molecular weight of about 9000 [1] and its chemical characteristics have been described in detail by Maeda and Ishida [2]. In several tumours, neocarzinostatin demonstrates strong anti-tumour activities. Among these are Ehrlich ascites tumour [3], mouse ascitic leukaemia SN-6 [4], and mouse leukaemia L1210 [5]. The drug has been also tried clinically. Its mode of action as an anti-tumour drug, however, is only partially known. The purpose of the present paper is to obtain the dose survival relationship with a murine epithelioma in which the response to neocarzinostatin has not yet been investigated and to determine the toxicity of this drug.

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MATERIAL AND METHODS

Mice

Male or female mice of strain WHT/Ht were used as tumour hosts in the TD50 experiments in order to construct the dose survival curve and male mice of strain WHT/Ht were used for the LD50 (30) assays.

Tumour

This was a transplantable keratinizing squamous carcinoma which arose spontaneously in a WHT/Ht mouse and has since been maintained by serial subcutaneous transplantation. A full description of the tumour has been reported by Hewitt, Chan and Blake [6] and by Hewitt and Sakamoto [7].

Neocarzinostatin treatment

The stock solution of neocarzinostatin was prepared at a concentration of 2·0 mg/kg in saline and kept frozen between experiments. For use, the stock solution was diluted with saline from 0·02 mg/kg to 1·0 mg/kg so that the volume of fluid containing the drug was not more than 0·03 ml. This was injected intraperitoneally.

Survival of tumour cells

Mice bearing a subcutaneous tumour in each axillary region were given a subcutaneous injection of 80,000 tumour cells into both axillary regions. After 10–11 days, when the tumour weighed 1·5–2·0 g, they were given a graded dose of neocarzinostatin intraperitoneally for one hour. Single cell suspensions of tumour cells were then prepared from the tumours by a method described previously [8]. Transplantation assays of counted cell suspensions were performed by the technique described by Hewitt, Chan and Blake [6]. TD50 (number of cells required for successful transplantation to half a group of injection sites) and the 95 per cent confidence limits were calculated from the results of an assay by the method of Litchfield and Wilcoxon [9]. A series of assays of cells from untreated tumours yielded a mean TD50 of 9·6 cells. The surviving fraction of cells from treated tumours was, therefore, obtained from the expression 9·6/TD50 for untreated cells.

*LD*50(30) *assay*

Neocarzinostatin doses of 0·5, 1·0, 1·5, 2·5, 5·0, and 10·0 mg/kg were given intraperitoneally to groups of 10 mice and deaths were recorded daily during the period of up to 30 days after drug administration.

RESULTS

Survival of tumour cells

The single dose survival curve of the squamous carcinoma cells exposed to neocarzinostatin is shown in Fig. 1. The curve is composed of two components and the first part bends at the dose level of approximately 2·5 mg/kg. The D_0 of the first component is 1·1 mg/kg and that of the second part is 8·5 mg/kg. The D_0 of the second part was 8 times greater than that of the first part. The reason why the survival curve of squamous carcinoma cells for neocarzinostatin showed a two component curve is not clear but it will be considered in the discussion.

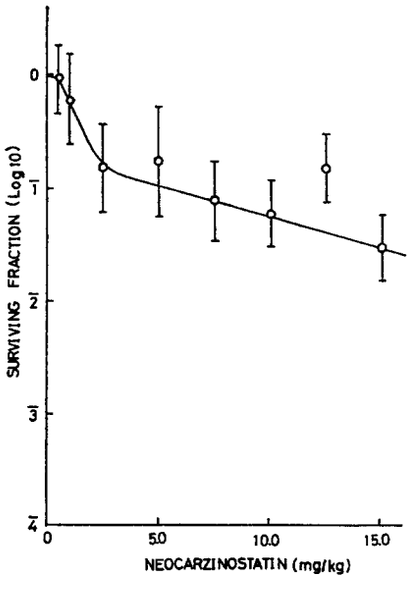

Fig. 1. *Survival of clonogenic squamous carcinoma cells treated with neocarzinostatin.*

*LD*50(30) *assays*

The result of the assay of the toxicity of neocarzinostatin is shown in Table 1. The LD50(30) for neocarzinostatin-treated mice was 2·4 ± 0·9 mg/kg. This dose level corresponds to the dose required to obtain a surviving fraction of around 0·1 in the squamous carcinoma cells. Autopsy of dead mice revealed abundant ascitic fluid and a marked shrinkage of the spleen and the liver.

Table 1. *Thirty-day survival of mice given neocarzinostatin intraperitoneally*

Dose of drug (mg/kg)	Deaths/total exposed (per cent)
10·0	10/10 (100·0)
5·0	8/10 (80·0)
2·5	7/10 (70·0)
1·5	4/10 (40·0)
1·0	3/10 (30·0)
0·5	2/10 (20·0)

LD50(30): 2·4 ± 0·9 mg/kg

DISCUSSION

Generally, the data for tumours irradiated *in vivo* suggest a compound curve representing the response of a mixed population of oxygenated and hypoxic cells as postulated by Hewitt and Wilson in 1959 [10] and demonstrated by many authors [11], [12], [13]. The dose response curve of squamous carcinoma cells to neocarzinostatin also showed a two component curve as mentioned previously. This may be considered as follows: (1) the squamous carcinoma cells surviving after receiving a neocarzinostatin treatment of over 4·0 mg/kg may be more resistant than the other cells. In other words, the squamous carcinoma cells used in the experiments may consist of two populations with different sensitivities to neocarzinostatin; (2) neocarzinostatin was more than enough to kill cells effectively at the dose level of over 4·0 mg/kg and a quantity of the drug given to mice might be wasted; that is, to kill a cell requires a given dose and, at this dose level,

cells die proportionally depending on the dose given, but, if the dose exceeds the level, some quantity of the drug may not be able to take part in killing cells; (3) another possibility is that drugs may not reach peripheral cells in the tumour due to decreasing blood flow; e.g. anoxic cells in tumour may have received a smaller amount of the drug as compared with oxygenated cells; (4) anoxic cells may be more resistant to neocarzinostatin treatment than oxic cells as a response in an irradiated tumour.

The LD50(30) of the drug was $2{\cdot}4 \pm 0{\cdot}9$ mg/kg. At this dose level, neocarzinostatin can kill 90% of the tumour cells. However, the repeated use of the drug will be dangerous because of its toxicity.

The bending of the survival curve of squamous carcinoma cells has also been observed in tumours treated with bleomycin (in preparation). In the survival curve of the tumour cells treated by bleomycin, the first component meets the second component at a surviving fraction of about 0·1. However, the dose of bleomycin required for killing 90% of the tumour cells was non-toxic.

In conclusion, neocarzinostatin does not seem to be a useful drug for the control of the squamous carcinomas as used in these experiments. The drug might be used for tumours more sensitive to neocarzinostatin.

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Some Thoughts on Experimental Models and their Clinical Correlations

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Abstract—*In a field as complex as cancer chemotherapy, where a very large number of antitumor agents may be candidates for clinical studies, clinicians must have the help of experimentalists in developing model tumor systems that will select the drugs having the greatest potential for clinical success. Consideration of experimental systems and their clinical correlations is particularly critical in view of the increasing importance of rationally designed combination drug regimens and the emphasis being placed on chemotherapy combined with other modalities in the treatment of solid tumors. This paper reviews the clinical experience with data obtained in the leukemia L1210 system, which has been the major screening tool in the drug development program of the National Cancer Institute, and its relationship to selecting new model systems for predicting drugs active against solid tumors. Within this context, some of the correlations between drug activity in the L1210 and B-16 melanoma systems and activity in human solid tumors are discussed.*

INTRODUCTION

THE DIVISION of Cancer Treatment (DCT) of the National Cancer Institute includes within the scope of its expanded operations, under the direction of the National Cancer Program, the drug development segment of the former chemotherapy program [1]. The new DCT functions involve the improvement of cancer treatment through the development of combined modality approaches to therapy. The greatest emphasis in combined modalities will be given to disease-oriented thrusts in the so-called solid tumors which are the major cause of cancer deaths in the United States and throughout the world. This new emphasis is being reflected in revised thinking about the methodology of drug development and clinical testing. It is the purpose of this paper to discuss the various kinds of experimental tumor testing in use or under consideration by the DCT from the point of view of clinical correlation and clinical interaction.

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CLINICAL RELEVANCE OF DRUG DEVELOPMENT PROGRAMS

A cancer chemotherapy drug development effort depends on equally important contributions by chemists, experimental chemotherapists and pharmacologists, and research clinicians, who must work in close collaboration to provide large numbers of potentially useful materials, evaluate them in meaningful preclinical systems, select the most promising agents, and evaluate these against the target disease(s) in man.

The two major approaches in selecting new drugs for preclinical evaluation, with an ultimate view to clinical trial, are (1) synthesis of congeners of known "active" chemicals using structure-activity relationship data and fundamental biochemical knowledge as guides and (2) broad screening of heterogeneous classes of chemicals, uncharacterized antibiotics, and plant products to obtain completely new classes of active drugs having different mechanisms of action than the known antitumor agents.

The purpose of a screening system is to eliminate the bulk of the negative materials and concentrate for further study those materials that have the greatest promise. It cannot be

designed to detect every compound that will have clinical activity without almost surely including an unbearable number of false positive compounds; conversely, it cannot be designed to eliminate every inactive compound without losing some that are active.

For many years, leukemia L1210 [2] has been the major screening tool of the NCI Drug Development Program. The leukemia P-388 system is also used for natural products since it has a greater sensitivity for crude fractions. Recently, we have added secondary testing of all L1210 and P-388-active materials in antitumor systems which hopefully will have a greater predictive capacity for drugs active against solid tumors. Initially, these systems will be the B-16 melanoma and Lewis lung carcinoma. The problems of evaluating a new model system for inclusion in the screening process will be discussed in a later part of this paper.

Once it is demonstrated that model systems for screening and quantitative evaluation can be created, usually taking into account various aspects of the host–tumor–drug interrelationship, a question of their clinical relevance arises. Do the selected model test systems identify compounds with a high likelihood of clinical activity? Obviously, if the predictive value of the system is nil, the screening evaluation effort becomes irrelevant to the clinic. In developing a mass screening program there is also the practical problem of designing a system which, besides being predictive, provides reproducible results and is economically feasible. We feel that leukemia L1210 meets these specifications better than any other system with which we have had experience.

In leukemia L1210 there is an approximately two day lag phase associated with transplantation. This is followed by a period of lag phase proliferation (generation time, 0·55 days) continuing until host death, at which time the host's leukemic cell population is of the order of one billion cells. Screening in L1210 begins on day 2 and is carried out under conditions far different from the usual clinical situation in acute leukemia; i.e. *in vivo* treatment of animals bearing relatively small numbers of leukemic cells. Still, the worth of a screen must be determined in the end by the clinical results obtained.

Any type of mass screening involves compromise and chemotherapy screening is no exception. It must be accepted that, of the materials that get through any screen, a certain percentage will be inactive clinically and, unfortunately, some active materials will be missed. The best screen is obviously one that misses the fewest active materials and selects the fewest inactive ones.

Any screen offers four possible results, as outlined in Fig. 1. In order to fully evaluate a screen, the data on all four possibilities should be evaluated. However, this is rarely possible, because it is not feasible to clinically test all negative materials to determine the numbers of true and false negatives. Therefore, the chemotherapy screen can only be judged by the positive drugs and the outcome in their clinical use.

	Screen	Clinical trial
True positive	+	+
False positive	+	−
True negative	−	−
False negative	−	+

Fig. 1. *Possible results with any screening system.*

The determination of clinical activity and its correlation with findings in the experimental screen depends significantly on an accepted definition of an adequate trial. Drugs selected by a highly quantitative system such as leukemia L1210 may only tentatively be classified as false positives if, because of poor absorption, systemic inactivation, or limiting toxicity in the modality of therapy employed, etc., they have not received an adequate trial. As a minimum, an adequate trial requires that a drug be given in the range of the maximum tolerated dose and on an appropriate schedule of administration.

The DCT conducts the clinical evaluation of a new agent in three phases which are specific modifications of those required by the Food and Drug Administration (Fig. 2) [3]. Phase II trials consist of a survey for clinical activity against a panel of ten signal tumor types. This survey is designed to thoroughly test for drug activity against tumors that are kinetically described as

Phase I: Clinical pharmacology
- Establish maximum tolerated dose at schedule(s) tested.
- Establish toxicity parameters and determine if toxicity is predictable, treatable and/or reversible.
- Pharmacologic evaluation.
- Antitumor activity not required.

Phase II: Screening for clinical activity
- Treat 20–30 evaluable patients with measurable disease in each of a range of "Signal" tumor types.
- Evaluate on the basis of objective response rate and characteristics of pharmacology, mechanism of action, and cell cycle sensitivity.

Phase III: Trial for recommendations of general use
- Controlled clinical trials.
- Combination studies.

Fig. 2. *Clinical trials of new drugs developed by the National Cancer Institute.*

"fast growing" (leukemia, lymphoma) and "slow growing" (colon, breast, lung, etc.). For each signal tumor type the objective response rate is examined in at least 20–30 evaluable patients in controlled cross-over designs [4]. Implicit in the use of the ten signal tumor types is a minimum definition of what constitutes an adequate evaluation of a new antitumor agent. As a minimum, it would be unfair to label a drug "inactive" until it has been adequately evaluated in at least 20–30 patients from each of 8 or 9 signal tumor types, and found to have insufficient clinical activity in all of them.

In line with this reasoning, the Cancer Therapy Evaluation Program (CTEP) has intensively reviewed all anticancer agents to see how many have been adequately evaluated by these standards and to provide a historical background for the data rapidly developing on the new investigational agents [5]. This review showed that most of these drugs have not been fully tested against all of the tumors. The data also revealed how tenuous a practice it is to list false positives for any particular preclinical screen for antitumor agents.

DEVELOPMENT OF EXPERIMENTAL SYSTEMS COMPATIBLE WITH CLINICAL NEEDS

The Division of Cancer Treatment currently is evolving a dual approach to combined therapy in solid tumors (Fig. 3). In disseminated (advanced) disease, the activity of single agents is determined and, from these, optimum combination chemotherapy regimens are designed.

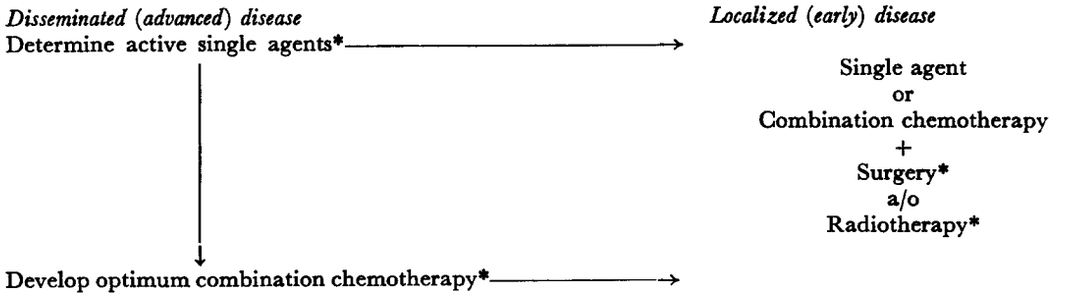

Fig. 3. Dual approach to combined therapy in solid tumors. Points at which model experimental systems could be used are shown by (*).

The active single agents or combinations are then joined with surgery and/or radiotherapy in a combined modality approach to initial treatment of local (early) disease. This dual approach includes four points where a model experimental system could be useful, three of them involving combined therapy.

The principal consideration in the development of an experimental model is how it is to be put to clinical use (Fig. 4). Among the several possibilities, probably the most feasible

To predict a high degree of activity by unknown compounds or combinations (screening).

To predict the success of refinements of therapy with existing compounds or combinations.

A. Elucidate principles behind successful clinical therapy.

B. Predict successful combinations among the myriad possibilities drawn from active agents and modalities.

Fig. 4. Clinical uses of an experimental model.

and worthwhile objective within present resources would be a system that could predict the success of combinations of existing agents and modalities. Of course, the validity of the system must be well established before undertaking large-scale testing. Also, testing of every combination would be a practical impossibility and selectivity will be required.

With these points in mind, the first critical question to be answered is whether the experimental systems commonly employed by the Division of Cancer Treatment will be useful in predicting active drug combinations. Certainly, a massive source of data already exists from studies in the leukemia L1210 system, the experimental tumor that has been so valuable in detecting active new single agents. Assuming that the L1210 system will be a valid model, the problem of identifying the drug combinations to be tested can be attacked by either a disease-oriented or a modality-oriented process of selection (Fig. 5).

The disease-oriented process approach may be taken by choosing the 10 (approx.) most active single agents employed against each of

Disease-oriented survey

1. Choose critical tumors
2. Isolate active agents
3. Prospectively test combinations of these agents

Modality-oriented survey

1. Choose critical drugs or modalities
2. Test these systematically in combination with all other available agents

Fig. 5. Possible approaches to selection of drug combinations for experimental studies.

the solid tumor types and hematologic malignancies. For example, Table 1 lists 9 agents for adenocarcinoma of the breast, the best dose schedule for each, and the 2-drug combinations (using any two of the 9 drugs) which have been clinically tested in the disease [6]. By selecting in this manner the most effective agents for each disease, 29 different drugs exhibit established activity when used alone against one or more of the 10 tumors shown in Table 2. One of the most striking points in these data is how few of the potentially effective 2-drug combinations, which could be devised with 9 or 10 most active drugs, have actually been tested in each tumor. Rarely does the number tested exceed 20% of the possibilities and it is much less in most of the diseases. Even if combinations that include drugs with similar mechanisms of action are excluded, which may or may not be a valid step, the gaps are still awesome. These gaps will never be filled with the available clinical resources and emphasize the need and the potential usefulness of testing combinations in a model that has some predictive value.

When the 2-drug combinations that have been clinically tested in each disease are reviewed, there are 38 combinations for solid tumors (Fig. 6) and 25 for hematologic malignancies

Table 1. Single agents active in breast cancer and clinically tested 2-drug combinations involving these agents

Drug	Schedule	Clinical 2-drug combinations
Adriamycin	Single dose of q 3 weeks	Adriamycin + vincristine
Cyclophosphamide	Various, all apparently equivalent	Cyclophosphamide + 5-FU; cyclophosphamide + prednisone
5-FU	Daily × 5, or weekly	5-FU + vincristine; 5-FU + vinblastine 5-FU + prednisone; 5-FU + melphalan
Methotrexate	Daily, daily × 5, or q 6h × 24	Methotrexate + thioTEPA
Vincristine	Weekly	See above combinations
ThioTEPA	Daily × 2–4	
Vinblastine	Weekly	
Prednisone	Daily	
Melphalan	Daily or daily × 4 q 3–4 weeks	

Cyclophosphamide + methyl-CCNU*
Cyclophosphamide + streptozotocin
Cyclophosphamide + 5-FU
Cyclophosphamide + methotrexate*
Cyclophosphamide + vincristine
Cyclophosphamide + hexamethylmelamine
Cyclophosphamide + prednisone*
Cyclophosphamide + DTIC
Cyclophosphamide + CCNU
BCNU + mitomycin C
BCNU + vincristine*
BCNU + DTIC
CCNU + hydroxyurea
CCNU + mechlorethamine
Vinblastine + bleomycin*
Vinblastine + melphalan
Methotrexate + mechlorethamine
Methotrexate + hexamethylmelamine
Methotrexate + thioTEPA

5-FU + streptozotocin
5-FU + BCNU*
5-FU + CCNU
5-FU + methyl-CCNU
5-FU + mitomycin C
5-FU + prednisone
5-FU + melphalan
5-FU + vinblastine
5-FU + vincristine
Vincristine + dactinomycin
Vincristine + DTIC*
Vincristine + adriamycin
Vincristine + CCNU
Vincristine + bleomycin*
DTIC + dactinomycin
DTIC + procarbazine
DTIC + methyl-CCNU
Dactinomycin + chlorambucil
Dactinomycin + mechlorethamine

Fig. 6. Two-drug combinations clinically evaluated in solid tumors. Evidence of synergism (*) indicated by some combinations.

Table 2. Single drugs most active in the major tumor types and disease-oriented comparison of potential and clinically- tested 2-drug combinations

Tumor types	Most active single drugs	Disease-oriented view of 2-drug combinations using the most active drugs			
		No. of active drugs	Potential combinations	Combinations actually tested	
				No.	%
Solid tumors					
Lung	Cyclophosphamide, mechlorethamine, methotrexate, adriamycin, CCNU, methyl-CCNU, BCNU, procarbazine, hexamethylmelamine, hydroxyurea	10	45	8	18
Breast	Adriamycin, cyclophosphamide, 5-FU, methotrexate, vincristine, thioTEPA, vinblastine, prednisone, melphalan	9	36	8	22
Gastrointestinal	5-FU, 5-FUDR, methyl-CCNU, CCNU, BCNU, cyclophosphamide, mitomycin C, methotrexate, adriamycin, streptozotocin	10	45	8	18
Ovary	Melphalan, cyclophosphamide, chlorambucil, 5-FU, methotrexate, dactinomycin, thioTEPA, hexamethylmelamine, adriamycin	9	36	2	6
Melanoma	DTIC, BCNU, methyl-CCNU, cyclophosphamide, dactinomycin, hydroxyurea, melphalan, vincristine, CCNU, procarbazine	10	45	10	22
Testicular	Dactinomycin, mithramycin, chlorambucil, vinblastine, melphalan, bleomycin, methotrexate, vincristine, mechlorethamine, adriamycin	10	45	7	15
Hematologic malignancies					
ALL	Vincristine, prednisone, 6-MP, methotrexate, daunorubicin, L-asparaginase, cyclophosphamide, cytosine arabinoside, adriamycin, BCNU	10	45	8	18
AML	Cytosine arabinoside, daunorubicin, adriamycin, 6-thioguanine, 6-MP, vincristine, prednisone, cyclophosphamide, BCNU, CCNU	10	45	7	15
Multiple myeloma	Melphalan, cyclophosphamide, prednisone, BCNU, CCNU, vincristine, adriamycin, procarbazine, bleomycin	9	36	6	17
Malignant lymphoma	Cyclophosphamide, mechlorethamine, vinblastine, vincristine, procarbazine, CCNU, BCNU, bleomycin, adriamycin, prednisone	10	45	9	20

(Fig. 7). For convenience, these 63 combinations are indicated on the quick-reference chart of Table 3. Unfortunately, few of these combinations have been directly compared with their single components in a prospective controlled fashion.

Currently, an analysis is in progress in the Cancer Therapy Evaluation Program on the clinical data available for 2-drug combinations. If possible, all of the experimental tumor data will be compiled for the clinically tested combinations so that comparisons and correlations between the two can be made. When this analysis is not possible because of schedule differences or gaps in the data, prospective experimental testing will be initiated to determine whether correlations exist. The results, when completed, will be the subject of a future publication.

In considering the evaluation of a new model system, one finds that the only compounds available for testing in it are those whose activity was predicted by older systems. The fact that all of these active compounds were not predicted by one system provides a heterogeneity that can be used to advantage in evaluating the new antitumor model. For example, Fig. 8 illustrates this approach in testing the new system against a number of drugs found active in L1210 and an equal number inactive in L1210 but active in other predictive models. The new system would be highly desirable if it showed activity by all 10 of the L1210-inactive materials and lack of activity for the 10 L1210-active compounds. On the other hand, the exact opposite result would be most unattractive. Most systems would fall somewhere between the two extremes but such an evaluation would be helpful in deciding whether to do further testing in a new experimental system.

Another approach could be taken by separating the drugs on the basis of their activity in hematologic malignancies or solid tumors. Figure 9 lists the ten most active drugs for leukemia and solid tumors, in the opinion of the CTEP. It is of great interest that only two drugs (methotrexate and cyclophosphamide) appear in both lists. If one is interested in uncovering a new experimental system for predicting drugs active in solid tumors, this approach might be helpful.

Within the DCT, the approaches in Figs. 8 and 9 have been merged to evaluate the potential of the B-16 melanoma system for inclusion

Prednisone + cyclophosphamide*
Prednisone + melphalan*
Prednisone + BCNU*
Prednisone + methotrexate*
Prednisone + 6-MP*
Prednisone + cytosine arabinoside
Prednisone + vincristine*
Prednisone + daunorubicin*
Prednisone + adriamycin
Prednisone + L-asparaginase
Vinblastine + vincristine
Vinblastine + BCNU
Cytosine arabinoside + cyclophosphamide*
Cytosine arabinoside + BCNU
Cytosine arabinoside + CCNU
Cytosine arabinoside + 6-MP
Cytosine arabinoside + 6-Thioguanine*
Cytosine arabinoside + Daunorubicin*
6-MP + methotrexate
6-MP + daunorubicin
BCNU + cyclophosphamide
BCNU + procarbazine
BCNU + melphalan
BCNU + bleomycin
Adriamycin + bleomycin

Fig. 7. Two-drug combinations clinically evaluated in hematologic malignancies. Evidence of synergism (*) indicated by some combinations.

L1210 Active	*L1210 Inactive*
Cyclophosphamide	Vincristine
Adriamycin	Bleomycin
Methotrexate	Hexamethylmelamine
5-Fluorouracil	Dactinomycin
Nitrosoureas	Vinblastine
Phenylalanine mustard	Streptozotocin
Cytosine arabinoside	Prednisone
6-Mercaptopurine	Dibromomannitol
Imidazole carboxamide	Mithramycin
Hydroxyurea	Myeleran

New system → 10 L1210+ / 10 L1210−

Fig. 8. Drug activity in L1210 as an approach to evaluating a new screening system.

Top ten leukemia drugs	*Top ten solid tumor drugs*
Vincristine	Cyclophosphamide
Prednisone	Adriamycin
Methotrexate	Methotrexate
6-Mercaptopurine	5-Fluorouracil
Cytosine arabinoside	Nitrosoureas
Daunorubicin	Phenylalanine mustard
L-Asparaginase	Hexamethylmelamine
6-Thioguanine	Mitomycin C
Cyclophosphamide	Bleomycin
Hydroxyurea	Dactinomycin

New system → 10 Leukemia drugs / 10 Solid tumor drugs

Fig. 9. Drug activity in leukemia and solid tumors as an approach to evaluating a new screening system.

Table 3. A quick reference chart of 2-drug combinations clinically evaluated in solid tumors and hematologic malignancies

Combinations evaluated in solid tumors ↓ / Combinations evaluated in hematologic malignancies →	Mechlorethamine	Cyclophosphamide	Melphalan	Chlorambucil	ThioTEPA	5-FU	Methotrexate	6-MP	6-Thioguanine	Cytosine arabinoside	Vincristine	Vinblastine	Mitomycin C	Dactinomycin	Adriamycin	Daunorubicin	Bleomycin	BCNU	CCNU	Methyl-CCNU	Streptozotocin	Hexamethylmelamine	Procarbazine	DTIC	Hydroxyurea	L-Asparaginase	Prednisone
Mechlorethamine																		H									Ⓗ
Cyclophosphamide										Ⓗ								H									Ⓗ
Melphalan		S	S																								
Chlorambucil																											
ThioTEPA	S	Ⓢ			S			H																			
5-FU										H						H											Ⓗ
Methotrexate										Ⓗ																	Ⓗ
6-MP		S				S						H						H	H								H
6-Thioguanine			S			S												H									Ⓗ
Cytosine arabinoside				S		S					S																
Vincristine											S						H										
Vinblastine											Ⓢ	Ⓢ	S					H									Ⓗ
Mitomycin C														S													H
Dactinomycin																H											Ⓗ
Adriamycin																		H									
Daunorubicin																											Ⓗ
Bleomycin																											
BCNU																											
CCNU																											
Methyl-CCNU																											
Streptozotocin																											
Hexamethylmelamine																											
Procarbazine																											
DTIC																											
Hydroxyurea																											
L-asparaginase																											
Prednisone																											

S = Combination evaluated in one or more solid tumors (Ⓢ = combination showing evidence of possible synergism)
H = Combination evaluated in one or more hematologic malignancies (Ⓗ = combination showing evidence of possible synergism)

Table 4. Evaluation of B-16 melanoma system by correlation of drug activity in L1210, B-16, and human solid tumors

Drug activity*		
L1210	B−16	Human solid tumors
L+ or L−	B+ or B−	S++ S+ S± S− SO

*L+, B+; ≧ 50% ILS in system concerned
L−, B−; < 50% ILS in system concerned
S++; Definite activity in at least 2 major solid tumors in man
S+; Clear activity in solid tumors but less than S++
S±; Some activity, but not clearly established. Includes drugs active in 1 solid tumor but clearly inactive in a wide range of tumors (e.g. DTIC, mithramycin, some hormones)
S−; Clearly inactive in at least 2 solid tumors
SO; Not adequately tested

in the drug development apparatus. In the analysis, the activity of each drug under consideration is defined in both experimental systems (L1210 and B-16) and in human solid tumors according to the scheme shown in Table 4. The ranking of the drugs in each category of activity in solid tumors is shown in Table 5.

An example of the results obtained in this type of analysis is given in Fig. 10. It can be seen that both experimental systems would have predicted 8 of the drugs that are most active in solid tumors, while L1210 alone predicted 6 and B-16 predicted 4. Both systems fail to predict the activity of 3 drugs (chlorambucil, hexamethylmelamine, and stilbestrol). A full presentation of this analysis is beyond the scope of this paper but it is being expanded to include P-388 leukemia and the Lewis lung carcinoma and will be the subject of a future publication.

DISCUSSION

The future of cancer therapy lies in the greater application of combined therapy. Combined therapy can mean either "combination chemotherapy" employing two or more drugs or it may involve "combined modality therapy" in which single-drug or combination chemotherapy is used as an adjuvant to surgery and/or radiotherapy. Viewing combined therapy strictly in the chemotherapeutic sense, it is quite evident that except for the cures with single drugs in choriocarcinoma and Burkitt's lymphoma, the major successes have been achieved by combination chemotherapy [7].

The overall progress of chemotherapy has been against the hematologic malignancies, especially acute lymphocytic leukemia and advanced Hodgkin's disease, but it has not been translated to the common solid tumors that are the chief cause of cancer mortality in the United States. The obvious reasons given for this failure are the differing cell kinetics in fast- and slow-growing tumors and the relative accessibility of tumor cells to effective drug concentrations. An additional factor, which is often neglected, is the point in the treatment strategy for a given disease at which chemotherapy is introduced. In this respect, it is significant that the local and regional modalities of surgery and radiotherapy are commonly used in primary treatment of solid tumors and chemotherapy is relegated to secondary use after their failure. When this fact is coupled with the knowledge that the therapeutic gains attainable by surgery and radiotherapy seem to

Table 5. Ranking of drugs by activity in solid tumors

Activity in solid tumors*				
S++	S+	S±	S−	SO
Cyclophosphamide	Vinblastine	DTIC	6-MP	6-Thioguanine
Adriamycin	Vincristine	Mithramycin	Cytosine arabinoside	Daunorubicin
Methotrexate	Chlorambucil	Streptonigrin	Camptothecin	Dibrommomannitol
5-FU	Bleomycin	Porfiromycin	TIC-Mustard	5-Azacytidine
CCNU	Dactinomycin	Hydroxyurea	L-asparaginase	Platinum salt
BCNU	Mitomycin C	Procarbazine	TMCA	ICRF-159
Methyl-CCNU	Dibromodulcitol	Myleran	L-acetyl-2-picolinoylhydrazine	Yoshi-864
Melphalan	Isophosphamide	TEM	Tubercidin	Chromomycin A_3
Mechlorethamine	Stilbestrol	Provera	Cycloleucine	Streptozotocin
Hexamethylmelamine		Testosterone	F_3TDR	
5-FUDR		Azotomycin	NSC 17256E	
ThioTEPA		Prednisone	6-Azauridine	

*See footnote to Table 4.

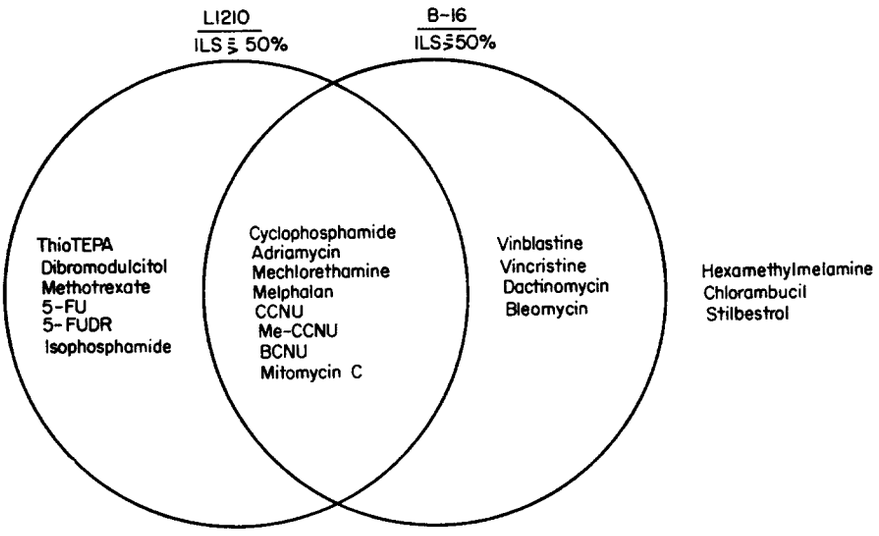

Fig. 10. *Relationship of the L*1210 *and B*-16 *systems in predicting drugs most active* (*S*++ *and S*+) *in human solid tumors.*

have plateaued and that secondary treatment is rarely curative in any tumor, it appears that a "combined modality therapy" would be advantageous in the primary treatment of solid tumors.

The National Cancer Institute's Division of Cancer Treatment has developed from the former Chemotherapy Program and naturally views combined modality therapy from the standpoint of the chemotherapy involved. In spite of the important clinical advances already made with combination chemotherapy, there is a pressing need to evaluate experimental systems that can provide predictive data of use in the rational design of combination regimens. Rather than attempting after-the-fact validation and understanding of successes in empirical studies, the correct role of the experimental therapist should be to obtain data in anticipation of clinical needs. The clinician and the experimentalist must work closely together if future successes are to come as rapidly as possible.

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Lymphocyte Toxicity Test in Clinical Melanoma

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Abstract—*Between July* 1970 *and April* 1972, *peripheral lymphocytes of* 35 *patients with malignant melanoma were tested for* in vitro *cytotoxicity to autologous or homologous melanoma cells. Twenty-one cases* (60%) *had lymphocytes with a specific cytotoxic activity. No correlation was found with sex, age or clinical stage of the disease. To evaluate the prognostic significance of the* in vitro *cytotoxic activity of peripheral lymphocytes,* 18 *patients at stage I and II, who had been subjected to radical surgery prior to the cytotoxic test, were divided into two groups according to the result of the test* (12 *positives and* 6 *negatives) and were followed up for a maximum period of* 32 *months. Twelve cases had recurrence of the disease:* 7 *of the* 12 *cases with positive lymphocytes, and five of the* 6 *patients with negative lymphocytes. The difference between the two groups was not significant. The tumour-free interval has been* 8·4 *months in the seven cases with cytotoxic lymphocytes and* 8 *months in the* 5 *cases with no reactive ones.*

INTRODUCTION

THE MAJORITY of the immunological *in vitro* studies have given convincing evidence that human malignant neoplasms possess tumor-associated antigens able to elicit an autocthonous immune response, and these antigens seem to be cross-reacting for tumors of the same histological type [1]. There is no proof, however, that the *in vitro* detected antigenicity can operate in the *in vivo* system, creating an immune response capable of rejecting tumor in a manner similar to that found for tumor-associated transplantation antigens in laboratory animals [2, 3]. In this respect, it is important to study the correlation between the results obtained with *in vitro* immunological tests and clinical course of the disease.

We have therefore considered a series of 35 patients suffering from malignant melanoma who have been treated at this Institute and whose peripheral lymphocytes have been tested for cytotoxic activity on melanoma cells. The experimental details of the tests of 14 cases were reported in previous paper [4] and 21 cases (unpublished) have since been added.

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MATERIAL AND METHODS

Between July 1970 and April 1972, peripheral lymphocytes of 20 stage I and II patients with malignant melanoma were tested for cytotoxicity on autologous or homologous melanoma cells within a month of surgery. In the same period, peripheral lymphocytes of 15 stage III patients were tested within a month of biopsy of superficial metastatic nodules. The method used to test the cytotoxic effect of lymphocytes was essentially similar to that proposed by Takasugi and Klein [5] and has been already described [4]. The assay was done in Falcon microplates No 3034. Control and experimental lymphocytes were purified with plasmagel and by passage through a nylon column. The target/effector–cell ratio was of 1/100 or 1/300. The effect of patients lymphocytes was compared with that of lymphocytes from healthy donors

and the difference was considered significant when P was 0·05 or less.

Nineteen patients were males and 16 were females; their age ranged from 21 to 76 years with a peak in the 4th decade, 19 being under 50 years and 16 over. With regard to the clinical extent of the disease, the patients were divided into 3 groups: (1) 6 cases with localized tumor and no evidence of metastases (stage I); (2) 14 cases with involvement of regional lymphnodes (stage II); (3) 15 cases with disseminated disease (stage III). Eighteen patients of stage I and II subjected to radical surgery had a monthly follow up for up to 32 months.

RESULTS

Twenty-one (60%) of the 35 patients examined had lymphocytes with a specific cytotoxic activity against autologous or homologous melanoma target cells. No correlation was found with sex, since 11 of the 19 males and ten of the 16 females had cytotoxic lymphocytes. Age did not effect the results, since the average age of the 21 patients with cytotoxic lymphocytes (51 ± 12 years) was not statistically different from that of the 14 patients whose lymphocytes had not such activity (47 ± 16 years). As shown in Table 1, no correlation was observed between the clinical stage and the lymphocyte activity.

Table 1. *Correlation between extent of the disease and cell mediated cytotoxicity* in vitro *tested within a month after surgery*

Malignant melanoma	No. of cases	No. of cases with active lymphocytes
Localized tumor (Stage I)	6	4
Regional metastases (Stage II)	14	8
Distant metastases (Stage III)	15	9
Total	35	21

To evaluate the prognostic significance of the *in vitro* cytotoxic activity of lymphocytes, 18 stage I and stage II patients submitted to radical surgery had a monthly follow up until recurrence. As reported in Table 2, 12 out of the 18 patients had a recurrence of the disease in a period of time from 1 to 16 months after surgery. In total, recurrence was observed in 7 out of 12 cases with positive lymphocytes

Table 2. *Relevant data of* 18 *patients submitted to radical surgery*

Sex	Age	Clinical stage	Lymphocyte cytotoxicity test	Tumor free interval (months)	Type of recurrence
F	72	I	Positive	1	Regional nodes metastases
M	37	I	Positive	7	Regional nodes metastases
M	50	I	Positive	8	Regional nodes metastases
M	60	I	Positive	16	Brain and lung metastases
M	44	I	Negative	12	Symptom free
M	59	I	Negative	23	Symptom free
F	65	II	Positive	5	Lung metastases
M	47	II	Positive	7	Bones and subcutaneous metastases
F	61	II	Positive	15	Subcutaneous metastases
F	42	II	Positive	12	Symptom free
M	40	II	Positive	13	Symptom free
M	42	II	Positive	32	Symptom free
M	31	II	Positive	32	Symptom free
M	37	II	Negative	5	Brain metastases
F	39	II	Negative	6	Brain metastases
M	57	II	Negative	9	Skin metastases
F	71	II	Negative	10	Ovarian metastases
F	71	II	Negative	10	Skin metastases

and in 5 out of 6 with negative ones. The incidence of recurrence in the two groups was not significantly different ($\chi^2 = 0{\cdot}281$, $P > 0{\cdot}5$). The average tumor-free interval was 8·4 and 8·0 months in the lymphocyte positive and negative cases, respectively.

It is interesting to note that if the group of patients is subdivided by stage, recurrence of the disease was observed in none of the 2 stage I patients and in all of the 5 stage II patients with negative lymphocytes, whereas among the lymphocyte positive patients all 4 of stage I and 3 out of 7 of stage II had recurrence.

DISCUSSION

Among human tumors malignant melanoma has been one of the most studied from an immunological point of view because some clinical features suggested an immune response [6, 7]. Investigations from different laboratories have demonstrated humoral and/or cellular immunity by several *in vitro* methods [1, 4, 8–12].

In our series neither sex or age influenced the immune response, whereas De Vries *et al.* [12] reported that patients with cytotoxic lymphocytes were older than those with non-reactive ones. As in other reports [12–14] we found no correlation between cell mediated reactivity and extent of the disease. On the contrary, cytotoxic antibodies have been found to be more frequently present in the serum of patients with localized disease than in that of those with widespread one [9, 15, 16].

Also, lymphocyte cytotoxicity test *in vitro* carried on within a month of surgery does not seem to be of prognostic value, since in our series the incidence of recurrences in the group of patients with cytotoxic lymphocytes and in that with unreactive lymphocytes was similar. In fact, although the results were slightly in favour of the cases with reactive lymphocytes, the statistical analysis showed that the difference was not significant. Moreover the tumor-free interval in the two groups was the same. Unfortunately, the groups were too small to attach a meaning to the observation that recurrence was less frequent among the stage II patients with reactive lymphocytes.

As demonstrated by several studies [12, 13, 17–21], blocking factors must play an important role in tumor immunity, and it has been shown that a blocking serum activity was associated with a progressive disease and with tumor recurrency within a relatively short period of time [20].

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Intra-Arterial Methotrexate in Head and Neck Tumors

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Abstract—*The authors report the results of a sequential trial demonstrating the superiority, in terms of tumor regression, of a treatment with high dose methotrexate (50 mg per day, in 10-hr infusion) over a treatment with low dose methotrexate in advanced cancers of the oral cavity and oropharynx.*

INTRODUCTION

IN CANCERS of the head and neck anti-neoplastic agents are at present most often administered by intra-arterial infusion. One of our main concerns is the improvement of the results already achieved by this technique [1].

On the basis of the studies of the dosage of methotrexate in the infused area and its elimination in urine [2, 3], which showed that large doses result in a high but transient rate in blood while low doses, on the contrary, give a lower but much more stable rate, two points remained under discussion: *the dose and the duration of the administration.* [4, 5, 6]. These led us to a consideration of the following two comparisons:

low dose without leucovorin vs high dose with leucovorin

continuous vs intermittent administration.

It was felt that the choice should depend on the patient's comfort and on the frequency of technical incidents such as obstruction or displacement of the catheter, most likely to occur at night when the patient is asleep.

Besides, intermittent intra-arterial infusion did not seem *a priori* to be inferior. Fairly satisfactory results have been obtained with this technique in more than 100 patients, on the one hand [7]; on the other hand, the synthesis phase of the cells of squamous-cell carcinomas, with which we are concerned here, lasts 8–16 hr for a cycle varying from 24 hr to 4 days on the average [8]. It is hardly probable, therefore, that a cell in activity may escape the action of methotrexate administered in this way. We obtained confirmation of this using marks made with intermittent intra-arterial tritiated thymidine.

For this reason it was decided as a first step—the only one we report here—to compare the following two treatment schedules:

5 mg/24 hr without leucovorin

50 mg/24 hr with leucovorin.

The same time schedule was used in both groups: intermittent 10–12 hr infusion, as it was decided to assess only one factor at a time.

A first sequential trial was therefore undertaken [9], in which the criterion of judgment was the tumor regression as measured *after* the chemotherapy. A pragmatic formulation was chosen [10]: it was merely decided to select the treatment schedule providing the best overall results (taking into account both the advantages and the drawbacks). Survival was the best criterion of assessment. However, the use of a sequential analysis required a more rapid judgment, and led to choice of the "tumor regression" as assessment criterion. (In a previous study [7] we found that patients with marked tumor regression had better survival. We assumed that this relation remained true for the patients of the present trial, as it had been demonstrated in patients with the same

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site and stage of cancer under the same type of treatment.)

MATERIAL AND METHODS

The trial was conducted in the Head and Neck Department of the Gustave-Roussy Institute from July 2, 1971 to March 22, 1972.

The patients entered into the trial conformed to our current criteria for the administration of intra-arterial chemotherapy. These were patients with an extensive (T4), histologically proven epidermoid cancer of the oral cavity or of the oropharynx.

The following patients were excluded:

- patients with badly irrigated tumors that were proof against stainers.
- patients having previously received treatment.
- patients with other tumor(s), with the exception of limited cutaneous epitheliomas.
- patients who were over 70 years of age.
- patients likely to cease treatment prematurely, because of their general condition or their psycho-social condition.

The treatments were as follows:

1. High dose chemotherapy (Treatment A)
   10–12-hr intra-arterial infusion of 50 mg of methotrexate, daily, for 8–10 days.
   The total dose varied from 400 to 500 mg depending on cutaneous toxicity.
   This infusion was combined with an intramuscular injection of 5 mg of folinic acid every 8 hr.
2. Low dose chemotherapy (Treatment B)
   10–12-hr intra-arterial infusion of 5 mg of methotrexate, daily, for 8–10 days.
   The average total dose was of 50 mg.
   No intra-muscular antidote.
3. *In both groups* chemotherapy was followed by a transcutaneous irradiation or a curietherapy, or by surgery, depending on the residual tumor and on the site.

The treatment was randomly allocated after the catheter had been placed and after checking the quality of local irrigation by a stainer-test. The route used was the superficial temporal artery. The infusion was administered by means of a syringe pushed by a non-portable electric pump. The infusion was interrupted at night; the catheter was shut while under pressure by means of a plastic tap.

The criterion of assessment was the tumor regression or progress expressed as a percentage (no regression = 0%—complete regression = 100%). Tumor volume was evaluated in a general and approximate manner. However, this evaluation was blind so that the same approximate method was used in both groups, avoiding any possible bias in the comparison. The criterion took into account both the effectiveness of the chemotherapy and the toxicity associated with interruption of the treatment at too low a dose.

A first assessment of the tumor volume was made before the treatment was begun. A second assessment was made 8 days after the end of the chemotherapy. These assessments were made by a physician ignorant of the treatment applied, and so of the patient's group.

A sequential *statistical analysis* was performed [6, 7].

In a sequential trial results are analysed as they become available. A graph of the "boundaries" is plotted from the basic data: mean value of the tumor regression achieved in our previous trial, the difference between treatment results worth consideration, risks of error that are allowed for. The boundaries form limits beyond which any difference between the compared treatments is considered to be statistically significant.

The results obtained in the course of the trial are plotted on the graph as they become available: so plotted they constitute a sample path. When the sample path crosses a boundary the trial is terminated and the resulting conclusion depends on which boundary has been reached (Fig. 1).

The plotting of the sample path is as follows: suppose A and B are the two treatments to compare. The patients are paired: one patient receives treatment A and the other treatment B. An assessment is made when the results of the two patients are available. Let us call these results x_A and x_B. The comparison is made for each pair by the difference: $x_B - x_A$. The sample path is traced by plotting, for each pair, the cumulative sum of the differences so far obtained.

When forming the pairs every effort should be made to select two patients as similar as possible in their disease, in particular concerning factors influencing prognosis. In this trial pairing took place within the following strata: site (gloss-tonsillar, pelvi-lingual, pelvi-gingival, and tongue), *T* status, *N* status, age (in 10-year groups), and sex.

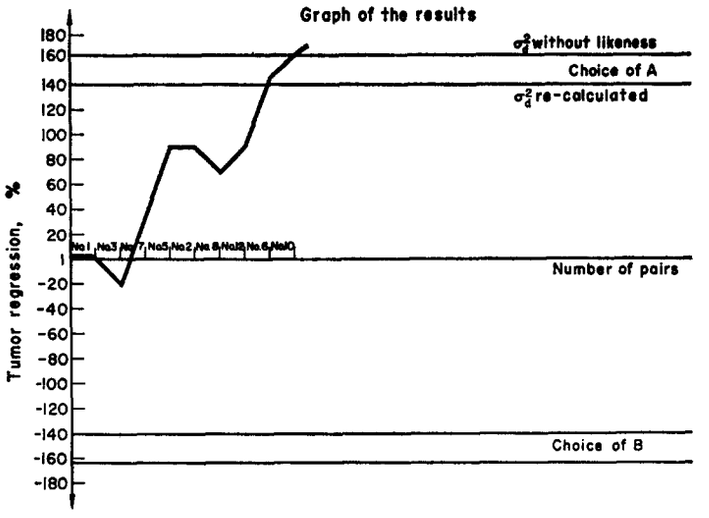

Fig. 1. *The upper line was obtained from previous data, assuming that the two patients in one pair are not alike. The lower line was calculated* a posteriori *from the data of this trial.*

The values used in an initial calculation of the boundaries were taken from our previous trial [1]. Then they were recalculated using as variance that obtained from the first few pairs providing comparisons in the trial. This made it possible to take into account the likeness between the two patients forming a pair.

The pragmatic design chosen was closed, quantitative, two-sided and symmetrical about 0.

Under these conditions the boundary equations obtained were as follows:

$$c = \frac{\sigma_d^2}{2\Delta} \log e \frac{1-\gamma}{\gamma}$$

where σ_d^2 is the variance of all the differences $(x_B - x_A)$ observed. The couple γ, Δ represents the maximum probability γ to adopt a treatment inferior to the other by Δ.

The calculation of σ_d^2 from previous data gave the value 1100. When calculated from the pairs in the trial the result was $\sigma_d^2 = 962$. Hence the calculation of the equations:

$$c = 142, \text{ when } \gamma = 5\% \text{ for } \Delta = 10\%.$$

The choice of the maximum number of pairs to include $=40$ (closed test) does not modify this estimation.

RESULTS

1. *Description of the patients in the trial*

The trial lasted 9 months. The results were obtained from nine pairs of patients. Five additional patients treated could not be paired, their results were, therefore, not used.

Table 1 gives the various characteristics of the 18 patients, whose treatment results led to a conclusion of the trial.

It can be seen that all the patients received the prescribed dosage. In the high dose group, in particular, no side effects necessitated termination of treatment before a dose of 400 mg was administered. The number of technical difficulties and the toxicity tolerated was not different in the two groups (see Table 1).

2. *Evaluation of tumor regression*

Table 2 shows how the sample path given in figure 1 was plotted.

The path crosses the upper boundary. This provides evidence of the superiority of the high dose treatment (A) over the low dose treatment (B). As we know that a prolonged survival is observed in patients with marked tumor regression, we may expect patients in group A to have better survival than those in group B: this will be checked after patient follow-up for at least one year.

DISCUSSION

By means of the sequential approach the initial aim was achieved quite rapidly and with a relatively small number of subjects. A total of 23 patients were entered into the trial while 2 years would have been needed to gather the 50 or so patients necessary for a classical analysis. Among these, 25 would have received the less satisfactory of the two treatments, while only 12 received it in our trial.

Table 1. *Reported cases*

Name	File Number	Site	*T*	*N*	Toxicity	Pair number	Patient's number in pair	Date	Dose (mg)
A	71 5128 GK	Glosso-tonsillar groove	4	3	Mucite—Erythema Interruption at the 9th day	1	2	7.31.71	425
A	71 541 ZL	Glosso-tonsillar	4	3	Temperature: 38°C on the 2nd day	1	1	7.2.71	70
B	71 5005 ZK	Pelvi-lingual	4	3		2	1	7.10.71	500
B	71 6606 KN	Pelvi-lingual	4	3		2	2	11.10.71	50
R	71 4812 FM	Glosso-tonsillar	4	3	Vomiting—Erythema False membrane mucite. Interruption at the 8th day	3	1	7.24.71	400
F	71 6022 ZD	Glosso-tonsillar	4	3		3	2	9.29.71	50
D	7106919 TT	Hemi-tongue	4	0	Interruption at the 9th day Temperature—Erythema Fall of WBC < 3000	5	2	10.16.71	450
G	71 5705 HF	Hemi-tongue	4	0		5	1	8.20.71	50
R	71 5895 WH	Hemi-tongue + floor of mouth	4	3		6	1	9.15.71	50
B	72 1456 HR	Pelvi-lingual	4	3		6	2	3.30.72	500
D	71 6370 AE	Glosso-tonsillar	4	3	Interruption at the 7th day Leakage—Thrombosis of the temporal artery	7	2	10.12.71	30
G	71 6376 PZ	Glosso-tonsillar	4	1		7	1	10. 8.71	500
T	71 7072 GX	Glosso-tonsillar	4	3	Erythema	8	1	10.27.71	50
G	71 7554 NH	Glosso-tonsillar	4	3	Leakage + purpura Interruption at the 9th day	8	2	11.10.71	450
S	72 9178 TU	Pelvi-lingual	4	2	Erythema	12	1	1.27.72	500
L	72 221 TD	Pelvi-lingual	4	2	Leakage—Obstruction	12	2	2. 8.72	50
R	72 2266	Pelvi-lingual + gum	4	3		10	2	3.22.72	500
M	71 7801	Pelvi-lingual + gum − Lower lip	4	3	Pain at the end of infusion	10	1	12.31.71	50

Table 2. *Evaluation of tumor regression*

Pair number	Treatment A % of regression (in positive value)	Treatment B % of regression (in negative value)	Algebraic difference between 1 and 2	Algebraic sum of differences
1	0	0	0	0
3	+30	−50	−20	− 20
7	+50	0	+50	+ 30
5	+80	−20	+60	+ 90
2	0	0	0	+ 90
8	−20	0	−20	+ 70
12	+50	−30	+20	+ 90
6	+25	+30	+55	+145
10	+70	−50	+20	+165

A = 50 mg (+ = regression − = aggravation).
B = 5 mg (− = regression + = aggravation).

This is to the credit of the sequential method. However, a number of limitations should be mentioned.

The assessment had to be made rapidly. Here 5 included patients were not used and out of these 3 had received the less satisfactory treatment.

Only one criterion of assessment could be used. Tumor regression was selected since it summarized both the efficacity and the toxicity. In fact, an interruption of the treatment due to toxicity resulted in a diminution of the dose, and may have also acted on the regression. Also, as previously noted, this criterion permitted an indirect assessment of the effect of the treatment on survival.

Lastly, the data necessary for the calculation of the boundaries came from our previous observations.

In considering the results, it seems that high doses administered according to our method are perfectly well tolerated by the patients. They result in better tumor regression than the low doses administered in the same conditions but without an antidote.

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RBE Values for Regrowth of C_3H Mouse Mammary Carcinomas after Single Doses of Cyclotron Neutrons or X-rays

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Abstract—*RBE values were obtained from the delay in regrowth of first-generation transplanted mammary carcinomas after irradiation with single doses of X-rays or neutrons. A decrease of RBE with increasing dose to a minimum value of* $2{\cdot}6 \pm 0{\cdot}6$ *at about* 600 *rads of neutrons was found. The results agree with those obtained previously for delay in regrowth and also for local control of tumours in multi-fraction experiments using small doses per fraction. The RBE values for this carcinoma were not significantly greater than RBE's for skin reactions at low doses per fraction, as was predicted from their shrinkage pattern after large doses of X-rays and their known reoxygenation kinetics.*

INTRODUCTION

THE PRESENT experiments were carried out to compare RBE values obtained from tumour regrowth with those from local control of tumours at 150 days, previously reported for single and fractionated exposures of cyclotron neutrons or 250 kV X-rays (1, designated paper I). In those experiments, 5 fractions of X-rays or neutrons were given in 9 days and the RBE values were found to be similar both for tumour control and for the production of skin reactions. Each neutron dose was about 330 rads. This result suggested that neutrons offered no therapeutic advantage over X-rays when used in this fractionation schedule for the treatment of C_3H mouse mammary tumours. When 9 fractions were given in 18 days however, each neutron dose being about 180 rads, the RBE for tumour control was $1{\cdot}2 \pm 0{\cdot}2$ times greater than for skin reactions. This fractionation schedule, employing smaller neutron doses per fraction, may therefore have some therapeutic advantage, although the advantage was not statistically significant.

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It was considered advisable to investigate further whether the therapeutic advantage was indeed so small as to be difficult to demonstrate in these tumours, using a different design of experiment.

In the present experiments the effects of *single small doses* were therefore investigated, for comparison with the multiple exposures of about the same size of dose per fraction (as in paper I). A wider range of doses can be used in regrowth than in tumour control experiments and results are obtained in a shorter time.

An increase of RBE with *decreasing* dose per fraction is expected because of the relatively smaller intracellular repair capacity after neutron irradiation, than after X-irradiation [2, 3]. In addition, for partially hypoxic tissues, such as tumours, at higher dose levels an increase of RBE with *increasing* dose per fraction is expected as the response of the hypoxic component becomes predominant.

MATERIAL AND METHODS

First-generation transplants of spontaneous mammary tumours in male and female C_3H mice from our inbred colonies were used as described in detail in Paper I (1). Two sets of

experiments were done, the first using 98 C_3H-HeH mice (obtained from the MRC Radiobiology Unit at Harwell in 1964 and bred at the Royal Postgraduate Medical School, Hammersmith Hospital), the second using 160 C_3H-He mice (obtained from MRC Carshalton in 1970 and inbred at the Gray Laboratory). In the first set two transplants were used and in the second set of experiments mice were drawn from 16 transplants.

The techniques used for local irradiation of the tumours were as described in Paper I; but for the second set of experiments a 240 kVcp Pantak X-ray machine was used with the same filtration (0·25 mm Cu + 1 mm Al) and HVT (1·3 mm), giving a dose rate of 230 rad/min instead of 180 rad/min. The scattered X-ray dose at the centre of the mouse's body was measured as 22 rads per kilorad received by the tumour. During irradiation all mice were surrounded by oxygen warmed to 25 ± 1°C as in Paper I, so that the results could be compared with those of Hill *et al.* [14].

Three perpendicular diameters of each tumour were recorded 2–5 times per week until they reached 6–7 mm average diameter when they were irradiated, and thereafter until the mean diameter of a tumour reached 12–15 mm, when the mouse was killed for ethical reasons. The method of using delay in regrowth as an end-point was as described by Thomlinson [4] and Thomlinson and Craddock [5]. The growth rates before and after irradiation and the delay in regrowth caused by a given X-ray dose were similar in the two sub-strains of mice.

RESULTS

Figures 1 and 2 show the tumour growth curves plotted as mean diameters versus time after irradiation for each of the nineteen dose groups including unirradiated controls, for the Hammersmith Hospital and Mount Vernon Hospital experiments respectively. Representative standard errors of the mean diameter for a group of mice are shown as vertical bars. They were calculated for each measurement day but are not all shown in the figures. The numbers of tumour-bearing mice in each dose group are shown in brackets. Where a fraction less than unity appears (e.g. 9/10 for 634 rads of neutrons) the regrowth curve shown is for 9 of the 10 tumours irradiated, because one grew so rapidly that it was considered as significantly different from the remainder of the group and the mouse was excluded from the analysis.

Values for RBE (i.e. ratio of doses to give the same effect), with approximate estimates of the

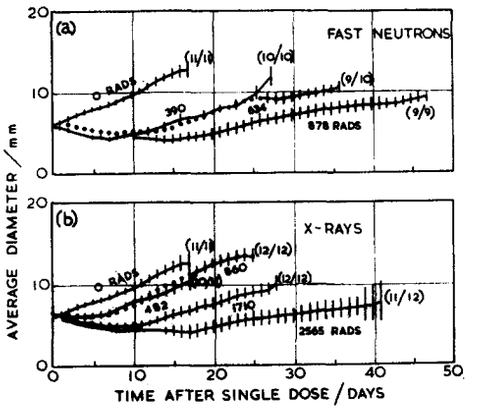

Fig. 1. Growth curves of C_3H mouse mammary first-generation transplanted tumours after the X-ray or fast neutron doses stated for each curve. Vertical lines give standard errors of the mean diameters; some are omitted to avoid overcrowding. The numbers of mice in each dose group analysed are shown in a bracket (Hammersmith Hospital experiments).

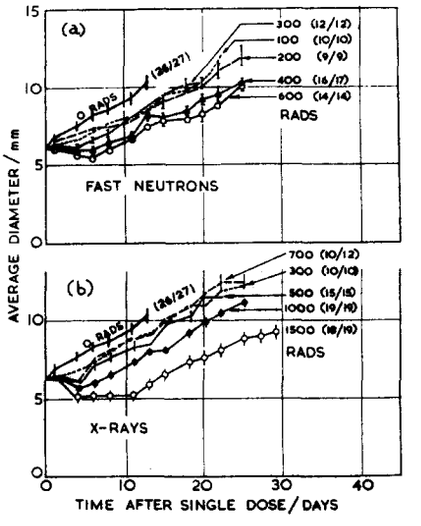

Fig. 2. As Fig. 1 but Mount Vernon Hospital experiments.

errors, can be obtained by finding the time at which the mean diameter of the regrowing tumours reaches an arbitrary size [6, 7]. Two sizes were chosen for analysis in the present work: 7·8 mm (250 mm^3) and 9·8 mm (500 mm^3).

Figure 3 shows a graph of the times at which the mean diameters reached 9·8 mm plotted against dose. The vertical error bars represent the intersections with the 9·8 mm abcissae of envelopes drawn through the ends of all the standard error bars on the regrowth curves. They are thus only approximately 70% confidence limits and are not necessarily symmetrical. The agreement between the first and second sets of experiments is good except for the 390 rad neutron point, so that the first set of experi-

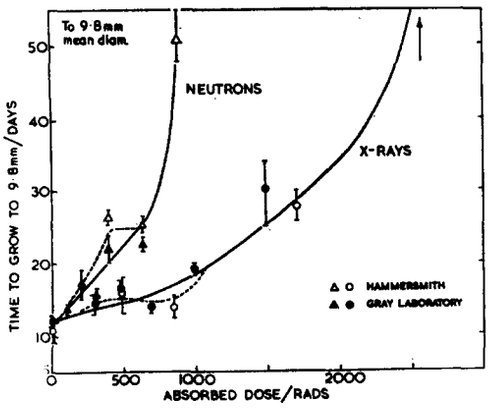

Fig. 3. Time required to grow to a mean diameter of 9·8 mm (500 mm^3) vs single dose to tumour, from Figs. 1 and 2. Full lines: smoothed curves between points. Dotted lines: less likely curves through more data points. Error bars were obtained from the intercepts of the envelope of the error bars in Fig. 1.

ments involving 98 mice would *by itself* have been inadequate. Curves have been drawn by eye between the data points. The deviations in Fig. 3 represented by the dotted lines suggest break-points or plateaux at doses consistent with 10% of hypoxic cells (see discussion), but because of the scatter of the data they cannot be considered significant.

In Fig. 4 RBE values derived from Fig. 3 (and from a similar plot where regrowth to 7·8 mm diameter was used) are plotted against neutron dose per fraction as proposed by Field [8]. The vertical lines show the range of RBE values obtained graphically, in the absence of a a rigorous statistical test for such data. Envelopes were drawn through the ends of the error bars in Fig. 3, and from these the range of

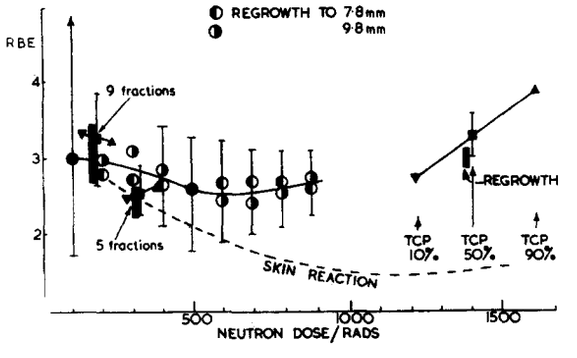

Fig. 4. RBE vs neutron dose. Circles show the present results. The error bars represent the whole range of spread of experimental values. In addition the following data from (1) Paper I are shown: ▲ = 90% probability of tumour control at 150 days; ■ = 50% probability of control (vertical line gives standard error); ▼ = 10% control. Thick vertical columns show range of values obtained from regrowth curves in Paper I. The data from Paper I are shown for single neutron doses (on right) and for 9F/18d and 5F/9d (on left). Dotted line: RBE for skin reactions in several species [9].

doses to produce a given regrowth delay was expressed as a percentage of the mean dose. A "root mean square standard error" of the RBE value was obtained from the square root of the sum of the squares of these percentages for corresponding neutron and X-ray doses. This gives a "maximum likely range" of values of RBE, and is similar to the range obtained by dividing the maximum X-ray dose by the minimum neutron dose and vice versa.

In Fig. 4 the RBE results from Paper I are also plotted against the neutron dose per fraction used in those experiments. The RBE's from the 10, 50 and 90% probabilities of tumour control are shown; also the ranges of RBE obtained from regrowth of those tumours which recurred. The groups of RBE's for the two low doses shown are from the 9-fractions-in-18-days and 5-fractions-in-9-days schedules respectively. Good agreement is demonstrated between the RBE results for multiple small fractions reported in Paper I and for the single doses of similar size used here.

For a normal-tissue comparison, RBE values for skin reactions in several species of mammal are shown dotted in Fig. 4 (from Fig. 2 in [9]).

DISCUSSION

No discrepancies between the RBE's for tumour growth delay and local control have been found in our results using this mammary carcinoma either with recurring tumours in the previous experiments (Paper I) or with similar tumours given smaller single doses in the present work. Field *et al.* [6] found a similar close agreement of RBE's measured by regrowth or local control in the rat fibrosarcoma RIB_5; Barendsen and Broerse [10, 11] however did not, in their rat rhabdomyosarcoma.

The variance in results is probably somewhat greater in this first-generation transplant system than in serially transplanted tumours, especially for small single doses. It appears that if more than one transplant has to be used, then a large number as in the second set of experiments gives more reliable results than just two transplants, probably because of the difficulty of ensuring an even distribution into dose groups.

A recent estimate of RBE obtained by comparing the rate of loss of ^{125}IUdR-labelled tumour cells *in vivo* has confirmed the present rather low values of RBE for neutron doses of 400 to 600 rads in these C_3H mammary tumours (Begg and Fowler [12] using a method developed by Pittner *et al.*, [13]).

Hill *et al.* [14], using oestrogen-induced mammary carcinomas in castrated male C_3H

mice of a different substrain and a modified form of tumour regrowth analysis, found RBE's of 2·6 and 2·3 for neutron doses of 1600 and 560 rads respectively, rising again to 2·8 at 300 rads (RBE's corrected from electron to X-ray doses by the factor 0·85). These values are in good agreement with the present results, but they did not investigate small single doses in detail.

A minimum value of RBE in Fig. 4 occurs between 500 and 700 rads of neutrons. This indicates the dose at which the response of the hypoxic cells in the tumours begins to be significant as the dose is increased; it is consistent with a proportion of 5–10% of hypoxic cells present before irradiation, assuming a conventional two-component cell survival hypothesis with D_0 = 130 rads, D_q = 400 rads and OER = 3 for the X-ray response. A similar estimate of about 10% comes from separate experiments on local control of tumours for 150 days: the difference in doses required to control 50% of tumours irradiated breathing warmed oxygen (as here) or clamped-off to make them anoxic being about 800 rads. Both estimates depend to some extent on the values assumed for the parameters, but for the same assumptions regrowth and control give consistent results.

Figure 5 shows the same results as in Fig. 4 plotted against the *reciprocal* of neutron dose. This was suggested by Alper [15], who showed that the slope ΔD_q of the steepest part of such curves, to the right of the trough in Fig. 5, is an indication of the *minimum* size of the shoulder of the survival curve for those cells

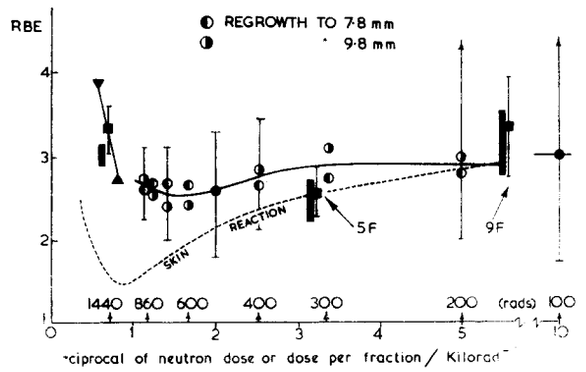

Fig. 5. RBE vs reciprocal of neutron dose. Circles show present results, as in Fig. 4. Squares and triangles show tumour control results and vertical columns the regrowth results from Paper I, as in Fig. 4. Dotted line: RBE for skin reactions, as in Fig. 4.

when irradiated with X-rays. However, the analysis is only valid for RBE values derived from the exponential region of survival curves, i.e. for doses of neutrons greater than about 300 rads. Also an error is introduced by the presence of hypoxic cells; in the present case this is apparent for doses greater than about 500 rads. Thus ΔD_q can only be derived for neutron doses between about 300 and 500 rads and will therefore be very imprecise. If doses outside these limits are used, this will tend to cause the estimate of ΔD_q to be *less* than the true value. No useful value of ΔD_q is obtainable.

It can be seen in Figs. 4 and 5 that the present single-dose RBE's are slightly greater than those for the five fractions in 9 days (Paper I) by about 10%. Although this difference is not significant it might represent a small amount of hypoxia affecting the single-dose experiments, whilst sufficient reoxygenation occurs during the 5-fraction-in-9-day schedules to give a reduction in tumour RBE. With nine fractions in 18 days the multi-fraction RBE was equal to or 10% higher than that for a single dose, implying inadequate reoxygenation with the smaller X-ray dose-fractions used. Inadequate reoxygenation was also found for two and five daily fractions in the rat fibrosarcoma RIB_5 by Field *et al.* [7].

The lack of therapeutic gain with neutrons (the tumour RBE's being similar to those for skin) for small doses per fraction [15a] is, in our opinion, correlated with the histology of the tumours and their immediate shrinkage after single doses of X-rays. Experimental carcinomas in general shrink rapidly after a substantial single dose of X-rays [17] and these tumours are no exception. This behaviour may correlate with a better blood supply than in sarcomas [16]. If shrinkage occurs sufficiently early during the course of the fractionated treatment, more reoxygenation is likely to occur [18].

In other histological types of experimental tumour, however, neutrons have been found to be advantageous even at fairly small doses per fraction, giving higher RBE's for delay in tumour regrowth than for injury to skin. Examples are the rat fibrosarcoma RIB_5 (7) and another sarcoma (type F) in CBA mice which has 50% of its cells hypoxic and shrinks less readily after irradiation than the C_3H mouse mammary carcinomas [20].

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The Inhibitory Effect of Proflavine and Ethidiumbromide on the Cell-free Transmission and the Growth of the Hamster Melanoma A Mel 3*

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Abstract—1. *The amelanotic hamster melanoma A Mel 3 can be induced by a subcellular fraction, which contains virus particles. The virus-induced tumour, arising 3 weeks after subcutaneous injection exhibits the same ultrastructural properties as the original tumour with respect to virus production.*

2. *The cell-free transmission of the A Mel 3 melanoma can be inhibited by a single injection of one of the mutagenic dyes—proflavine or ethidiumbromide.*

3. *Tumour production is prevented by these dyes when applied during the first 12 h after inoculation with tumour cells. Furthermore, the growth of manifested melanoma (on the 4th day of inoculation) can be inhibited strongly by a daily injection of proflavine or ethidiumbromide. The survival time of the treated animals amounts to more than 3 months whereas the untreated tumour-bearing animals die in between 18–20 days after inoculation with tumour cells.*

INTRODUCTION

THE AMELANOTIC hamster melanoma A Mel 3 shares many morphological, histological and "clinical" characteristics with human melanoma [1,2] and may thus serve as a convenient experimental model for this type of human cancer.

In earlier studies it has been shown that mitochondria of the A Mel 3 melanoma produce proteins which are not synthesized by mitochondria of normal tissue [3], although they exhibit no differences with respect to oxidative phosphorylation [4] and cytochrome oxidase activity [5]. In addition, tumour-specific proteins have been detected in the hamster melanoma, the synthesis of which can be inhibited by the mutagenic dyes proflavine or ethidiumbromide [6]. These findings were tentatively explained by the presence of an additional messenger in this tumour and a possible candidate may be a viral nucleic acid.

The assumption for the presence of viral information in this tumour has been supported by the detection of a virus-specific 70S RNA [7] and a RNA-directed DNA-polymerase activity [8] as well as by the electron microscopic observation of virus-like particles [9]. These particles were identical to the so called "R"-viruses first discovered by Bernhard and Tournier [10].

The main questions arising from these results are the following: (a) are the virus particles, present in the A Mel 3 melanoma, causatively related to this type of cancer? and (b) do the mutagenic dyes proflavine and ethidiumbromide, which have been shown to inhibit the synthesis of tumour-specific proteins in the

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melanoma [6], inhibit the growth of the A Mel 3 melanoma? In other words, can the inhibition of certain proteins be correlated with the inhibition of the growth of this tumour?

The aim of this paper is to give an answer to these questions.

MATERIAL AND METHODS

The tumour used in our studies is the amelanotic hamster melanoma A Mel 3, described first by Fortner [11]. It is a very rapidly growing tumour, which metastasizes to lymph-nodes and many other organs and which leads to the death of the animal after 18–20 days. By subcutaneous injection of about 10^6 cells it has been carried in our laboratory for more than forty generations.

Isolation of virus particles

Tumours were carefully dissected, suspended in 0·25 M sucrose in TNE buffer, pH 8·3, (10 mM Tris-HCl, 150 mM NaCl and 2 mM EDTA) and homogenized with an Ultraturrax at low speed. The tumour cells were then broken up in a glass teflon potter by 10 strokes. Nuclei and cell debris were removed by centrifugation for 5 min at $3000 \times g$. Crude mitochondria were sedimented after 10 min at $10{,}000 \times g$. The mitochondrial supernatant was filtrated through a 1μ millipore filter, layered on a cushion of 10 ml 20% glycerol in TNE, pH 8·3, and centrifuged for 1 hr at 27000 rev/min in a Beckmann SW 27 rotor. The pellet denoted as Mc-P100, was suspended in a minimal volume of TNE and layered on a linear sucrose gradient ranging from 25 to 50%. Velocity sedimentation was performed for 5 hr at 40,000 rev/min in a Beckmann SW 40 rotor. The tubes of the gradient were eluted by pumping through 60% sucrose from the bottom. Simultaneously the optical density at 280 nm was recorded in a flow-through cuvette of a Gilford 2400 S spectrophotometer. Fractions of 0·4 ml were collected and pooled for each protein peak. The protein of each peak was pelleted by centrifugation for 1 hr at $100{,}000 \times g$ and examined for RNA-directed DNA-polymerase ("reverse transcriptase") activity [7]. The fraction of the gradient with the highest reverse transcriptase activity was monitored in the electron microscope for the presence of virus particles and used as "virus" preparation for the cell-free transmission experiments.

Preparation for electron microscopy

The pellets of the subcellular fractions were fixed in 1% OSO_4 in 0·1 M cacodylate buffer, pH 7·2, for 1 hr, post-fixed for 1 hr in 0·5% aqueous uranyl acetate, dehydrated in ethanol and embedded in Epon 812. Thin sections were cut on a LKB Ultrotome III with a diamond knife, mounted on copper grids covered with collodium and carbon films, stained with uranyl acetate [12] and lead citrate [13] and examined in a Siemens Elmiskop IA or 101 at 80 kV with a 50 μm objective aperture.

Cell-free transmission

The "virus" preparation of the A Mel 3 melanoma was subcutaneously injected into newborn hamsters. Doses ranging from 1·0 to 15·0 mg per animal on a protein basis were used.

Chemicals

The dyes proflavine and ethidium bromide were purchased from Serva, Heidelberg (Germany), and recrystallized in ethanol before use. Stock solutions of 10 mg/ml of both dyes were prepared in sterilized TNE, pH 8·3, kept in the dark and in the cold and used for all experiments.

RESULTS

Cell-free transmission of the A Mel 3 melanoma

In the amelanotic hamster melanoma A Mel 3, virus particles are present in appreciable number either free in the cytoplasm or in cisternae of the endoplasmic reticulum (Fig. 1). The ultrastructural appearance of these particles is characterized by an electron dense nucleoid from which fine projections extend to the limiting triple-layered membrane. Their average size is 120 nm and they mature by budding through the membrane of the endoplasmic reticulum (Fig. 1). This maturation process is one of the morphological characteristics of oncogenic RNA-viruses [14].

The virions are enriched in the subcellular fraction, in which the reverse transciptase has a maximum. Electron microscopic examination of this fraction reveals numerous particles (Fig. 2). Some of them are still enclosed in vesicles and a few are in the process of maturation as can be seen by their budding through a membrane (Fig. 2). Subcutaneous injection of this virus preparation into hamsters leads to the formation of tumours at the site of inoculation. In newborn animals, a 100% induction rate was achieved with a dose of at least 10 mg on a protein basis. Lesser amounts of protein have also been tested. Using 5 mg of the viral fraction an induction rate of 20% was obtained whereas with 1 mg no tumour formation was observed up to 6 months.

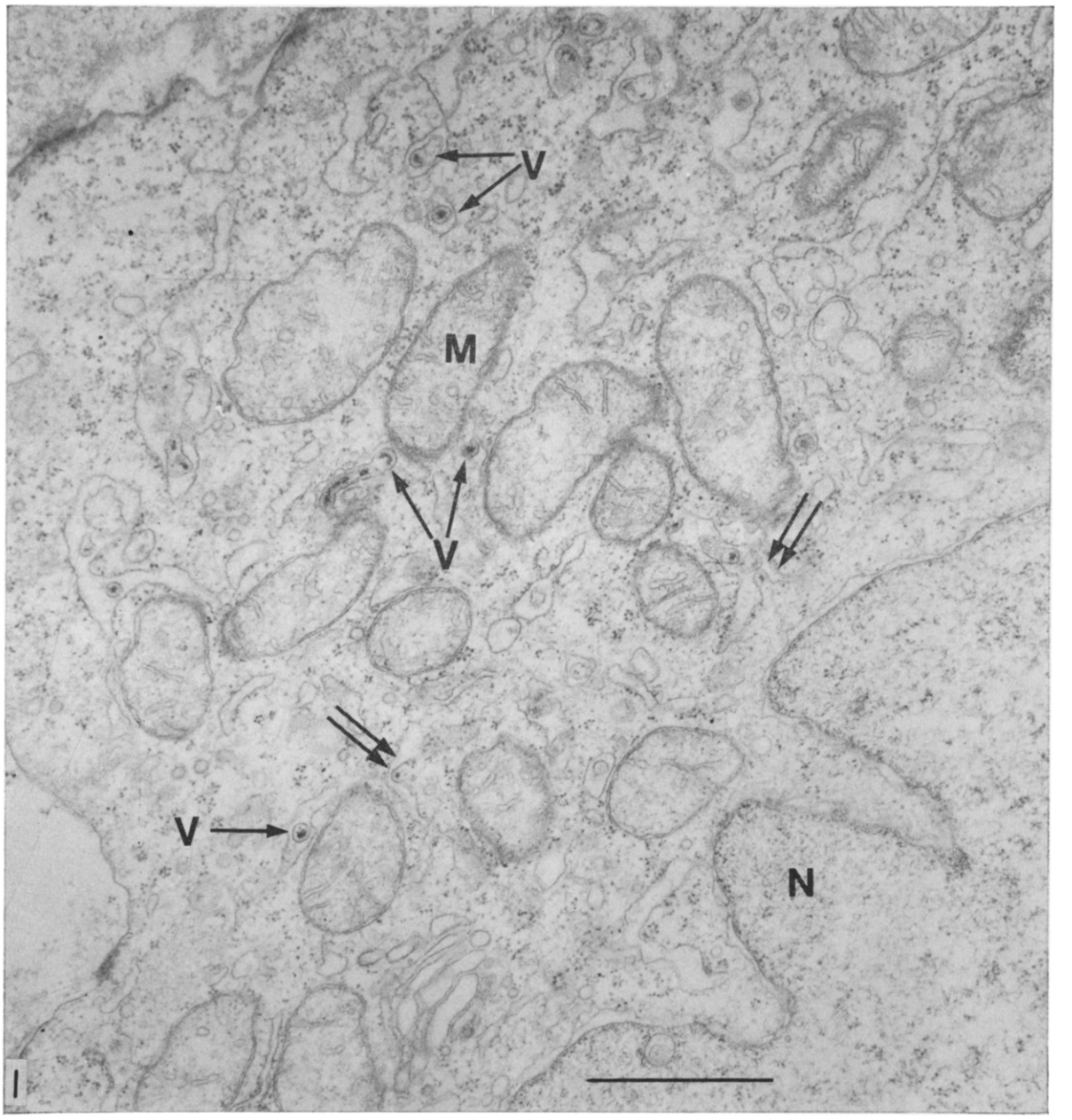

Fig. 1. *Thin section of tumour tissue from the amelanotic hamster melanoma A Mel* 3. *Virus particles (arrow) present free in the cytoplasm or enclosed within vesicles of the endoplasmic reticulum. They mature by budding through the membrane of these vesicles (double arrow). N = nucleus; M = mitochondria; V = Virus particles; Scale line = 1 μm.*

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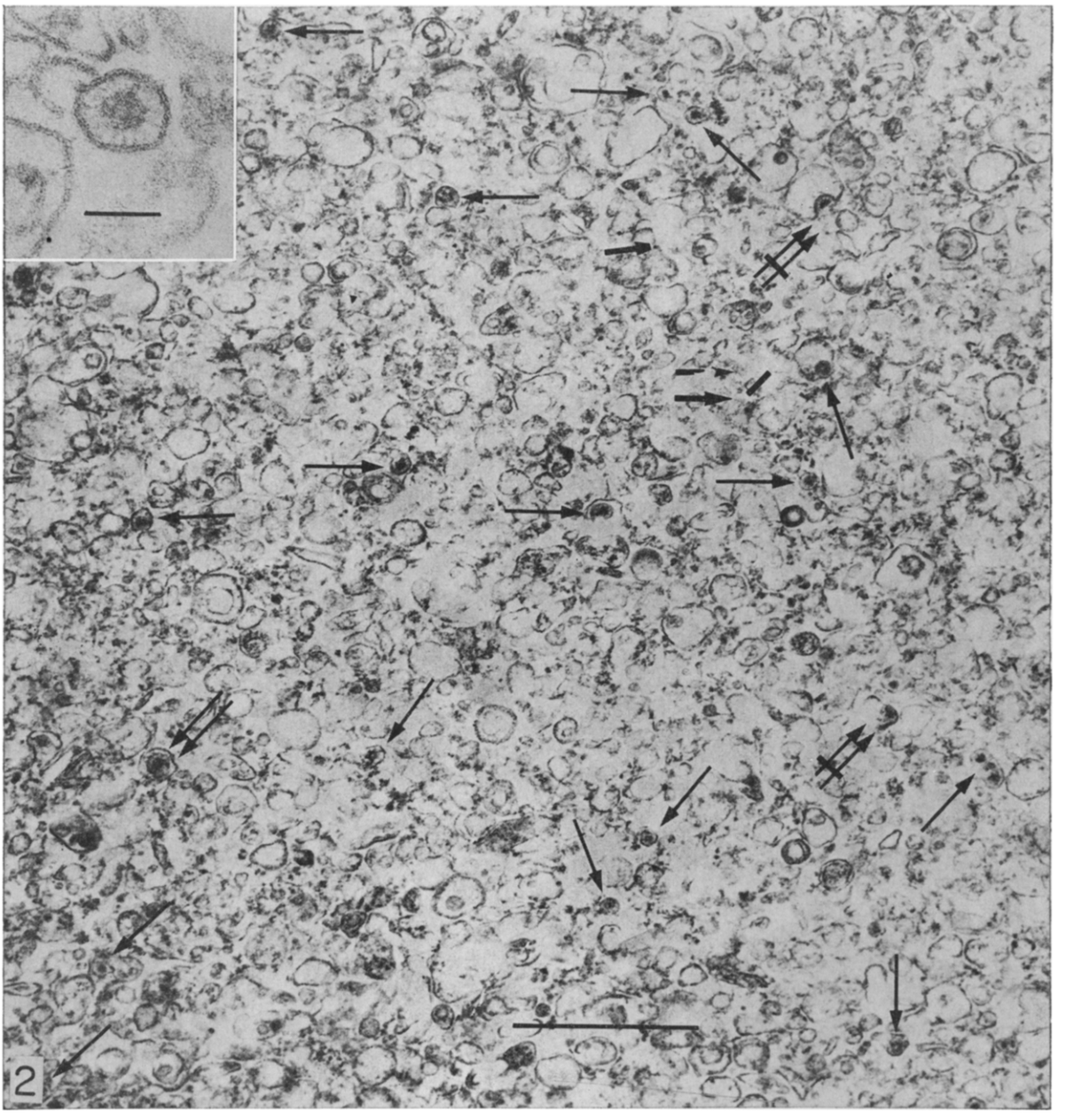

Fig. 2. Thin section of a pelleted viral fraction isolated from the amelanotic hamster melanoma A Mel 3. Numerous particles (V, arrows) are present, some are surrounded by membranes of the endoplasmic reticulum (double arrows) a few are just budding through these membranes (double arrows with bar). Scale line = 1 μm.

Inset shows a particle of the viral fraction at higher magnification. The fine radial projections toward the limiting triple-layered membrane give these particles a wheel-like appearance. Scale line = 0·1 μm.

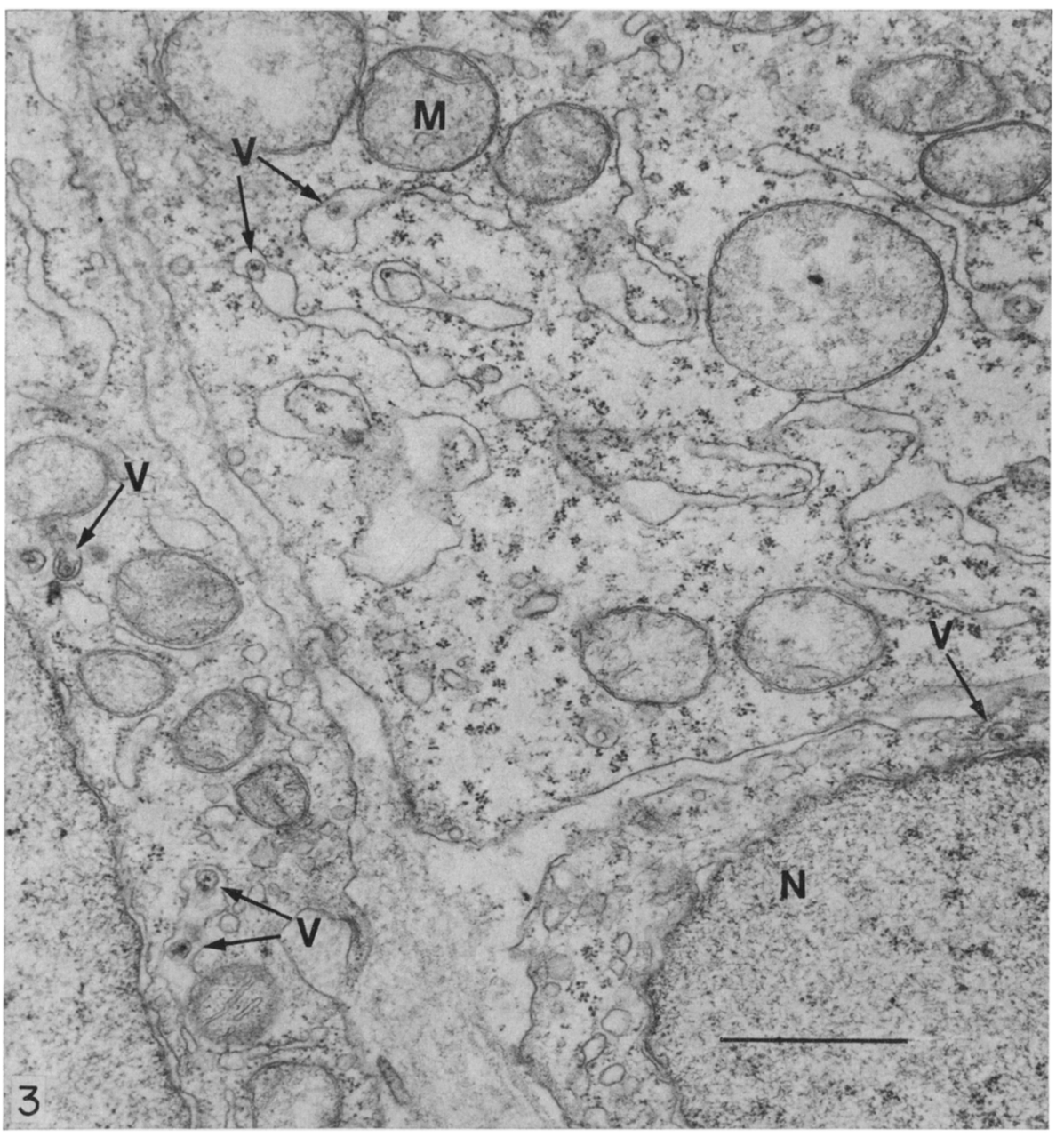

Fig. 3. Thin section of virus-induced tumours. Virus particles present in 3 adjoining cells. N = nucleus; M = mitochondria; V = virus particles; Scale line = 1 μm.

From the protein content and the volume of the fraction after dehydration and embedding (as determined by a micro-voluminometer) as well as from the numbers of virus particles in a defined area of the thin sections, an approximate value of the number of virus particles injected can be calculated. About 10^8 virus particles are necessary to induce a melanoma. With this number tumours have also been produced in animals up to 4 weeks old.

One of the main properties of an oncogenic virus is the ability to replicate in the cell-free induced tumour [15]. To test this possibility, thin sections of the newly formed tumour were examined in the electron microscope. Numerous virus particles were found in the cell-free induced tumour, exhibiting their characteristic wheel-like structure (Fig. 3). From these results it can be concluded that the virions present in the melanoma A Mel 3 are oncogenic agents.

Effect of proflavine and ethidiumbromide on the cell-free transmission of the melanoma A Mel 3

As shown in Table 1, the mutagenic dyes proflavine or ethidiumbromide prevent the induction of melanomas by a virus preparation. In this experiment 3 groups of 10 animals each received 10 mg of the viral fraction and 2·5 mg per animal of proflavine, ethidiumbromide or chloramphenicol. In the control animals sterilized TNE buffer, pH 8·3, was injected. In these animals tumours were produced which reached a weight of 3000 mg on the 20th day after inoculation. The induction rate amounts to 90%. Neither in the proflavine nor in the ethidiumbromide-treated animals can tumour formation be observed for several months. Chloramphenicol treatment, however, has no effect on the cell-free transmission of the melanoma A Mel 3 (Table 1). Thus it seems that the effect of proflavine or ethidiumbromide is a rather specific one, and is very probably not due to the interference of these dyes with mitochondrial protein synthesis.

The antitumour effect of proflavine and ethidiumbromide on the melanoma A Mel 3

When a suspension of 10^6 cells is incubated for 30 min at room temperature with proflavine (4·0 mg/animal) or ethidiumbromide (same dose) prior to subcutaneous injection, no tumour will be produced, whereas the control cell suspension gives rise to a melanoma that becomes first visible after 4 days.

Hence, these dyes interfere either with the transforming capacity of the melanoma virus or impede with the growth of the tumour cells. To distinguish between these possibilities, an experiment was designed in which the effect of these dyes on pre-existing melanomas could be tested. Tumours were produced by inoculation of 10^6 cells. At the time of macroscopic appearance of a tumour (usually at the 4th day after inoculation) the animals received a daily subcutaneous injection of proflavine or ethidiumbromide, until the first untreated control animals died. The weight of the tumours from the control as well as from the drug treated animals was measured on the 4th day and on the 14th day (at which time-point 50% of the untreated control animals die). The ratio of the weight on the 14th day to that on the 4th day is a parameter from which the growing rate of the tumour can be deduced and which demonstrates whether a tumour grows or regresses;

Table 1. *Effect of proflavine and ethidiumbromide on the cell-free transmission of the amelanotic hamster melanoma A Mel 3*

Group	Dose/animal (in mg)	Number of animals	Mean weight of tumour on 20th day of inoculation* (in mg)	% Tumour induction
Tumour-bearing animals	No treatment	10	3000	90
Proflavine	2·5	10	0·0 (no tumour production)	0
Ethidiumbromide	2·5	10	0·0 (no tumour production)	0
Chloramphenicol	2·5	10	2500	100

*Average value of 5 experiments with 10 animals each.

in the latter case the factor is smaller than one, in the former it is larger than one. As can be seen in Table 2 the parameter of the growing rate of the melanoma A Mel 3 is around 10, which implies that it is growing rapidly. Treatment of the 4-day old tumours (which weigh 300 mg at this time) by proflavine or ethidiumbromide (a daily subcutaneous injection of 4·0 mg per animal for 10 days) leads to a partial regression of the tumour as can be deduced from the fact that the growing parameter becomes smaller than one. Lower doses of the very toxic dyes (1·0 mg per animal per day) reduce the growth of the tumour remarkably but do not stop it completely, as can be seen from the growing parameter which becomes smaller than that of the untreated animals but is still larger than one. This finding indicates that a critical amount of dyes is necessary to lead to a regression of the tumour.

On the other hand, the antibiotic chloramphenicol seems to promote the growth of the tumour. The average weight of the chloramphenicol-treated tumours is greater by about 25% than that of the untreated tumours (Table 2, last row). A variety of other antibiotics exhibits a similar effect as chloramphenicol.

Table 2. Antitumour effect of proflavine and ethidiumbromide on the amelanotic hamster melanoma A Mel 3

Group	Dose/animal (in mg)	Number of animals	Mean weight of tumour on 4th day of inoculation* (in mg)	Mean weight of tumour on 14th day of inoculation*	Growth factor†
Tumour-bearing animals	No treatment	6	300	3000	10
Proflavine	4·0	8	300	200	0·66
Proflavine	1·0	8	300	450	1·5
Ethidiumbromide	4·0	8	300	180	0·60
Ethidiumbromide	1·0	8	300	400	1·33
Chloramphenicol	4·0	8	300	4000	13·3

*Average value of 5 experiments with 8 animals each.
†Growth factor = ratio of the mean weight of tumour on the 14th day and on the 4th day.

DISCUSSION

The result presented in this communication makes it very likely that the virus particles occurring in the amelanotic hamster melanoma A Mel 3 are etiologically related to this type of cancer. Morphological identical particles have been detected in various other hamster tumours [16, 17, 18], some of which have been successfully transmitted by a cell-free ultrafiltrate [19]. Their assumed oncogenic nature has been confirmed by our experiments. Meanwhile, a single-stranded high molecular weight RNA, sedimenting at about 70 S, and a RNA-directed DNA-polymerase activity have been associated with these viruses [7, 8, 20]. Because these properties are the most characteristic features of RNA tumour viruses, it can be assumed that the virus particles present in the melanoma A Mel 3 belong to the oncorna group.

The aim of our investigation was to elucidate the role of the mutagenic dyes proflavine and ethidiumbromide on the cell-transforming capacity of oncorna viruses as well as their influence on the growth of the tumour cells. In the cell-free transmission experiments the injected virions have to transform the cells first. If the production of a virus-induced tumour is prevented by certain drugs, the drug must interact at the level of cellular transformation. This seems to be the case with proflavine and ethidiumbromide in the hamster melanoma system. The question arises at which point in the very complex process of malignant cell-transformation these mutagenic dyes act. Although the mechanism of cell-transformation by oncogenic RNA viruses is not completely understood, it is generally accepted that at least two virus-specific components are involved in this process, namely the 70 S RNA [21] and the reverse transcriptase [22]. Viral mutants defective in one of these components are not able to transform normal cells into tumour cells [21, 22].

In earlier studies, we showed that proflavine or ethidiumbromide strongly depress the synthesis of the 70 S RNA present in the amelanotic hamster melanoma *in vivo* [7]. In addition, we found that the reverse transcriptase activity, associated with the 70 S RNA and the virus particles of the melanoma A Mel 3, can be inhibited *in vitro* up to 95% by these dyes [8, 23]. From these observations some characteristic points of interaction of these drugs in the cell-transformation can be deduced, which are summarized in a schematic drawing (Fig. 4). Two main inhibitory functions have to be considered. One block seems to lie at the transcription of the viral 70 S RNA by the reverse transcriptase (Fig. 4a). This assumption is supported by our own findings as well as by the findings of Hirschmann [24], who showed a similar inhibitory effect of proflavine and ethidiumbromide *in vitro* on reverse transcriptase from other oncogenic RNA viruses.

On the other hand, the hitherto known effects of these dyes suggest that they interfere with the replication and transcription of the virus-specific DNA, which codes for virus- and tumour-specific proteins (Fig. 4b). Indirect evidence for this assumption is provided from our earlier findings of tumour-specific proteins in the hamster melanoma, the synthesis of which is sensitive to proflavine or ethidiumbromide [6]. In addition, the strong inhibition of growth of this tumour by these dyes might be due to an interference with protein synthesis by impeding the binding of transfer RNA to messenger RNA on the ribosome [Fig. 4c].

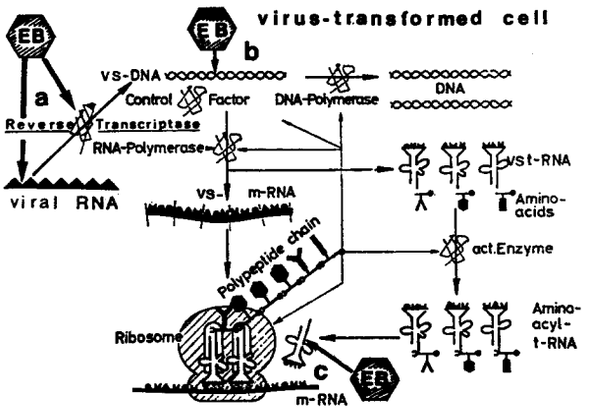

Fig. 4. Possible points of action of proflavine and ethidiumbromide at the molecular level in virus-transformed cells (A Mel 3 melanoma). EB = abbreviation for ethidiumbromide; Proflavine acts at identical points. vs = abbreviation for virus-specific.

Our findings are strongly supported by the observations of Richert and Hare [25], who showed that ethidiumbromide inhibits the malignant transformation of chicken embryo fibroblasts by Rous sarcoma virus. Work is now in progress to study the role of these cytoplasmic mutagenes on the virus-coded processes in the hamster melanoma system in more detail.

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Chromosomal Examination of Various Cell Clones (*In Vitro*) Derived from a DMBA (7-12 Dimethyl-α-Benzanthracene)-Induced Male Rat Breast Tumour

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Abstract—*A tumour originating in the breast of a male rat was set up in culture and the cells cloned thirteen times. The chromosomes of the different clones differed slightly as to chromosomal mode, but they all contained two constant marker chromosomes. Ten of the thirteen clones were re-injected into cortisone prepared rats, but only five of these subsequently formed tumours in the animals. The chromosomes of the latter tumours were studied and they showed no significant shift in modal numbers or change in the number of marker chromosomes.*

INTRODUCTION

ALTHOUGH there is now general agreement that a relationship exists between chromosomal abnormalities and malignant tumours, the nature of this relationship has yet to be clarified [1]. Abnormal chromosome configurations have been found in many tumours, and it has been also established that there is a general optimum for abnormal chromosomes including both numerical and morphological types e.g. markers [2]. The latter provide convincing evidence for the existence of a main stem line which is believed to be responsible for the existence and progression of the tumour [3].

Examination of the chromosomes of mammalian tumours would show that most cancers are composed of a heterogeneous population of cells [4], with each population being physiologically and immunologically different [5]. Utilization of the single cell (*in vitro*) technique has allowed many established clones from a single tumour to be examined karyologically, in order that interdependance between chromosomes, cell selection, and tumour formation and progression may be established. The following article provides data concerning some karyological differences among the cells from clones derived from a tumour of the breast from a male rat.

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MATERIAL AND METHODS

Establishment of the tumour

The tumour originated in one male of a group of 72 treated male Sprague–Dawley rats, all about 8 weeks old. The animals were force-fed with 7–12 dimethyl-α-benzanthracene (20 mg in 1 ml oil) and the dose repeated after two weeks. The animals were maintained on a special diet with an occasional vitamin supplement administered in the drinking water. Within two months of the first treatment, one animal developed a mammary tumour which was specially selected for further transplanta-

tion and study, since it was the only breast tumour that developed in the batch of treated animals. Details of the tumours in the other animals have been reported [6].

Utilising the method described previously [6], transplantation of the tumour was carried out at frequent intervals of two months for the last eight years.

Culture methods

The tumour was first set up in culture by both the explant and cell suspension methods, about five years after originating in the animal. The former method followed the principles, with some modifications, published by Harnden [7], while the latter involved treating the minced tissues with 0·25% trypsin for 20 min, and then seeding the dis-aggregated cells in sterile falcon flasks. The cell innoculum was usually in the region of 1×10^6/ml of medium, which consisted of 10% foetal calf serum with 90% Eagle's minimum essential medium and added penicillin, streptomycin and glutamine. All cultures were incubated at 37°C and the media changed routinely every second day.

Cloning procedures

Cloning of the cells from the original tumour was carried out by a technique perfected through modifications of previously published methods [8–10]. Dilutions of cells in suspension from the original culture were made up to give approximately 100 cells/ml and then one hundred such cells were introduced into a 25 cm^2 falcon flask containing tissue culture medium. Normally, from this number of cells, about 20–25 clones are formed and they are usually widely scattered in the flask.

Trypsinisation of an individual colony involved first of all washing the flask with several rinses of pre-warmed sterile Hank's solution to remove all loose cells and cell debris. A colony which was the only one present in the field of a low power ($\times 4$) objective of an inverted microscope, and which was observed to be separate from the others, was located. By means of a very finely-drawn out sterile pasteur pipette, a small drop of 0·25% trypsin solution was lowered onto the colony of cells. As soon as the cells in the colony became dissociated, as seen under the inverted microscope, another fine pipette containing sterile foetal calf serum was introduced into the flask and two small drops of serum dropped onto the dissociated cells before the cell suspension is sucked up into the pipette. This entire procedure is carried out under observation by the inverted microscope all the time.

The pipette with the cell suspension is withdrawn and then, utilising the $\times 10$ objective, single cells are planted into several individual flasks (falcon 25 cm^2), by gently squirting the suspension into the flask. Whenever more than one cell is extruded, the flask is discarded. This procedure absolutely ensures that each colony, when formed, has done so from a single cell as observed under the microscope. Each culture flask then receives 4 ml culture medium, is gassed and incubated for 10–14 days. When colonies are formed, they are dispersed in the flask by a fine pasteur pipette and then allowed to grow to confluency for chromosomal examination and re-injection into animals.

Injection of cultured cells

Confluent cultures from ten clones were trypsinised, washed in Hank's solution and re-suspended in saline before intraperitoneal injection into cortisone prepared male animals, of an innoculum of approximately 8×10^6 cells. The controls (5 animals in each case) received 2 ml saline. The animals were examined at regular intervals for tumour formation.

Chromosome preparation

Preparation of cells for chromosome examination involved incubation of monolayer cultures about 22 hr after subculture, with colchicine (0·5 μg/ml), followed by trypsinisation (0·25%) for three minutes and hypotonic treatment for 20 min. The cells were then fixed in a mixture of acetic acid/alcohol (1:3), flame dried and then stained with acetic orcein. Chromosomal examinations were carried out as soon as the cultures became confluent.

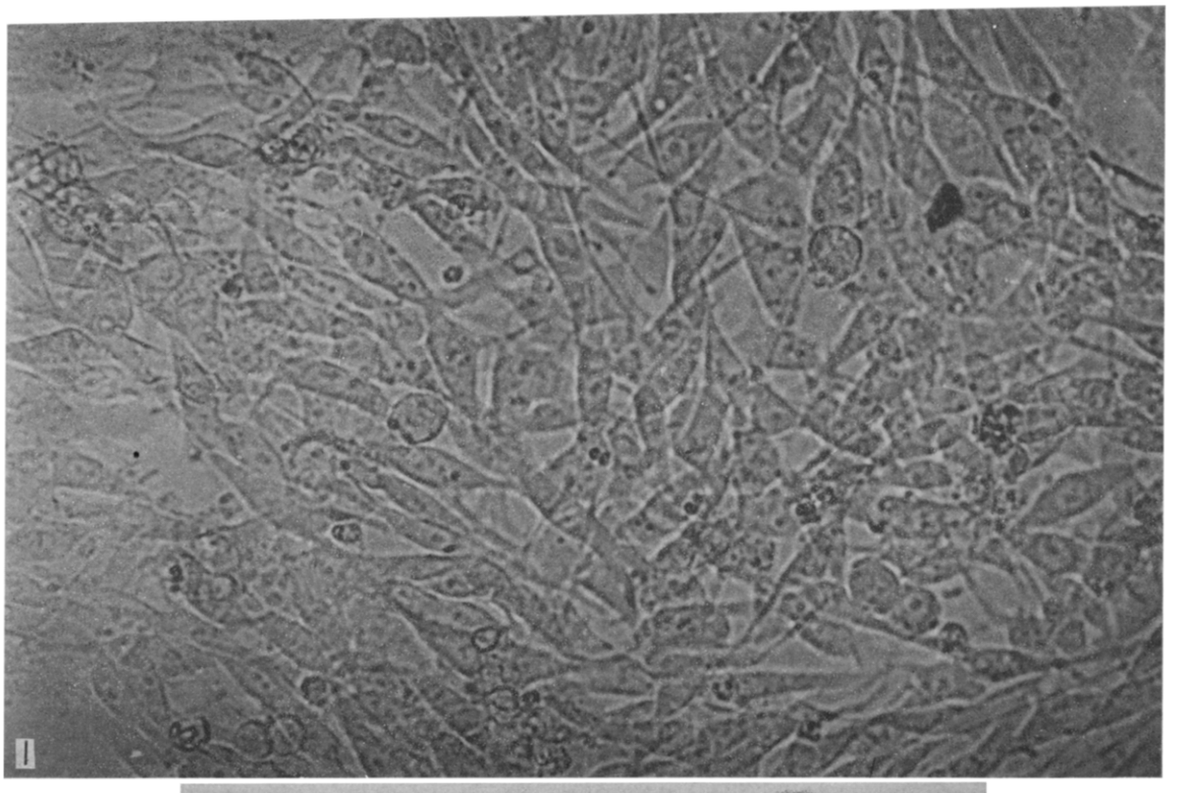
Systematic examination of the metaphases then followed established standard procedures [11, 12]. In the assessment of chromosomal breaks, a fragment was counted as one break, a ring as two breaks and an exchange arrangement also as two breaks.

Attempts to identify the banding pattern of the marker chromosomes were carried out using a modification [13] of the original method described by Sumner and colleagues [14].

RESULTS

Cell culture and morphology

Both the explants and minced tissues grew very well. The cells were mainly rounded with distinct nuclei and well defined borders (Fig. 1).



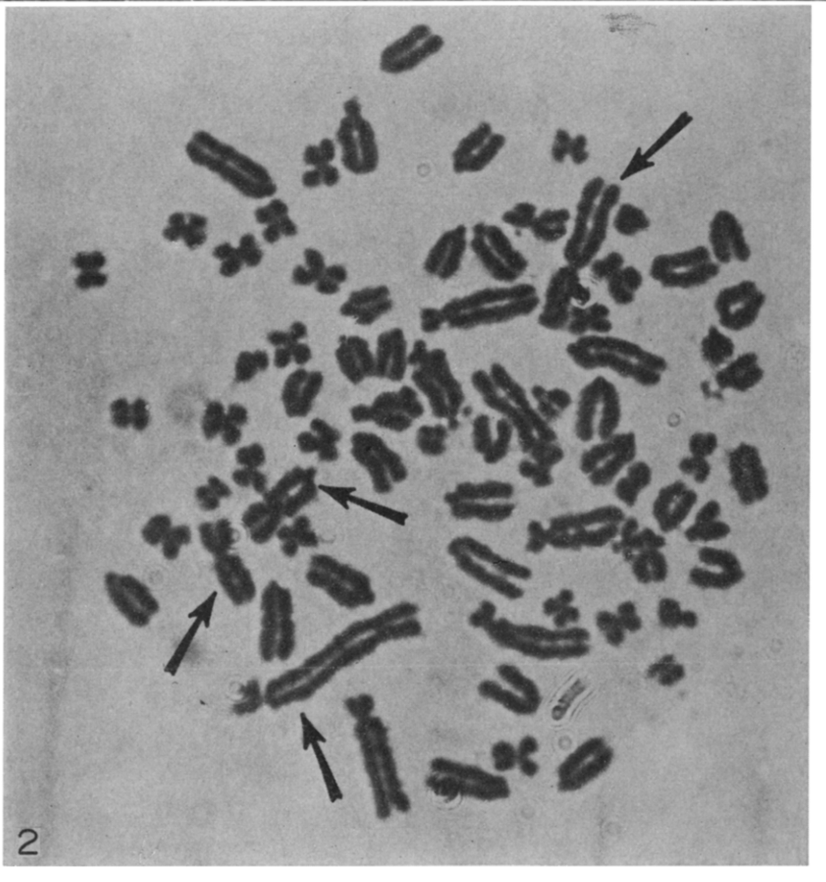
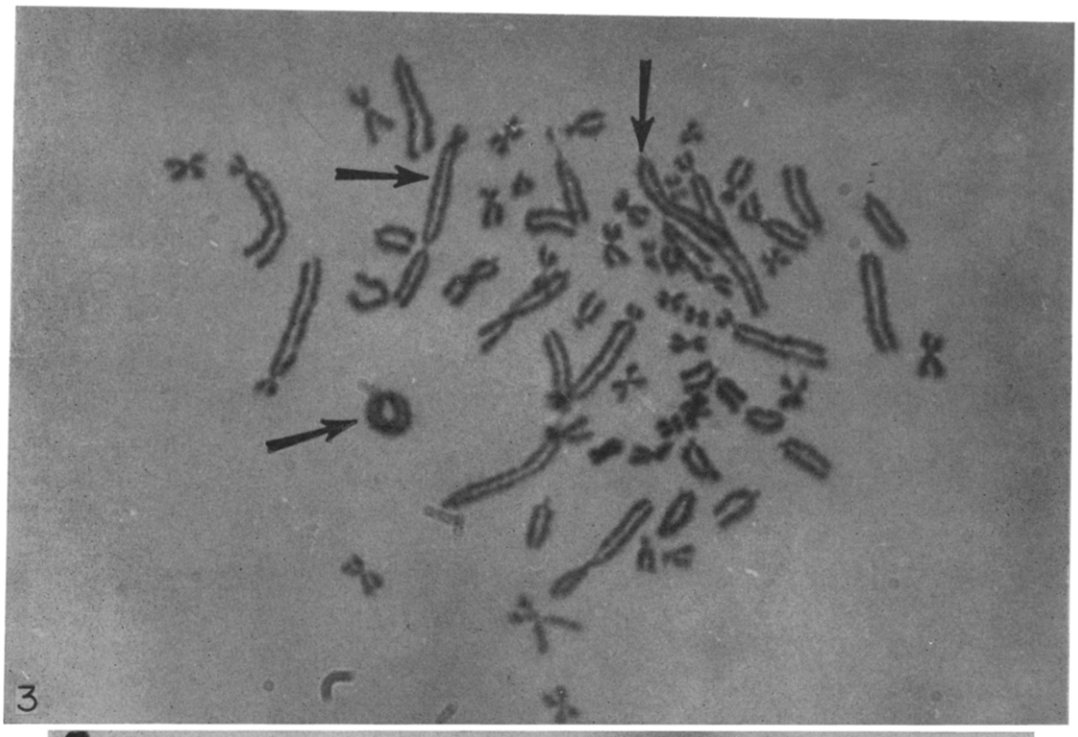


Fig. 1. *A confluent culture of the original tumour, showing the morphology of the cells and the pattern of growth.* × 500.

Fig. 2. *Metaphase spread from clone 5b, showing one telocentric and three submetacentrics (one large and two small arrowed).* × 2500.

(to face page 866)



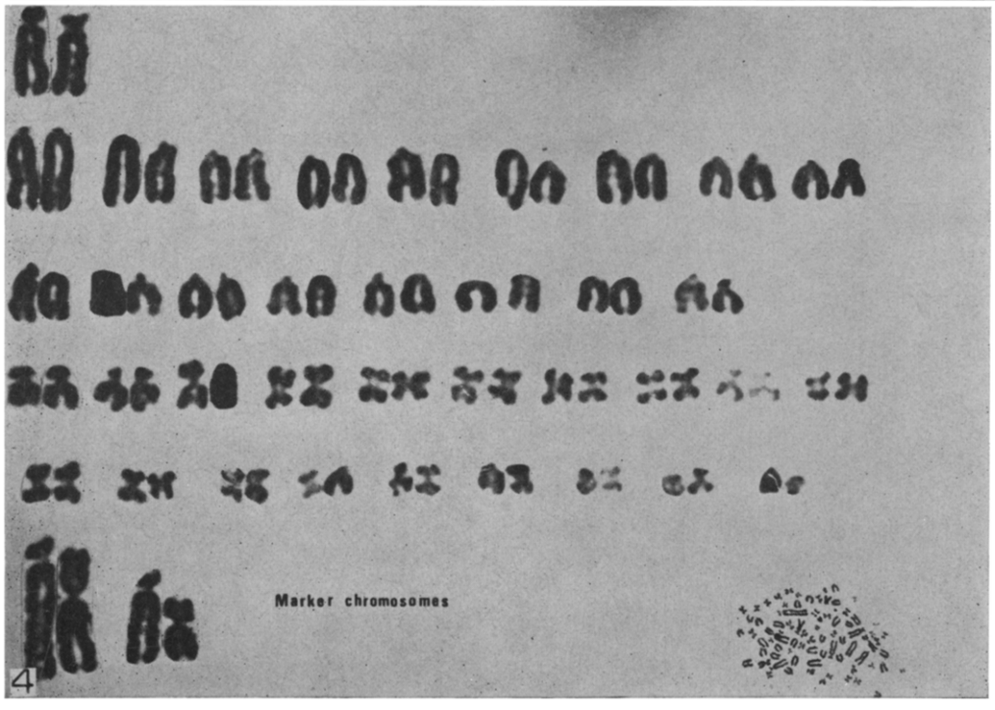


Fig. 3. Metaphase spread from clone 6b after re-injection into prepared animals. Note ring and other marker chromosomes arrowed. ×2500.

Fig. 4. Karotype of a metaphase cell (6*b*), *showing abnormal chromosome number and various marker chromosomes.* ×2500.

They were epithelial-like and grew as confluent sheaths. On several occasions these epithelial-like cells had patches of fibroblast-like cells which were long, pointed at both ends and showed a typical spindle shape morphology. The latter, however, were never prevalent and eventually died out.

Using the cloning technique described, several clones were identified from the original tumour cells in culture, the latter being designated as MCT (0) in Table 1. Chromosome counts of parental cells and those from tumours of cells re-injected into animals, are shown in Tables 1 and 2. Details of the tumours are shown in Table 3.

Chromosomes

The chromosomal mode in the original tumour was centered around 65–79 chromosomes, although various smaller modes are detectable on scrutinisation of Table 1. One telocentric and one submetacentric chromosome were the main markers. In the various clones examined, distinct modal numbers were centered around 70–79 chromosomes, with the same two main marker chromosomes present in all the cells. In clones 9b, 10b, 11b, a few additional marker chromosomes (2 small submetacentrics) were present.

Ten clones were re-injected into animals, (MCT 4a, MCT 5a, MCT 5b, MCT 6a, MCT 6b, MCT 7b, MCT 8b, MCT 9b, MCT 10b, MCT 11b), but only those in Table 2 formed tumours (Figs. 2, 3 and 4).

Banding pattern

The Giemsa banding patterns of the two main marker chromosomes (the telocentric and the submetacentric) were investigated in two clones and the patterns were found to be identical in these clones.

The short arm of the telocentric marker is darkly stained, but a single dark band can be consistently distinguished on it. Close to the centromere, the long arm has a dark area which can generally be separated into three bands. Beside this region is a wide pale area with a single light band, which is particularly an obvious feature of this marker in both clones. There is then another dark region, centered slightly above the mid-point of the long arm, which is divisible into two main dark stained bands plus a subsidiary one. The remaining section of this chromosome has six bands of which the second stands out as the most prominent feature.

The submetacentric marker generally appeared dark staining, although four dark bands can be determined on the short arm, in addition to a paler terminal region. The second and third of these bands often appear as one band in the central region of the arm. Four dark bands appear in the long arm of this chromosome and can be seen sometimes as a pair of bands together with a wider dark region. A paler area with two dark bands follows these and there is a terminal dark region containing a group of three narrow bands.

These features were prominent in the main markers (the telocentric and submetacentric chromosomes) in both the clones examined.

DISCUSSION

According to the stem line theory of carcinogenesis, cancer is formed from a single cell [2, 3]. Marker chromosomes occurring in premalignant lesions and in cancers provide some evidence in support of the theory (15). Since the chromosomes in the present investigation were not studied when the present tumour was induced, one cannot infer that the DMBA was responsible for the formation of the marker chromosome initially.

The experimentation involved in cloning the cells from the original tumour may provide further evidence in support of the stem line theory of cancer. Each clone was undoubtedly formed from a single cell which carried the two marker chromosomes. When these cells were re-injected, they formed tumours which, apart from some variations, still carried the marker chromosomes and did not show a significant shift in chromosomal mode.

Recent experimental studies, due mainly to the improvement of cytological techniques, have shown that the chromosome constitution of tumours also reflect the existence of a heterogeneous population of cells, in which the number of normal diploid cells may be very small or may be entirely absent [9]. However, this may depend on the length of survival of the cancer cell *in vivo*, provided that the tumours are kept alive by suitable transplantation techniques [6].

The tumour reported here had been transplanted for about eight years [6], and did not retain any normal diploid cells, but had a modal number of between 65–79 chromosomes on first (MCT 0) chromosomal examination of the cells in culture. The main marker chromosomes which were present in all the cells examined, were a long submetacentric and a long subtelocentric chromosome respectively.

Table 1. Chromosome analysis of parental clones

Clone	Chromosome counts														
	35–39	40–44	45–49	50–54	55–59	60–64	65–69	70–74	75–79	80–89	90–99	100>	Breaks	Other abnormalities	Total cells
MCT 0	4	2	4	2	1	2	12	20	23	5	2	3		Main markers	80
MCT 1									41					Main markers	41
MCT 2	2	1	1					2	33		1		3	Main markers	40
MCT 3							1	7	20	1				Main markers	29
MCT 4a	2							11	10					Main markers	23
MCT 5a					1	1	3	10	30					Main markers	45
MCT 5b								18	27					Main markers	45
MCT 6a	2	2	1	1			3	20	21		1			Main markers	51
MCT 6b	1	1				2	1	15	19		1	1	1	Main markers	41
MCT 7b							5	8	27					Main markers	40
MCT 8b	1						1	1	25					Main markers	28
MCT 9b	2	2						2	38	10				Various markers	54
MCT 10b		1	1					10	40				1	Various markers	52
MCT 11b	2	2	1	1		8	7	20	5		1	1	2	Various markers	48

Table 2. Chromosomal analysis of cells from tumours formed by injection of parental clones

Clone	Chromosome counts														
	35–39	40–44	45–49	50–54	55–59	60–64	65–69	70–74	75–79	80–89	90–99	100>	Breaks	Other abnormalities	Total Cells
MCT 56	2		1		1			35	1		2			2 markers. One telocentric, one sub-metacentric.	42
MCT 6b					1	1	33	3	1		1	2	7	Mainly 2 markers, one telocentric and one sub-metacentric.	42
MCT 7b	2	1				22	17	3			1	1	13	Two markers, one telocentric and one sub-metacentric.	47
MCT 10b					1	1	2	38	10				3	Varying number of markers.	52
MCT 11b														Varying number of markers.	40

Table 3. Details of tumour formation from injection of parent clones

Clone	Test animals Injected	Period before tumour formation (weeks)	Comment on tumours
MCT 5b	5	6	Tumour fast growing and invaded alimentary canal, spleen, kidneys and diaphragm. Tumour was also nodular and had ascitic fluid. Tumours were found in all animals.
MCT 6b	5	10	Tumours not very nodular and found on one side of testis, on spleen, diaphragm, liver, body wall, kidney and stomach. There was scanty ascitic fluid. Tumour found in all animals.
MCT 7b	5	12	Tumour very nodular and apparently transparent. It was found all over alimentary canal, liver and diaphragm. Tumour was found in all animals.
MCT 10b	5	9	Fast growing tumour found mainly on the liver but small nodular tumours found throughout the bodies of all the animals injected.
MCT 11b	5	9	Tumour was not very nodular and found on the body wall, spleens and kidneys of all the animals injected.

Morphologically, they were the same in all the clones examined, in addition to their banding patterns being identical in the two clones investigated [13]. These features provide evidence for the clonal evolution and progression of the reported tumour.

The original tumour showed chromosome constitutions centered mainly around 65–79 chromosomes. However, on further scrutinisation of Table 1, this tumour also showed a certain percentage of cells in almost all the groups, thereby exhibiting the characteristic heterogeneity of cell population. This feature is further exemplified by the fact that the isolated clones showed definite modes and were concentrated mainly around groups 70–79 chromosomes.

By utilising this cloning technique, we sought to separate the cell population and investigate the cancer producing potential of individual cells in the original tumour. We have paid special attention to the shift in the chromosomal modes and the occurrence of the original marker chromosomes. A slight shift (7b, 10b) was noticed in only two injected clones, but the two original marker chromosomes were present in all the cells examined from all the clones.

Apart from a higher incidence of breaks, gaps etc. seen in the cultured cells of the tumours formed from injected clones of cells, there was no appreciable difference in the chromosome constitutions of clones before and after injection. The cause of the breaks and gaps can only be speculative, since there were no obvious signs of virus or any other agent affecting these cells before re-injection. Further, there was no evidence of the animals being injected with or having received chromosome-breaking chemicals.

In conclusion, the experimental procedure has produced evidence for the existence of a stem line theory and the tumour being formed from a single cell; the tumours may then develop into a heterogeneous cancerous population of cells. Although it can be argued that the cloned cell was already "tumourous", the whole idea, in our opinion, of cancer being formed from a single cell, is based on the assumption that originally, minute genomic changes are involved before the more bizarre anomalies like marker chromosomes occur. Utilisation of the tissue culture system in conjunction with animal experimentation therefore provides this useful system for the study of the biology of cancer.

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Effect of Heat on the C_3H Mouse Mammary Adenocarcinoma Evaluated in Terms of Tumor Growth*†

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Abstract—*The effect of single and fractionated doses of wet heat was evaulated* in vivo *on the C_3H mouse mammary carcinoma by means of growth curves. Response of tumor growth to variable doses of single exposures indicated that a dose-response relationship exists* in vivo. *Response to four fractioned doses of heat administered at 24 hr intervals suggested that part of the original heat damage was repaired in the fractionation intervals.*

INTRODUCTION

RECENTLY there has been a renewal of interest in the possibility of utilizing heat as a modality to treat cancer. The older literature contains many references to the therapeutic applications of heat and reports with extensive literature reviews are available [1, 2]. A selective thermal sensitivity of neoplastic cells *in vitro* has been reported [1, 3, 4]. Similar levels of sensitivity of normal and neoplastic cells to heat has also been reported [5]. Quantitation of the heat effect *in vitro* revealed a sigmoidal relationship between survival and duration of heat treatment [6, 7]. Mitotic [7] and S phase cells [7, 8] seem to be most heat sensitive relative to other phases of the cell cycle. The heat inactivation rate of cells has been shown to be temperature dependent [7, 9]. Protein denaturation [7, 10] nucleic acid synthesis arrest [11] and lysosomal injury [12] have been implicated as contributing to heat induced lethality.

Data describing the effects of heat *in vivo* are not as numerous as *in vitro* studies. Some spontaneous tumors in dogs and man have been selectively destroyed by heat without damage to surrounding normal tissue but heat-sensitive tumors were more rare than heat-resistant ones [13]. The tumor growth inhibition induced by heat was found to be dose dependent, and an accompanying abscopal tumor effect has also been reported [14]. An extensive study of the effect of short wave treatment of the mouse mammary carcinoma *in situ* [2] revealed that selective cure of the tumor without extensive damage to normal tissue was possible in up to 25% of the animals treated.

In this report the response of the C_3H mouse mammary carcinoma to both single and fractionated doses of heat is presented. Evaluation was based on perturbations of tumor growth.

MATERIAL AND METHODS

The tumors were third generation transplants derived from a single spontaneous

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*In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care", as promolgated by the Committee on the guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Research, National Academy of Sciences, National Research Council.

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‡This work is to be submitted to the faculty of the Department of Radiology and Radiation Biology, Colorado State University in partial fulfillment of the requirements for the Ph.D. degree.

mammary adenocarcinoma which occurred in a female C_3H/Bi mouse.

The original tumor was removed utilizing aseptic technique. Viable appearing areas were minced in a small volume of ice-cold Hank's balanced salt solution. The mincate was diluted approximately 1:4 with Hank's solution which contained 10% glycerol and 5% fetal calf serum. Approximately 20 ml of the final product was placed in vials, frozen, and stored in liquid N_2 for future use. Another quantity was injected into the flanks of both male and female C_3H/Bi mice. Four weeks later 91% of the mice had developed tumors. These first generation transplants were harvested and stored in liquid nitrogen as described above. Vials of the first generation transplants were removed at regular intervals and injected into male and female C_3H/Bi mice to provide a source of second generation tumors. Overall tumor take rate of the second generation tumors was approximately 80%.

To prepare the experimental tumors the second generation transplants were removed utilizing aseptic technique and minced in a small volume of ice cold Hank's solution. The mincate was collected in two large plastic tubes and Hank's solution was added to the top of the tubes. The contents were mixed well and allowed to settle for 30 min to allow the larger tissue fragments to settle to the bottom of the tube. The supernatent, which contained the viable tumor cells, was removed with a syringe and needle, filtered through a Swinney filter into centrifuge tubes and centrifuged for 5 min at approximately 4500 rev/min. The supernatant was decanted and a volume of Hank's, equal to the volume of material sedimented in the bottom of the centrifuge tubes, was added and the material thoroughly mixed. This procedure resulted in an average cell concentration of approximately 2×10^4 cells/μl. Using a microliter syringe approximately 10^5 cells (5 μl) were injected into the right gastrocnemius muscle of C_3H mice. Cell counts refer to viable cells as determined by their capacity to exclude trypan blue. These mice harboring third generation transplants were utilized as the experimental animal. Tumor take rate of the third generation tumors was approximately 95%.

All heat treatments were administered by immersion of the leg into a precision controlled circulating water bath (accuracy greater than $\pm$ 0·05°C). The water temperature was 44·5°C.

Intratumor temperature was measured with a 25-gauge thermister probe positioned so that the sensitive area of the probe was located in the center of the tumor. Following immersion, the intratumor temperature rose rapidly and stabilized in 3 min at 44·1°. Peripheral areas of the tumor were warmer than the center.

Mice were anesthetized for heating by intraperitoneal injection of pentobarbital sodium at a dose of 0·065 mg/g body weight.

Growth curves were constructed for both control and heated tumors by daily calculation of tumor volume following initial detection of the tumor. Three orthogonol diameters were measured and the tumor volume calculated by the formula $V = \pi/6 \times D_1 \times D_2 \times D_3$ [15]. Mice were randomized to a treatment schedule and were given their respective heat treatment on the day the tumor reached an average diameter of 8 mm.

RESULTS

Results of the exposure of 8 mm average diameter tumors to single heat treatments of 0 min, 15 min, 39 min, 51 min and 60 min immersion are shown in Fig. 1. From this data, it can be seen that the delay in tumor growth produced by heating is dependent upon the magnitude of the heat treatment. No tumor cures were observed in any of the mice receiving heat treatment.

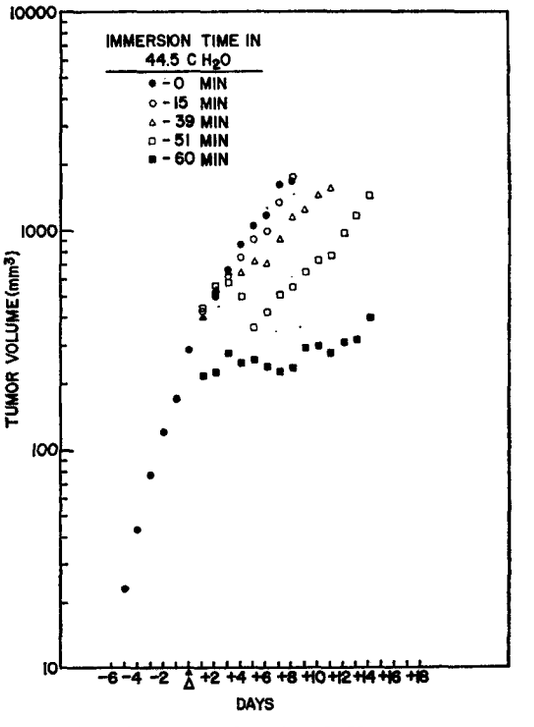

Fig. 1. Growth curves for the C_3H mouse mammary carcinoma showing the effect of single exposures of heat administered for variable amounts of time. Heat was administered at the time specified by the arrow.

Results of the exposure of 8 mm diameter tumors to a fractionated schedule of 4 treatments of 15 min duration separated by 24 hr

are shown in Fig. 2. Comparing the growth curve for the fractionated schedule with the control growth curve and a curve representing an equivalent single exposure (51 min) indicates that fractionation of the heat treatment reduces the heat effect evaluated in terms of tumor regrowth.

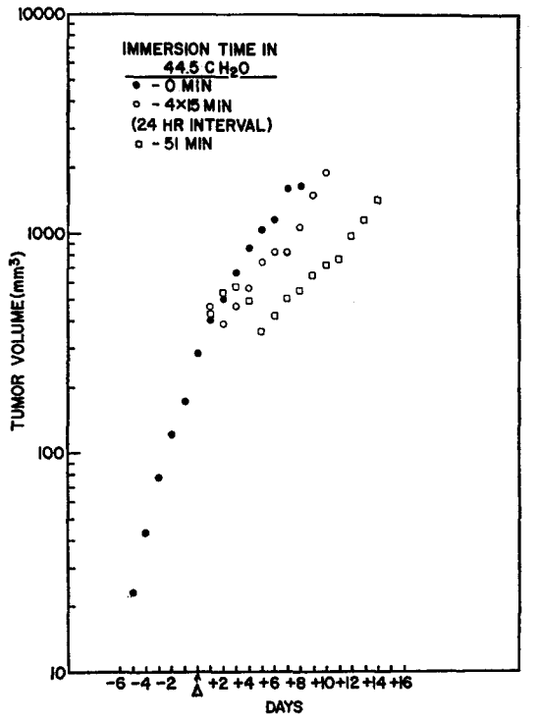

Fig. 2. Growth curves for the C_3H mouse mammary carcinoma showing the effect of four heat treatments of 15 min immersion in relation to control tumors and a comparable single dose. Heat was administered at the time specified by the arrow.

DISCUSSION

Utilization of tumor cure to evaluate the effectiveness of cytocidal agents is well accepted, however previous experiments in this laboratory have shown that doses of wet heat sufficient to cause tumor control also produce a very high percentage of leg losses [16]. The high percentage of leg loss introduces the possibility that the tumor cures observed were due to amputation rather than to cell killing. We therefore chose growth curve analysis to observe the effect of heat on the C_3H mouse mammary carcinoma.

The water temperature of 44·5°C was chosen because this was the highest temperature that could be used without producing loss of the heated leg, especially at long treatment times. At this temperature only three leg losses occurred and all were mice receiving 60 min immersions. Also, approximately 6·5% of all mice succumbed within 24 hr of heating presumably as a result of the heat treatment.

Utilization of an *in vivo* endpoint, such as perturbation of growth, to evaluate the effect of single doses of heat on the C_3H mammary carcinoma is difficult and suffers from inaccuracy. This is true because factors such as intercellular edema, division cycle delay and the cell loss factor, which may become altered by the heat treatment, exert a direct effect upon tumor volume and can not be separated from cell killing.

The growth curves demonstrating the effect of variable doses of heat certainly suggest that cell killing is dependent upon length of heat treatment as shown *in vitro* [6, 7, 9, 10]. Growth curves representing the response of the C_3H mouse mammary carcinoma to irradiation have been used to construct survival response curves [17]. The technique of developing such curves from growth curves would be useful in that parameters of cell survival response to the lethal agent could be estimated. The uncertainties of analyzing growth curves in this way have been discussed [17]. The one uncertainty that limits the application of such a technique to this study is the lack of knowledge concerning the magnitude of division delay produced by the heat. It has been shown *in vitro* that heat produces a great amount of division delay [7, 9] and this fact, of course, eliminates obtaining estimates of cell killing from growth curve changes until the effect is more fully quantitated.

The split dose administration of heat was undertaken to evaluate the effectiveness of a heat repair mechanism. Reversibility of a heat treatment of 46°C for 15 min [5] and recovery from potentially lethal heat lesions [18] have been shown *in vitro*. Repair of sublethal heat damage as well as a fluctuation in survival compatible with redistribution induced by the heat has also been shown *in vitro* [9]. A graphical representation of the previously mentioned data [9] (Fig. 3) agrees well with radiation data both *in vitro* [19] and *in vivo* [20, 21] which demonstrate a rapid rise in survival due to the repair of sublethal radiation damage followed by a dip in survival attributable to redistribution of partially synchronized cells into a more sensitive phase of the cell cycle. The results shown in Fig. 2 demonstrate that 4 treatments of 15 min, each separated by 24 hr, produces a delay in growth less than a comparable single treatment of 51 min and suggests partial but not complete repair of the damage produced by the heat. Selection of 51 min as a comparable single dose is derived from the temperature rise kinetics

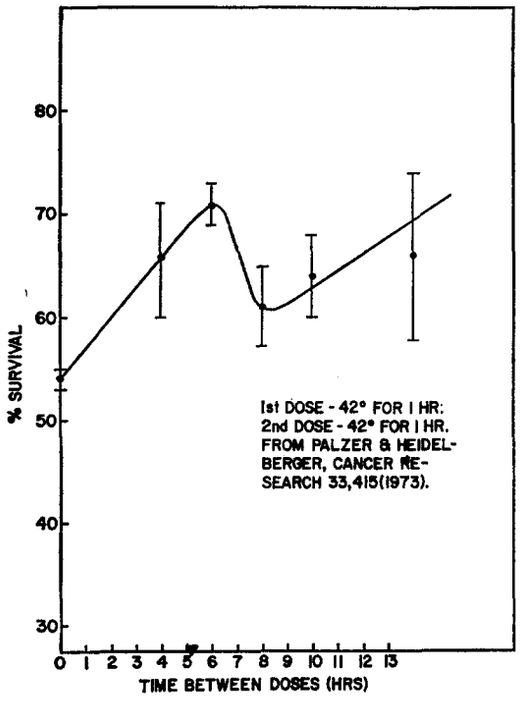

Fig. 3. *Graphical representation of previously reported data of a split dose experiment showing an initial rise in survival attributable to repair and a subsequent drop thought due to redistribution of the partially synchronized cells.*

observed when an 8 mm tumor is immersed into 44·5°C H_2O. The 15 min treatment is composed of a 3 min warm up period followed by 12 min at a constant temperature (44·1°C). Due to the fact that no knowledge of the effect of the heat during the warm up period is available, the single dose comparable to four 15 min fractions was taken as one warm up period followed by four 12 min constant periods or in other words a (3+12+12+12+12) 51 min exposure. This approach would minimize any effect of repair in relation to taking 60 min as the comparable single dose.

Due to the variable rate at which tumors regrew following heating, standard errors of the mean values have not been shown in the figures. For example, the standard error of the mean values for the control tumors ranged in magnitude from approximately 30%–40% of the mean value itself. Standard error values of treated tumors were even higher. This extreme in variation is another reason why this type of analysis may not be carried too far in terms of extracting data from it. We feel, however, that the variability does not preclude the evaluation of trends as has been done.

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Letter to the Editor

Cross-Immunity Between Chemically-Induced Sarcomas, Detected by Transplantation in Restricted Genetic Conditions*

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AS A RULE, chemically-induced murine sarcomas do not show cross-immunity in transplantation tests, contrarily to what happens in the case of virus-induced tumours [1]. The exceptions to this rule can, in many cases, be attributed to contamination of such tumours by a virus.

On the other hand, we reported that the immune reactions against the tumour associated transplantation antigens (T.A.T.A.) of strain-specific chemically-induced sarcomas can be more intense in certain semi-syngeneic F_1 hybrids than in the strain of origin, in some tumour–host systems [2, 3].

We decided to try to detect an eventual cross-immunity in some tumour combinations, in such "hyperreactive" F_1 hybrids, by comparison with syngeneic hosts. The results presented here, which concern a system comprising 3 long transplanted sarcomas (from 30 to 80 graft passages), show the existence of a cross transplantation immunity, detectable in at least one system.

Three sarcomas, TP8, TP4 and TP10 [3], were chemically induced in the inbred Swiss/B murine strain. TP8 is highly immunogenic, whereas the two others are of lower antigenicity [3]. Although TP8 is less antigenic (and more rapidly growing) at its last graft passages than it was at its first ones, it can be used as a test tumour, sensitive enough to T.A.T.A. specific immunizations throughout all the experiments described here.

Immunizations are performed by subcutaneous injections of one or two fragment(s) of tumour, calibrated 1 mm^3 and taken from heavily irradiated tumours (8000 rad, given *in vivo*). Challenges with viable tumour consist of fragments (1 mm^3) injected subcutaneously in the dorsum. Control animals are either untreated, or pretreated with either 5×10^5 splenic cells or 1 mm^3 thigh muscle fragments from healthy Swiss/B donors. Immunizations and pretreatments are given twice: four weeks and two weeks before challenge. These experiments were reproduced several times in both syngeneic Swiss/B, (Swiss/B × C3H/He) F_1 and (XVII × Swiss/B) F_1 hybrid mice. The results, expressed as the number of tumour takes divided by the number of grafted mice, are highly reproducible. They are pooled in Fig. 1.

It can be observed that specific immunization (i.e. immunization with TP8 and challenge with TP8) always gives a strong immunity. As previously shown [2, 3], this immune reaction is more intense in the hybrids than in the Swiss/B strain of origin. In two cases, the strict specificity of this reaction is demonstrated by the fact that immunizations with other tumours (i.e., a mixture of TP4 + TP10) are not able to immunize Swiss/B and (XVII × Swiss/B) F_1 hybrids against TP8. However, in (Swiss/B × C3H) F_1 hybrids, this mixture of TP4 + TP10 also elicits a strong immunity against TP8, while normal Swiss/B tissues do not.

It is further shown that each of these individual tumours can elicit some cross-reaction towards another, in the (Swiss/B × C3H) F_1 hybrids (Table 1).

In summary, cross-immunity between 3 chemically-induced Swiss/B specific tumours is demonstrable in one genetic system [(Swiss/B × C3H) F_1] whereas it cannot be detected in two

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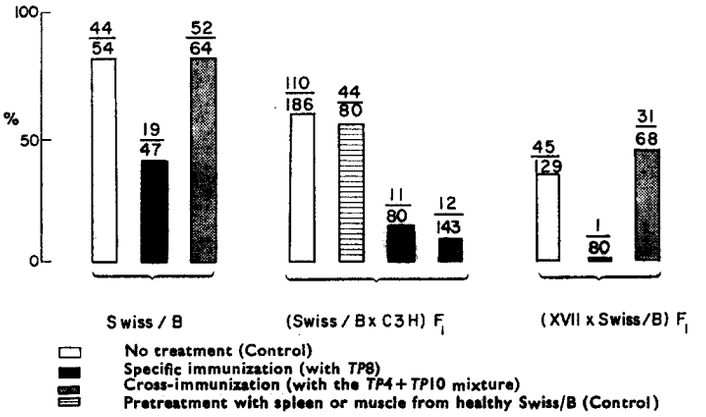

Fig. 1. Tumour incidence (per cent) after grafting TP8 in Swiss/B, (Swiss/B × C3H) F_1 and (XVII × Swiss/B) F_1 hybrids, as a function of their pretreatment. The fractions indicate the number of mice killed by TP8, divided by the number of grafted mice. Each of these values represents the pool of 3–12 similar, mutually confirming, separate experiments. All transplantations of viable tumour are performed with a calibrated fragment.

Table 1. Testing cross immunity between TP4, TP8 and TP10 in (Swiss/B × C3H) F_1 hybrids

Immunization with	Challenge with (one fragment of viable tumour)	Tumour incidence (%)	P*
Nothing (control)	TP8	110/186 (59%)	—
TP4	TP8	22/77 (28%)	< 0·001
TP10	TP8	22/69 (32%)	< 0·001
Nothing (control)	TP4	14/20 (70%)	—
TP8	TP4	3/21 (14%)	< 0·01
Nothing (control)	TP10†	29/31 (94%)	—
TP8	TP10†	18/30 (60%)	< 0·01

*Calculated by χ^2 corrected for continuity; each experimental group is compared with its control.
†Challenged with 5×10^5 cells instead of a fragment.

other genetic backgrounds [(Swiss/B and (XVII × Swiss/B) F_1], where specific immunity is fully demonstrable.

A possible explanation of this fact is that these tumours contain at least two sorts of T.A.T.A.: a first sort, unique for of each individual tumour, which is easily demonstrable in all cases, and a second sort, possibly due to contamination with a common virus [4] (and probably weaker than the first one), which is recognized only in those animals which are not genetically tolerant to them.

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Letter to the Editor

Low Dose Tolerance Preventing Tumor Immunity*

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THE IMMUNE surveillance concept [1, 2, 3] states that the body's immune system plays a decisive role in the defence against malignancies. Direct proof of this hypothesis is still lacking. The two major difficulties which encounter its acceptance are: (1) immune suppression in man does not lead to a general enhancement of tumor incidence, but rather to a selective increase in reticulum cell sarcomas and lymphomas [4], and (2) tumor latency in mice is unrelated to the overall immunocompetent status of the host [5]. Since tumorigenesis starts from very low numbers of cells, a theoretical objection to the immune surveillance theory is possible. Low concentrations of tumor cells or tumor antigens, too small to elicit an immune reaction, might induce immunological tolerance in the initial phase of neoplastic proliferation before immunogenic doses of antigen have been accumulated. The experiments reported here have been set up to test whether low dose tolerance [6] can be induced and might counteract tumor immunity.

Balb/c Str. mice were inoculated three times at weekly intervals with various amounts of lethally irradiated Balb/c mastocytoma cells kept frozen between the injections. The animals were challenged with 1×10^5 living mastocytoma cells, which from preliminary experiments were expected to induce tumors in 50% of the animals within a month. Figure 1 shows the mean body weight of the animals of the groups. Significant immunity has been reached by pretreatment with 10^4–10^7 irradiated tumor cells. The control animals challenged with living tumor cells show an increase in body weight which is, however, not as high as in the animals pretreated with 10^2–10^3 irradiated cells. Individual data are given in Table 1. Animals pretreated with subimmunogenic doses of antigen have a significantly higher incidence of tumors than the animals receiving one or 10 irradiated cells. In another experiment (Table 2), the animals were pretreated for 7 weeks and challenged with 3×10^4 living tumor cells, a dose of tumor cells too low to induce any mastocytomas in the controls within a month. However, tumors did arise in animals pretreated with 10^1–10^3 irradiated tumor cells. The

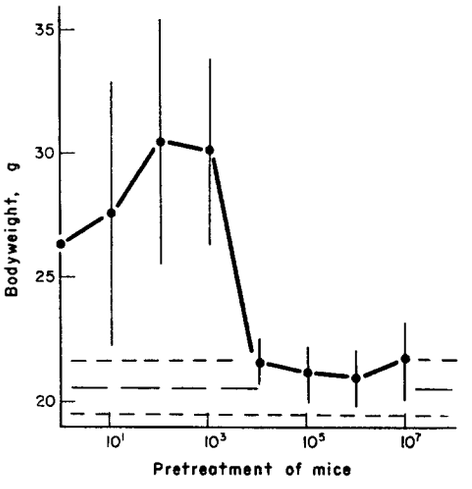

Fig. 1. Tumor growth in Balb/c Str. mice pretreated with subimmunogenic and immunogenic doses of irradiated mastocytoma cells. Groups of 10 animals each were injected with graded doses (10^0–10^7) of irradiated (1400 rads) tumor cells 3 times at weekly intervals and challenged with 1×10^5 living mastocytoma cells. Animals were weighed 15 days later. Mean body weight (●) and standard deviations (vertical bars) are given. Mean body weight (—— ——) and standard deviations (– – – –) of control animals, which received neither irradiated nor living tumor cells, are indicated.

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strongest growth was found in animals pretreated with 10^2 cells; this is exactly the dose which was optimal for tumor development in the experiment described in Table 1. In both experiments, we have been unable to demonstrate circulating cytotoxic or enhancing [7, 8] antibodies in the animals of the different groups (R.M. and E.K., to be published). Cytotoxicity as well as unresponsiveness could be demonstrated in an *in vivo* ^{51}Cr-release assay (Table 3); the cytotoxicity seems to be mediated *in vitro* by macrophages (R. M. and E. K., to be published). In experiments summarized in Table 4, we have inoculated untreated animals with various concentrations of living tumor cells, and the total tumor incidence was followed up for over two months. The tumor frequency gradually decreases from 100 per cent after injection of 6×10^6 living mastocytoma cells until a plateau of 20 per cent is reached when 10^4–10^3 cells are injected. If, however, less than 10^3 cells are injected, the frequency of tumors increases again. This preferential take of very low numbers of tumor cells has been described

Table 1. *Tumor incidence in Balb/c mice pretreated with irradiated mastocytoma cells*

	Body weight (g)	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39
Group	Pre-treatment (irradiated cells)	Challenge (1×10^5) living cells)																					
1	0	–	2	2	3	3	4	1															
2	$10^0 - 10^1$	+		2	1	3	1	2				2		2	1		2	3					1
3	$10^2 - 10^3$	+				1	1			1		1	1	1	3	4	2	1	1	2			
4	$10^4 - 10^6$	+		5	6	11	11	6															
					No tumors					Tumors													

Animals were injected intraperitoneally with various concentrations of Balb/c mastocytoma cells, irradiated with a lethal dose of 1400 rad. Batches of the diluted cells were frozen in balanced salt solution and animals were reinjected with the same dose once a week for 3 weeks. They were challenged one week later uniformly with 1×10^5 living mastocytoma cells injected into the peritoneal cavity. Tumor growth was measured by weighing the animals. The data from day 15 are given. The Balb/c mastocytoma was given to us originally by Dr. Hilz, Institute for Physiological Chemistry, University of Hamburg, and maintained in ascites form by serial transfer. It is strain-specific and has surface antigens which can be detected by a rabbit antiserum absorbed with Balb/c spleen cells. Balb/c Str. mice originate from the Salk Institute, La Jolla, USA, and were bred by brother–sister mating in our institute. Only Balb/c ♀, 6–8 weeks old, were used for the experiments. Groups 2 and 3 are significantly different at $P < 0{\cdot}03$ (χ^2-test).

Table 2. *Tumor incidence in Balb/c mice pretreated with irradiated mastocytoma cells*

	Increase in body weight (g)	0	1	2	3	4	5	6	7	8	9	10–16
Group	Pre-treatment (irradiated cells)	Challenge (3×10^4 living cells)										
1	10^0	+	9	2	1							
2	10^1	+	10	1			1*			1*		1*
3	10^2	+	6	4								3*
4	10^3	+	11	2						1†		
5	10^4	+	10	4								
6	10^5	+	12	2								
7	10^6	+	9	4								
8	—	+	21	7	1	1‡						
					No tumors		Tumors					

*On day 18, all dead by day 22.
†On day 28, dead by day 32.
‡This animal was definitely tumor-free; it started with a low weight at the beginning of the experiment and weighed 22·6 g on day 28 and remained at this weight.
All other weights determined on day 28.
The experimental procedure was essentially the same as in Table 1 except that the animals were pretreated by weekly injections for 7 weeks. They were uniformly challenged with 3×10^4 living mastocytoma cells.
Groups 2, 3 and 4 are significantly different from group 8 at $P < 0{\cdot}03$ (χ^2-test).

Table 3. In vivo *cytotoxicity in Balb/c mice pretreated with irradiated mastocytoma cells*

Group	Pretreatment (irradiated cells)	Challenge (1×10^5 living cells)	Radioactivity recovered (counts/min)	Mean radioactivity recovered (counts/min)
1	0	–	10705, 9228, 7556, 5427	8223 ± 2098
2	0	+	7509, 6534, 5236, 3074, 2525	4974 ± 2028
3	2×10^2	+	11322, 10577, 8748, 8230, 4367	8643 ± 2555
4	1×10^7	+	3073, 593, 529, 520, 446	1029 ± 1085

Balb/c Str. mice were injected with saline or 2×10^2 or 1×10^7 irradiated (1400 rad) mastocytoma cells 7 times at weekly intervals. The animals were then left for 35 days and challenged afterwards with 1×10^5 living mastocytoma cells. Seven days later five animals per group were injected intraperitoneally with $2{\cdot}5 \times 10^4$ living mastocytoma cells, labelled with Na $^{51}CrO_4$ (44000 counts/min per $2{\cdot}5 \times 10^5$ cells). Twenty-four hours later the cells were recovered from the peritoneal cavity, washed three times, and the radioactivity in the cell sediment was determined for each individual animal. Mean and standard deviation from the mean are also given. Resting the mice for 35 days before challenge with living cells served at the same time as control for the experiments in Tables 1 and 2, showing that pretreatment with low or high doses of irradiated cells does not *per se* lead to tumors. The tumors arise from the challenging cells. Groups 2 and 3 are significantly different at $P < 0{\cdot}05$ and groups 2 and 4 at $P < 0{\cdot}01$ (t-test).

as "sneaking through" phenomenon [9, 10, 11]. It may not be by chance that this occurs in our experiments with exactly the same numbers of cells which induce increasing susceptibility to challenging doses of tumor cells.

Our data show that antigen in concentrations too low to immunize the host against the tumor is able to induce a status which facilitates the growth of tumor cells. The extremely small and subimmunogenic number of tumor cells involved suggests a clear discrimination between this process and enhancement [7, 8] by antibodies. The situation is comparable with the induction of low dose tolerance shown for humoral [6] and homograft reactions [12].

Induction of tolerance by low doses of tumor antigens would explain, among other findings, two as yet poorly understood observations: (1) the lack of correlation between tumor latency and general immunocompetence, and (2) the preferential manifestation of tumors in animals injected with low numbers of cells considered from limiting dilution experiments to be too low for the tumor to grow. If our concept is correct, then immunological intervention in cancer is primarily the problem of preventing or breaking low dose immunological tolerance.

Table 4. *Incidence of Balb/c mastocytomas in Balb/c animals injected with a single dose of living cells*

Group	Number of living cells	Tumor incidence	Percentage of tumors
1	2×10^1	2/12	16
2	1×10^2	7/22	32
3	5×10^2	7/12	58
4	1×10^3	2/12	17
5	$2{\cdot}5 \times 10^3$	3/12	25
6	6×10^3	2/10	20
7	8×10^3	3/22	14
8	1×10^4	6/30	20
9	$1{\cdot}6 \times 10^4$	3/12	25
10	$3{\cdot}2 \times 10^4$	10/22	45
11	6×10^4	13/22	59
12	1×10^5	58/77	75
13	$1{\cdot}25 \times 10^5$	19/22	86
14	$2{\cdot}5 \times 10^5$	11/12	92
15	5×10^5	19/20	95
16	6×10^6	20/20	100

Tumor incidence was followed up over two months and in some cases up to 4 months. No new tumors came up after 2 months.

Tumor frequency after low dose (groups 1–3) and after medium dose (groups 4–8) inoculum shows a significant difference at an error probability $P < 0{\cdot}025$ (χ^2-test).

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Letter to the Editor

Four Drug Sequential Chemotherapy for Metastatic Neuroblastoma

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INTRODUCTION

IN PATIENTS with disseminated neuroblastoma, administration of 3 drugs in sequence (vincristine, cyclophosphamide and daunomycin) resulted in a median survival of 18 months, but did not affect the cure rate [1]. The addition of a fourth drug, dactinomycin, and substitution of adriamycin for daunomycin was incorporated in a treatment program in an attempt to improve the response rate and the cure rate.

Between November 1970 and December 1972, 10 children with disseminated neuroblastoma were entered in this study. Clinical–pathologic staging was determined after tumor resection or biopsy [1]. After surgery (8/10), supervoltage radiation therapy (7/10) was applied to the primary tumor site and to large or critically located metastatic deposits. Concomitant chemotherapy was given as long as the patient's peripheral white blood cell (WBC) count remained greater than 1500 cells/mm^3. One course of chemotherapy consisted of adriamycin, dactinomycin, vincristine and cyclophosphamide. On days 1–5 dactinomycin 450 ug/m^2 per day via rapid i.v. injection was given followed by adriamycin 20 mg/m^2 per day via rapid i.v. injection on days 20, 21, 22, 38, 39, and 40. On day 55 vincristine 1·5 mg/m^2 via rapid i.v. injection followed by cyclophosphamide (1200 mg/m^2 or 40 mg/kg in 10 ml of water) via rapid i.v. injection followed by electrolyte solution (400 ml/m^2 at 100 ml/hr) was given. On days 62 and 69 the patients received vincristine (2·0 mg/m^2) via rapid i.v. injection, and on day 76 vincristine 2·0 mg/m^2 via rapid i.v. injection followed by cyclophosphamide (1200 mg/m^2 or 40 mg/kg in 10 ml of water) via rapid i.v. injection, followed by electrolyte solution (400 ml/m^2 at 100 ml/hr) was administered.

Five patients received previous chemotherapy with vincristine, cyclophosphamide, and daunomycin and were considered resistant to these agents.

Twenty-four hour urinary excretion of vanilmandelic acid (VMA) and catecholamines was measured before therapy. Successive collections at daily, weekly, or greater intervals were measured, and the values were compared with those from age-matched control patients [2, 3, 4]. Urinary cystathionine excretion was determined in 8 children during the course of their disease (Table 1) [5].

Patients were monitored for drug induced toxicity with determination of hemoglobin, WBC and platelet levels once weekly or more frequently if necessary. The extent of disease was determined with skeletal roentgenograms and bone marrow aspiration. The responses and their duration were evaluated according to the Karnofsky categories of response [5].

Nine patients had recurrence of disease during or following therapy and died (Table 1). Clinically beneficial responses occurred in 5 patients and lasted 4 to 16 months. The median survival time was 20 months (range seven to 40 months). Most patients who ceased

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Table 1. Response to chemotherapy consisting of dactinomycin, adriamycin, cyclophosphamide and vincristine in 10 patients with disseminated neuroblastoma

Patient No.	Age at diagnosis (months)	Disease stage at diagnosis	Number of chemo-therapy courses	VMA and catecholamines levels* before therapy	VMA and catecholamines levels* after therapy	Cystathio-nine urinary excretion u mol/day†	Responses category (duration)	Survival time to death (months)
1	11	IV	1	A	A	122	O–A (1 mo.)	23
2	11	IV	1	A	A	1562	II (4 mos.)	7
3	18	III	2	A	A	205	I–A (10 mos.)	20
4	19	IV	2	N	A		I–A (4 mos.)	18
5	24	IV	4	N	N	0	I–A (16 mos.†)	
6	26	III	1	N	N	0	O–O	16
7	27	IV	1	A	A	398	O–O	7
8	34	IV	2	A	A	23	I–A (6 mos.)	28
9	55	IV	1	N	N		O–O	25
10	130	IV	2	N	N	0	I–A (10 mos.)	40

*A = abnormal: N = normal—for values see text.
†The upper limits of normal urinary cystathionine excretion were previously determined to be 17 u mol/day [6].

to or did not respond to this therapeutic regimen subsequently received blood and platelet transfusion was severe enough to require platelet transfusion in one patient. The nadir of leukopenia

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MOLECULAR BASE OF MALIGNANCY; NEW CLINICAL AND THERAPEUTIC EVIDENCES

Vienna, October 30–31, 1974

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