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Depletion of Proteins of Phasic and Tonic Muscles in Tumour-bearing Rats*†

CATHERINE M. CLARK and G. A. J. GOODLAD

Department of Biochemistry, St. Salvator's College, University of St. Andrews, St. Andrews, U.K.

THE PRESENCE of a growing tumour has a pronounced effect on the protein metabolism of the host, causing a general wasting of tissues which is particularly reflected in a loss of muscle protein [1]. Whether this loss of protein is due to a decreased rate of synthesis or an increased rate of catabolism has not been established.

In muscles of normal animals, the rate of protein synthesis is dependent on the type of fibre constituting the muscle, tonic muscles with predominantly dark or red fibres exhibiting a more rapid rate of protein synthesis than phasic muscles containing a high proportion of white fibres [2, 3] and therefore the response of these two types of muscle to the presence of a growing tumour might be expected to differ. Accordingly in the present work a study was made of changes occurring in the amounts of various protein fractions of gastrocnemius and soleus muscles and in the amino acid incorporation into these fractions in rats bearing the Walker 256 carcinoma.

METHODS

Female Wistar rats of fasting body weight, 200–210 g, were injected subcutaneously under aseptic conditions with 0·2 ml of a suspension of Walker 256-carcinoma cells. These animals were paired with control animals on the basis of initial body weight. The control and inoculated rats were housed individually and fed daily 14 g finely ground rat cake (North Eastern Agricultural Co-operative Ltd.). Both sets of animals consumed their daily food allowance throughout the experiment. After 12–14 days, by which time the tumour had grown to 20–33 g, the animals were killed in the fasting state by stunning and decapitation. The gastrocnemius and soleus muscles were excised, dissected free of nerves and connective tissue and weighed.

Some of the rats were injected intraperitoneally 12 hr before sacrifice with L-^{14}C-U-valine (260 mC$_i$/mM) obtained from the Radiochemical Centre, Amersham, at a dose of 20 μC$_i$/kg body weight.

Muscle proteins were separated into myofibrillar, sarcoplasmic and extracellular fractions by the method of Dickerson [4]. Muscle ribonucleic acid (RNA) was estimated by the method of Margreth and Novello [5] and deoxyribonucleic acid (DNA) according to Burton [6]. The proteins were estimated by the method of Gornall, Bardawill and David [7] or by micro-Kjeldahl nitrogen [8].

Protein samples for counting were washed 5–6 times with 10% (w/v) trichloracetic acid at 0°C, twice with ethanol and twice with ether. Protein was dissolved in 0·3 N NaOH, volume of digest was adjusted to give a final normality of 0·05 N with respect to NaOH and aliquots of this solution counted in a Beckman Scintillation

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Counter (SL100) with a 66% efficiency using NE220 (Nuclear Enterprises, U.K., Ltd.) scintillator. Differences in quenching were corrected by the external standard method.

Statistical differences between tumour-bearing and control animals were tested using Student's paired *t*-test.

RESULTS

Table 1 shows that in the tumour-bearing rat both the gastrocnemius and soleus muscles lost weight. From a comparison of the values of the ratio of gastrocnemius weight to soleus weight the relative weight loss of the soleus was significantly less than that of the gastrocnemius. In neither muscle was the weight loss associated with any significant alteration in the DNA content, a finding which suggests that the mean nuclear number was not altered by the action of the tumour.

The total protein content of both muscles was significantly reduced in the presence of the tumour but the value of the ratio of protein-content of gastrocnemius/protein-content of soleus was lower in tumour-bearing animals than in control animals, indicating that the gastrocnemius was being depleted of protein to a greater extent than was the soleus. Compared to levels in control animals, in tumour-bearing rats the amount of RNA per mg DNA was significantly reduced in the gastrocnemius but was not altered in the soleus.

The data on the incorporation of ^{14}C-valine into total muscle proteins is shown in Table 2.

Table 1. *The effect of the Walker* 256 *carcinoma on the weight, DNA, RNA and protein content of right gastrocnemius and paired soleus muscles*

	Control	Tumour-bearing	*P**
wt. gastrocnemius (wet weight in g) per 100 g initial body weight	0·61 ±0·006	0·50 ±0·016	<0·001
wt. soleus (wet weight in g) per 100 g initial body weight	0·096±0·002	0·086±0·003	<0·01
wt. gastrocnemius/wt soleus	6·40 ±0·14	5·83 ±0·13	<0·01
mg total protein per gastrocnemius per 100 g initial body weight	120·3 ±1·30	96·1 ±3·90	<0·001
mg total protein per soleus per 100 g initial body weight	17·05 ±0·275	14·82 ±0·576	<0·01
total gastrocnemius protein/total soleus protein	7·06 ±0·077	6·50 ±2·08	<0·025
mg DNA per gastrocnemius per 100 g initial body weight	0·523±0·010	0·506±0·017	N.S.
mg DNA per soleus per 100 g initial body weight	0·154±0·004	0·142±0·010	N.S.
mg RNA/mg DNA (gastrocnemius)	2·07 ±0·143	1·71 ±0·113	<0·02
mg RNA/mg DNA (soleus)	1·73 ±0·061	1·66 ±0·057	N.S.

Results are expressed as the mean±S.E. of 9 rats except for the data on muscle DNA where the results are the mean ±S.E. of 7 rats. Mean tumour weight was 26·3±2·52 g.

*In this and following tables, *P* refers to the statistical significance of the differences between control and tumour-bearing rats.

Table 2. *The effect of the Walker* 256 *carcinoma on the incorporation of* ^{14}*C-valine into total gastrocnemius and soleus protein*

	Counts/min/mg protein		Specific activity gastrocnemius protein
	Gastrocnemius	Soleus	Specific activity soleus protein
Control	51·4±1·08	69·0±4·79	0·75±0·046
Tumour-bearing	32·2±1·48	65·3±1·46	0·49±0·012
P	<0·02	N.S.	<0·02

Results are expressed as the mean±S.E. of 3 rats. Mean tumour weight was 29·9±3·76 g.

Table 3. The effect of the Walker 256 carcinoma on amounts of myofibrillar, sarcoplasmic and extracellular protein in gastrocnemius muscle

	mg protein per g gastrocnemius			mg protein/gastroc./100 g initial body wt.		
	Myofibrillar	Sarcoplasmic	Extracellular	Myofibrillar	Sarcoplasmic	Extracellular
Control	134·4±2·75	40·65±1·75	14·58±1·15	80·53±1·85	24·44±1·47	8·78±0·823
Tumour-bearing	123·7±1·99	49·33±2·83	19·42±1·19	57·00±2·49	22·61±1·24	8·91±0·510
P	<0·005	<0·01	<0·001	<0·001	N.S.	N.S.

Results are expressed as the mean±S.E. of 6 rats. Mean tumour weight was 29·4±2·32 g.

In tumour-bearing rats the specific activity of gastrocnemius protein was reduced by approximately 37% whereas the specific activity of soleus protein was not significantly affected.

When the proteins of the gastrocnemius were separated into myofibrillar, sarcoplasmic and extracellular fractions it was found that in tumour-bearing rats there was a significant decrease in the amount of myofibrillar protein per g muscle while the concentrations of sarcoplasmic and extracellular protein increased (Table 3). The total amount of myofibrillar protein per gastrocnemius was also significantly decreased in the tumour-bearing animals but the total amounts of sarcoplasmic and extracellular proteins were not significantly altered from control levels. Thus it would appear that the action of the tumour on gastrocnemius protein is directed to a greater extent at the proteins of the contractile system. This was confirmed when the effect of the tumour on the incorporation of ^{14}C-valine into myofibrillar and sarcoplasmic protein was compared. In the tumour-bearing animals, the specific activity of the myofibrillar proteins was reduced by approximately 50% while that of the sarcoplasmic protein was reduced by 26·6% (Table 4). When a similar study was made on the soleus of tumour-bearing animals no change was found in the concentration of any of the protein fractions in the tumour-bearing rats but the total content of myofibrillar protein was reduced (Table 5). The specific activity of the myofibrillar and sarcoplasmic fractions of soleus were not altered significantly from control levels in the tumour-bearing animals (Table 6).

A comparison was made of the uptake of amino acids into protein of the medial and lateral heads of the gastrocnemius and Table 7 shows that although the specific activity of the total protein of both the medial and lateral head was reduced in the tumour-bearing rats, that of the latter was reduced to a greater extent.

DISCUSSION

Compared to the gastrocnemius, the soleus muscle suffered a relatively smaller loss in weight and total protein in the tumour-bearing rat. Incorporation of labelled amino acids into soleus protein appeared to be unaffected by tumour growth when incorporation into gastrocnemius protein was markedly inhibited. Soleus is composed predominantly of dark or red fibres whereas the gastrocnemius is composed mainly of light or white fibres [9].

Thus it would appear that red muscle-fibres are not affected by tumour growth to the same extent as white. However the degree to which they have the ability to resist the action of the tumour appears to vary since the amino acid incorporation into the proteins of the medial head of the gastrocnemius, which is composed of red fibres, is decreased in tumour-bearing animals whereas incorporation into the proteins of the soleus in these animals is unaffected. Inhibition of uptake of ^{14}C-valine into the proteins of the lateral head, which is composed of white fibres, was nevertheless more pronounced (Tables 6, 7).

The greater resistance of the soleus to the cachectic effect of the tumour is in keeping with its behaviour in other circumstances where depletion of muscle protein is occurring. Thus in adult mice fed an inadequate food intake, the soleus lost less weight than various phasic muscles and the mean diameter of its fibres was not altered although those of the phasic muscles were considerably reduced [10]. In hypophysectomised rats given cortisone, plantaris muscle, which consists predominantly of white fibres, suffered a greater weight loss and showed a larger increase in rate of protein catabolism than did the soleus [11, 12].

The loss of total protein from the soleus of the tumour-bearing rat in the absence of a measurable change in amino acid incorporation suggests that loss of protein in this muscle

Table 4. The effect of the Walker 256 carcinoma on incorporation of ^{14}C-valine into myofibrillar and sarcoplasmic proteins of rat gastrocnemius

	Counts/min/mg protein		Specific activity myofibrillar protein
	Myofibrillar	Sarcoplasmic	Specific activity sarcoplasmic protein
Control	54·8±3·38	92·1±5·72	0·60±0·050
Tumour-bearing	26·9±0·89	67·5±2·89	0·40±0·006
P	<0·02	<0·05	<0·02

Results are expressed as the mean±S.E. of 3 rats. Mean tumour weight was 29·9±3·76 g.

Table 5. The effect of the Walker 256 carcinoma on the amounts of myofibrillar, sarcoplasmic and extracellular protein in soleus muscles.

	mg protein per g soleus			mg protein/soleus/100 g initial body wt		
	Myofibrillar	Sarcoplasmic	Extracellular	Myofibrillar	Sarcoplasmic	Extracellular
Control	117·6±2·89	36·2±1·88	24·2±4·40	11·8±0·31	3·6±0·19	2·4±0·46
Tumour-bearing	115·6±4·43	37·2±1·49	22·3±0·92	10·1±0·54	3·3±0·16	2·0±0·13
P	N.S.	N.S.	N.S.	<0·025	N.S.	N.S.

Results are expressed as the mean±S.E. of 4 rats. Mean tumour weight was 29·4±2·32 g.

Table 6. The effect of the Walker 256 carcinoma on the incorporation of ^{14}C-valine into myofibrillar and sarcoplasmic proteins of rat soleus

	Counts/min/mg protein		Specific activity myofibrillar protein
	Myofibrillar	Sarcoplasmic	Specific activity sarcoplasmic protein
Control	70·7±3·36	116·1±14·81	0·62±0·045
Tumour-bearing	64·9±9·29	113·4±11·97	0·57±0·024
P	N.S.	N.S.	N.S.

Results are expressed as the mean±S.E. of 4 rats. Mean tumour weight was 25·8±3·05 g.

Table 7. The effect of the Walker 256 carcinoma on the incorporation of ^{14}C-valine into total protein of medial and lateral heads of gastrocnemius

	Counts/min/mg protein		Specific activity of medial head
	Medial head	Lateral head	Specific activity of lateral head
Control	73·41±4·73	56·85±2·14	1·30±0·099
Tumour-bearing	39·42±4·40	18·85±1·86	2·10±0·060
P	<0·025	<0·001	<0·01

Results are expressed as the mean±S.E. of 4 rats. Mean tumour weight was 25·8±3·05 g.

may be mainly due to an increase in the rate of catabolism of protein. This was also indicated in the fractionation studies on this muscle which showed that only the myofibrillar protein showed a decrease in the tumour-bearing animals and there was no evidence of any decrease in amino acid incorporation into this fraction. In the case of the gastrocnemius the loss of total protein was accompanied by a decreased rate of amino acid incorporation. In the absence of data on any effect of the tumour on the entry of amino acids into the free amino acid pools of muscle, it is not possible in the present instance to say whether this decreased labelling of protein in gastrocnemius is due to a decrease in the rate of protein synthesis *per se*. However the decrease in the ratio of RNA/DNA and the differential effect of the tumour on the uptake of ^{14}C-valine by myofibrillar and sarcoplasmic fractions of this muscle suggests that some inhibition of protein synthesis may be involved in this instance.

SUMMARY

In rats bearing the Walker 256 carcinoma both gastrocnemius and soleus muscles exhibited a loss of protein, the gastrocnemius being more severely depleted than the soleus. The ratio of RNA/DNA was decreased in the gastrocnemius but was unchanged in the soleus. In both muscles the loss of protein appeared to be mainly from the contractile elements. The incorporation of ^{14}C-valine into the contractile and sarcoplasmic proteins of the gastrocnemius was decreased in the tumour-bearing animals, the incorporation into the contractile proteins being reduced to a relatively greater extent. The tumour had no significant effect on ^{14}C-valine incorporation into either protein fraction of the soleus. Incorporation into proteins of the medial head of the gastrocnemius was reduced to a lesser extent than incorporation into proteins of the lateral head in the tumour-bearing rats.

REFERENCES

1. C. D. Sherman, Jr., J. J. Morton and G. B. Mider, Potential sources of tumour nitrogen. *Cancer Res.* **10,** 374 (1950).
2. A. L. Goldberg, Protein synthesis in tonic and phasic skeletal muscles. *Nature (Lond.)* **216,** 1219 (1967).

3. F. A. SHORT, Protein synthesis by red and white muscle *in vitro*: effect of insulin and animal age. *Am. J. Physiol.* **217,** 307 (1969).
4. J. W. T. DICKERSON, The effect of growth on the composition of avian muscle. *Biochem. J.* **75,** 33 (1960).
5. A. MARGRETH and F. NOVELLO, Observations on the chemical determination and distribution of ribonucleic acid in several striated muscles. *Expl. Cell Res.* **35,** 38 (1964).
6. K. BURTON, A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem. J.* **62,** 315 (1956).
7. A. G. GORNALL, C. J. BARDAWILL and M. M. DAVID, Determination of serum proteins by means of the biuret reaction. *J. biol. Chem.* **177,** 751 (1949).
8. J. S. MA and G. ZUAZAGA, Micro-Kjeldahl determination of nitrogen. A new indicator and an improved rapid method. *Ind. Engng. Chem. Analyt. Edn.* **14,** 280 (1942).
9. F. A. SRETER and G. WOO, Cell water, sodium and potassium in red and white mammalian muscle. *Am. J. Physiol.* **205,** 1290 (1963).
10. R. W. D. ROWE, Effect of low nutrition on size of striated muscle fibres in the mouse. *J. exp. Zool.* **167,** 353 (1968).
11. A. L. GOLDBERG and H. M. GOODMAN, Relationship between cortisone and muscle work in determining muscle size. *J. Physiol.* **200,** 667 (1969).
12. A. L. GOLDBERG, Protein turnover in skeletal muscle II. Effects of denervation and cortisone on protein catabolism in skeletal muscle. *J. biol. Chem.* **244,** 3223 (1969).

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Factors Determining Cell Killing by Chemotherapeutic Agents *In Vivo*—II.

Melphalan, Chlorambucil and Nitrogen Mustard

L. M. VAN PUTTEN and P. LELIEVELD
Radiobiological Institute TNO, Rijswijk Z.H., The Netherlands

INTRODUCTION

A STUDY on the mechanism of action of cyclophosphamide on haemopoietic stem cells *in vivo*, as measured by their capacity to form colonies upon inoculation into lethally irradiated isogenic recipients, was described earlier [1]. The resulting dose-effect curves for stem cell survival led us to postulate a mechanism which could explain the cycle specificity of the action of difunctional alkylating agents. Our finding of a lower sensitivity of normal haemopoietic stem cells with increasing time interval between drug administration and assay was interpreted to be associated with repair of the alkylation lesion in the cell DNA before DNA synthesis leads to irreversible damage. The absence of a similar repair effect after delayed assay of rapidly-proliferating haemopoietic stem cells was in accordance with this hypothesis since for these cells the duration of the time available for repair (that is the time between alkylation of DNA and the next DNA synthesis period) is independent of the interval between drug administration and assay for surviving cells.

In support of the hypothesis a number of data from the literature were cited which indicated the occurrence of repair of alkylation lesions in DNA [2, 3] and the protection afforded by blocking DNA synthesis in sulphur mustard-exposed micro-organisms [4]. However, there remained some unsolved problems. If our findings on the effect of cyclophosphamide were to be interpreted as generally valid for bis-chloro-aethyl-amines, the finding of differential sensitivity towards normal and rapidly-proliferating haemopoietic stem cells should also be obtainable with other agents of this type. This was not in agreement with the classification of nitrogen mustard by Bruce and Valeriote [5, 6] as a type I agent, that is, an agent which is equally effective on resting cells and on cells in mitotic cycle. For these reasons it seemed useful to expand our studies to include a number of bis-chloro-aethyl-amines. The results of these studies are presented in this report. They indicate a similarity of action for all bis-chloro-aethyl-amines studied and they demonstrate that nitrogen mustard is a type III agent, that is, its activity towards cells is depending on the rate of proliferation but not cell phase specific.

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MATERIAL AND METHODS

Cell survival curves were made as described earlier [1]; 16 hours after the subcutaneous administration of various doses of chemotherapeutic agents cell suspensions for spleen colony assay were prepared from haemopoietic tissues of control and treated mice. Bone marrow was collected from the femurs of mice by scraping and flushing the bone marrow with a syringe and needle from the cavity of the shaft. Rapidly-proliferating haemopoietic stem cells were collected from the spleens of

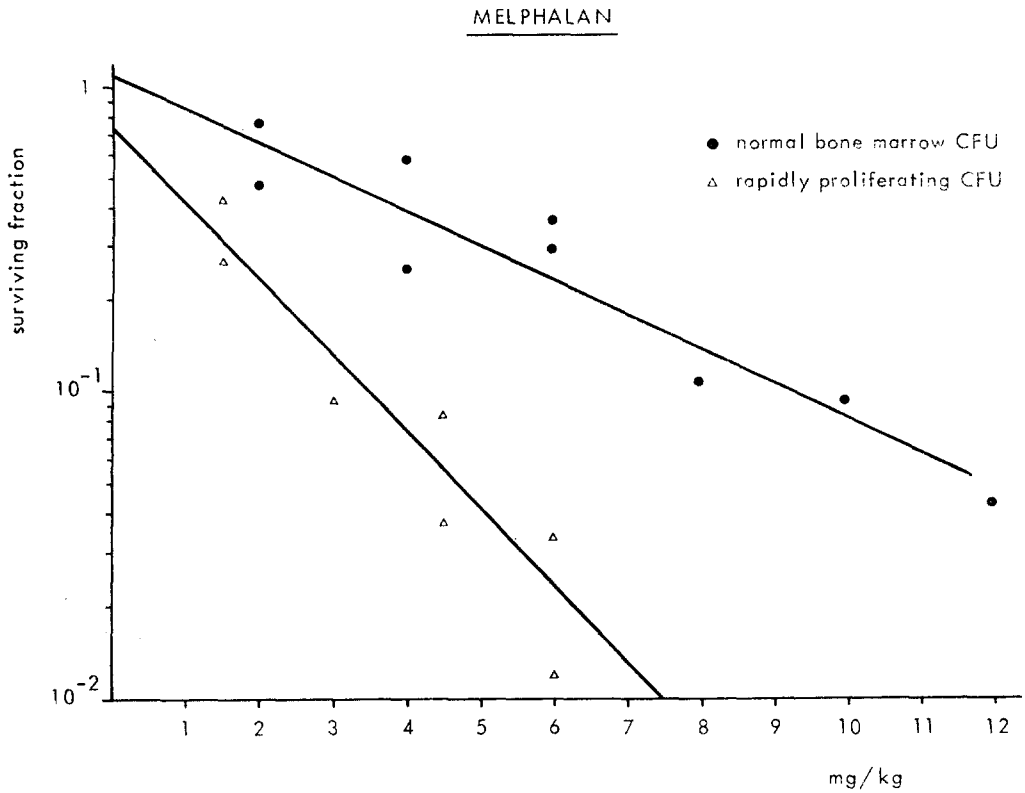


Fig. 1. *Survival of spleen colony-forming cells from normal bone marrow and from rapidly repopulating spleen* 16 *hr after subcutaneous injection of the donor with melphalan.*

mice that had been lethally irradiated and injected with a standard amount of bone marrow cells one week earlier. Each experiment was performed by simultaneous assay of the number of colony-forming units in the pooled femurs or spleens of groups of animals treated with different drug doses.

Usually for each treatment dose and for untreated control animals, a group of five mice was used. Assays for surviving colony-forming units were performed at two cell dose levels in 5 recipient mice per cell dose. The results were expressed as fractional survival of CFU per femur or per repopulating spleen by comparison with the simultaneously studied untreated control cell donors. Exponential survival curves were fitted to the resulting dose-response graphs by the method of least squares. For the individual results as given in the graphs, no standard derivations were presented since such estimates only reflect the theoretical accuracy based on the number of colonies counted and this variation is much smaller than the additional variations caused by the differences in donor animals and suspension technique between experiments. The spread of the points around the best fitting lines should give an adequate impression of the reproducibility of the results.

Mice

The majority of the data were obtained in 10–13-week old male C57BL/Rij × CBA/Rij F_1 hybrid mice, but for most drugs duplicate studies were performed in C57BL/Ka mice of both sexes. Since no differences in fractional survival of colony-forming cells between these animals were noted, the results were pooled.

Drugs

The following chemotherapeutic agents were used: melphalan* (L-phenyl alanine mustard); chlorambucil* (phenyl butyric acid mustard) and nitrogen mustard (methyl-bis-chloroaethylamine).

All drugs were administered subcutaneously, in volumes of 0·1–0·3 ml. Nitrogen mustard was dissolved in saline; the other agents were suspended in 2% carboxy-methylcellulose.

RESULTS

The cell survival data for melphalan, chlorambucil and nitrogen mustard are presented in Figs. 1, 2 and 3. Each of these graphs indicates that rapidly-proliferating CFU are more sensitive than the CFU in normal

*These agents were kindly made available by Burroughs Wellcome & Co., London.

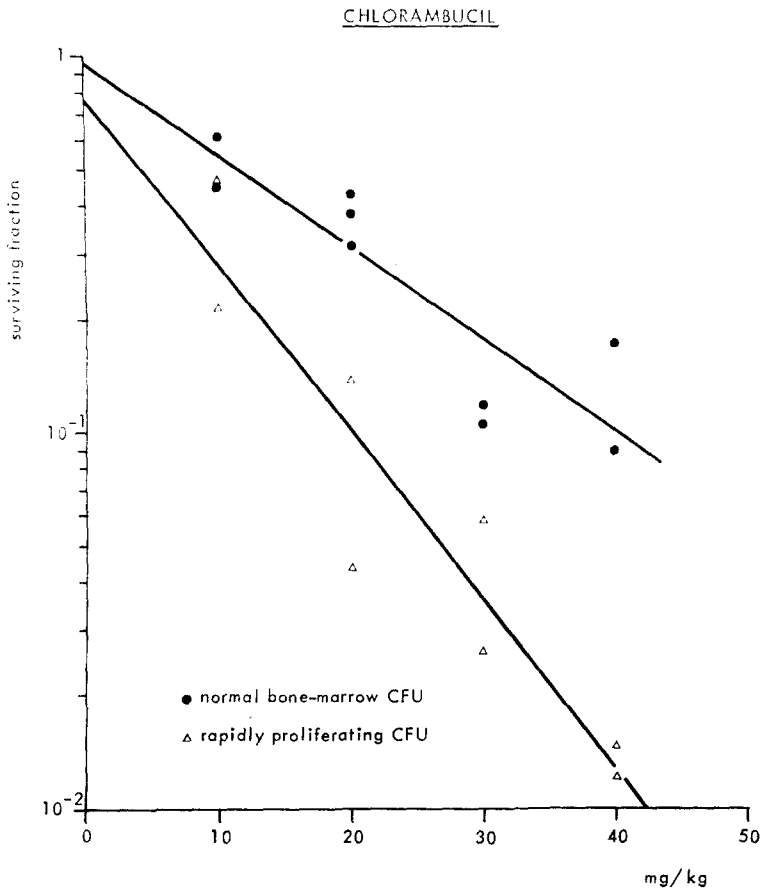


Fig. 2. Survival of spleen colony-forming cells from normal bone marrow and from rapidly-repopulating spleen 16 hr after subcutaneous injection of the donor with chlorambucil.

bone marrow. Since there is no indication of a plateau in the curves, this effect does not seem to be related to a phase specific sensitivity and these drugs should be classified as type III agents according to the classification of Bruce and Valeriote [6]. For nitrogen mustard this result was unexpected in view of the data of Bruce *et al.* [5], who had found identical slopes of the survival curves of normal bone marrow CFU and rapidly-proliferating lymphoma cells. In order to confirm that the difference in sensitivity between resting and rapidly-proliferating CFU is real, a few confirmatory tests were performed. In the first place it was verified that CFU in repopulating femur bone marrow had the same sensitivity as in repopulating spleen and in the second place assays were performed after exposure of CFU from different sources in the CFU recipients. For this purpose irradiated mice were injected subcutaneously with nitrogen mustard and simultaneously either normal femur-bone marrow cells or repopulating spleen cells were given intravenously. The result of these studies are indicated in Fig. 3. The difference in survival for the two types of CFU is evident and this rules out an alternative explanation, namely that the difference was due to different drug metabolism in the two types of donor. (The fact that the open circle in Fig. 3 lies clearly below the line for normal-bone marrow CFU is in accordance with the shorter time available for repair when exposure to the drug occurs in the recipient; under these conditions the interval till the next DNA synthesis period in which the lesions become irreversibly fixed is shorter than in the case of a 16-hr interval in the cell donor. The essential result lies in the significant difference between the open circle and the line for rapidly-proliferating CFU.)

The slopes of the best fitting exponential survival curves, calculated by the method of least squares are presented in Table 1 for the present study as well as for a cyclophosphamide study in which CFU survival was assayed after a similar time interval [1]. The ratio of the D_0 values (the reciprocal of the slope) for the CFU in the two different states of proliferation is relatively similar for the four drugs, varying between 1·8 and 2·4, indicating that the gain in cell sensitivity with more rapid proliferation is of similar magnitude for these agents.

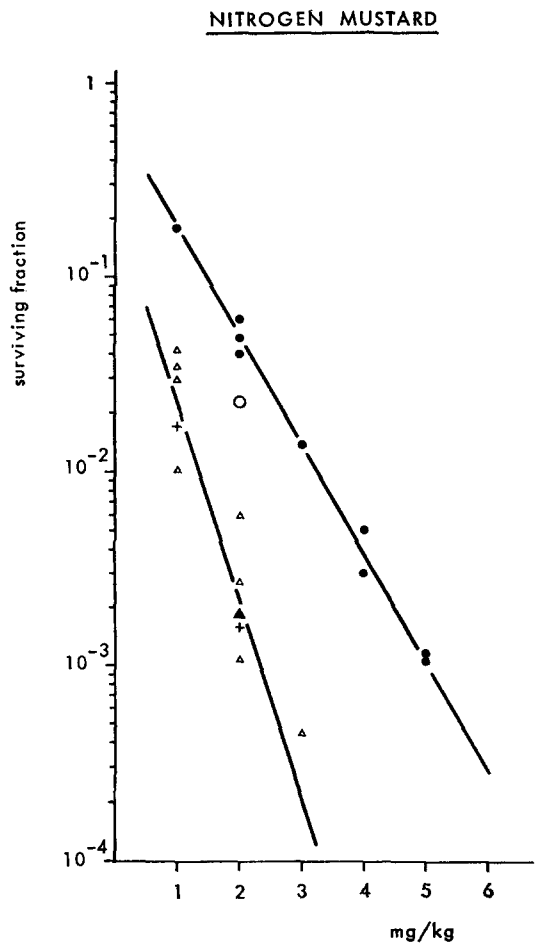


Fig. 3. Survival of spleen colony-forming cells from normal bone marrow (●) and from rapidly repopulating spleen (Δ) and rapidly repopulating femur bone marrow (+) 16 hr after subcutaneous injection of the donor with nitrogen mustard. The points indicated by O and ▲ respectively indicate the survival of spleen colony forming cells from normal bone marrow and repopulating spleen respectively, if the drug was administered to the spleen colony-assay recipients simultaneously with the cells. The different survival of the two cell types under these conditions indicates that the observed differences in survival reflect true differences in cellular sensitivity to the agents.

DISCUSSION

The finding that an increased proliferation rate leads to a similar degree of sensitization to each of the bis-chloro-aethyl-amines studied supports the validity of the hypothesis referred to in the introduction of this paper. It is only seemingly at variance with the results of Bruce *et al.*, reported earlier on nitrogen mustard [5]. Apparently, in their study, the faster proliferation of the lymphoma cells must have been compensated by a lower intrinsic cellular sensitivity to produce coincidentally the same survival slope.

In addition the finding is of more general

Table 1. Slopes of survival curves of normal and rapidly proliferating CFU when assayed 16–20 hr after the administration of different agents

Agent	Resting CFU $D_0(R)$ (mg/kg)	Rapidly-prolif. CFU $D_0(P)$ (mg/kg)	Ratio $D_0(R)/D_0(P)$
Melphalan	3·8	1·7	2·2
Chlorambucil	18	9·8	1·8
Nitrogen Mustard	0·79	0·43	1·8
Cyclophosphamide	126	54	2·4

interest; it indicates that all chemotherapeutic drugs which have so far been tested on haemopoietic stem cells are more effective on rapidly-proliferating cells.

Since these drugs contain many of the best presently available chemotherapeutic agents, it would seem that this specific greater effectiveness on rapidly-proliferating cells might reflect an essential feature of a good chemotherapeutic agent. This is somewhat puzzling as the evidence grows that the turnover of tumour cells is rarely or never faster than that of the most rapidly-proliferating normal cell renewal systems. On one side it may reflect the regrettable fact that many human tumours are not very sensitive to chemotherapy, but alternatively it may be that there are other factors involved in the greater sensitivity of tumour cells to those drugs, which are more effective on rapidly-proliferating cells. In the introduction evidence was cited, which indicates that for the alkylating agents repair of the cross linking lesion was responsible for the descreased sensitivity of resting normal cells. Sensitivity of tumour cells could then be explained either by a more rapid turnover, which would leave little time for repair before the lesion becomes irreversible in the next DNA-synthesis phase, or by a deficient repair-enzyme system. The latter possibility is supported by the finding that tumour-cell sensitivity to cyclophosphamide was found to be much greater relative to the sensitivity of haemopoietic stem cells than could be explained in the basis of cell-cycle time [1]. This indicates that apart from the variation in sensitivity on the basis of cell-cycle times, there is a wide variation in intrinsic cellular sensitivity which could be based upon differences in cell permeability to the drug or on differences in repair capacity. The possibility that limited repair capacity might be the cause of the specific drug sensitivity of tumour cells is suggested by the finding of a variation in repair capacity between different strains of micro-organisms [3].

The isolation of di-guaninyl-alkyl compounds from mammalian cells during repair of the alkylation lesion [2] has indicated that an excision of damaged bases from DNA is involved in the repair of the lesion. In this context the present study indicates that since there is a similar ratio of effectiveness of the four agents on the two types of cells, the repair of the lesion has, in each instance, a similar time constant and is therefore likely to be associated with the same enzymatic process for each of the drugs studied. Roberts *et al.* [7] have already pointed out the similarity between repair of the alkylation lesions and the repair of u.v. radiation induced damage in DNA of micro-organisms. The similarity of the activity of the repair process for the four alkylating agents studied in this paper points to the probability that one single enzymatic base excision process is responsible for repair of a multiplicity of different lesions. In addition the uniformity of the repair mechanism might be the reason why Schmidt *et al.*, in their systematic study [8] of a large number of alkylating agents on a number of tumours, found a lack of specificity in anti tumour activity as well as lack of specificity in toxicilogical characteristics. Further studies will indicate whether other types of alkylating agents than the bis-chloro-aethyl-amines studied, show similar pattern of stem cell survival, and may consequently be assumed to share the same repair mechanism.

Acknowledgement — Our thanks are due to Miss L. Idsenga and Mrs. E. Koning-Jonker for technical assistance and to Mrs. H. Uilenreef and Mrs. L. van den Burg for irradiation of mice.

SUMMARY

The sensitivity of resting spleen colony-forming cells in normal mouse bone marrow and of rapidly-proliferating spleen colony-forming cells in repopulating mouse spleen to a number of bis-chloro-aethyl-amines was determined. The rapidly-growing cells were more sensitive to each of the agents than the resting cells. The equal degree of sensitization to these agents by an increased rate of proliferation of the cells points to a common mechanism for all agents of this type. It is suggested that repair of damaged DNA, by excision of alkylated bases or oligonucleotides before the damage becomes irreversible during the next DNA synthetic period, is the most likely explanation of the uniform difference in sensitivity caused by the modified rate of cell proliferation.

REFERENCES

1. L. M. VAN PUTTEN and P. LELIEVELD, Factors determining cell killing by chemotherapeutic agents *in vivo*. I. Cyclophosphamide. *Europ. J. Cancer*, **6**, 313 (1970).

2. A. R. Crathorn and J. J. Roberts, Mechanism of cytotoxic action of alkylating agents in mammalian cells and evidence for the removal of alkylated groups from deoxyribonucleic acid. *Nature (Lond.)* **211,** 150 (1966).
3. P. D. Lawley and P. Brookes, Molecular mechanism of cytotoxic action of difunctional alkylating agents and of resistance to this action. *Nature (Lond.)* **206,** 480 (1965).
4. B. Papiermeister and C. L. Davison, Unbalanced growth and latent killing of *Escherichia Coli* following exposure to sulphur mustard. *Biochem. biophys. Acta (Amst.)* **103,** 70 (1965).
5. W. R. Bruce, B. E. Meeker and F. A. Valeriote, Comparison of the sensitivity of normal hematopoietic and transplanted lymphoma colony-forming cells to chemotherapeutic agents administered *in vivo*. *J. nat. Cancer Inst.* **37,** 233 (1966).
6. W. R. Bruce and F. R. Valeriote. Normal and malignant stem cells and chemotherapy. In *The Proliferation and Spread of Neoplastic Cells*, p. 409. Williams & Wilkins, Baltimore (1968).
7. J. J. Roberts, T. P. Brent and A. R. Crathorn, The Mechanism of the cytotoxic action of alkylating agents on mammalian cells. In *Interaction of Drugs and Subcellular Components* (Edited by P. N. Campbell), p. 5. J. & A. Churchill, London (1968).
8. L. H. Schmidt, R. Fradkin, R. Sullivan and A. Flowers, Comparative pharmacology of alkylating agents. *Cancer Chemother. Rep.*, Supplement 2 (1965).

Europ. J. Cancer Vol. 7, pp. 17–24. Pergamon Press 1971. Printed in Great Britain

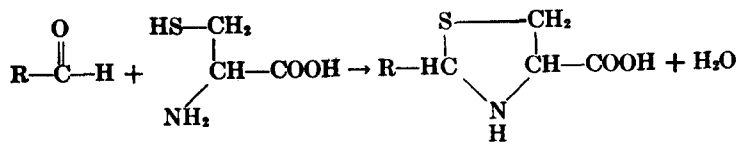
Studies on the Anti-Tumour Activity of Aliphatic Aldehydes—IV.

Radiosensitizing Properties of L-*erythro*-α, β-dihydroxybutyraldehyde on *E.coli* B and Ehrlich Ascites Tumour Cells

E. CIARANFI, A. PERIN, A. SESSA, A. ARNABOLDI, G. SCALABRINO and A. CASTELLANI

Institute of General Pathology and C.N.R. Centre for Research in Cell Pathology, University of Milan, Italy, and C.N.E.N., Laboratory of Animal Radiobiology, Casaccia Nuclear Study Centre, Rome, Italy

PREVIOUS studies carried out in this laboratory showed that aliphatic aldehydes inhibit amino acid incorporation into proteins of surviving mammalian and bacterial cells [1–3]. It has been suggested [2] that this effect might depend on the depletion of intracellular free cysteine consequent to its condensation with the aldehydes to form a thiazolidine-4-carboxylic acid, as follows:



Under physiological conditions of temperature and pH, this reaction occurs very rapidly and its equilibrium lies far to the right [2, 4, 5]. Recently, the formation of thiazolidine-4-carboxylic acids in normal and neoplastic tissues upon addition of aldehydes has been reported [6].

Moreover, preliminary experiments indicate that aliphatic aldehydes depress the translation of a synthetic RNA messenger in ribosomal cell-free systems [7].

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Among the aliphatic aldehydes hitherto studied, L-*erythro*-α,β-dihydroxybutyraldehyde (DHBA) has been found to inhibit protein synthesis without any direct interference with the mechanisms of energy supply such as respiration and glycolysis [2]. Additional *in vitro* studies with this aldehyde have shown that protein synthesis is more affected in neoplastic than in normal tissues [8], and this might explain the carcinostatic and carcinolytic effect found in *in vivo* experiments [9]. In turn, a depletion of the intracellular cysteine could increase the sensitivity of the cells to radiation, since it is well known that the blockage of SH-groups renders the cells more vulnerable to radiation [10].

It is the purpose of this paper to find out whether DHBA acts as a radiosensitizer for mammalian tumour cells in *in vitro*/*in vivo* experiments. In order to study the radiosensitizing effect of DHBA in a simpler model, the substance was initially tested on bacterial cells.

MATERIALS AND METHODS

Bacterial cells

E.coli B were collected from an overnight culture in nutrient broth (DIFCO) during the exponential phase of growth. Cells were washed twice with 0·067 *M* sodium phosphate buffer (pH 6·8) and then resuspended in the same buffer at a concentration of 5×10^7 cells/ml. The incubation with DHBA was carried out for 15 min at 37°C. Immediately after the incubation, the cells were washed with buffer and an aliquot of cell suspension was taken for toxicity assay.

Survival was measured by colony counts after plating the cells on nutrient agar and incubating them at 30°C for 24 hr.

The radiosensitizing effect of DHBA was studied measuring the survival curves of cells preincubated with various concentrations of DHBA (from 1 to 6×10^{-3} *M*) and irradiated at 4°C with γ-rays up to a dose of 4 krads.

The irradiation was carried out using αγ-Cells (Atomic Energy of Canada) mod. 200 giving a dose-rate of 1 krads/min. Survival was measured by colony counts as reported above.

Tumour cells

Ehrlich ascites tumour cells (nearly 50% diploid and 50% tetraploid cells) were collected from the peritoneal cavity of Swiss/CAS mice 5 days after transplantation of 10^7 cells.

The cells were washed three times by suspending them in saline at 5°C and centrifuging them at $400 \times g$ for 10 min and finally resuspending them in Krebs-Ringer phosphate [11].

Tumour cells (12 ml of suspension, 10^7 cells/ml) were incubated in a "Perspex" capsule (10 cm of diameter) contained in a "Perspex" rotating container (60 rev/min) to ensure complete gas diffusion (air or nitrogen) through the cell suspension. The container was set in a thermostatic room at 37°C. Routine experiments were performed incubating cells for 30 min in the absence or in the presence of 5 mM DHBA (final concentration). In additional experiments, the concentration of the aldehyde ranged from 1 to 10 mM and the incubation lasted 30–120 min.

Some samples of tumour cells were then irradiated at room temperature in the same rotating apparatus used for the incubation. Irradiation was carried out using a therapeutical X-ray apparatus at 220 kV, 18 mA, 0·5 mm Cu and 1 mm Al; the dose-rate was 98 rads/min. The dose delivered to tumour cells was determined by means of thermoluminescence dosimeters (LiF-Teflon Micro-Rods).

In some experiments, tumour cells were treated with DHBA after the irradiation. In this case, pre-irradiated cells were incubated for 30 min in the absence or in the presence of non-irradiated or irradiated 5 mM DHBA.

After the described treatments the various samples of cells, were collected by centrifugation, washed twice with cold saline to remove DHBA when present and resuspended in saline at the concentration of 2×10^6 cells/ml. 0·5 ml of this suspension (1×10^6 cells) was injected into the peritoneal cavity of Swiss/CAS female mice (20–22 g body weight) to study the effect of the various *in vitro* treatment on the kinetics of tumour growth. Tumour growth was measured by counting the total number of cells present in the peritoneal cavity at various time-intervals after the inoculation of treated or untreated cells. Five animals per day were sacrified by cervical dislocation and the cells were collected from the peritoneal cavity of each animal according to Klein and Révész [12]. The number of tumour cells in the peritoneal fluid was established by counting the number of nucleated cells in a Bürker hemocytometer and calculating the percentage of non-tumour cells (i.e. endothelial and monocytoid cells) from Feulgen-green light stained smears. To test the experimental error of such a technique, 10^6 and 10^7 tumour cells were injected into the peritoneal cavity of mice and immediately afterwards the cells were collected, diluted and counted. Under such conditions the experimental error was less than 5%. Groups of 20–30 animals for each treatment were kept alive to study survival.

In experiments where the tumour growth was studied as a function of inoculum size, 10^6, 10^5, 10^4, and 10^3 untreated cells were inoculated into the peritoneal cavity of mice.

In order to take into account whether immunological processes play a role in our experimental systems, 10^6, 10^5, 10^4, 10^3, 10^2 living tumour cells were respectively inoculated together with 10^6 cells killed by combined treatment of 5 mM DHBA for 30 min and 950 rads in air.

All the *in vitro* procedures described above were carried out under aseptic conditions.

RESULTS

Bacterial cells

The 15-min treatment with 2×10^{-3} *M* DHBA gave no or very low toxic effects on *E.coli* B cells (cell surviving 95–100%). Under these conditions a radiosensitizing effect with a

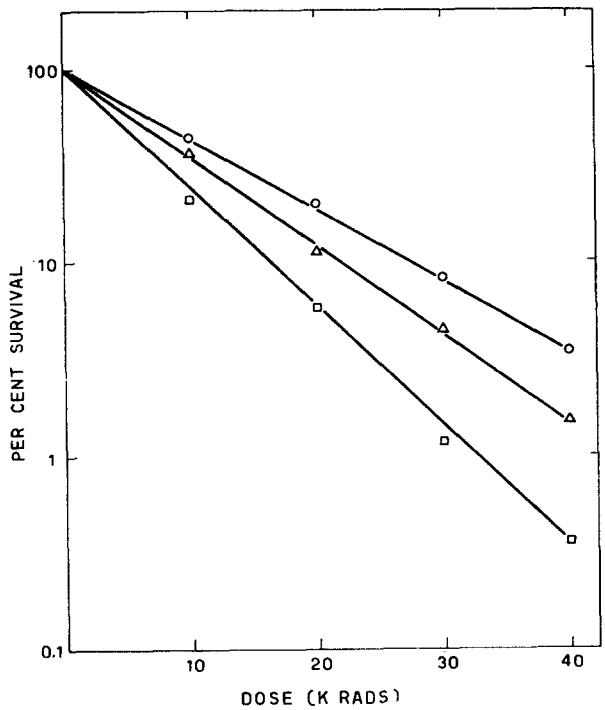


Fig. 1. Radiosensitization of E.coli *B by DHBA. Bacterial cells were irradiated after pre-incubation at 37°C for 15 min in the absence (○) or in the presence of* $2 \cdot 10^{-3}$ *M (Δ) and* $3 \cdot 10^{-3}$ *M (□) DHBA. Each point is based on 6 determinations of 400–500 colonies count each. The data refer only to the bacteria which survived the DHBA treatment.*

Dose Modifying Factor, DMF [13], of 1·3 (Fig. 1) has been found. The toxic effect was related to the DHBA concentration. In fact 15-min treatment with 3×10^{-3} *M* DHBA reduced the survival of the bacterial population to 60%. Under these conditions a radiosensitization with a DMF of 1·7 was observed. The DMF were calculated from Fig. 1, in which survival curves of treated and untreated cells are reported.

Tumour cells

Figure 2 shows the growth curves of Ehrlich ascites tumour cells for inocula of 10^6, 10^5, 10^4, and 10^3 cells. On a log scale the curves ran parallel and may be fitted by an exponential line during the first days. A progressive decline in the growth rate is observed after 3, 4, 6 and 7 days with inocula of 10^6, 10^5, 10^4, and 10^3 respectively. The survival data corresponding to the above mentioned inocula are reported in Table 1. On the basis of these results the subsequent experiments were performed with inocula of nearly 1×10^6 cells which represented the minimal cell number that could be easily counted 24 hr after the inoculum with the technique described above.

The incubation of tumour cells in the presence of DHBA might result in a modification of

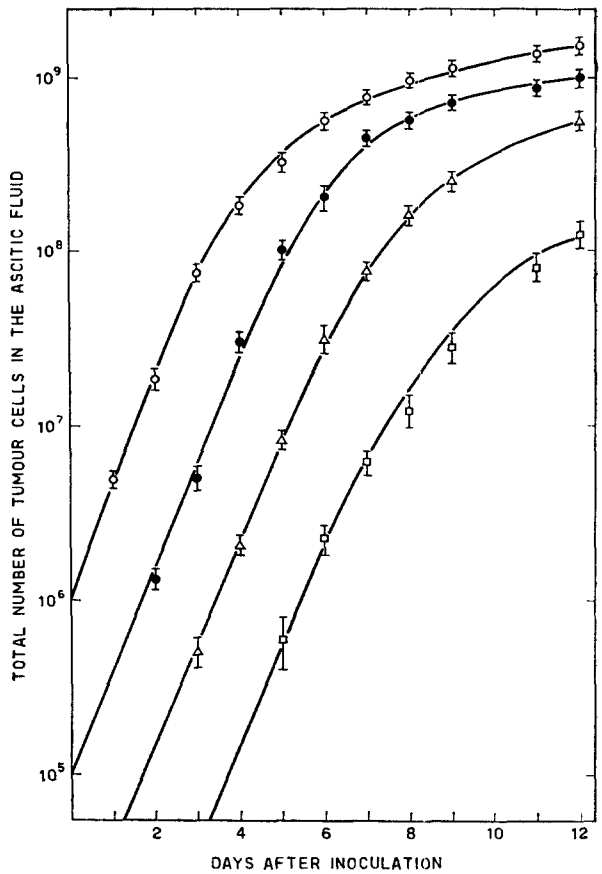


Fig. 2. Growth of Ehrlich ascites tumour in mouse. Four different cell doses were used for inoculation: 10^6 (○), 10^5 (●), 10^4 (Δ), *and* 10^3 (□) *cells. Each point is the mean of 5 animals±S.E.*

tumour growth. Figure 3 shows the effect of various aldehyde concentrations at constant period of treatment and Fig. 4 the effect of various times of treatment for a given aldehyde concentration.

As shown in Fig. 3, negligible or no effects were obtained treating the cells for 30 min with 1, 2·5 and 5 mM DHBA. Under these conditions the growth curve of cells treated with 1 mM DHBA overlapped the control and those of cells incubated with 2·5 and 5 mM aldehyde ran parallel to the control curve, reaching the

Table 1. Survival of mice intraperitoneally injected with various number of Ehrlich ascites tumour cells

Cell inocula	Survivors* treated	Survival time of tumour-bearing mice (days)†
1×10^6	0/30	19·3±0·2
1×10^5	0/20	23·2±0·8
1×10^4	0/26	25·2±0·8
1×10^3	0/30	26·6±0·3

*Alive and healthy after 4 months.
†Mean±S.E.

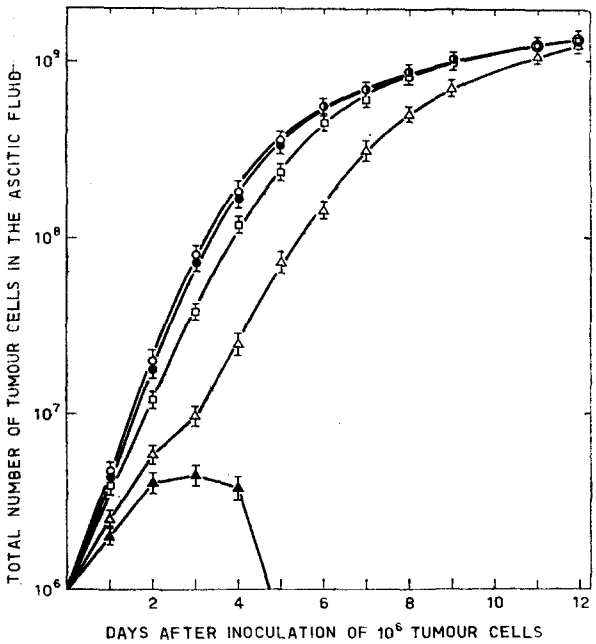


Fig. 3. Growth of Ehrlich ascites tumour in the mouse. Animals were inoculated with cells incubated in Ringer phosphate at 37°C for 30 min in the absence (O) or in the presence of 1 (●), 2·5 (□), 5 (Δ), and 10 (▲) mM DHBA. Each point is the mean of 5 mice±S.E.

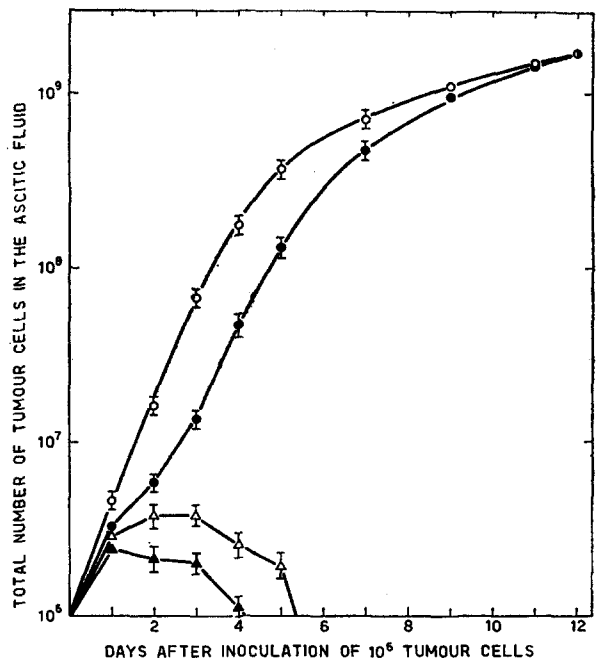


Fig. 4. Growth of Ehrlich ascites tumour in the mouse. Animals were inoculated with cells incubated in Ringer phosphate at 37°C in the absence (O) or in the presence of 5 mM DHBA for 30 (●), 60 (Δ), and 120 (▲) min. Each point is the mean of 5 mice±S.E.

latter after 9 and 12 days respectively. Treatment with 10 mM DHBA for 30 min caused a disappearance of tumour cells from the peritoneal fluid 4 days after the inoculation. All the above reported treatments, with the exception of 10 mM DHBA, did not modify the survival of the injected mice which died at the same time as the controls (Table 2).

As shown in Fig. 4, treatments with 5 mM DHBA for 60 and 120 min brought about irreversible damage leading to cell death.

Growth curves of tumour cells irradiated with various doses of X-rays in air and in nitrogen are shown in Figs. 5 and 6. The irradiation brought about a slow-down or a suppression of tumour growth depending on the dose of X-rays. A resumption of the exponential rate of growth took place after 5, 3 and 2 days after the inoculum for cells irradiated in air with 950, 500 and 280 rads respectively.

*Table 2. Survival of mice injected with Ehrlich ascites tumour cells incubated in the presence of L-erythro-α,β-dihydroxybutyraldehyde (DHBA) at various concentrations**

DHBA concentration (mM)	Survivors†/ treated	Survival time of tumour-bearing mice (days)‡
0	0/20	18·6± 0·4
1·0	0/32	18·9± 0·2
2·5	0/20	19·0± 0·3
5·0	0/36	19·5± 0·3
10·0	16/20	57·5±14·0

*Tumour cells were incubated for 30 min at 37°C in Krebs-Ringer phosphate containing DHBA at various concentrations and then 1×10^6 cells were injected into the peritoneal cavity of each mouse.
†Alive and healthy after 4 months.
‡Mean±S.E.

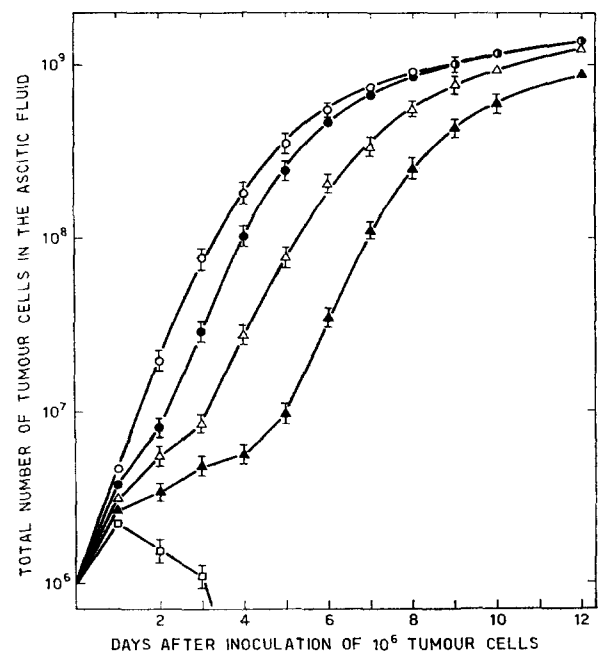


Fig. 5. Growth of Ehrlich ascites tumour in the mouse. Animals were inoculated with unirradiated cells (O), cells X-irradiated in vitro in air with 280 (●), 500 (Δ), 950 (▲), and 1780 (□) rads. Each point is the mean of 5 mice±S.E.

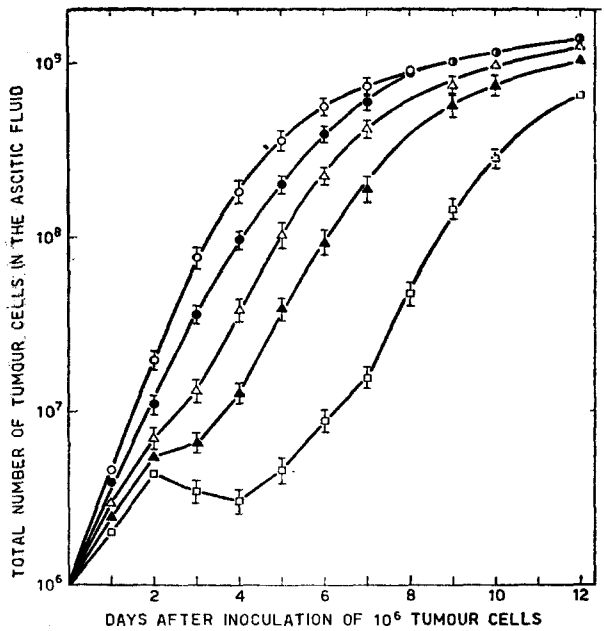


Fig. 6. Growth of Ehrlich ascites tumour in the mouse. Animals were inoculated with unirradiated cells (O), *cells X-irradiated* in vitro *in nitrogen with* 700 (●), 1237 (Δ), 1790 (▲), *and* 2340 (□) *rads. Each point is the mean of* 5 *mice*±*S.E.*

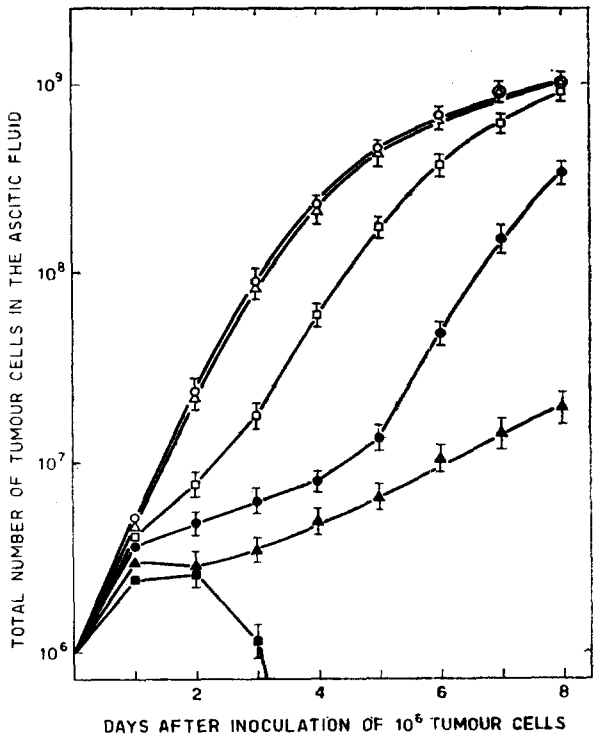


Fig. 7. Radiosensitizing effect of DHBA in air on the growth of Ehrlich ascites tumour in the mouse. Animals were inoculated with untreated cells (O), *cells incubated for* 30 *min at* 37°C *in the presence of* 1 (Δ) *and* 5 (□) *mM DHBA, cells X-irradiated with* 950 *rads* (●), *cells X-irradiated with* 950 *rads after incubation for* 30 *min at* 37°C *in the presence of* 1 (▲) *and* 5 (■) *mM DHBA. Each point is the mean of* 5 *mice*±*S.E.*

Similar modifications of growth rate were observed when the cells were irradiated in nitrogen using a "dose increasing factor" of nearly 2·5.

The effect of X-rays on tumour cells pre-incubated in the presence of DHBA are reported in Figs. 7 and 8. As shown in Fig. 7, a radiosensitizing effect was evident for the treatment with 1 mM aldehyde: under this condition there was no obvious toxic effect of the aldehyde and the irradiation led to a longer and greater slow-down of the growth curve as compared to that of irradiation alone. This result was confirmed by a significant increase in survival time of animals injected with these radiosensitized

Table 3. Survival of mice inoculated with Ehrlich ascites tumour cells treated in vitro *with X-rays, L*-erythro-α,β-*dihydroxybutraldehyde (DHBA) and X-rays*

Cell treatment	Gas phase	Survivors*/Treated	Survival time of tumour-bearing mice (days)†
500 rads	Air	0/20	18·9±0·4
DHBA+ 500 rads‡	Air	0/27	25·6±0·1§
950 rads	Air	0/20	27·8±0·3
DHBA+ 950 rads‡	Air	30/30	—
700 rads	Nitrogen	0/23	17·1±0·5
DHBA+ 700 rads‡	Nitrogen	0/21	23·1±0·8§
1790 rads	Nitrogen	0/23	20·3±1·0
DHBA+1790 rads‡	Nitrogen	6/30	31·6±2·7§
2340 rads	Nitrogen	7/25	25·6±2·3
DHBA+2340 rads‡	Nitrogen	30/30	—

*Alive and healthy after 4 months.
†Mean±S.E.
‡Cells were pre-incubated for 30 min at 37°C in the presence of DHBA and then irradiated.
§$P<0·01$. Significance has been tested between survival time of mice inoculated with irradiated cells and mice inoculated with cells pre-incubated in the presence of DHBA and then irradiated with the same X-rays dose.

cells (34 days against 27 days of the control). The 5 mM aldehyde or the irradiation with 950 rads, by themselves, led to a slow-down in the growth rate followed by a resumption of the tumour growth as previously described. Combined action of 5 mM DHBA and 950 rads led to a decrease in the number of the tumour cells followed by their total disappearance from the ascitic fluid (Fig. 7). The latter result was confirmed by the lack of tumour growth in all animals injected with cells treated with DHBA plus X-rays (100% survival 4 months later; Table 3).

An immediate and progressive decrease in the number of the tumour cells followed by their disappearance from the ascitic fluid was obtained in nitrogen using 5 mM aldehyde treatment plus irradiation with 2340 rads (Fig. 8). No tumour growth was observed in animals injected with these cells (100% survival 4 months later; Table 3).

A radiosensitizing effect of DHBA was observed also with lower doses of X-rays down to 270 rads in air and 330 rads in nitrogen. At these X-ray doses the growth curves of the

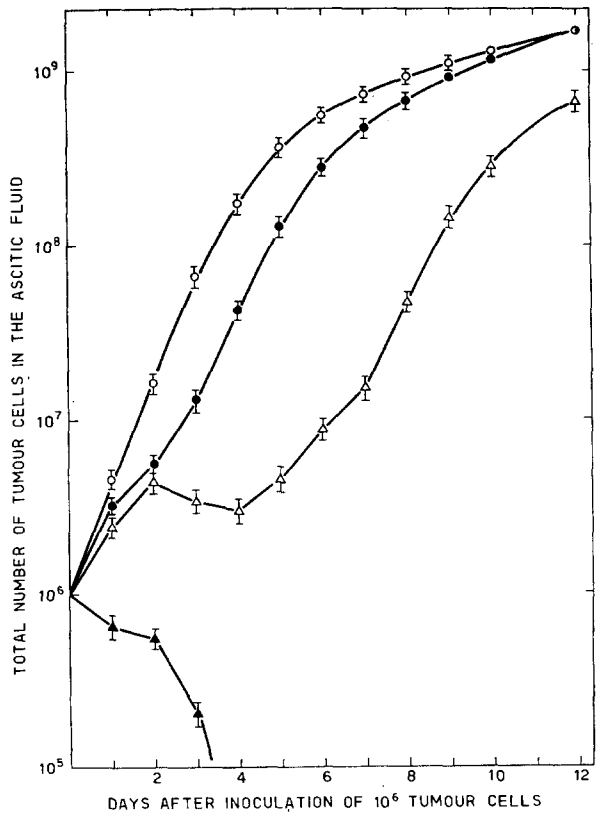


Fig. 8. Radiosensitizing effect of DHBA in nitrogen on the growth of Ehrlich ascites tumour in the mouse. Animals were inoculated with untreated cells (O), *cells incubated in the presence of 5 mM DHBA for 30 min at 37°C* (●), *cells X-irradiated with 2340 rads* (Δ), *and cells incubated in the presence of 5 mM DHBA for 30 min at 37°C and then X-irradiated with 2340 rads* (▲). *Each point is the mean of 5 mice ± S.E.*

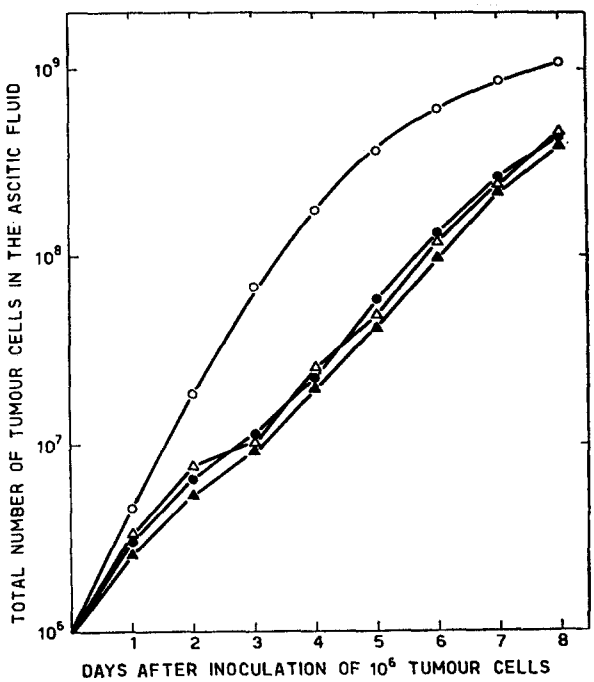


Fig. 9. Growth of Ehrlich ascites tumour in the mouse. Animals were inoculated with untreated cells (O) *cells X-irradiated with 500 rads* (●), *cells X-irradiated with 500 rads and then incubated for 30 min at 37°C in the presence of non-irradiated* (Δ) *or X-irradiated* (▲) *5 mM DHBA. Each point is the mean of 5 mice. Standard error ranged from 4 to 10%.*

5 mM DHBA pre-treated cells showed a greater and longer slow-down in the growth rate as compared to that caused by irradiation alone. An increase in the survival time was observed in animals injected with cells pre-incubated in the presence of 5 mM DHBA and irradiated with 500 rads in air or 700 and 1790 rads in nitrogen (Table 3).

The incubation of pre-irradiated cells with either non-irradiated or irradiated DHBA did not result in an enhancement of the irradiation damage. In fact the growth curves of so treated cells overlapped that of irradiated cells alone (Fig. 9).

Results of the experiments relating to the role of the immunological processes in our experimental system are reported in Table 4. No difference in tumour take and survival was observed in animals injected with living and killed cells as compared to the animals injected with the same number of living tumour cells alone.

DISCUSSION

Results reported in this paper show that DHBA enhances the effect of radiation on *E.coli* B both in non-toxic and toxic conditions, giving a DMF of 1·3 and 1·7 respectively.

In Ehrlich ascites tumour cells a true radiosensitizing effect is demonstrated irradiating

Table 4. Survival of mice intraperitoneally injected with various inocula of Ehrlich ascites tumour mixed or not with killed cells

Number of living cells inoculated	Inoculation of 1×10^6 killed cells*	Survivors†/Treated	Survival time of tumour-bearing mice (days)‡
0	+	20/20	—
10^5	—	0/20	24·8±0·7
10^5	+	0/28	25·6±0·4
10^4	—	1/20	25·9±0·6
10^4	+	1/28	24·5±1·3
10^3	—	0/20	27·6±1·1
10^3	+	3/23	30·3±2·1
10^2	—	14/30	30·0±1·6
10^2	+	13/26	38·3±4·0

*Tumour cells were killed by 5 mM L-*erythro*-α,β-dihydroxybutyraldehyde for 30 min at 37°C plus 950 rads.
†Alive and healthy after 4 months.
‡Mean±S.E.

cells pre-incubated for 30 min in the presence of 1 mM DHBA which, by itself, is completely void of toxicity.

In the case of 5 mM DHBA treatment we must also consider the biochemical action of the aldehyde on cells consisting of a depression of protein synthesis [2]. In this case, however, survival data and the correct analysis of the growth curve suggest that the action of DHBA on tumour cells is reversible. Consequently 5 mM DHBA treatment for 30 min at 37°C has to be considered of low toxicity for tumour cells.

It is important to note that to have a radiosensitization of tumour cells, DHBA must be present during the radiation. In fact no enhancement of radiation effect is observed when DHBA is added to irradiated cells. Under these conditions neither radiosensitization nor summation of toxic effect takes place.

A radiosensitizing effect is demonstrable at different doses of X-rays both in aerobic and anaerobic conditions. According to the given doses, the effect consists of a slow-down of tumour growth with an increased survival of animals, or of a lack of tumour take and development with a 100% survival of animals.

According to our experimental data showing that 10^3 cells give a tumour in 100% of the injected animals and that 10^2 cells give a tumour in 50% of treated animals, we may conclude that after radiosensitizing treatments with 5 mM DHBA and 950 rads in air or 2340 rads in nitrogen the number of survived cells must be less than 0·01% of the inoculum (1×10^6).

Experiments carried out by injecting animals with viable cells together with an aliquot (1×10^6) of killed cells demonstrate that the presence of killed cells does not induce any variation of tumour takes and of mice survival. These data suggest that immunological processes play a negligible role in our experimental conditions. Consequently, the effects described in this paper are due to a radiosensitizing phenomenon.

Acknowledgements—We wish to express our gratitude to Professor A. Ratti for permitting X-irradiation at the Institute of Radiology of the University of Milan, to Dr. A. E. Sichirollo and Dr. G. Fava of the Health Physics Department of the National Cancer Institute of Milan who carried out dosimetric determinations.

SUMMARY

The aim of the present paper was to see if L-erythro-α,β-*dihydroxybutyraldehyde (DHBA) which interacts with intracellular cysteine, may act as a radiosensitizing compound on bacteria and tumour cells.*

A test of radiosensitivity carried out with conventional procedures on E.coli *B indicated that DHBA enhances the effect of radiation both in non-toxic and toxic conditions, giving respectively a DMF of* 1·3 *and* 1·7.

In vitro/in vivo *experiments, carried out with the adenocarcinoma Ehrlich ascites cells, have shown that DHBA displays a marked radiosensitizing action at non-toxic doses* (1 *or* 5 *mM*), *when the irradiation is carried out in air as well as in nitrogen. The*

combined action of DHBA and X-rays leads to a slow-down of tumour growth with increased survival of the animals and, in convenient X-rays doses, brings about a dramatic decrease in the number of the tumour cells from the ascitic fluid with their total disappearance confirmed by the lack of tumour growth in all animals.

DHBA must be present during the radiation. The addition of aldehyde to irradiated cells appeared to be without effect on the cytokynetics of tumour growth.

Immunological processes play a negligible role under our experimental conditions.

The results are discussed.

REFERENCES

1. G. G. Guidotti, A. Fonnesu and E. Ciaranfi, Inhibition of amino acid incorporation into protein of Yoshida ascites hepatoma cells by glyceraldehyde. *Cancer Res.* **24,** 900 (1964).
2. G. G. Guidotti, L. Loreti and E. Ciaranfi, Studies on the antitumour activity of aliphatic aldehydes. I. The mechanism of inhibition of amino acid incorporation into protein of Yoshida ascites hepatoma cells. *Europ. J. Cancer* **1,** 23 (1955).
3. A. Sessa, A. Arnaboldi, A. Perin and A. Angelotti, Unpublished results.
4. M. P. Shubert, Compounds of thiol acids with aldehydes. *J. biol. Chem.* **114,** 341 (1936).
5. S. Ratner and H. T. Clarke, The action of formaldehyde upon cysteine. *J. Am. chem. Soc.* **59,** 200 (1937).
6. L. Loreti, M. E. Ferioli, G. C. Gazzola and G. G. Guidotti, Studies on the antitumour activity of aliphatic aldehydes. III. Formation of thiazolidin-4-carboxylic acid in tissues. *Europ. J. Cancer*, to be published in 1971.
7. A. F. Borghetti, B. Giglioni, S. Ottolenghi and G. G. Guidotti, Effects of aliphatic aldehydes on protein synthesis in cell-free systems. *Biochem. J.* **117,** 67 (1970).
8. A. Perin, A. Sessa, A. Arnaboldi, G. Scalabrino and E. Ciaranfi, Unpublished results.
9. E. Ciaranfi, A. Perin and A. Arnaboldi, Attività antineoplastica della L-*eritro*-α,β-diidrossibutiraldeide su alcuni tumori sperimentali. *Tumori* **53,** 29 (1967).
10. A. Bridges, Sensitization of organism to radiation by sulphydryl-binding agents. *Adv. Radiat. Biol.* **3,** 123 (1969).
11. W. W. Umbreit, R. H. Burris and J. F. Stauffer, Manometric Techniques (4th edition). Burgess, Minneapolis (1964).
12. G. Klein and L. Révész, Quantitative studies on the multiplication of neoplastic cells *in vivo*. I. Growth curves of the Ehrlich and MC1M ascites tumours. *J. nat. Cancer. Inst.* **14,** 229 (1953).
13. M. C. Pike and A. T. Alper, A method for determining dose-modification factor. *Brit. J. Radiol.* **35,** 458 (1964).

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Urine and Blood Serum Muramidase (Lysozyme) in Patients with Urogenital Tumors

G. KOVANYI and K. LETNANSKY

Institute for Medical Chemistry and Institute for Cancer Research, University of Vienna, Austria

INTRODUCTION

MURAMIDASE (N-acetylmuramidglycanohydrolase, E.C. 3.2.1.17), an enzyme which cleaves certain glucosidic linkages in mucopolysaccharides, is widely distributed in the mammalian organism. It is a basic protein with a molecular weight of 15,000. High concentrations of this enzyme are found in kidneys, tears, human milk and blood serum. In urine, on the other hand, no appreciable activity of this enzyme can be detected [for reviews see refs. 1–8].

During the last years, the significance of the muramidase for clinical purposes has increased rapidly. According to Noble *et al.* [9] and Perillie *et al.* [10] serial assays of serum muramidase activity furnish a valuable tool in the diagnosis, classification, and management of patients with various forms of leukemia. After kidney transplantation, high urine muramidase levels indicate a rejection of the transplant [11] and some diseases of the kidneys are also paralleled by elevated urine levels [12–15]. It was not known, however, if muramidase is also found in the urine as a result of neoplastic alterations within the genito-urinary system. Therefore, the objective of the present paper was to investigate muramidase concentrations in urine and serum of patients suffering from tumors of the urogenital tract and to evaluate the usefulness of these determinations for diagnosing neoplasms of this region.

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MATERIAL AND METHODS

Urine and serum from untreated (not operated) patients* were used in most of the determinations. In addition, determinations were made following operations and during therapy in a small group of patients, in order to investigate the possibility of using these determinations for controlling the progress of the disease.

Muramidase concentrations were determined by measuring the lytic capacity of the enzyme against micrococcus lysodeicticus suspensions. This method was introduced by Smolelis and Hartsell [16] and modified by Litwack [17] and Parry *et al.* [18]. Lyophilized bacteria, obtained from Worthington (Freehold, New Jersey), were suspended in 60 mM Sørensen phosphate buffer, pH 6·2, containing 0·1% sodium chloride. A concentration of 10 mg/100 ml corresponded to an absorption of 0·350 at 645 mμ. After the addition of 1 ml sample to 2 ml of this suspension, the decrease in absorbancy was registered at 33°C in a Beckman DB spectrophotometer. The decrease during the first 30 sec of the incubation was compared with a standard curve, obtained with egg white muramidase (Fluka, Basel). Determinations should be made at standardized time intervals after the beginning of the incubation, since reaction velocity is not constant over long

*We wish to express our gratitude to Professor Dr. R. Übelhör, director of the Department of Urology, University of Vienna Hospitals, Vienna, Austria, for making the material available.

time periods. Concentrations, obtained by this method, are relative values, as the specific activities of human and egg white muramidase differ by a factor of 3 [3, 19].

Muramidase activity is pH-dependent to a high degree. The optimum was found at pH 6·2 [20]. Therefore, urine samples, having a pH too high for being buffered satisfactorily, were titrated before the test to about pH 6·2 with 1 NH_3PO_4.

RESULTS

Muramidase activity could not be demonstrated in the *urine* of 21 out of 28 healthy persons. There were concentrations of 0·1 µg/ml (2 samples), 0·3 µg/ml (3 samples), 0·5 µg/ml, and 0·7 µg/ml (1 sample each) in the remaining samples. Assuming that normal concentrations do not exceed 1·0 µg/ml, then elevations above the normal were found in the urine of 51 out of 53 patients with neoplasms of the urogenital region (29 bladder carcinomas, 9 carcinomas of the prostate, 6 kidney tumors, 3 carcinomas of the ureter, 2 carcinomas of the uterus, 1 carcinoma each of the ovary, vagina, and penis, and 1 seminoma) (Fig. 1). No elevation was found (1) in the urine of one patient with a 13-year old carcinoma of the prostate, which had been treated with high doses of estrogens, and (2) in another patient with a non-infiltrating bladder papilloma undergoing malignancy which could be removed completely by partial resection of the bladder. Enzyme concentrations were found substantially elevated in some cases e.g. 19·4 µg/ml in 1 patient with bladder carcinoma. On the other hand, benign tumors caused no elevation of the urine muramidase concentration above the normal. The same was observed with inflammations of different localization,

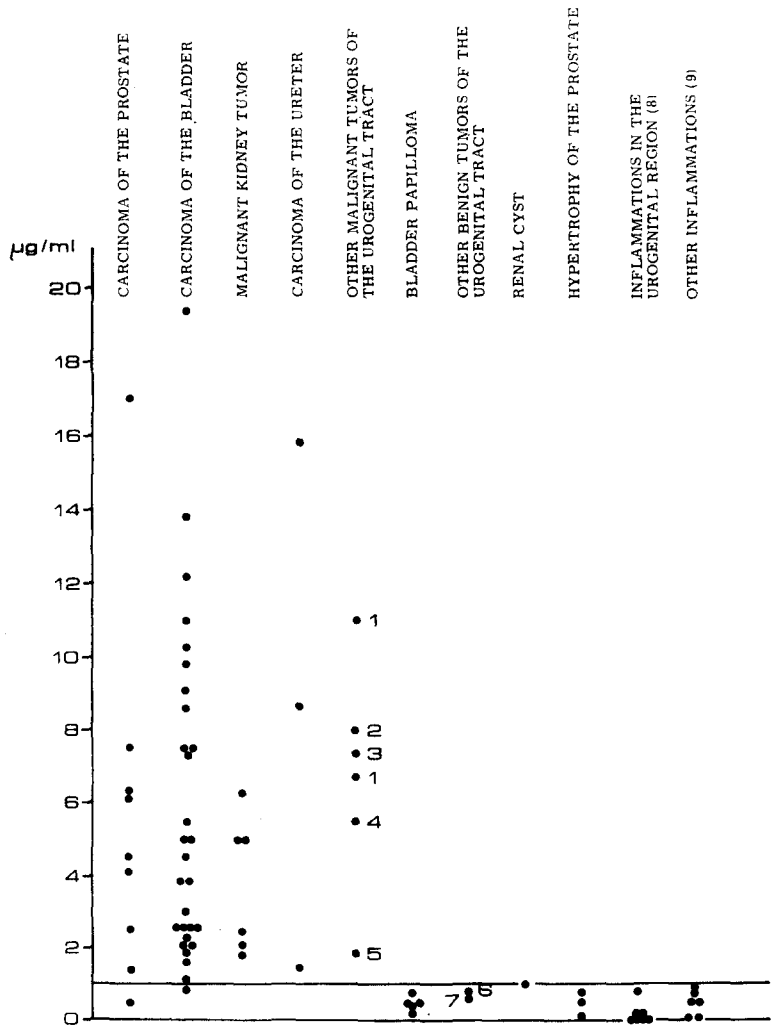


Fig. 1. Urine muramidase concentration from patients with different diseases of the genito-urinary system and inflammations. (1) *carcinoma of the uterus,* (2) *carcinoma of the vagina,* (3) *seminoma,* (4) *carcinoma of the ovary,* (5) *carcinoma of the penis,* (6) *multiple myoma of the uterus,* (7) *adenoma of the prostate,* (8) *adnexitis* (3 *cases*), *inflammatory infiltration of the adnexa, prostatitis* (2 *cases each*), (9) *appendicitis, acute cholecystitis, chronic polyarthritis* (2 *cases each*). *Normal range:* 0–1 µg *muramidase/ml urine.*

including the urogenital region. However, suppurative processes frequently result in significant elevations of the muramidase concentration. In 1 case of pyelonephritis, for example, we found an enzyme concentration of 29·2 µg/ml. Specimens with high leukocyte counts were therefore discarded.

Similar observations were made concerning *serum* muramidase concentrations. The mean value from 17 healthy persons was 7·0 µg/ml, with a standard deviation $\sigma = \pm 1 \cdot 1$. The concentration was elevated substantially in the sera of 4 patients with carcinomas of the prostate (9·6–14·3 µg/ml), 19 patients with carcinomas of the bladder (7·4–29·2 µg/ml), 3 with neoplasms of the kidney (13·4–18·2 µg/ml), 1 with seminoma (15·8 µg/ml), 1 with carcinoma of the cervix (9·7 µg/ml), and 2 with carcinomas of the ureter (8·7 and 9·8 µg/ml) (Fig. 2). All except 1 of these 30 cases exhibited values higher than 8·1 µg/ml, which was considered the upper limit of normal values. The exception was a tumor of the bladder which had been diagnosed histologically as "papilloma undergoing malignancy" (it is of interest that the muramidase concentration, found in the urine of this patient, amounted to 1·0 µg/ml). On the contrary, non-malignant affections of urogenital organs (3 bladder papillomas, 2 hypertrophies of the prostate, 1 renal cyst and 1 adenoma of the prostate) were within the normal range. Only 1 bladder papilloma exhibited slightly elevated serum muramidase, although the urine concentration of this enzyme was found to be normal. It might be possible, however, that these discrepancies between determinations in serum and urine are already an expression of beginning malignization. This would strongly suggest the performance of the determination both in serum and in urine.

The elevation of muramidase concentrations

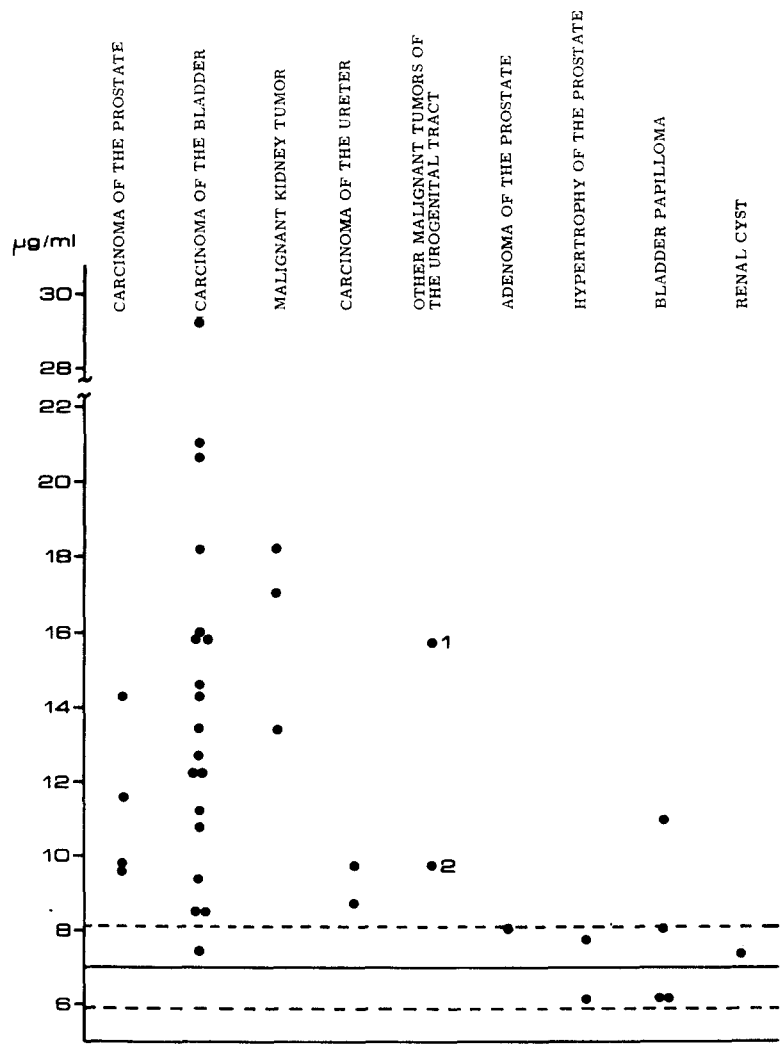


Fig. 2. Serum muramidase concentration from patients with different diseases of the genito-urinary system. (1) *seminoma*, (2) *carcinoma of the cervix. Normal range:* 7·0 (*mean value from* 17 *healthy individuals*) ±1·1 µg/ml *serum* (*standard deviation* σ).

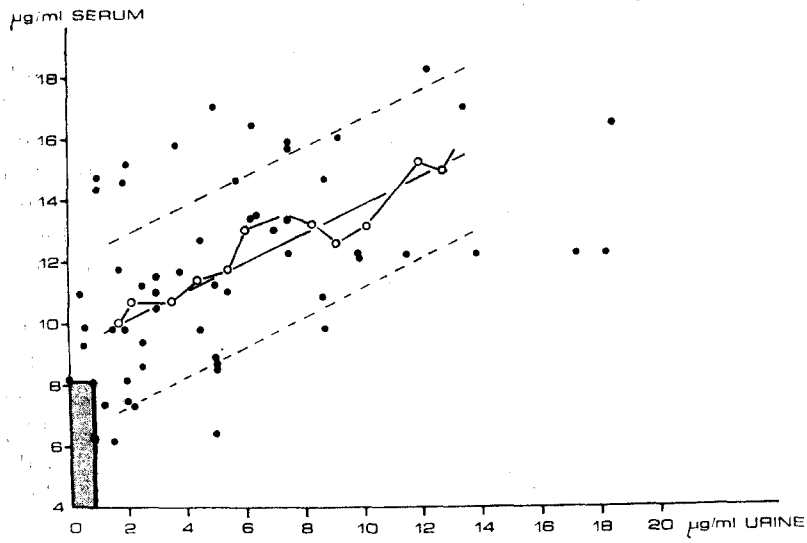


Fig. 3. Relationship between muramidase concentrations in urine and in serum. Between 0 *and* 15 µg/ml 13 *overlapping classes with a class width of* $\Delta x = 3$ µg/ml *were used and the mean values (o) within these classes were calculated ("overlapping means"* [21]). *Broken line: standard deviation; hatched area: normal region.*

in serum as well as in urine is a strong argument for the existence of a malignant tumor, even despite possibly negative clinical or histological evidence. This is demonstrated with 2 bladder papillomas which, although having been diagnosed histologically as benign tumors, gave positive reactions in the enzyme test (2·0 and 2·5 µg/ml urine and 15·2 µg/ml serum). In the further progress of the disease (2 and 7 months after our first determination) histological methods succeeded in demonstrating the malignancy of the tumors.

There is evidence of a relationship between serum and urine muramidase concentrations, although the degree of the relationship is obviously not very high. Despite the widely scattered values of the scatter-diagram (Fig. 3), one can observe the tendency for low blood muramidase concentrations to be associated with low urine muramidase concentrations and high blood concentrations with high urine concentrations. Calculation of "overlapping means" according to Gebelein and Heite [21] results in a polygon, coming very close to a regression line, representing linear relationship between the muramidase levels of these two body fluids.

No correlation, however, seems to exist between muramidasuria and routine tests of renal function, as is demonstrated with serum creatinin in Fig. 4. In many cases elevations of the urine muramidase concentration was observed, although creatinin and BUN were in the normal range. In addition, urine muramidase concentrations changed with time in some cases without being paralleled by changes in serum creatinin or BUN. These observations are consistent with the results of other authors [12, 13, 22].

DISCUSSION

Elevated urine muramidase concentration has been found primarily in certain forms of leukemia [3, 9, 10, 23, 24], cardiac infarction [25], and several diseases and toxic lesions of the kidney (glomerulo-nephritis [13, 14], nephrotic syndrom [12, 13], Fanconi syndrom [14], Cd-intoxication [14], and urinary calculus [15]). It has also been found in association with the rejection of kidney transplants [11]. The present paper describes a significant elevation both of urine and serum muramidase concentrations in patients with neoplasms of urogenital organs. Serum muramidase levels of patients with malignant tumors of different localizations had been found elevated in some cases also by Fogelson and Lobstein [26]. The elevations reported by these authors, however, were not significant.

In general, benign tumors of the genito-urinary region are characterized by normal muramidase levels in serum and in urine. This could be of considerable interest in differential diagnosis of neoplasms, particularly since

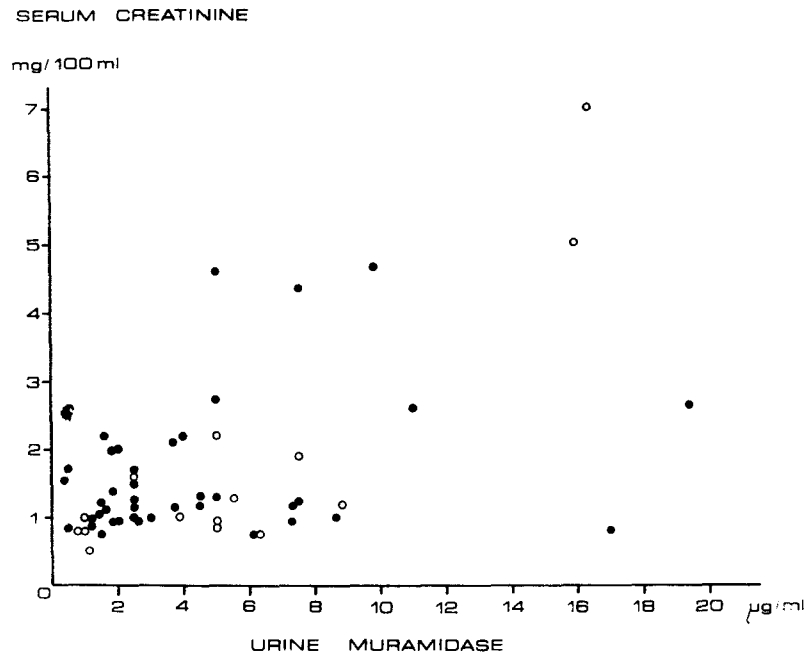


Fig. 4. Relationship between serum creatinin and urine muramidase. ● male patients; ○ female patients.

determinations of LDH and alkaline phosphatase activity, which are frequently used for diagnosing malignant tumors, are not of great value with tumors of this localization [27].

Figure 3 demonstrates a relationship between concentrations of muramidase in urine and in serum. This is in accordance with the findings of Prockop and Davidson [13]. On the other hand, Burghartz and Boosfeld [22], having investigated a rather small number of different diseases of the kidney, were not successful in demonstrating an interrelation of these parameters.

The high muramidase content of kidney cells is well known [28]. Therefore, one explanation of muramidasuria following tumor progression in this area might be the destruction of renal tissue. However, the observations that malignant tumors of different localization within the urogenital region also cause elevations of the urine level, and that benign tumors of the same localization fail in doing so, are at variance with this assumption. The contemporary elevation of serum muramidase would indeed be more in favour of an elevated production or secretion of the enzyme from tissues, not restricted to the kidney. According to Rather [29] and Hardwicke and Squire [30] protein molecules with molecular weights similar to that of muramidase may pass the glomerular membrane. Normally these proteins subsequently undergo tubular reabsorbtion. It would be more likely, therefore, to assume impaired renal tubular function causing the excretion of the enzyme in the urine — an explanation being compatible with the findings of Harrison *et al.* [14].

Serum and urine concentrations are generally lowered after surgical removal of the neoplasm. Immediately after the operation, however, very high enzyme levels are frequently found. This is supposedly caused by the operation itself. It is suggested to investigate this observation in more detail with a greater number of patients in order to clarify, whether the determination of muramidase concentrations might be used for postoperative control. The results reported in the present paper, however, clearly demonstrate the usefulness of muramidase determinations in diagnosing malignant tumors of the genito-urinary system.

SUMMARY

Serum and urine muramidase levels of patients suffering from carcinomas of the urogenital tract have been determined. The concentration in the urine amounted to 1·1–19·4 µg/ml *in* 51 *out of* 53 *cases, whereas in healthy persons and in patients suffering from benign tumors, hypertrophy of the prostate, kidney cysts or inflammations, concentrations did not exceed* 1·0 µg/ml. *Serum muramidase concentration was between* 8·5 *and* 29·2 µg/ml

in 29 out of 30 cancer patients. In 17 healthy individuals we found 7·0±1·1 μg/ml and concentrations in the serum of patients of the control group were found in the same range in 7 out of 8 cases.

The simultaneous determination of muramidase concentrations both in urine and in serum has proved to be a valuable test for the diagnosis of malignant tumors in the genito-urinary region.

REFERENCES

1. K. Grossgebauer and H. Langmaack, Lysozyme: Ergebnisse und Probleme. *Klin. Wschr.* **46,** 1121 (1968).
2. P. Jolles, Lysozyme: In *The Enzymes* (edited by P. D. Boyer, H. Lardy and K. Myrbäck), p. 431. Academic Press, New York (1960).
3. E. F. Osserman and D. P. Lawlor, Serum and urinary lysozyme (muramidase) in monocytic and monomyelocytic leukemia. *J. exp. Med.* **124,** 921 (1966).
4. P. Jolles, Neuere Untersuchungen an Lysozymen. *Angew. Chem.* **76,** 20 (1964).
5. P. Jolles, Chemie und Biochemie der Lysozyme. *Chimia* **19,** 176 (1965).
6. R. C. Skarnes and D. W. Watson, Antimicrobial factors of normal tissues and fluids. *Bact. Rev.* **21,** 273 (1957).
7. M. R. J. Salton, The properties of lysozyme and its action on microorganisms. *Bact. Rev.* **21,** 82 (1957).
8. P. Jolles, Lysozyme: Ein Kapitel Molekularbiologie. *Angew. Chem.* **81,** 244 (1969).
9. R. E. Noble and H. H. Fudenberg, Leukocyte lysozyme activity in myelocytic leukemia. *Blood* **30,** 465 (1967).
10. P. E. Perillie, S. S. Kaplan, E. Lefkowitz, W. Rogaway and S. C. Finch, Studies of muramidase in leukemia. *J. Am. med. Ass.* **203,** 317 (1968).
11. R. E. Noble, J. S. Najarian and H. D. Brainerd, Urine and serum lysozyme measurement in renal homotransplantation. *Proc. Soc. exp. Biol. Med.* **120,** 737 (1965).
12. A. T. Wilson and W. P. Hadley, Urinary lysozyme III. Lysozymuria in children with the nephrotic syndrom. *J. Pediat.* **36,** 199 (1950).
13. D. J. Prockop and W. D. Davidson, A study of urinary and serum lysozyme in patients with renal disease. *New Engl. J. Med.* **270,** 269 (1964).
14. J. F. Harrison, G. S. Lunt, P. Scott and J. D. Blainey, Urinary lysozyme, ribonuclease, and low-molecular weight-protein in renal disease. *Lancet* (I), 371 (1968).
15. G. Gasser and G. Kovanyi, in preparation.
16. A. N. Smolelis and S. E. Hartsell, The determination of lysozyme. *J. Bact.* **58,** 731 (1949).
17. G. Litwack, Photometric determination of lysozyme activity. *Proc. Soc. exp. Biol. Med.* **89,** 401 (1955).
18. R. M. Parry, R. C. Chandan and K. M. Shahani, a rapid and sensitive assay of muramidase. *Proc. Soc. exp. Biol. Med.* **119,** 384 (1965).
19. J. F. Petit and P. Jolles, Purification and analysis of human saliva lysozyme. *Nature (Lond.)* **200,** 168 (1963).
20. K. Letnansky and P. Hübl, Die Lysozymaktivität des menschlichen Magensaftes. *Wien. klin. Wschr.* **74,** 46 (1962).
21. H. Gebelein and H.-J. Heite, Statistische Urteilsbildung. Springer Verlag, Berlin (1951).
22. N. Burghartz and E. Boosfeld, Bericht über Lysozymuntersuchungen im Urin. *Klin. Wschr.* **32,** 182 (1954).
23. P. E. Perillie, S. S. Kaplan and S. C. Finch. Significance of changes in serum muramidase in megaloblastic anemia. *New Engl. J. Med.* **277,** 10 (1967).
24. E. F. Osserman, Crystallisation of human lysozymes. *Science* **155,** 1536 (1967).
25. G. Kovanyi and H. Karobath, Lysozymaktivität in Serum und Harn nach Herzmuskelinfarkt. *Verh. dtsch. Ges. Kreisl-forsch.* **35,** 389 (1969).
26. S. J. Fogelson and O. E. Lobstein, A statistical comparison of the blood lysozyme activity of normal adults and of patients with localized and generalized carcinomatosis. *Amer. J. dig. Dis.* **21,** 324 (1954).
27. U. C. Dubach, Diagnostischer Wert von Enzymbestimmungen im Urin bei Tumoren des Urogenitalsystems. *Oncologia* **19,** 254 (1965).

28. T. E. MILLER, C. M. CAMERON and J. D. K. NORTH, Distribution of lysozyme in the rat kidney and the role of this enzyme in experimental pyelonephritis. *Proc. Soc. exp. Biol. Med.* **128** 749 (1968).
29. L. J. RATHER, Filtration, resorption and excretion of protein by the kidney. *Medicine (Baltimore)* **31,** 357 (1952).
30. J. HARDWICKE and J. R. SQUIRE, The relationship between plasma albumin concentration and protein excretion in patients with proteinuria. *Clin. Sci.* **14,** 509 (1955).

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Growth Inhibiting Effects of Cytotoxic Agents on Human Tumor and Tumor-Bearing Normal Tissue *in vitro*

F. CH. IZSAK, E. EYLAN, A. GAZITH, J. SHAPIRO, S. NAHARIN and CH. RAANANI

Government Hospital, Jaffa, Tel-Aviv University, and Israel Atomic Energy Commission, Israel

MODERN cancer chemotherapy is based on compounds developed on the basis of their known pharmacological effects and their modifications, which were tested in a wide range of systems and innumerable experimental tumors *in vivo*. The successful translation of the accumulated information into clinical application, however, still depends mainly on the knowledge derived from clinical observations on a large series of cases [1–3]. In spite of the extensive accumulated experience, there are a large number of patients who do not benefit from the use of cytotoxic agents because of individual differences in the sensitivity of the tumors and the endless variation, from patient to patient, of tumor-host relationship patterns [4, 5]. A substantial proportion of cancer patients do not therefore enjoy the benefits that chemotherapy can offer, and in a number of instances cytotoxic agents have been shown even to stimulate tumor growth [6–8].

It seems justified, therefore, to seek alternative criteria and guiding principles in the choice of the drug to be employed in each individual case. Several investigators have shown that valuable information about the sensitivity of a given tumor to a series of cytotoxic agents can be obtained with tissue-culture techniques [9–12]. Short-term organ cultures of freshly removed human tissues were recently employed in the evaluation of anti-cancer compounds [13–16]. These procedures do not however offer a solution to the important problem of the toxic, and sometimes lethal effect of the chemotherapy on the normal tissues of the tumor-bearing host [15, 17, 18]. To fill this gap, Eagle and Foley have tested the effects of cytotoxic agents on tissue cultures of cells derived from tumors and from normal tissues [19].

The present communication describes the results of a study performed on human tumors and on adjacent normal tissue, obtained from the tumor-bearing organ. The aim of this work was to achieve a rapid clear-cut response to the cytotoxic agents employed.

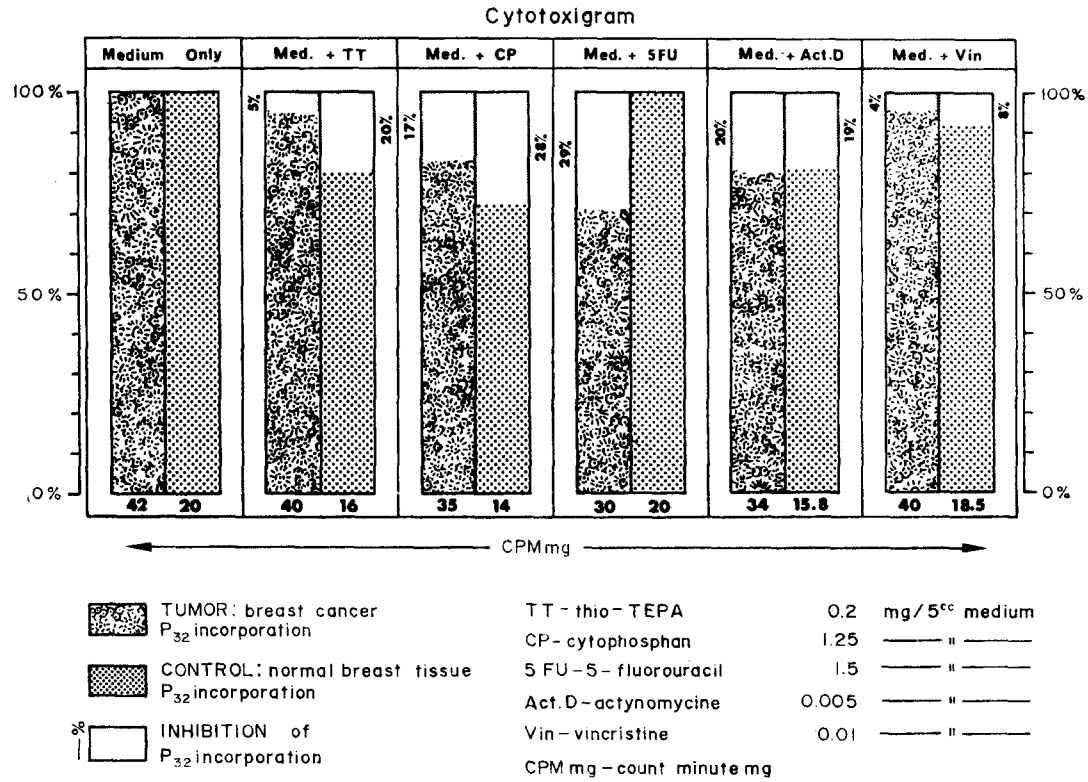
MATERIAL AND METHODS

Fourteen unselected patients, 11 of whom were operated upon for breast cancer and the remaining three for cancer of the stomach, were studied. In 3 cases the results obtained in the tissue cultures were applied to the treatment of the patients and a brief summary of these, is also reported here. A sizeable biopsy specimen was obtained from the tumor as well as from the adjacent normal tissue, verified by histological examinations. The biopsy specimens were immediately cut into small fragments with a scalpel, and were placed in triplicates into the culture medium described. The method is basically that of Ambrose with a slight modification [9, 13, 16].

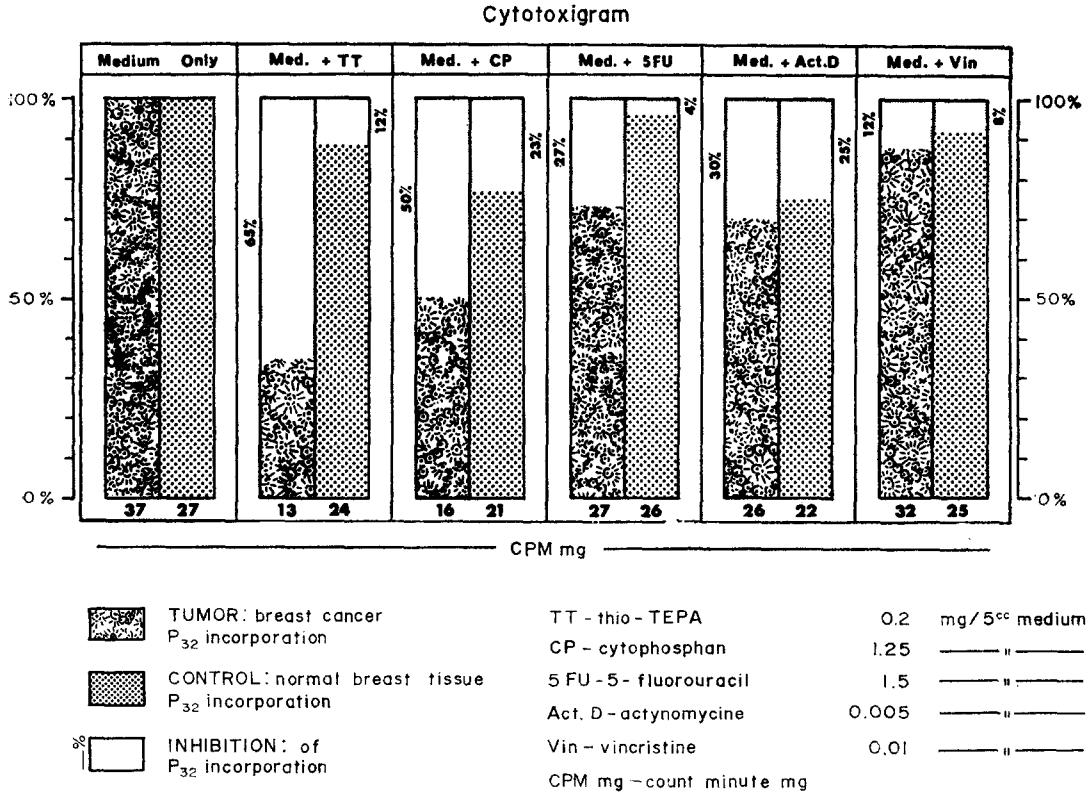
In the laboratory, the pieces were put into petri dishes containing modified Eagle's

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medium with 10% calf serum [20]. The cultures were incubated at 37°C, in an atmosphere of 5% CO_2 for 30 min, and the cytotoxic drug was then added. A control set without cytotoxic drug was also included. After incubation for 18 hr 0·2μC P^{32}/2·5 cm^3 medium was added to the culture, which was then incubated for a further 24 hr. The frag-



Figs. 1 and 2. Cytotoxigrams illustrate the quantitative and qualitative effect of various cytotoxic agents on cancer of the breast and normal breast tissue, in tissue culture, in two patients. The comparative activity in CPM/mg/tissue is given on the horizontal axis, while the inhibiting effects are expressed as a percentage of the control cultures (i.e. grown in the absence of cytotoxic agents).



ments were then treated with 95% ethylalcohol, washed twice with TCA (fluid concentration 10%), and finally extracted with chloroform–ethanol–ether (2:2:1) for 15 min at 70°C.

After extraction the fragments were dried in an oven at 80°C, weighed and dissolved in Soluen 100 (Packard Instrument Company). The radioactivity was determined in a Liquid Scintillation Counter (Packard Model: 3003). The results were expressed as CPM per mg dry weight of the tissue. The following drugs were used, in the concentrations indicated, per 5 ml medium: thio-TEPA 0·2 mg; cytophosphan 1·25 mg; 5-fluorouracil 1·5 mg; actinomycine D 0·005 mg; vincristine 0·01 mg. The concentrations employed were those suggested by previous investigators [16].

In order to express the results in an easily surveyable form and to render them comparable to each other, we established a graphic system which we have called a cytotoxigram Such cytotoxigrams are illustrated in Figs. 1 and 2. In the evaluation of the cytotoxic action, an inhibitory effect of 50% or more on the incorporation activity was considered very good, between 25% and 49% good, while less than 25% was taken as a poor response.

RESULTS

Breast cancer

The incorporation activity, observed in 11 cultures (Table 1) obtained from breast cancer, was found to be low. There were no marked differences in this activity between tumors with various histological appearances. The incorporation activity of the normal tissue in a given patient was similar to that observed in the cultures of the tumor from the same patient. Table 1 also shows that thio-TEPA produced a very good response in two cases, a good response in four cases, while the effect was poor in the remaining five cultures.

Table 1. Inhibition of ^{32}P incorporation by different cytotoxic agents of breast cancers and normal breast tissue of the same patient

Patient	Age	Histological diagnosis	CPM without drug	Inhibition percentage				
				TT	CP	5FU	ACT.D	VIN
1		Infiltrating duct carcinoma	24	38	46	20	42	34
Z.Z.	120 69	Normal breast tissue	10	40	40	58	18	70
2		Infiltrating duct carcinoma	37	65	50	27	30	12
A.B.	115 44	Normal breast tissue	27	12	23	4	25	8
3		Infiltrating duct carcinoma	30	50	ST	24	18	12
S.R.	119 42	Normal breast tissue	24	0	ST	34	20	40
4		Infiltrating duct carcinoma	18	23	34	47	34	25
K.E.	124 53	Normal breast tissue	16	75	6	ST	18	7
5		Infiltrating duct carcinoma	88	42	42	37	48	0
H.K.	209 74	Normal breast tissue	35	34	84	82	54	61
6		Papillary carcinoma	42	5	17	29	20	4
M.CH.	114 54	Normal breast tissue	20	20	28	0	19	3
7		Papillary carcinoma	22	23	0	27	12	
G.L.	112 41	Normal breast tissue	23	18	40	70	10	
8		Colloid carcinoma	19	30	27	20		
CH.L.	117 73	Normal breast tissue	21	5	0	41		
9		Colloid carcinoma	40	25	56	30	48	50
M.M.	115 42	Normal breast tissue	37	25	43	30	14	48
10		Colloid carcinoma	75	24	15	33	23	33
DI.CH.	137 35	Normal breast tissue	63	43	6	34	7	40
11		Undifferentiated cancer	40	13	10	35	20	
R.S.	131 50	Normal breast tissue	34	0	15	15		27

CPM — Counts per minute per milligram; TT — thio-TEPA; CP — cytophosphan; 5 FU — 5-fluorouracil; ACT.D — actinomycine; VIN — vincristine; ST — stimulation.

Marked suppression was obtained in the normal tumor-bearing tissue in one case, moderate suppression in four and a poor effect was seen in six cases. It should be stressed, however, that there was no correlation between tumor tissue and its host tissue with regard to their response to the cytotoxic agents added to the culture medium.

When the other agents such as cytophosphan, 5-fluorouracil, actinomycine D and vincristine were added to the culture medium, an individual reaction pattern of the tissue could be observed. It should be noted that each tumor or normal tissue showed a different sensitivity to the various agents employed. It follows, therefore, that by comparing the cytotoxigram for each tumor and its normal host tissue, a valuable guide-line may be obtained for the choice of the chemotherapeutic agent to be used in each individual case. It is noteworthy that cytotoxic agents occasionally seemed to have stimulated rather than depressed the incorporation activity (Cases no. 3 and 4, Table 1)

Cancer of stomach

The incorporation activity of both the tumor and the normal stomach tissue was higher than that seen in the breast tissue. Here too, a highly individual pattern characterized each tumor (Table 2).

DISCUSSION

A great deal of work has been published, in which attempts to gauge the effects of chemotherapy have been described. Explanted tumor tissue, kept alive under a variety of conditions, has been exposed to cytotoxic agents, and their effects assessed. The cultures employed were monolayers [9, 18, 19, 21–25], explants organ cultures and others [13, 16, 26, 27]. Criteria of effectiveness of the drugs have varied from analysis of their morphological effects [23, 28, 29], the number of cells destroyed per unit of time, the reduction of O_2 consumption [30], the inhibition of DNA, RNA [31–33] or protein synthesis [11, 13] by the tumor cells, and other methods.

Employing these methods Wright *et al.* showed good correlation between the *in vitro* results and the *in vivo* effects of the chemotherapeutic agents in 26 of 40 cancer patients, on whom these tests were performed [34]. Similar observations were reported in other studies [17, 19, 22, 30, 35]. Other investigators have found no such correlation at all [26], while a large number of workers have reported observations ranging widely between these two extremes [14, 22, 36].

The data reported in the present communication support the contention that the growth pattern of each tumor, or each normal tissue, as well as its response to cytotoxic agents *in vitro* is extremely variable and highly individual. Thus, for each cancer patient, a cytotoxigram must be produced which may enable the physician to choose the drug most likely to injure the tumor and least likely to cause damage to the normal tissue. This conclusion raises the question of the extent to which the tumor-bearing normal tissue represents all the vulnerable tissues of the body such as bone marrow, intestinal epitheliums, liver and skin. While the answer to this question cannot be supplied on the basis of available knowledge both the topographical, biological and genetic proximity of the tumor to its host tissue makes the comparative cultures employed here relevant.

The practical consequences of the above considerations are well illustrated by preliminary data in three patients. In patient no. 1, Table 2, thio-TEPA caused a 59% inhibition in the tumor, and only a 28% depression in the host tissue. A much more pronounced effect was elicited against the tumor by cytophosphan,

Table 2. Inhibition of ^{32}P incorporation by different cytotoxic agents in stomach cancer

Patient no.	Age	Histological diagnosis	CPM without drug	Inhibition percentage				
				TT	CP	5 FU	ACT.D	VIN
1	72	Adenocarcinoma	324	59	79	76	45	60
		Normal stomach tissue	100	28	33	50	30	42
2	61	Adenocarcinoma	100	20	0	20	18	30
		Normal stomach tissue	280	52	72	25	21	12
3	69	Adenocarcinoma	99	18	1	18	0	
		Normal stomach tissue	40	0	42	58	42	

CPM — Counts per minute per milligram; TT — thio-TEPA; CP — cytophosphan; 5 FU — 5-fluorouracil; ACT.D — actinomycine; VIN — vincristine.

5-fluorouracil or even vincristine (79, 76, 60% respectively), but their higher relative "toxic" effect on the normal tissue would favour the therapeutic use of thio-TEPA despite its lower anti-tumor effect. Accordingly thio-TEPA was employed with remarkably good results on the disseminated tumor, with very little untoward effect on the vulnerable normal tissues.

In case no. 2 (Table 1, Fig. 1), the good response to thio-TEPA was not surprising in view of the results depicted in the cytotoxigram. It can be seen that the drug had a marked (65%) inhibitory effect on the tumor, and a very low effect (12%) on the normal tissue. The administration of this compound to the patient brought good response as could be expected on the basis of the cytotoxigram. This tumor also showed remarkable sensitivity to a number of other compounds employed in the test. This cytotoxigram suggests that once the tumor becomes resistant to thio-TEPA, other drugs such as cytophosphan and 5-fluorouracil may be effective.

Another example is provided by the third patient (Case no. 6, Table 1, Fig. 2), where there was only one drug which depressed the P^{32} incorporation activity of the tumor tissue slightly more than that of the normal breast tissue. The other four compounds inhibited the normal tissue much more than they did the cancer cells. This would indicate that treatment with any of these compounds may not only not benefit the patient, but may in fact produce harmful effects. In accordance with these findings, 5-fluorouracil was given to the patient with markedly beneficial effects.

In a number of instances the cytotoxic drug elicited a stimulatory rather than depressing effect on the tissue against which it had been tested. Similar observations were also made by other investigators [6–8]. Whether this phenomenon is due to the abolition of an inhibitor of DNA replication, as suggested by Lajtha in connection with radiation effect, cannot be stated on the basis of the data available [37]. It may be relevant to mention the frequent clinical observation that certain parts of tumor masses regress during chemotherapy, while other areas continue their growth apparently unhindered by the presence of the cytotoxic agents [38].

All these observations suggest that the preparation of a cytotoxigram in each patient may be justified since only large scale correlative studies of this nature will finally allow us to reach meaningful conclusions concerning the possiblity of an *in vitro* system capable of predicting *in vivo* response.

SUMMARY

The inhibitory effects of a selected group of cytotoxic agents on the incorporation of P^{32} into nucleic acids by cancer tissue and adjacent normal tissue were studied in short term cultures. A comprehensive schematic illustration of the results, termed a cytotoxigram, was presented.

These procedures were employed in 11 *patients with cancer of the breast, and* 3 *patients with cancer of the stomach. Both the tumor and the non-tumorous tissues showed a highly individual sensitivity pattern to the agents that were added to the culture medium.*

REFERENCES

1. E. Boesen and W. Davis, Cytotoxic Drugs in the Treatment of Cancer, p.2. Ed. Arnold, London. (1961).
2. C. G. Zubrod, S. Schepartz, G. Leitner, V. M. Endicott, L. M. Carrese and C. G. Barker, The chemotherapy program of the National Cancer Institute. Part III. Proposal for the National Cancer Institute Cancer Chemotherapy Program. *Cancer Chemother. Rep.* **50,** 461 (1966).
3. A. Gellhorn, Invited remarks on the current state of research in clinical cancer chemotherapy. *Cancer Chemother. Rep.* **5,** 1 (1959).
4. H. Eagle and G. E. Foley, The cytotoxicity of anti-tumor agents for normal human and animal cells in first tissue culture passage. *Canc. Res.* **18,** 1012 (1958).
5. M. M. Murray, E. R. Peterson, E. Hirschberg and J. L. Pool, Metabolic and chemotherapeutic investigation of human glioblastoma *in vitro*. *Ann. N.Y. Acad. Sci.* **58,** 1147 (1954).
6. J. E. Byfield and J. J. Stein, A simple radiochemical assay of inhibition by chemotherapeutic agents of precursor incorporation into biopsy samples effusions and leukocyte preparations. *Canc. Res.* **28,** 223 (1968).
7. G. O. McDonald, J. M. McIver and A. S. Llamas, Tumor cytotoxicity. *Arch. Surg.* **92,** 541 (1966).

8. C. M. Southam, S. Tankara, I. Arata, D. Simhovic, M. Miura and S. F. Petropulos, Enhancement of responses to chemical carcinogens by non-carcinogenic viruses and anti-metabolites. *Progr. Exp. Tumor Res.* **11,** 192 (1969).
9. E. J. Ambrose, R. D. Andrews, D. M. Easty, E. D. Field and J. H. T. Wylie, Drug assays on cultures of human tumor biopsies. *Lancet* No. 1, p. 24 (1962).
10. I. V. Bichies, I. W. O. Wenderson and H. S. Nostel, Biochemical studies of human tumors *in vitro* estimation of individual tumor sensitivity to anti-cancer agents. *Canc. Res.* **19,** 103 (1966).
11. J. A. Di Paolo and J. E. Dowd, Evaluation of inhibition of human tumor tissue by cancer chemotherapeutic drugs with an *in vitro* test. *J. Nat. Cancer Institute* **27,** 807 (1961).
12. G. O. McDonald, A. R. Stround, A. M. Brunes and J. P. Cole, *In vivo* and *in vitro* assay for drug effect on cancer cells. *Ann. Surg.* **157,** 785 (1963).
13. E. J. Ambrose, R. D. Andrews and D. M. Easty, Drug assay on organ culture of biopsies. *Brit. med. J.* **2,** 490 (1964).
14. D. W. Easty and J. H. T. Wylie, Screening of 12 gliomata against chemotherapeutic agents *in vitro*. *Brit. med. J.* **4,** 1589 (1963).
15. D. A. G. Galton, Selective toxicity of anti-tumor agents. *Proc. Int. Symp. Chemotherapy of Cancer*, Lugano (Edited by P. A. Plattner), p. 234 (1964).
16. R. Tchao, D. W. Esty and E. J. Ambrose, The use of P^{32} labelled orthophosphate for the assay of chemotherapeutic agents on tumors maintained in organ culture. *Brit. J. Cancer* **21,** 821 (1967).
17. H. Gerharzt, Clinical results with cyclophosphamid and trisethylene iminoquinone. *Proc. Int. Symp. Chemotherapy of Cancer*, Lugano (Edited by P. A. Plattner) p. 62 (1964).
18. P. Loustalot and P. R. Desaulles, Characterisation of specificity of action of tumor inhibitory compounds. *Ann. N.Y. Acad. Sci.* **76,** 383 (1958).
19. H. Eagle and G. E. Foley, Susceptibility of human cells to cytostatic agents. *Ann. N.Y. Acad. Sci.* **66,** 534 (1958).
20. I. McParson and M. Stroker, Polyoma transformation of hamster cell clones. An investigation of genetic factors affecting cell competence. *Virology* **16,** 147 (1962).
21. J. P. Cole, O. G. Walker and J. C. Wright, Comparative chemotherapy studies on primary short term cultures of human normal benign, and malignant tumor tissues: A five-year study. *Canc. Res.* **21,** 583 (1961).
22. H. Lazarrus, W. Tegeder, H. M. Mazzone, J. Y. Leroy, B. A. Boone and J. E. Foley, Determination of sensitivity of individual biopsy specimens to potential inhibitory agents. Evaluation of some explant culture methods as assay system. *Cancer Chemother. Rep.* **50,** 543 (1966).
23. G. O. McDonald and J. P. Cole, The use of human tumors in primary culture for testing anti-cancer compounds. *Surg. Forum* **10,** 67 (1959).
24. O. G. Walter and J. C. Wright, Cytotoxical alteration in primary explant cultures of human neoplasms exposed to vincaleucoblastine. *Canc. Res.* **22,** 1267 (1962).
25. S. Tannenberger and G. Bacigalupo, The use of cell cultures for determining the sensitivity of human tumors to cytostatics. *Dtsch. Gesundh.-Wes.* **22,** 11 (1967).
26. J. J. Biescle, Tumor specific cytotoxicity of anti-tumor agents. *Ann. N.Y. Acad. Sci.* **78,** 530 (1958).
27. O. O. Throwell, Organs in a synthetic medium. *Expl. Cell Res.* **16,** 118 (1958).
28. I. Palyi, Effect of anti-tumor agents on cell morphology in tissue cultures. *Neoplasma (Bratisl.)* **14,** 159 (1967).
29. M. R. Roller, P. S. Owen and C. Heidelberger, Studies on the organ culture of human tumors. *Canc. Res.* **26,** 626 (1966).
30. J. A. Di Paolo and J. E. Dowd, *In vitro* assessment of chemotherapeutic activity in cancer. *N.Y. St. J. Med.* **62,** 2127 (1962).
31. D. Andrysek, K. Motycka, O. Dvorak, H. Jandova and E. Stramska: The estimation of human tumorous sensitivity to cytotoxic treatment. *Neoplasma (Bratisl.)* **16,** 57 (1969).
32. P. B. Dannenberg, J. Montag and C. Heidelberger, Studies on fluorinated pyrimidines effect on nucleic acid metabolism *in vivo*. *Canc. Res.* **18,** 329 (1958).

33. G. O. McDonald, J. M. McIver, A. S. Llamas and M. H. Sky-Pech, Tumor cytotoxicity change in DNA synthesis as an inhibition. *Arch. Surg.* **92,** 541 (1966).
34. J. C. Wright, Y. P. Cob, F. M. Golomb, S. L. Gumport and D. Safad, Investigation of the relation between clinical and tissue culture responses to chemotherapeutic agents on human cancer. *New Engl. J. Med.* **237,** 1207 (1957).
35. J. D. Hurley and L. Y. Young, Selection of anti-cancer drugs for palliative using tissue culture sensitivity studies. *Amer. J. Surg.* **39,** 109 (1965).
36. H. Eagle and G. E. Foley, The cytotoxic action of carcinolytic agents in tissue culture. *Amer. J. Med.* **21,** 739 (1956).
37. L. G. Lajtha, The effect of ionizing radiations and tumor-chemotherapeutic agents on the bone marrow. *Progr. Biophys.* **11,** 99 (1961).
38. J. F. Holland, Chemotherapy and chemopraxis of cancer. *Cancer Res.* **21,** 1086 (1961).

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Evidence for Tumor-Specific Immunity in Human Malignant Melanoma

GERD A. NAGEL*†‡, WILLY F. PIESSENS‡, MAGDA M. STILMANT§ and FERDINAND LEJEUNE§

‡*Service of Medicine and Laboratory of Clinical Investigation,* §*Service of Pathology and* §*Service of Surgery, Institut J. Bordet, Cancer Center of the University of Brussels, Brussels, Belgium*

INTRODUCTION

THAT tumor cells contain neoantigens strong enough to elicite tumor-specific immune reactions of the host can be considered an established fact for all experimental tumors adequately studied. This has comprehensively been reviewed by several authors [1–5].

The confirmation in animal systems of the old concept of a host–tumor relationship and the development of new techniques to study it consequently revived the search for comparable immunological specificities of human neoplasms [6, 7]. Although some favorable arguments were considered for the majority of human tumors, convincing results were presented only for a minority of them. Malignant melanoma ranks high in this list along with Burkitt's lymphoma [8], the leukemias [9, 10], osteosarcomas [11], and intestinal carcinomas [12].

We wish to report here a study of a case of human malignant melanoma in whom we observed that specifically sensitized lymphocytes might circulate in the peripheral blood in the absence of detectable disease, while it was impossible to find specific immune lymphocytes when the disease was clinically manifest.

MATERIAL AND METHODS

The test system which we designed and called MLTC (mixed lymphocyte–tumor cell culture) is basically a modification of the MLC (mixed leucocyte culture) [13] which allows the reproduction *in vitro* of the reaction of transplantation immunity *in vivo* [14] by the selective measurement of antigenic incompatibilities for important foreign antigens.

The principle of the *in vitro* studies is shown in Fig. 1. Tumor cells and lymphocytes were collected at the time of surgery, slowly frozen in a Linde BF-4-1 biological freezer and stored separately in liquid nitrogen with 10% dimethylsulfoxide as preservative. At various intervals postoperatively sufficient cells for further *in vitro* studies were obtained by rapid thawing of the stored lymphocyte and tumor-cell suspensions. The preparation of cell suspensions, culture conditions and quantitative estimation of the reaction by means of DNA labelling with 3H-thymidine followed by liquid scintillation counting have been previously described [15]. Some modifications of the original technique will be described below. Pieces of tumor and corresponding normal tissue were minced with scissors and blades in tissue-culture medium (TCM) immediately after operation. The crude tissue suspension was placed between two closely fitting sieves (0·3 mm meshes) which were twisted in opposite directions without further pressure. After passing it through several layers of sterile surgical gauze the filtrate was washed three times in TC 199 and its cell content counted. This purely mechanical procedure gives satisfactory single cell suspensions, the overall

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*Present address: Head, Oncology Service, Department of Internal Medicine, Bürgerspital, 4000 Basel, Switzerland.
†Research fellow of the Swiss Cancer Ligue.

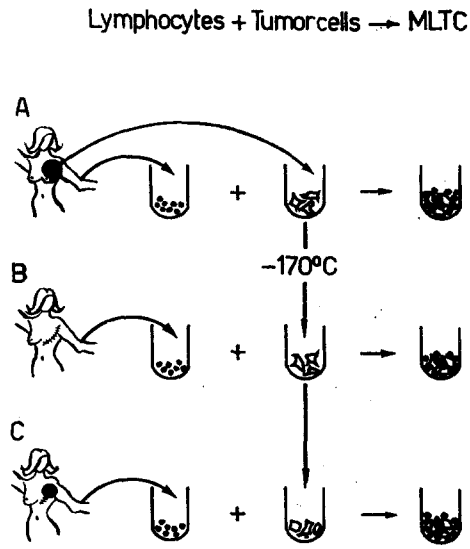


Fig. 1. *The principle of the horizontal follow-up of cellular immunity at various disease stages with the mixed lymphocyte tumor-cell culture (MLTC). (A) Disease manifestation. (B) Remission or cure. (C) Relapse. Storage of the tumor cells at* −170°*C.*

counting error due to cell clumps being ±13%. Cell viability was checked either by Trypan blue exclusion and/or FDA (fluorescein-diacetate) uptake. Over 90% pure lymphocyte suspensions were obtained from venous blood after heparin sedimentation (30 U/ml blood) and adsorption of granulocytes on cotton wool columns.

The culture media were either TC 199 or RPMI 1640 supplemented with 15% fresh plasma of the donor of responding lymphocytes. Antibiotics were omitted.

The MLTC with lymphocytes as responding (*R*) and tissue cells as stimulating (*S*) cells was carried out at different *R*/*S* ratios ranging from 25:1 to 1:25. The control MLC was done at *R*/*S* ratios 5:1 to 1:5. The ratios were obtained by keeping the number of the *R* cells constant at 1×10^6/culture tube, adding *S* cells in increasing concentrations but constant volume. These ratios were chosen on the basis of previous experience with the MLC indicating optimal results within the range used here [16]. In the MLC *S* lymphocytes were preincubated with mitomycin C (0·05 mg/ml) for 30 min and washed twice in TC 199 thereafter, in order to make the reaction strictly one-way.

Since preliminary experiments had shown that tumor or normal control cells had no or little DNA synthesis *in vitro* at the end of the culture period under the experimental conditions, these cells were not preincubated with mitomycin before their use as stimulants in the MLTC.

The cell mixtures were incubated at 37°C in 2·5 ml of culture medium in tightly sealed siliconized test tubes. After 130 hr 2 μCi 3H-thymidine (spec. act. 5 Ci/mM, Radiochemical Centre, Amersham) was added and the mixtures incubated for an additional 14 hr period. To end the reaction the cells were centrifuged and separated. The method of DNA precipitation and counting, as described previously [16], was changed in favor of the following procedure: the cell pellets were precipitated with 5% cold trichloroacetic acid (TCA) and passed through millipore filters (pore size 0·45 μ) by means of vacuum suction which eliminates the acid soluble fraction. The acid insoluble material containing the labelled DNA remained on the filter and was washed twice with 10 ml of 5% TCA. Thereafter the filters were put into counting vials and dried overnight at 60°C. After addition of 10 ml of liquid scintillation solution (6 g butyl-PBD in 1000 ml toluol) to each vial the radioactivity was determined in a Philips liquid scintillation analyzer with correction for quenching by the channels ratio method. The results are expressed as median count per min (CPM) of experiments done in triplicate.

Bacteriological controls of representative samples of all cell suspensions at the beginning and several random samples after 144 hr of culture were done and were negative.

CASE REPORT

1. *Clinical history*

The previous history of patient VS, a 68-year old male (IB 48601), was unremarkable until February 1968 when he first noted a pigmented lesion on the right foot. This

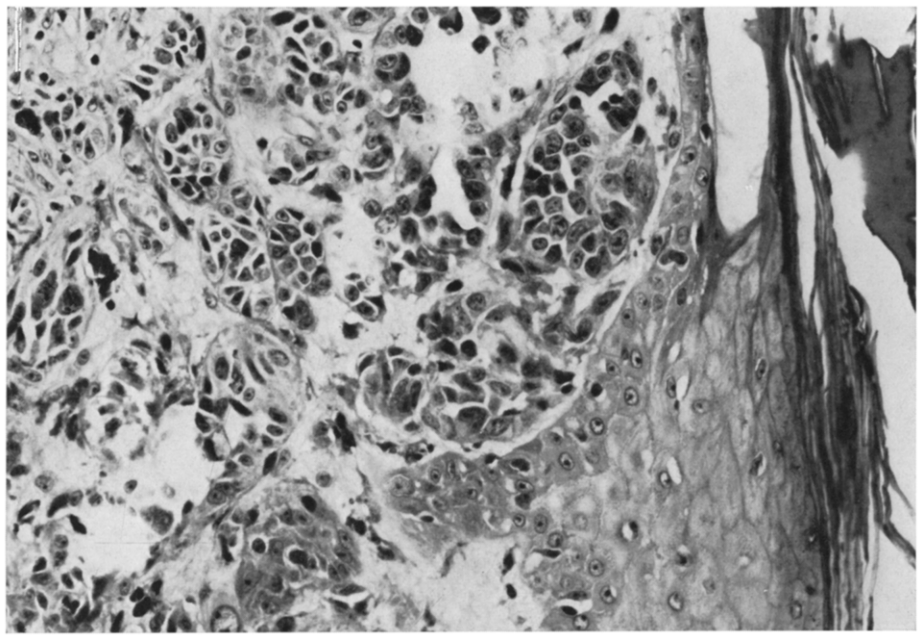


Fig. 2. *Histological section of the primitive site. Hematoxylin-eosin* × 600.

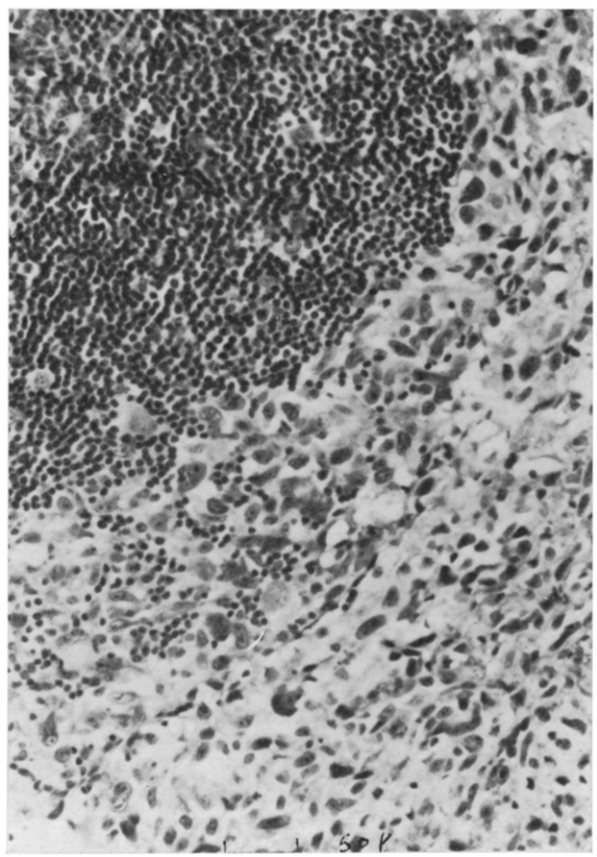


Fig. 3. *Lymph node metastasis. Hematoxylin-eosin* × 600.

(to face p. 42)

enlarged progressively and ulcerated in June 1969. At that time a biopsy taken at another hospital gave the diagnosis of melanocarcinoma. The patient was then referred for treatment at this Institute. There was no familial history of melanoma.

Physical examination was normal except for the ulcerated lesion measuring 22×21 mm and surrounded by satellite nodules of 2×2 mm. Laboratory tests were all normal and melanuria was absent. X-ray examination of the chest and the squeleton was normal. Bilateral lymphangiograms of the legs were normal. The tumor was thus classified as T3NO (Internal classification).

On July 30th the patient underwent local excision of the lesion and right inguino-iliac lymph node dissection. Recovery was uneventful, and at the time of submisison of this report the patient is still free of overt disease.

2. *Pathological examination*

Pathological examination of the surgical specimens confirmed the diagnosis of malignant melanoma of the epithelial cell-like type (Fig. 2). No particular lymphoplasmatic infiltration was seen in the primitive tumor site.

There were several unexpected microscopic lymph node metastases (Fig. 3). These lymph nodes showed also reticular hyperplasia and lymphangiectasia. Pigment-loaded plurinuclear macrophages and plasmatocytes were abundant on several sections. This could be interpreted as *in vivo* evidence for a tumor–host reaction but is more probably related to the previous lymphography.

3. *Culture studies*

Day 0. The primary tumor minus a representative specimen for histology was processed as outlined above. Sufficient cells were retained for the immediate culture studies, and the rest of the tumor cells was stored at −170°C. Plasma of the patient was stored at −20°C. Lymphocytes were prepared likewise from the patient and an unrelated normal control. Part

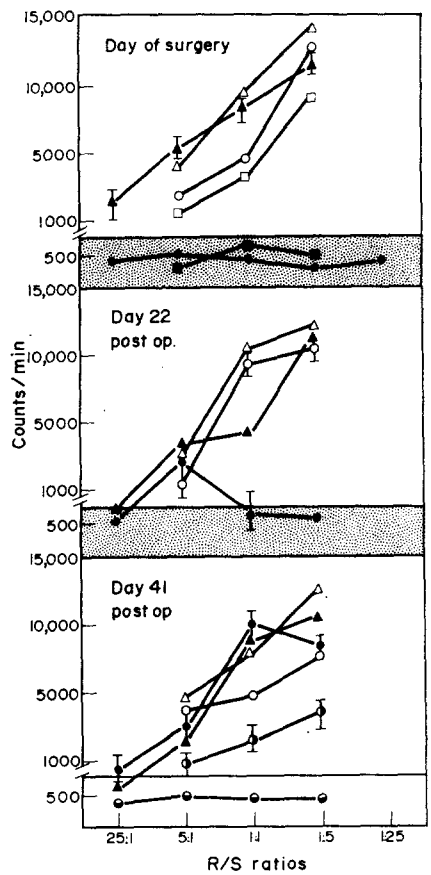


Fig. 4. Results of the MLTC and MLC at the day of surgery, 22 and 41 days thereafter. The autochthonous mixture changes the pattern from negative to positive. MLTC: ●----●: autochthonous mixture; ◑----◑: autochthonous mixture with plasma from the day of surgery. ◒----◒: idem with lymphocytes from day 0. ▲----▲: allogeneic control mixture. □----□: allogeneic mixture, tumor cells pretreated with mitomycin C. ■----■: allogeneic mixture, both lymphocytes and tumor cells pretreated with mitomycin C. MLC: O----O: patients lymphocytes as responding cells. △----△: control donor's lymphocytes as responding cells. Stimulating cells were pretreated with mitomycin C.

Table 1. *Control experiments in addition to results in Fig.* 4

Day of surgery

Cells alone, 2×10^2/tube:

N, Nm, V, Vm, tu, tum.

Mixed cell cultures:

N/Nm, V/Vm, V/tum, (N/tum and N/tum *vide* Fig. 4), V/tu in N plasma.

PHA-P cultures, $1 \cdot 0 \times 10^6$/tube:

N=28·732 counts/min. V=24·369 counts/min. tu=388 counts/min. tum=471 counts/min.

Day 22 postop.

Cells alone, 2×10^6/tube:

N, Nm, nm, V, Vm, v, vm, tu.

Mixed cell cultures:

N/Nm, N/n, V/v, Nm/Vm, n/Vm=13·722 counts/min, v/Nm=8·416 counts/min, Nm/tu, Vm/tu, v/tu (5:1 and 1:1), n/tu=2·936 counts/min.

PHA-P cultures, $1 \cdot 0 \times 10^6$ cells/tube:

N=32·100 counts/min, V=28·610 counts/min, n=22·376 counts/min, v=18·443 counts/min, tu=229 counts/min.

Day 41 postop.

Cells alone, 2×10^6/tube:

N, n, V, v, tu.

Mixed cell cultures:

Nm/Vm, n/Nm, N/n, V/v (v/tu *vide* Fig. 4) V/tu in N plasma=8·872 counts/min, n/Vm=8·250 counts/min, v/Nm=4698 counts/min, v/tu in N plasma. N/tu in stored V plasma day 0=10·759 counts/min (*R/S* ratio 1:1) and 12·334 counts/min (*R/S* ratio 1:5).

PHA-P cultures:

N=26·987 counts/min, V=27·692 counts/min, n=10·545 counts/min, v=21·377 counts/min, tu=498 counts/min.

V: patient's lymphocytes freshly prepared.
v: patient's lymphocytes stored at −170°C from the day of surgery.
N: control donors lymphocytes.
n: idem, stored since day 0.
tu: tumor cells.
m: pretreatment with mitomycin C.
R/S ratio: responding /stimulating cell ratio.
Controls were done at 1:1 ratio unless otherwise specified. Exact counts are given only if stimulation above background was obtained, or exact information is desired.

of the lymphocytes to be used as stimulating cells was pretreated with mitomycin C as previously described.

Cell viability (as judged by Trypan blue exclusion test) gave the following values: untreated tumor cells: 67±3%; mitomycin treated tumor cells: 59±2%; lymphocytes of both donors untreated: 99%; idem mitomycin treated: 94±3%.

The *R/S* ratios of the various culture combinations are shown in Fig. 4.

Day 22. Stored lymphocytes and tumor cells were thawed, washed, counted and assayed for viability. The Trypan blue results were: tumor cells: 44±4%; patients lymphocytes: 52±3%, control lymphocytes: 43±2%.

MLTC were done at *R/S* ratios (based upon viable cell counts) 5:1 and 1:1 with stored lymphocytes and 25:1, 5:1, 1:1, 1:5 with freshly obtained lymphocytes. The MLC ratios were 5:1, 1:1 and 1:5.

Day 41. Forty-one days after surgery, the MLTC were performed with freshly obtained lymphocytes and lymphocytes stored since day 0 at *R/S* ratios 25:1, 5:1, 1:1, 1:5. The MLTC with day 41 fresh lymphocytes was

done on two identical parallel experiments except that in one experiment fresh plasma was replaced by plasma stored since the day of surgery. As many controls were included as possible, in order to find all the stimulating combinations, to exclude other mitogens than *S* cells and to assess the responsiveness of the *R* cells. The control combinations tested are shown under results in Table 1.

RESULTS

The results of the MLTC and MLC for this patient with melanoma are given in Fig. 4. It is evident that the MLTC with autologous tumor cells was negative at the day of surgery but positive in at least 3 out of the 4 *R*/*S* ratios tested 42 days later with fresh lymphocytes as responding cells. Twenty-two days after surgery, counts clearly above background were obtained at the 5:1 *R*/*S* ratio only.

Positive stimulation of allogeneic lymphocytes was obtained with fresh or deep frozen tumor cells on all occasions, although fresh tumor cells gave higher counts.

Lymphocytes obtained at the day of surgery were never stimulated by autochthonous tumor cells, either when tested immediately or after storage, or whether in autologous or allogeneic plasma. They showed however blastic transformation throughout the experiments in the presence of allogeneic mitomycin C treated lymphocytes or when stimulated by PHA-P.

Cultures were carried out routinely in culture medium supplemented with fresh autochthonous plasma. The MLTC at day 41 was done in addition with plasma stored from the day of surgery. With this plasma the responsiveness of autochthonous lymphocytes was considerably reduced but the reactions were not abolished however. When in the allogeneic MLTC normal plasma was replaced by stored "day of surgery" plasma the counts remained unchanged.

The DNA synthesis of tumor cells with or without pretreatment by mitomycin, even after "stimulation" with PHA-P remained within background counting values. Moreover, when both lymphocytes and allogeneic tumor cells were pretreated with mitomycin and thereafter cultured together, no stimulation was found. Mitomycin treatment of tumor cells in allogeneic mixtures resulted in depression of the counts.

DISCUSSION

Malignant melanoma has attracted much interest because of its unusual biological variability. Two particularities of its clinical course, spontaneous regression [17–18] and fulminant aggressiveness, even crossing histocompatibility barriers in a case of fatal homotransplantation [19], have tentatively been explained on an immunological basis. In addition, the existence of melanoma-directed antibodies has been clearly demonstrated by several investigators [20–25].

Lymphocyte transformation as a response of sensitized cells to the foreign antigens of melanoma tissue was first demonstrated in an isogeneic situation [15]. The present data extend this observation by the demonstration of lymphocyte transformation induced by autochthonous tumor cells during the weeks following surgical excision of the malignant melanoma. The controls included in the experimental set-up exclude extrinsic mitogens as the causative agents as well as spontaneous lymphocyte transformation.

It might be possible that this patient developed skin auto-immune disease postoperatively. No controls with normal skin or pigmented benign naevi were done which properly could exclude this possibility, but clinically no sign of skin disease was present. Furthermore, normal control tissue from the corresponding organ was included in the controls of two other cases studied as outlined above (one hypernephroma and one breast carcinoma) and failed to induce significant lymphocyte transformation (unpublished results).

An alternative explanation is that specific immunity developed against the tumor after its removal. Such a change of the reactivity of the host's lymphocytes against autochthonous tumor cells after the tumor has been removed is well known from animal experiments [26] and evidence for specific immune paralysis recovering postoperatively was recently presented for animal tumors [27].

In our study "day of surgery" (day 0) lymphocytes never transformed in the presence of autochthonous tumor, whether the culture was done in autochthonous or allogeneic plasma. Serum factors, perhaps enhancement antibodies, might play a role by blocking the reaction by competitive interaction at the antigenic sites [28, 29]. Indeed, "day of surgery" plasma reduced the MLTC counts as compared to fresh postoperative plasma, but did not abolish the transformation process. Since it has been shown that antibodies possibly decline postoperatively [27] one could speculate that in our case the circulating antibody titer was sufficiently low 41 days after surgery as to leave the antigenic sites unmasked, but high enough to mask them at

the day of surgery. However, if coating of the tumor with antibody would be the only explanation of the failure to induce lymphocyte transformation, stored tumor cells of the same batch would not be expected to stimulate at a later stage unless some of the coating material was washed off during the storage process. In this case day 0 lymphocytes should then also transform in the presence of these "uncoated tumor cells" since the freezing process does not alter their ability to transformation in the presence of allogeneic lymphocytes.

It seems therefore that the failure of tumor cells to induce blastic transformation of autologous lymphocytes obtained at the day of surgery could be tentatively explained by specific immune paralysis from which the patient recovered postoperatively.

As yet it cannot be ascertained whether the presence of humoral and/or cellular tumor-specific immunity is associated with better prognosis or slower tumor growth. Therefore, as long as there is no answer as to the importance of humoral and cellular antibody, their relationship and importance, clinical trials of immunotherapy in the early phase of the disease seem obsolete.

SUMMARY

A case of cutaneous malignant melanoma metastatic to regional lymph nodes is presented. Blastoid transformation of lymphocytes in the presence of autochthonous tumour cells failed to occur when the lymphocytes were taken at the day of the operation. Lymphocytes, however, obtained 41 *days postoperatively were stimulated by autochthonous tumor cells and the reaction was suppressed by autologous stored serum. It is concluded that lymphocyte transformation indicates cell bound tumor specific immunity which becomes demonstrable only after the removal of the primary tumor whereas the day of operation serum possibly contains blocking antibodies.*

REFERENCES

1. G. Klein, Tumor specific transplantation antigens. *Cancer Res.* **28,** 625 (1968).
2. H. O. Sjögren, Transplantation methods as a tool for detection of tumor-specific antigens. *Progr. exp. Tumor Res.* **6,** 289 (1965).
3. R. T. Smith, Tumor-specific immune mechanisms. *New Engl. J. Med.* **278,** 1207, 1268, 1326 (1968).
4. B. Benacerraf, Cell-associated immune reactions. *Cancer Res.* **28,** 1392 (1968).
5. L. W. Law, Studies of the significance of tumor antigens in induction and regression of neoplastic diseases. *Cancer Res.* **29,** 1 (1969).
6. C. M. Southam, Evidence for cancer specific antigens in man. *Progr. exp. Tumor Res.* **9,** 1–39 (1967).
7. G. H. Fairley, Immunity to malignant diseases in man. *Brit. med. J.* **ii,** 467 (1969).
8. E. Klein, P. Clifford, G. Klein and C. A. Hamberger, Further studies on the membrane immunofluorescence reaction of Burkitt lymphoma cells. *Int. J. Cancer* **2,** 27 (1967).
9. J. H. Burchenal, Long-term remissions of acute leukemia, spontaneous and induced. *Scand. J. Haematol.* **1,** 47 (1965).
10. J. F. Doré, R. Motta, L. Marholev, Y. Hrsak, H. Colas de la Noue, G. Séman, F. de Vassal and G. Mathé, New antigens in human leukaemia cells and antibody in the serum of leukaemic patients. *Lancet* **ii,** 1396 (1967).
11. D. L. Morton and R. A. Malmgren, Human osteosarcomas: immunologic evidence suggesting an associated infectious agent. *Science* **162,** 1279 (1968).
12. P. Gold and S. O. Freedman, Specific carcinoembryonic antigens of the human digestive system. *J. exp. Med.* **122,** 467 (1965).
13. B. Bain, M. R. Vas and L. Lowenstein, The development of large immature mononuclear cells in mixed leucocyte cultures. *Blood* **23,** 108 (1964).
14. F. Bach and K. Hirschhorn, Lymphocyte interaction: a potential histocompatibility test *in vitro*. *Science* **143,** 813 (1964).
15. G. A. Nagel, G. St. Arneault, J. F. Holland, R. Kirkpatrick and D. Kirkpatrick, *Cancer Res.* in press.
16. G. A. Nagel and J. F. Holland, submitted to *Transplantation.*
17. T. C. Everson and W. F. Cole, *Spontaneous Regression of Cancer.* W. B. Saunders, Philadelphia (1966).

18. W. BOYD, *The Spontaneous Regression of Cancer.* C. C. Thomas, Springfield, Ill., USA (1966).
19. E. F. SCANLON, R. A. HAWKINS, W. W. FOX and W. S. SMITH, Fatal homotransplanted melanoma. *Cancer, N.Y.* **18,** 782 (1965).
20. A. W. KOPF, I. SILBERBERG and N. S. COOPER, Immunohistochemical study of human malignant melanoma for the presence of gamma-globulin. *J. invest. Derm.* **47,** 83 (1966).
21. D. L. MORTON, R. A. MALMGREN, E. C. HOLMES and A. S. KETCHAM, Demonstration of antibody against human malignant melanoma by immunofluorescence. *Surgery* **64,** 233 (1968).
22. N. M. MUNA, S. MARCUS and C. SMART, Detection by immunofluorescence of antobodies specific for human malignant melanoma cells. *Cancer* **23,** 88 (1969).
23. M. G. LEWIS, R. L. IKONOPISOV, R. C. NAIRN, T. M. PHILLIPS, G. HAMILTON-FAIRLEY, D. C. BODENHAM and P. ALEXANDER, Tumor-specific antibodies in human malignant melanoma and their relationship to the extent of the disease. *Brit. med. J.* **ii,** 547 (1969).
24. J. W. FINNEY, E. H. BYERS and R. H. WILSON, Studies in tumor autoimmunity. *Cancer Res.* **20,** 351 (1960).
25. I. HELLSTRÖM, K. E. HELLSTRÖM, G. E. PIERCE and J. P. S. YANG, Cellular and humoral immunity to different types of human neoplasms. *Nature, Lond.* **220,** 1352 (1968).
26. Z. B. MIKULSKA, C. SMITH and P. ALEXANDER, Evidence for an immunological reaction of the host directed against its own actively growing tumor. *J. nat. Cancer Inst.* **36,** 29 (1966).
27. G. BARSKI and J. K. YOUN, Evolution of cell-mediated immunity in mice bearing an antigenic tumor. Influence of tumor growth and surgical removal. *J. nat. Cancer Inst.* **43,** 111 (1969).
28. K. T. BRUNNER, J. MAUEL, J. C. CEROTTINI and B. CHAPUIS, Quantitative assay of the lytic action of immune lymphoid cells on 51Cr labelled allogeneic target cells *in vitro*; inhibition by isoantibodies and by drugs. *Immunology* **14,** 181 (1968).
29. I. HELLSTRÖM, K. E. HELLSTRÖM, C. A. EVANS, G. D. HEPPNER, G. E. PIERCE and P. S. YANG, Serum mediated protection of neoplastic cells from inhibition by lymphocytes immune to their tumor-specific antigens. *Proc. nat. Acad. Sci. (Wash.)* **62,** 362 (1969).

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Feulgen-Microspectrophotometric Determination of Different Evolutionary Patterns in serial Cultures of Cells Derived from Human Skin Tumours

C. D. DEWSE and C. G. POTTER

Cytogenetics Unit, Nuffield Wing: School of Medicine, University of Liverpool, England

INTRODUCTION

MIXED cell populations incorporating aneuploid cells have been shown in a number of neoplastic systems to evolve *in vitro* by elimination of either the normal or the aneuploid lines. Serial selection for different cell lines has been demonstrated in a mixed aneuploid culture of Novikoff hepatoma by Hsu and Klatt [1]. Levan and Biesele [2] found that the proportion of diploid cells decreased in mouse skin cultures until only an aneuploid strain capable of tumour production remained. Data on the chromosome complements of HeLa cell strains were tabulated by Vogt [3], showing that strains and substrains of these cells can be maintained with widely differing modal chromosome numbers. Auersperg [4] has reported evolution of a near-hexaploid culture derived from invasive carcinoma of human cervix, in which spontaneous decrease to hypotetraploid was observed. However Sandberg *et al.* [5] have shown conversely that the proportion of abnormal dividing cells in bone marrow from acute leukaemia patients decreased after only 10 hr *in vitro*. Neu *et al.* [6] showed that long-term cultures of reticulum cell carcinoma of the lymph node yielded only karyotypically normal cells whereas short-term cultures of the same material contained cells which had a marker chromosome and other, irregular, chromosomal abnormalities.

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An important disadvantage of karyotypic analysis as employed in the previously mentioned studies is demonstrated by the work of Stich and Steele [7], who showed that not all cells in a tumour *in vivo* can divide and that some cells can begin but are unable to complete mitosis. Similarly, the presence *in vitro* of some lines not represented in chromosome counts has been shown by microspectrophotometric examination of Ehrlich ascites carcinoma [8].

Many workers have estimated DNA-level distributions directly in specimens of a wide variety of neoplasms using different microspectrophotometric techniques [7–13]. DNA-levels in a number of skin neoplasms have been directly measured more recently by these techniques [14–20]. Kint [17] examined differentiation of skin tumours and both Rabotti [21] and Meek [22] considered metastatic growth to be essentially secondary and compared the DNA-level distribution in nuclei of such specimens with that of their primary tumours. However, we have found no report that the evolution of tumour-derived cell populations *in vitro* has been examined using microspectrophotometry.

This paper presents microspectrophotometric data on the DNA of cells of disaggregated and cultured basal cell carcinomata (BCC) and other skin tumours. Feulgen-DNA distributions in cell samples at successive stages of serial culture are compared. Evolutionary patterns are indicated by the number, size and relative position of modes in the histograms.

MATERIAL AND METHODS

Tissue culture

Tumour biopsies, collected as therapeutic excisions, were divided into two; one part was fixed in 10% formol saline for histological examination and final diagnosis, the other was put into TC 199 (Glaxo) and refrigerated overnight at 4°C. Explants about 1 mm^3 from the diametrically cut face of the tumour were attached to coverslips in Leighton tubes by human plasma (1:1 in Alsever's solution), clotted with a fifth part of a solution of 10 mg% thrombin (Bovine B grade, Calbiochem) in 2% $CaCl_2$. The coverslips were inverted to reduce drifting of the explants due to enzymic degradation of the clot. Growth was fibroblastic. Subculturing was done as required, removing the cells from the glass with 0·025% trypsin (×2 crystalline, Worthington Biochemicals) and 0·025% versene in phosphate buffered saline. Fresh tubes containing coverslips were seeded with cell suspension. All cultures were incubated at 37°C, fed three times weekly with medium comprising 80% TC 199, 15% pooled human AB serum and 5% chick embryo extract and were gassed with 5% CO_2 in air.

Preparations

Cells from primary cultures were harvested after an average of 14 days from setting up, or 5–7 days from initial growth. By this time, enough cells to give a good sample had migrated from the explant, but few cells had begun the active division cycle. Secondary cultures were harvested after 4–10 subcultures. The cells were harvested on coverslips by washing three times with phosphate buffered saline and fixing with 95% ethanol. Minced tumour was disaggregated in trypsin-versene solution overnight and the suspension shaken vigorously. The cells were spun down, fixed in suspension with 95% ethanol, spread on ice-cold slides and air-dried.

Staining

Two-hr staining in commercial Feulgen-Schiff reagent (G. E. Gurr) after hydrolysis in 5N HCl/25 min at room temperature was found to give the most intensive, uniform staining [23].

DNA estimations

Individual nuclei were scanned without selection on a Barr and Stroud integrating microspectrophotometer, type GN2, at 550 mμ. The cellular absorptions, representing DNA values, were measured in arbitrary units. These were plotted on a $\log_2$ scale so that an increase of one unit would denote a doubling of the DNA value.

In normal cell preparations the small error involved by the methods used indicated that there was little variation of staining intensity over different parts of the same slide. Therefore changes and characterization of the DNA value patterns can be validly compared although variation in staining between different slides and different staining batches precluded any statistical comparison of modal means.

RESULTS

Clinical details of the cases investigated are given in Table 1. Studies on 10 lesions were completed; cases of basal cell carcinoma and 1 each of Bowen's disease, Paget's disease and seborrhoeic wart.

Figure 1 shows histograms of Feulgen-DNA values observed in specimens of seborrhoeic wart (29 MT) and BCC (33 TG), where the distributions at all stages compare with that

Table 1. *Clinical details of the* 10 *cases investigated*

Case	Age of patient	Sex	Lesion	Site of lesion	Size of lesion (mm)	Age of lesion (months)	Rate of growth *in vivo*
21 HS	64	M	Paget's disease	Scrotum	15×20	30	Slow
26 HM	80	M	BCC*	Face	8×8	24	Slow
29 MT	55	F	Seborrhoeic wart	Shoulder	12×20	3	Very fast
32 TG	73	M	BCC	Hand	5×5	2	Fast
33 TG	73	M	BCC	Nose	3×3	2	Fast
41 GJ	45	F	Bowen's disease	Thigh	25×25	36	Moderate
59 WJ	69	M	BCC	Groin	10×15	24	Slow
63 EM	50	F	BCC	Forehead	3×3	6	Slow
66 JT	44	M	BCC	Cheek	5×15	6	Fast
70 HT	55	F	BCC	Central back	25×30	36	Moderate

*Basal cell carcinoma.

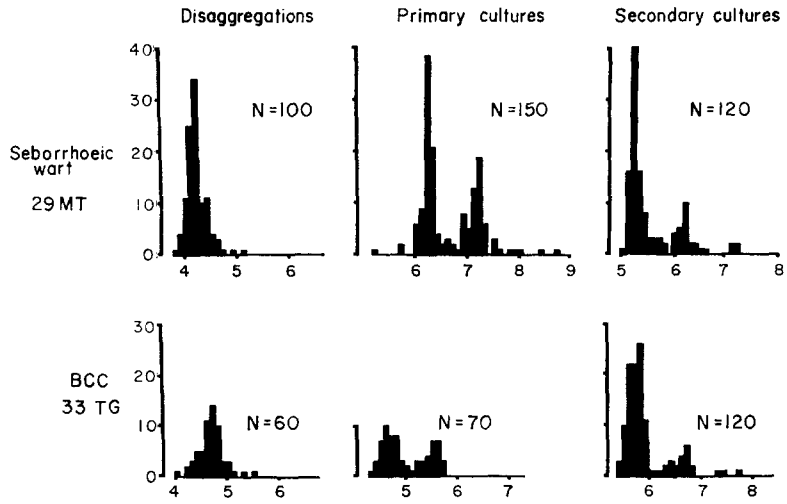


Fig. 1. Frequency distributions of Feulgen-DNA values of individual interphase nuclei in disaggregated cells and in primary and secondary cultured cells derived from seborrhoeic wart and basal cell carcinoma. These cases show distributions comparable to that of normal cells at each stage examined. Ordinate: number of cells. Abscissa: DNA level in arbitrary units on a $\log_2$ scale. N=number of cells in the sample.

expected from a normal population. In a system of normal dividing cells there would be a single main peak representing the pre-synthetic (G_1) diploid DNA value with a second smaller peak one $\log_2$ unit greater representing the post-synthetic (G_2) cells. Between these peaks would be a few cells whose DNA level was elevated due to current DNA synthesis. All the histograms in Fig. 1 show such distributions. Both disaggregation histograms have a single wide peak, revealing a high error. This reflects the presence of pycnotic cells at the lower range and DNA-synthesizing cells at the higher range, and also possibly is due to cell damage and inadequate flattening of cell nuclei during preparation. The error due to S-period cells would be expected to be small however, as Frindel *et al.* [24] showed the number of cells actually synthesizing DNA in BCC *in vivo* was less than 1%. The distributions of DNA in primary cultured cells of the two cases illustrated in Fig. 1 each show a marked second peak at twice the value of the main peak, accounted for by a combination of G_2 diploid and G_1 tetraploid cells. These two types of cell were not separable by the techniques used. There is a number of cells outside the main peaks in 29 MT in both the pycnotic (2%) and polyploid (6% over 4n) ranges. In our experience the numbers of cells in these categories are not inconsistent with distribu-

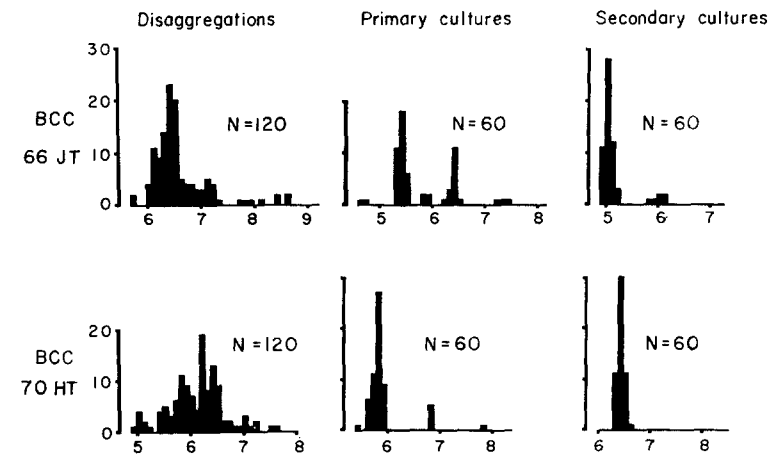


Fig. 2. Frequency distributions of Feulgen-DNA values of individual interphase nuclei in disaggregated and in primary and secondary cultured cells derived from basal cell carcinomas. These cases show the multimodality of the distributions in disaggregated tumours to be lost at the primary stage of culture. Ordinate: number of cells. Abscissa: DNA level in arbitrary units on a $\log_2$ scale. N=number of cells in the sample.

tions in normal primary cultured cells. The histograms from secondary cultured cells show normal distributions with a tiny peak at the octoploid value. The histograms of DNA levels in each stage for these two lesions therefore have distributions which correspond with those expected from normal populations of cells.

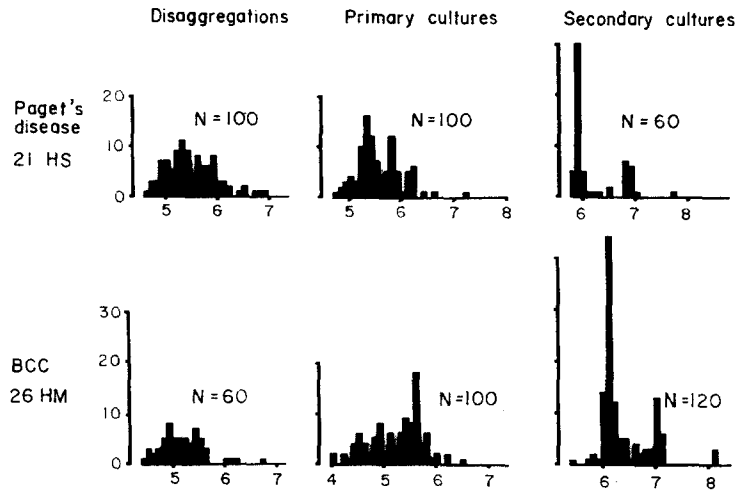


Fig. 3. Frequency distributions of Feulgen-DNA values of individual interphase nuclei in disaggregated and in primary and secondary cultured cells derived from Paget's disease and basal cell carcinoma. These cases show the multimodality of the distributions in disaggregated tumours to persist in primary culture but to be lost at the secondary stage of culture. Ordinate: number of cells. Abscissa: DNA level in arbitrary units on a $\log_2$ scale. N=number of cells in the sample.

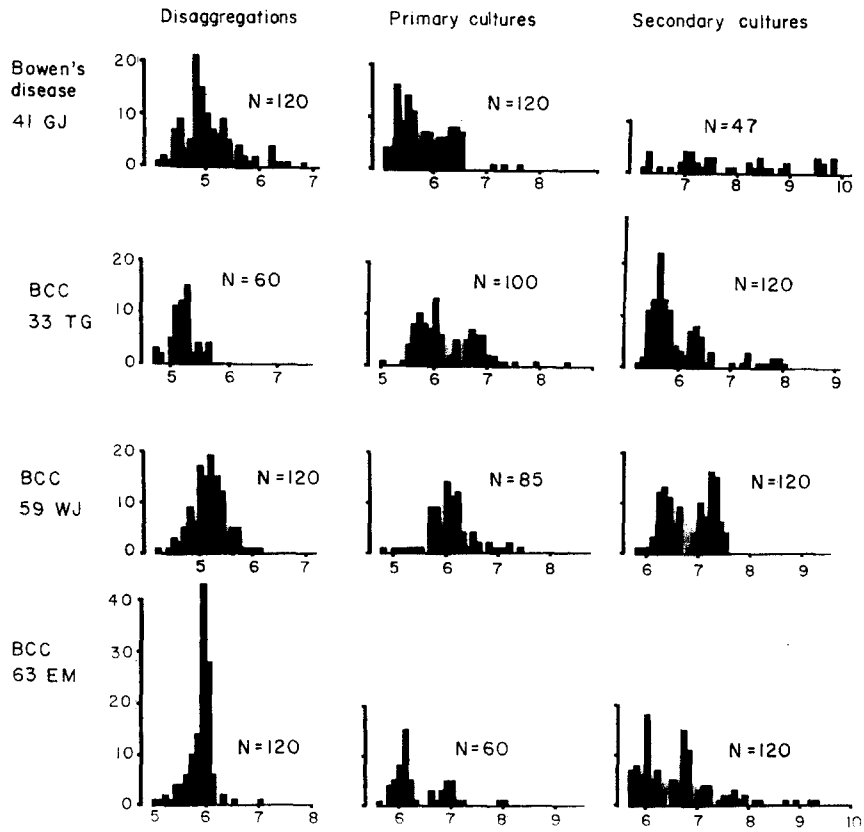


Fig. 4. Frequency distributions of Feulgen-DNA values of individual interphase nuclei in disaggregated and in primary and secondary cultured cells derived from Bowen's disease and basal cell carcinoma. These cases show the multimodality of the distributions in disaggregated tumours to persist throughout primary and secondary culture. Ordinate: number of cells. Abscissa: DNA levels in arbitrary units on a $\log_2$ scale. N=number of cells in the sample.

Figure 2 contains histograms showing a second pattern, where distributions consistent with those of normal cells are present in the histograms of both primary and secondary cultured cells, but where the disaggregations show abnormal distributions of DNA levels. The two disaggregated BCC specimens (66 JT and 70 HT) have multiple-moded histograms in which the peaks do not fit the normal diploid-tetraploid+G_2 diploid pattern already described. Thus aneuploid lines present in the tumours *in vivo* failed to grow *in vitro*.

A third pattern is shown in Fig. 3, where abnormal distributions are evident in the histograms of disaggregated cells from specimens of Paget's disease (21 HS) and BCC (26 HM). However, in these cases abnormal distributions are also seen in the histograms of primary cultured cells, demonstrating survival of aneuploid lines in early culturing. The distributions of DNA levels in each of the histograms of secondary cultured cells are consistent with the normal, even with the few (4%) pycnotic cells in 26 HM secondary and a small representation at the octoploid level.

The final pattern is shown in Fig. 4, where the distributions of DNA levels in the histograms of cells from all stages are abnormal. The 4 cases comprise 1 of Bowen's disease (41 GJ) and 3 of BCC (32 TG, 59 WJ and 63 EM). The histograms of 59 WJ primary and of both primary and secondary cultured cells of 63 EM could be considered to show distributions consistent with the normal. However, in interpreting these histograms, some account has been taken of the quality of preparations read. These preparations were particularly good and the consequent small error in reading allows more weight to be given to the minor peaks present. Thus these two cases are included in the class where all stages are abnormal.

No obvious connection was found between the degree of persistence of abnormal lines and the age of the patient, the duration of the lesion, the rate of growth *in vivo*, or the growth rate *in vitro*.

DISCUSSION

The results presented show that the histograms of DNA levels in individual nuclei of cells derived from some skin tumours may follow different patterns over a period of time *in vitro*. Four patterns of evolution emerge from comparison of such histograms from the lesions studied. Feulgen-DNA level distributions at each stage of culture may be similar (either single moded or multiple moded) to that found in the corresponding disaggregation, or the multimodality as seen in the majority of disaggregation histograms may be lost at either the primary or secondary stage of culture.

Apparent reversion, as demonstrated by the patterns of evolution where some peaks are lost in cultured cells is considered to show selection of cytogenetically similar cells from the mixed cell population originally present in the excised tumour rather than modification of cells so present. The novel conditions *in vitro* may impose selective pressures on the migration of some cells from explants or on the subsequent rate of multiplication of the cells of different cell lines. Thus individual cell lines of a mixed cell population may be eliminated in either the primary or secondary culture, leaving a population whose DNA distribution histograms are consistent with the normal. Such a single population may however be of normal diploid or of aneuploid cells and by the methods employed here a distinction cannot be made. If however the conditions *in vitro* favour none of the different cell lines, and if the cells in these lines divide at a comparable rate, equilibrium may be reached whilst several lines are still present.

The multiple modes in the histograms of the majority of disaggregations and most primary cultures represent the normal plus the abnormal lines. These distributions would give elevated DNA-levels if pooled, in accordance with the results of Laquerrière *et al.* [14] (as cited by Kint [16], and also of Kint [16] who both used microdensitometry in their estimations of DNA in skin tumours. Fluorimetric estimations by de Bersaques [25] showed that BCC cells have an average of three times and Paget's disease cells have twice the quantity of DNA present in normal skin cells.

Evolution *in vitro* shown by these results contrasts with the patterns outlined by others, where cultures of normal mammalian cells developed aneuploidy [2, 26–28] or became tumorigenic [2, 29–31]. Integrity of the modal chromosome pattern has been maintained, however, in long-term cultures of neoplastic origin, despite regular variation around the modal chromosome number [3, 32]. Karyotypically normal cells have also been maintained for long periods in cultured mammalian tissues [28, 33–36]. Moreover, German *et al.* [37] have reported keeping three aneuploid lines under identical conditions without change in chromosome count and several biochemical parameters over a number

of years. The loss *in vitro* of other neoplastic characteristics, such as tumorigenic potential, has been demonstrated extensively in mammalian tumour cultures [1, 38–52].

A parabiotic tissue culture technique was used by Katsuta and Takaoka [53] to show how rat ascites tumour cells increased in numbers whilst normal cells in a common bathing medium, or more pronouncedly, in the same tube were inhibited or destroyed. Such cultures are a good model for mixed cell populations found in or derived from clinical neoplastic material and gave results which those presented here only partly support.

Clearly, as in any evolutionary competition, the supplanting of one population by another depends on the nature of the environment and on the quality of difference or abnormality possessed by the competitive lines. Thus the apparent paradox can be rationalized whereby normal cells can be the more successful in some abnormal environments, e.g. *in vitro*, whilst some abnormal lines can thrive and even outstrip competitive lines (including the normal)in a normal environment, e.g. tumour growth *in vivo*.

The non-specific nature of the cytogenetic changes causing or resulting from malignant development of ECC allows diversity of behaviour in the same culturing conditions and consequently with the same selection pressures in this clinically defined lesion. Thus this one type of tumour is represented in each of the four classes of evolutionary growth described here.

Because of the evident instability of cells in the highly unnatural culturing environment, the reliability of long-term tissue culture techniques (or of any culturing at all) has been questioned with regard to diagnostic practice [5, 6, 54].

The variable behaviour *in vitro* of basal cell carcinomas demonstrated in this paper precludes the use of this particular culturing system in connection with diagnosis or prognosis of this lesion. Further work to compare the behaviour of cultures from a single tumour in identical and in different conditions is proceeding in order to find a system suitable for such investigation.

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SUMMARY

Distributions of DNA-levels were estimated in cells from seven cases of basal cell carcinoma, and one each of Paget's disease, Bowen's disease and seborrhoeic wart by means of Feulgen-microspectrophotometry. Four patterns of evolution were revealed by the distributions from primary and secondary cultured cells and from disaggregated tumour cells. All stages may show a DNA-level distribution consistent with the normal, or a tumour whose disaggregated cells show a multiple-moded distribution may give rise to cultures which either show similar distributions or lose their multi-modal distributions at the primary or secondary stage of culture. Thus aneuploid lines may be selected against in vitro *at the stage of migration from the explant or by subsequent differential growth. Alternatively the abnormal lines may persist in secondary cultures. Specimens of basal cell carcinoma showed all four patterns of cell population evolution.*

REFERENCES

1. T. C. Hsu and O. Klatt, Mammalian chromosomes *in vitro*. *X.* Heteroploid transformation in neoplastic cells. *J. nat. Cancer Inst.* **22,** 313 (1959).
2. A. Levan and J. J. Biesele, Role of chromosomes in cancerogenesis, as studied in serial tissue culture of mammalian cells. *Ann. N.Y. Acad. Sci.* **71,** 1022 (1958).
3. M. Vogt, A genetic change in a tissue culture line of neoplastic cells. *J. cell. comp. Physiol.* **52,** 271 (1958).
4. N. Auersperg, Karyotype changes of near hexaploid carcinoma cells during adaptation *in vitro*. *Nature (Lond.)* **212,** 635 (1966).
5. A. A. Sandberg, T. Ishihara, L. H. Crosswhite and T. S. Hauschka, Chromosomal dichotomy in blood and marrow in acute leukaemia. *Cancer Res.* **22,** 748 (1962).
6. R. L. Neu, T. Kaji and L. I. Gardner, Neoplastic tissue-culture cells. *Lancet* **i**/7480, 51 (1967).

7. H. F. Stich and H. D. Steele, DNA content of tumour cells. III. Mosaic composition of sarcomas and carcinomas in man. *J. nat. Cancer Inst.* **28,** 1207 (1962).
8. J. J. Freed and D. A. Hungerford, DNA content of nuclei and chromosome number in sublines of the Ehrlich ascites carcinoma. *Cancer Res.* **17,** 177 (1957).
9. S. Bader, Distribution of desoxyribose nucleic acid in tumour nuclei. *Proc. Soc. exp. Biol. (N.Y.)* **82,** 312 (1953).
10. C. Leuchtenberger, R. Leuchtenberger and A. M. Davis, A microspectrophotometric study of desoxyribosenucleic acid content in cells of normal and malignant human tissues. *Amer. J. Path.* **30,** 65 (1954).
11. N. B. Atkin and B. M. Richards, Deoxyribonucleic acid in human tumours as measured by microspectrophotometry of Feulgen stain: A comparison of tumours arising at different sites. *Brit. J. Cancer* **10,** 769 (1956).
12. G. Causey and C. J. Stratman, Differences in deoxyribonucleic acid content of nuclei from Schwann cells, endoneural cells and related tumour cells. *Brit. J. Cancer* **13,** 318 (1959).
13. N. B. Atkin and A. Ross, Polyploidy in human tumours. *Nature (Lond.)* **187,** 579 (1960).
14. R. Laquerriere, R. Laumonier and W. Stewart, Essai d'étude histophotométrique des tumeurs cutanées. *X° Congrés des Derm. et Syph. de langue française, Alger,* (1959).
15. R. Laumonier and R. Laquerriere, Quelques applications de l'histophotométrie en anatomie pathologique. *Ann. Histochim.* **8,** 313 (1963).
16. A. Kint, Histophotometric investigation of the nuclear DNA-content in normal epidermis, seborrhoeic keratosis, keratosis senilis, squamous cell carcinoma and basal cell carcinoma. *J. invest. Derm.* **40,** 95 (1963).
17. A. Kint, Contribution à l'étude des acides nucléiques au cours de la differenciation epidermique normale et pathologique. *Arch. belges Derm.* **20,** 243 (1964).
18. W. Knoth and W. Sandritter, Messungen des DNS-Gehaltes und morphologische Untersuchungen bei Reticulose, Reticulosarkomatose und Hodgkin-Sarkom der Haut. *Arch. klin. exp. Derm.* **223,** 217 (1965).
19. A. Kint, Les acides nucléiques dans la kératose sénile, la maladie de Bowen et la maladie de Paget. *Arch. belges Derm.* **22,** 102 (1966).
20. G. Ehlers, Vergleichende quantitativ-cytochemische Untersuchungen über den Deoxyribonucleinsäure- und Nucleohiston-Gehalt von Basalzellepitheliomen. *Arch. klin. exp. Derm.* **232,** 102 (1968).
21. G. Rabotti, Ploidy of primary and metastatic human tumours. *Nature (Lond.)* **183,** 1276 (1959).
22. E. S. Meek, The cellular distribution of deoxyribonucleic acid in primary and secondary growths of human breast cancer. *J. Path. Bact.* **82,** 167 (1961).
23. J. J. Decosse and N. Aiello, Feulgen hydrolysis: Effect of acid and temperature. *J. Histochem. Cytochem.* **14,** 601 (1966).
24. E. Frindel, E. Malaise, F. Vassort and M. Tubiana, La cinétique de prolifération cellulaire des tumeurs humaines *in vivo* et *in vitro*. *Bull. Ass. franç. Cancer* **55,** 51 (1968).
25. J. de Bersaques, Teneur en acide désoxyribonucléique des épitheliomas basocellulaires. *Arch. belges Derm.* **21,** 503 (1965).
26. T. C. Hsu, Chromosomal evolution in cell populations. *Int. Rev. Cytol.* **12,** 69 (1961).
27. T. C. Hsu, D. Billen and A. Levan, Mammalian chromosomes *in vitro*. XV. Patterns of transformation. *J. nat. Cancer Inst.* **27,** 515 (1961).
28. R. E. Wallace, E. V. Orsi, H. B. Ritter and A. W. Moyer, Observations during *in vitro* growth of cells derived from rabbit kidney. *Exp. Cell Res.* **25,** 671 (1961).
29. K. K. Sanford, G. D. Likely and W. R. Earle, The development of variations in transplantability and morphology within a clone of mouse fibroblasts transformed to sarcoma-producing cells *in vitro*. *J. nat. Cancer Inst.* **15,** 215 (1955).
30. A. E. Moore, C. M. Southam and S. S. Sternberg, Neoplastic changes developed in epithelial cell lines derived from normal persons. *Science* **124,** 127 (1956).

31. V. J. Evans, G. A. Parker and T. B. Dunn, Neoplastic transformation in C3H mouse embryonic tissues *in vitro* determined by intraocular growth. I. Cells from a chemically defined medium with and without serum supplement. *J. nat. Cancer Inst.* **32,** 89 (1964).
32. N. Auersperg, Long-term cultivation of hypodiploid human tumour cells. *J. nat. Cancer Inst.* **32,** 135 (1964).
33. L. Hayflick and P. S. Moorhead, The serial cultivation of human diploid cell strains. *Exp. Cell Res.* **25,** 585 (1961).
34. G. Yerganian and M. J. Leonard, Maintenance of normal *in situ* chromosomal features in long-term tissue cultures. *Science* **133,** 1600 (1961).
35. E. H. Y. Chu, Chromosomal stabilisation of cell strains. *Nat. Cancer Inst. Monogr.* **7,** 55 (1962).
36. R. S. Krooth, M. W. Shaw and B. K. Campbell, A persistent strain of diploid fibroblasts. *J. nat. Cancer Inst.* **32,** 1031 (1964).
37. J. L. German, V. J. Evans, J. A. Cortner and B. B. Westfall, Characterisation of 3 human cell lines by chromosomal complement and by certain biochemical parameters. Reversible alterations of isoenzyme patterns by different media. *J. nat. Cancer Inst.* **32,** 681 (1964).
38. F. Jacoby, Longterm cultivation *in vitro* of a dibenzanthracene mouse sarcoma. *Nature (Lond.)* **152,** 299 (1943).
39. W. M. de Bruyn, *In vitro* cultivation of malignant lymphoblasts of transplantable mouse lymphosarcoma MB(T86157) without typical mesenchyme cells. *Bijdr. Dierk.* **28,** 77 (1949).
40. W. R. Earle, E. Shelton and E. L. Schilling, Production of malignancy *in vitro*. XI. Further results from reinjection of *in vitro* cell strains into strain C3H mice. *J. nat. Cancer Inst.* **10,** 1105 (1950).
41. W. M. de Bruyn and G. O. Gey, Further studies *in vitro* of transplantable mouse lymphosarcoma MB (T86157). *Acta Un. int. Cancr.* **7,** 772 (1952).
42. G. O. Gey, F. B. Bang and M. K. Gey, Responses of a variety of normal and malignant cells to continuous cultivation, and some practical applications of these responses to problems in the biology of disease. *Ann. N.Y. Acad. Sci.* **58,** 976 (1954).
43. W. R. Earle (Discussion of) Tissue culture in the study of animal and human tumours, author—A. M. Brues. *J. nat. Cancer Inst.* **19,** 781 (1957).
44. C. J. Dawe, M. Potter and J. Leighton, Progression of a reticulum-cell sarcoma of mouse *in vivo* and *in vitro*. *J. nat. Cancer Inst.* **21,** 753 (1958).
45. I. S. Johnson, Cancer research: Cell production versus cell function in tissue culture. *Cancer Res.* **18,** 367 (1958).
46. T. C. Hsu, Reduction of transplantability of Novikoff hepatoma cells grown *in vitro* and the consequent protecting effect to the host against their malignant progenitor. *J. nat. Cancer Inst.* **25,** 927 (1960).
47. E. E. Deschner and B. R. Allen, *In vitro* culture of Ehrlich ascites tumour cells. *Science* **131,** 419 (1960).
48. L. F. Guerin and S. F. Kitchen, Adaptation of 6C3HED tumour cells to culture *in vitro*. *Cancer Res.* **20,** 344 (1960).
49. M. Takahashi, Alteration of virulence of MN ascites sarcoma after continuous culture *in vitro*. *Gann* **54,** 295 (1963).
50. S. Mori, Y. Harada and I. Yamaoka, Establishment of MN-lymphosarcoma as a permanent cell strain in tissue culture. *Gann* **54,** 251 (1963).
51. G. E. Foley and B. P. Drolet, Loss of neoplastic properties *in vitro*. I. Observations with S-180 cell lines. *Cancer Res.* **24,** 1461 (1964).
52. G. E. Foley, A. H. Handler, P. M. Lynch, S. R. Wolman, C. S. Stulberg and H. Eagle, Loss of neoplastic properties *in vitro*. II. Observations with KB sublines. *Cancer Res.* **25,** 1254 (1965).
53. H. Katsuta and T. Takaoka, Parabiotic cell culture. IV. Interaction between normal and ascites tumour cells of rat. *J. nat. Cancer Inst.* **32,** 963 (1964).
54. D. T. Hughes, The role of chromosomes in characterisation of human neoplasms. *Europ. J. Cancer* **1,** 233 (1965).

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Anti-Oestrogens and Breast Cancer

LARS TERENIUS
Department of Pharmacology, University of Uppsala, Uppsala, Sweden

INTRODUCTION

In about a third of patients with metastatic breast cancer, remission of the disease can be brought about by oophorectomy, adrenalectomy or hypophysectomy or by administration of androgens, oestrogens or corticosteroids [1–3].

There is some evidence that none of these treatments completely eliminates the endogenous production of oestrogens [4, 5], and for this reason, substances which antagonize the actions of oestrogens (the anti-oestrogens) deserve attention as potential remedies against the disease.

The object of the present study was to examine the possibility of using such therapy in patients with tumours that might be oestrogen responsive. The obvious choice of an experimental model was the Huggins tumour of the rat which, in most instances, is oestrogen dependent [6]. The effect of U-11100A, one of the most potent antioestrogens known, on the growth of these tumours was investigated. During the course of this work, an abstract appeared in which it was reported that clomiphene (another anti-oestrogen) inhibited the growth of these tumours [7]. The effect of U-11100A and clomiphene on the specific binding of oestrodiol-17β to "receptors" in both the Huggins tumour and in biopsy material from human breast cancers was also investigated.

MATERIAL AND METHODS

Tumours

Breast tumours (Huggins tumour) were induced in 40 rats by the intravenous administration of 7,12-dimethylbenz(a)anthracene as described by Huggins *et al* [8]. The animals were divided into two groups, one with 25 animals for anti-oestrogen treatment and the remaining 15 for control. In each group 2 animals died early after the treatment with carcinogen. The remaining animals were inspected twice weekly and the tumour diameters recorded starting 30 days after the injection. When a rapidly growing tumour had reached about 0·8 cm in diameter, treatment of the experimental animals with the anti-oestrogen U-11100A was started. Three animals in the anti-oestrogen group never developed tumours of this size and were excluded from the series. Every other day appropriate amounts of a solution of U-11100A in olive oil (0·1 mg/ml) were injected subcutaneously at a dosage of 1 mg per kg body weight. The therapy was continued for 3 weeks after which time the animals were further kept for 5–7 weeks. A few animals (particularly in the control group) had to be killed earlier when the tumours were very big, and ulcerating and death appeared imminent.

Tumours for the *in vitro* experiments (Table 2) were obtained from animals not treated with anti-oestrogen. These tumours were rapidly growing at the time of excision.

Human tumour material was obtained from radical mastectomy specimens. A series of 14 unselected patients treated at the Department of Surgery, the University Hospital of Uppsala, was investigated. The interval between the excision of the tumour and the start of the experiment varied but was always less than 3 hours. During this period the tumour specimens were stored on ice.

Hormones

Tritium-labelled oestrodiol-17ß (oestra, 1, 3, 5(10)triene-3, 17ß-diol, labelled in the 6 and 7

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Table 1. *Response of the Huggins mammary carcinoma to treatment with the anti-oestrogen U-11100A*

Treatment	No. of rats	No. of tumours/rat			Tumour volume (mm^3)/rat		
		Start	End	Difference	Start	End	Difference
Untreated control	13	4·5±0·5	7·6±2·6	3·2±0·6	1110±160	16100±4100	15000±4100
U-11100A 1 mg/kg every other day	20	4·7±0·5	2·0±0·3*	−2·7±0·5*	680±130†	620± 260*	−53± 210*

Mean values±s.e.m. are given. Significance is tested against control. *$=P<0·001$; †$P<0·05$.

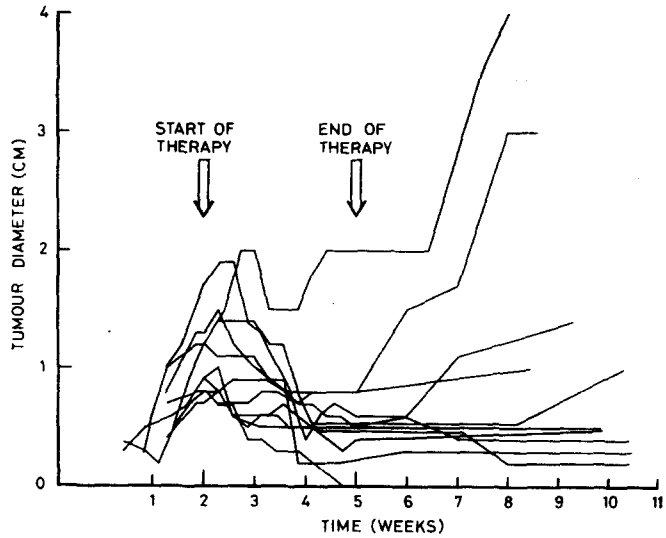


Fig. 1. Response of the Huggins tumour (tumour diameter) to anti-oestrogen (U-11100A) treatment. Large tumours (0·8 cm or larger during observation period) occurring singly in each rat are shown.

positions) was obtained from The New England Nuclear Corp., Dreieichenhain, W. Germany. Its specific activity was 40 C/mmol. The radiochemical purity was over 97% on thin-layer chromatograms. Non-radioactive oestradiol-17ß was purchased from Sigma Chemicals Company, St. Louis, Mo., U-11100A (1-[2-[*p*-3,4-dihydro-6-methoxy-2-phenyl-1-1-naphthyl) phenoxy] ethyl]-pyrrolidine hydrochloride) and clomiphene (Clomid®, [2-[*p*-[2-chloro-1,2-diphenylvinyl] phenoxy] triethylamine dihydrogen citrate) were obtained through the Upjohn Company, Kalamazoo, Mich. and the Wm. S. Merrel Company, Cincinnati, Ohio, respectively. These compounds were used as supplied from the manufacturers.

In vitro experiments

The experimental conditions have been described in detail elsewhere [9]. Briefly, tumour slices of about 10 mg and less than 0·5 mm thick were incubated in Krebs-Ringer phosphate buffer, pH 7·4, with 2% bovine albumin, 0·25% glucose and hormones as described below. The tumour slices were randomly distributed into different incubation solutions which all contained 2×10^{-9} M labelled oestradiol-17ß. The "control" solution received no further addition, the "carrier" solution contained 2×10^{-7} M non-labelled oestradiol-17ß and solutions with anti-oestrogen either contained 2×10^{-5} M U-11100A or $8{\cdot}5\times10^{-5}$ M clomiphene. The slices were

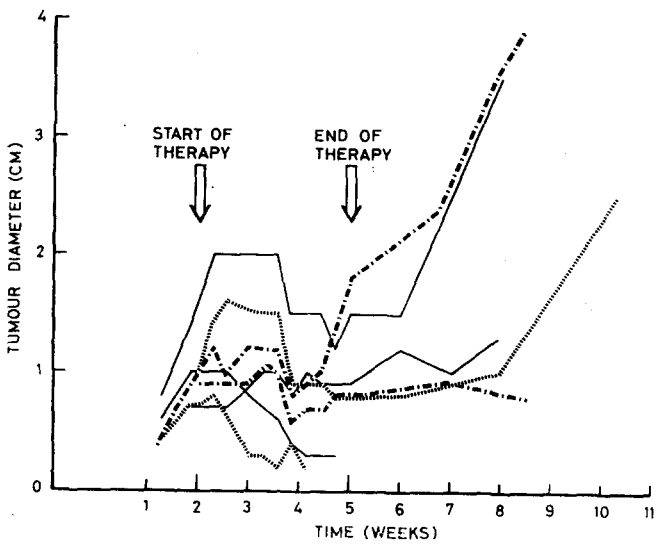


Fig. 2. Response of the Huggins tumour (tumour diameter) to anti-oestrogen (U-11100A) treatment. Large tumours (0·8 cm or larger during observation period) are shown. The symbols refer to individual rats, each with multiple tumours.

incubated for 1 hr at 37°C in these solutions after which they were washed in the buffer with 2×10^{-7} M non-labelled oestradiol-17ß for 1 hr at 25°C.

The tritium content of each tumour slice was measured separately after dissolving in hyamine by the liquid scintillation technique. The accumulation of radioactivity is expressed as $\frac{\text{DPM/mg wet tissue}}{\text{DPM/µl medium}}$ where "medium" is the solution to which the radioactivity was added.

Significance was tested by Student's *t*-test.

RESULTS

U–11100A and tumour growth

The effect of the anti-oestrogen U–11100A on the growth of the Huggins tumour is illustrated in Figs. 1 and 2. For the sake of clarity only representative examples are given and only tumours which reached at least 0·8 cm diameter during the observation period are included. Figure 1 shows the effect of U–11100A on 11 tumours growing in 11 rats with only 1 tumour each of this size. Figure 2 shows the effect on tumours in 3 other rats which each had multiple tumours (of the critical size). In the rats with single tumours all ceased to grow and most of them regressed (Fig. 1). After the end of the therapy 4 tumours showed a rapid recurrence while 7 showed no tendency to grow. In fact, several of the latter remained just palpable. In the rats with multiple tumours, most of the tumours responded to therapy (Fig. 2). These results also show that the variable response to therapy (see Fig. 1) is largely due to differences between the tumours and not between the hosts. For instance in the rat bearing 3 tumours (solid lines) 1 tumour regressed during the therapy after which it showed a rapid recurrence, 1 tumour was essentially unaffected by the therapy and continued to increase in size very gradually while the third tumour was extinguished during the therapy.

Table 1 gives a comparison between the growth of tumours of all sizes in the control and the anti-oestrogen treated groups. There was a marked response to the hormonal treatment. In only 3 of the 20 treated rats the total tumour value increased slightly. However, this was due to a single tumour in each rat. In all, about 4% of the tumours in the treated rats continued to grow during therapy.

Treatment with U–11100A only slightly affected the increase in body weights during the 3-week period. In the treated group body weights increased by 5 ± 5 g as against in the control group 15 ± 10 g. In 10 rats not bearing tumours but treated with U–11100A, body weights increased by 12 ± 2 g as against in the corresponding controls the increase was 22 ± 4 g. The difference between these two latter groups is probable ($P<0·05$). U–11100A produced vaginal dioestrus at the dosage level; vaginal smears contained leucocytes only or mixed with <50% nucleated epithelial cells as well.

Effect of U–11100A on oestrogen binding to breast tumours

Huggins tumours from rats not treated by anti-oestrogen were used for oestrogen binding experiments *in vitro*. The tumours chosen were steadily growing in young animals. Such tumours are almost without exception oestrogen dependent and bind oestradiol–17ß in a specific manner [10, 11]. Oestrogen binding to these tumours can be demonstrated also *in vitro* [10, 11]. Table 2 shows the tissue/medium radioactivity ratios of the tumour slices after incubation with labelled oestradiol–17ß either

Table 2. Effect of unlabelled oestradiol-17β and of anti-oestrogens on the binding of tritium-labelled oestradiol-17β to rat mammary tumours

Rat no.	Bound oestradiol-^{3}H (Tumour/Medium)			
	Control	With carrier	With U–11100A	With clomiphene
1	4·2± 0·4	0·42±0·04	0·95±0·06	0·61±0·04
2	3·7± 0·4	0·42±0·02	1·03±0·03	0·51±0·02
3	3·5± 0·2	0·39±0·02	0·80±0·04	0·43±0·02
4	2·9± 0·3	0·67±0·05	1·32±0·05	0·82±0·04
5	1·6±0·09	0·62±0·04	0·95±0·04	0·76±0·04

Mean values ± s.e.m. are given. There were 6 slices per group. All differences between control and experimental groups were significant at a $P<0·001$

Table 3. Effect of unlabelled oestradiol-17β (=carrier) and of anti-oestrogens on the binding of tritium-labelled oestradiol-17β to biopsies of human breast cancer

Patient no.	Menopausal stage	Histopathology of tumour	Anti-oestrogen	Bound oestradiol-^{3}H (Tumour/Medium)					
				Control		With carrier		With anti-oestrogen	
57	Post-	Scirrhous, l	U-11100A	1·07±0·18	(6)	0·22±0·02*	(10)	0·42±0·09†	(6)
54	Post-	Scirrhous, l	U-11100A	1·02±0·09	(9)	0·46±0·04*	(10)	0·60±0·03*	(10)
65	Post-	Scirrhous, m	U-11100A	0·63±0·04	(6)	0·21±0·01*	(10)	0·30±0·01*	(6)
50	Pre-	Solid, l	U-11100A	0·45±0·04	(6)	0·37±0·02	(12)	0·38±0·02	(10)
56	Post-	Ductal, m	U-11100A	0·26±0·03	(6)	0·28±0·05	(10)	0·32±0·02	(6)
62	Post-	Scirrhous, l	U-11100A	0·55±0·05	(6)	0·68±0·05	(10)	0·74±0·05‡	(5)
72	Post-	Ductal, m	U-11100A	0·12±0·02	(6)	0·33±0·08	(12)	0·27±0·05	(6)
78	Post-	Medullary, m	Clomiphene	5·48±1·01	(6)	0·70±0·08*	(10)	1·89±0·22*	(6)
77	Pre-	Scirrhous, l	Clomiphene	1·96±0·24	(6)	0·39±0·05*	(10)	0·61±0·10*	(6)
81	Post-	Solid, l	Clomiphene	1·64±0·14	(6)	0·55±0·19*	(10)	0·51±0·12*	(6)
73	Pre-	Ductal, h	Clomiphene	1·29±0·15	(5)	0·40±0·03*	(10)	0·57±0·10*	(5)
76	Pre-	Adenocarc., l	Clomiphene	0·69±0·03	(6)	0·23±0·02*	(11)	0·31±0·05*	(7)
74	Post-	Scirrhous, l	Clomiphene	0·51±0·13	(4)	0·30±0·06	(4)	0·50±0·07	(4)
84	Post-	Scirrhous, l	Clomiphene	0·21±0·02	(6)	0·16±0·01	(10)	0·14±0·01	(6)

Mean values±s.e.m. are given. Values in brackets are number of slices analysed. *=$P<0·001$; †=$P<0·01$; ‡=$P<0·05$; l=low, m=medium and h=high differentiation.

alone (control), with a 100-fold excess of unlabelled oestradiol–17ß (carrier) or with either unlabelled anti-oestrogen. At the used concentrations, the unlabelled compounds act as competitors for oestrogen binding sites. It was found (Table 2) that the binding of labelled oestradiol–17ß was markedly inhibited by carrier as well as by the anti-oestrogens.

Similar experiments were carried out with biopsies of human breast cancer (Table 3). This notoriously heterogenous material presents difficulties when a quantitative estimation of the oestrogen binding capacity is to be done, since only a part of the oestrogen bound can be considered to occupy sites which fulfil criteria for receptors. The oestrogen binding capacity is also generally lower than that of the Huggins tumours. The discrimination between "specific" and "unspecific" binding is based on the assumption that only the specific binding can be saturated within the given concentration range, cf. Table 2. (A detailed discussion of this problem is given in [9]).

Tumour slices from one group of patients were incubated with U–11100A, the other group with clomiphene. The results are shown in Table 3. Within each group of patients the tumours are arranged in descending order according to the "specific" (= saturable) oestrogen binding capacity. It was observed that in some cases either U–11100A or clomiphene were capable of inhibiting the binding of oestradiol–17ß. However, an inhibition was only observed in the tumours which showed specific (= saturable) oestradiol–17ß binding.

DISCUSSION

U–11100A and clomiphene are not pure oestrogen antagonists, but also possess some weak oestrogenicity in several tests [12, 13], which complicates the interpretation of the results. However, U–11100A was anti-oestrogenic at least on the vagina at the dosage used. It is also known that U–11100A inhibits oestrogen dependent growth of a renal carcinoma in the hamster and that its tumour inhibitory effect is abolished by simultaneous administration of oestrogen [14]. In intact rats clomiphene inhibits uterine growth rather than stimulates although not to the extent of castration [13]. It is therefore probable that U–11100A and clomiphene inhibit tumour growth by virtue of their anti-oestrogenic properties.

Clomiphene is also clinically mainly antagonistic and only weakly oestrogenic [15]. Thus it has been found that clomiphene inhibits endocervical secretion induced by endogenous oestrogen and reverses benign or malignant endometrial hyperplasia to atrophic endometrium [16, 17]. In castrated women clomiphene clearly antagonizes exogenous oestrogen and gives no oestrogenic response to antagonistically active doses [18]. As for U–11100A no clinical data are available to the author.

Anti-oestrogens such as U–11100A [10, 11] and clomiphene [19] inhibit oestrogen binding in reproductive organs and probably act by this mechanism. The present experiments clearly show that these anti-oestrogens inhibit oestradiol–17ß binding to the Huggins tumour as well as to certain human breast tumours. As to the human tumours an inhibition was only observed in those cases which showed a significant (= saturable) oestrogen binding. This is a support for the specific nature of the observed inhibition. The data also show the advantage of doing these studies *in vitro* since the effect of an anti-oestrogen on oestrogen binding can be established for each individual tumour separately.

In the hormonal therapy of advanced breast cancer androgens are frequently used. Androgens antagonize several actions of oestrogens on peripheral tissues. Two groups have studied the effect of androgens on oestrogen uptake by malignant breast tumours [20, 21]. Deshpande *et al.* [20] claim that the androgen dromostanolone inhibits *in vivo* uptake of oestradiol–17ß in breast tumours while Braunsberg *et al.* [21] found no significant effect neither by dromostanolone nor by testosterone. Both groups of investigators injected the hormones into the patients and had to use one group of patients as controls and other groups for androgen treatment. In view of our previous experience [9] their conflicting results are not unexpected since we found that only a third of human breast tumours bound oestrogen significantly. A possible effect would therefore be difficult to detect against a "background" of nonbinders. They also found very considerable individual variations in oestrogen uptake in the different groups.

In a previous report [9] the capacity of tissue obtained from biopsies of human breast tumours to bind oestradiol–17ß *in vitro* has been studied with the present method. This report and additional data gathered since show that on about 90 cases of breast cancer approximately one third show significant binding of oestradiol–17β of different degrees. Since oestrogens are only bound to target tissues in experimental animals as well as in the human being (for a review see 22) it is highly suggestive that tumours with oestrogen binding sites are oes-

trogen responsive. The clinical experience also demonstrates that roughly, in three women, one can be helped by hormone therapy (and it may very well be that these patients are those with breast tumours containing oestrogen receptor sites). A direct link between oestrogen accumulation in breast cancer tissue and the clinical response to combined oophorectomy/adrenalectomy was obtained by Folca *et al.* [23]. These workers injected the oestrogen *meso*-hexoestrol labelled with tritium into 9 breast cancer patients and obtained biopsies of metastatic tumour tissue. In patients which responded favourable to the therapy, the concentration of oestrogen was higher in the tumour than in muscle.

The place of clomiphene in the therapy of benign and malignant endometrial hyperplasia is fairly well established [15, 16]. Incidentally, biopsies of such specimens have high oestrogen binding capacity (unpubl.) . The place of anti-oestrogens in the therapy of human breast carcinoma remains to be established. It is highly suggestive, however, that at least tumours of high oestrogen binding capacity should be sensitive to treatment with oestrogen antagonists. The experimental technique described in this communication could be used for the selection of patients. The technique is simple enough to be used routinely.

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SUMMARY

An attempt has been made to evaluate the use of anti-oestrogenic compounds for the treatment of breast cancer in women.

It was found that the anti-oestrogen U-11100A was a powerful inhibitor of the oestrogen responsive Huggins mammary carcinoma in the rat. Such tumours bind oestradiol-17β specifically in vitro *and both U-11100A and clomiphene are effective inhibitors of this binding.*

About a third of 14 *patients with breast cancer had tumours which, like the Huggins tumours, bind oestradiol-17β specifically. U-11100A and clomiphene inhibit this binding in biopsy specimens* in vitro.

It is suggested that anti-oestrogen therapy might be considered for use for patients who have breast tumours of high oestrogen binding capacity.

REFERENCES

1. C. Huggins and D. M. Bergenstal, Inhibition of human mammary and prostatic cancer by adrenalectomy. *Cancer Res.* **12,** 134 (1952).
2. B. J. Kennedy, Hormone therapy for advanced breast cancer. *Cancer (Philad.)* **18,** 1551 (1965).
3. E. F. Lewison, Castration in the treatment of advanced breast cancer. *Cancer (Philad.)* **18,** 1558 (1965).
4. R. D. Bulbrook and F. C. Greenwood, Persistence of urinary oestrogen excretion after oophorectomy and adrenalectomy. *Brit. med. J.* **1,** 662 (1957).
5. E. F. Scowen, Oestrogen excretion after hypophysectomy in breast cancer. In: *Endocrine aspects of breast cancer* pp. 208, edited by A. R. Currie. E. & S. Livingstone, Edinburgh (1958).
6. C. Huggins, L. C. Grand and F. P. Brillantes, Mammary cancer induced by a single feeding of polynuclear hydrocarbons and its suppression. *Nature (Lond.)* **189,** 204 (1961).
7. K.-D. Schultz, B. Haselmeier and F. Hölzel, The influence of clomid and its isomers upon dimethylbenzanthracene-induced rat mammary tumours. *Acta endocr. (Kbh.) Suppl.* **138,** 236 (1969).
8. C. Huggins, L. Grand and R. Fukunishi, Aromatic influences on the yields of mammary cancers following administration of 7,12-dimethylbenz(a)anthracene. *Proc. nat. Acad. Sci. (Wash.)* **51,** 737 (1964).
9. H. Johansson, L. Terenius and L. Thorén, The binding of estradiol-17β to human breast cancers and other tissues *in vitro*. *Cancer Res.* **30,** 692 (1970).
10. L. Terenius, Selective retention of estrogen isomers in estrogen-dependent breast tumours of rats demonstrated by *in vitro* methods. *Cancer Res.* **28,** 328 (1968).

11. E. V. JENSEN, E. R. DESOMBRE and P. W. JUNGBLUT, Estrogen receptors in hormone-responsive tissues and tumors. In *Endogenous factors influencing host-tumor balance*, (edited by R. W. WISSLER, T. L. DAO and S. WOODS, JR.), p. 15 University of Chicago Press, Chicago (1967).
12. G. W. DUNCAN, S. C. LYSTER, J. J. CLARK and D. LEDNICER, Antifertility activities of two diphenyl-dihydronaphthalene derivatives. *Proc. Soc. exp. Biol. (N.Y.)* **112,** 439 (1963).
13. S. ROY, V. B. MAHESH and R. B. GREENBLATT, Effects of clomiphene on the physiology and reproduction in the rat. I. *Acta endocr. (Kbh.)* **47,** 645 (1964).
14. H. J. G. BLOOM, F. J. C. ROE and B. C. V. MITCHLEY, Sex hormones and renal neoplasia, *Cancer (Philad.)* **20,** 2118 (1967).
15. R. J. THOMPSON and R. C. MELLINGER, The effects of clomiphene citrate in patients with pituitary-gonadal disorders. *Amer. J. Obstet. Gynec.* **92,** 412 (1965).
16. J. A. WALL, R. R. FRANKLIN and R. H. KAUFMAN, Reversal of benign and malignant endometrial changes with clomiphene. *Amer. J. Obstet. Gynec.* **88,** 1072 (1964).
17. R. W. KISTNER, J. L. LEWIS and G. J. STEINER, Effects of clomiphene citrate on endometrial hyperplasia in the premenopausal female. *Cancer (Philad.)* **19,** 115 (1966).
18. J. VAN CAMPENHOUT, R. SIMARD and B. LEDUC, Antiestrogenic effect of clomiphene in the human being. *Fertil. and Steril.* **19,** 700 (1968).
19. S. ROY, V. B. MAHESH and R. B. GREENBLATT, Effects of clomiphene on the physiology and reproduction in the rat. III. *Acta endocr. (Kbh.)* **47,** 669 (1964).
20. N. DESHPANDE, V. JENSEN, R. D. BULBROOK, T. BERNE and F. ELLIS, Accumulation of tritiated oestradiol by human breast tissue. *Steroids* **10,** 219 (1967).
21. H. BRAUNSBERG, A. E. CARTER, W. T. IRVINE and V. H. T. JAMES, Relative oestrogen concentrations in breast tumour tissue following androgen therapy. *Acta endocr. (Kbh.) Suppl.* **138,** 235 (1969).
22. H. BRAUNSBERG and V. H. T. JAMES, Mathematical analysis of experiments with labelled hormone tracers: problems of interpreting tissue radioactivity. *J. clin. Endocr.* **27,** 1174 (1967).
23. P. J. FOLCA, R. F. GLASCOCK and W. T. IRVINE, Studies with tritium-labelled hexoestrol in advanced breast cancer. *Lancet* **ii,** 796 (1961).

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Effect of Anti-oestrogens on Initiation of Mammary Cancer in the Female Rat

LARS TERENIUS
Department of Pharmacology, University of Uppsala, Sweden

INTRODUCTION

HORMONE dependence is not an uncommon characteristic of cancer of the breast in women. A widely used animal model for this type of cancer is the Huggins breast tumour, a hormone-dependent adenocarcinoma in the rat induced by polycyclic hydrocarbons, e.g. DMBA*.

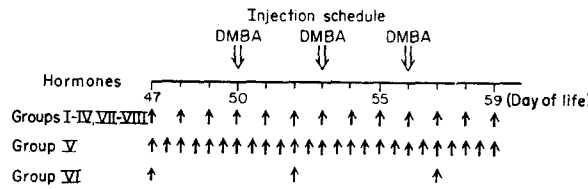
In recent years, research efforts concerning the hormonal etiology of human breast cancer have been reported. Hyperoestrogenism seems to be common in breast cancer patients [1]. Bulbrook and Hayward [2] give evidence that women with low levels of androgen metabolites have high risks of developing the disease while Lemon [3] reports that women with low levels of oestriol subsequently develop breast cancer. Both androgens and oestriol act as anti-oestrogens. The purpose of the present investigation was to test whether anti-oestrogens (including androgens and oestriol) could interfere with the early phases of DMBA carcinogenesis in the rat. It has been reported that spayed animals and animals treated with androgen over long periods of time do not develop tumours [4, 5]. In the present investigation the hormone treatment was confined to the induction period so that initiation and out growth phases were separated.

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*Abbreviations: DMBA, 7,12-dimethylbenz(a)anthracene; testosterone, 17β-hydroxy-androst-4-en-3-one; oestriol, 1,3,5(10)-triene-3,16α,17β-triol; progesterone, pregn-4-ene-3,20-dione; MER-25, 1-*p*-(2-diethylaminoethoxy)phenyl-2-(*p*-methoxyphenyl)-1-phenylethanol.

MATERIAL AND METHODS

Female rats of the Sprague–Dawley strain were used in all experiments. They were kept under uniform conditions. Food and water were available without restriction. The induction of breast tumours was performed according to Huggins *et al.* [6] by the intravenous administration of DMBA. Hormones were administered subcutaneously in olive oil or in the case of polyoestriol phosphate in neutral saline (0·1 ml per 100 g body weight) according to the following schedule:



Testosterone, oestriol (grade I) and progesterone were purchased from Sigma Chemicals Co., St. Louis, Mo. MER-25 was a gift from Wm. S. Merrell Co., Cincinnati, Ohio, while polyoestriol phosphate was donated by Leo AB, Hälsingborg, Sweden. This polyoestriol phosphate contains about 80% oestriol. According to the manufacturers, contamination from oestradiol-17β or oestrone is less than 0·1%.

Vaginal smears were recorded at least every other day from day 47 to day 59 of life. Vaginal smears were also taken each day about one month after the last hormone injection during a period sufficient to establish normal cyclicity. The animals were inspected for the appearance of palpable tumours twice weekly starting 3 weeks after the last DMBA-treatment. The

Table 1. *Experimental groups and influences of treatment on survival, body weights and vaginal cytology at different days of life*

	Group, treatment	Start	No. of surviving rats Day 70	Day 120	Day 150	Change in body weights (g) from Day 47† Day 59	Day 105	Vaginal smears during DMBA-treatment* (Days 49–59)
I	Oil	15	13	13	—	+18 (11–27)	+69 (63–84)	Normal cycles
II	Testosterone 10 mg/kg/day	15	15	15	—	+27 (17–38)	+74 (46–92)	Dioestrus
III	MER-25 20 mg/kg/day	15	13	13	13	+2·2 (−8 to 10)	+ 67 (54–82)	Dioestrus
IV	Oestriol 1 mg/kg/day	14	8	8	8	+9·9 (−16 to 30)	+ 74 (58–87)	Dioestrus—(prooestrus)
V	Oestriol 100 μg/kg/12 hours	14	11	11	11	−3·3 (−31 to 13)	+ 66 (51–88)	Dioestrus—(prooestrus)
VI	Polyoestriol phosphate 5 mg/kg/5th day	15	11	11	10	+26 (20–35)	+ 80 (66–103)	Prooestrus—(oestrus)‡
VII	Progesterone 20 mg/kg/day	15	15	15	15	+28 (20–35)	+ 77 (62–98)	Dioestrus
VIII	Castration	14	14	14	12	+18 (8–23)	+124 (99–151)	Dioestrus

*The column only gives values for animals which survived day 70.
†Mean values and ranges.
‡1 animal in permanent dioestrus. This animal had the lowest tumour number and by far the lowest tumour volume at day 150.

Table 2. Influence of hormones on tumour induction

	Group, treatment	Appearance of palpable tumours (day)	No. of tumours/rat Day 100	No. of tumours/rat Day 120	No. of tumours/rat Day 150	Tumour volume/rat Day 100	Tumour volume/rat Day 120	Tumour volume/rat Day 150
I	Oil Control	90 (81–101)	3 (1–7)	6 (3–15)		401 (4–6745)	2670 (175–39,276)	
II	Testosterone 10 mg/kg/day	96 (85–115)	2 (0–8)	6 (0–9)		67 (0–4207)	4370 (0–45,444)	
III	MER-25 20 mg/kg/day	112* (92–153)	0* (0–4)	1* (0–7)	4 (1–11)	0* (0–1435)	31* (0– 8834)	780 (4–19,513)
IV	Oestriol 1 mg/kg/day	102† (88–115)	1† (0–4)	5·5 (3–8)	7 (4– 8)	32† (0– 63)	610† (40– 3006)	3160 (58–30,381)
V	Oestriol 100 µg/kg/12 hr	108† (81–133)	0* (0–3)	3* (0–6)	5 (2–10)	0* (0–116)	110* (0–2362)	3540 (36–28,716)
VI	Polyoestriol phosphate 5 mg/kg/5th day	96 (85–105)	1† (0–4)	4‡ (2–9)	9 (5–13)	14 (0–3911)	1180 (508–37,792)	7400 (854–28,386)
VII	Progesterone 20 mg/kg/day	92 (85– 99)	3 (1–6)	7 (5–14)	11 (7–19)	170 (14–1080)	1460 (675– 9450)	6320 (1144–17,179)
VIII	Castration	—	—	—	—	—	—	—

Median values and ranges are given.
*$P<0·002$.
†$P<0·02$.
‡$P<0·05$ as compared with group I.
"Day"=day of life.

investigation was terminated for groups I and II at 120 days of age (tumours causing mortality) and for the other groups at 150 days of age. The number of tumours, and their volume, was recorded. At days 100, 120 and 150, caliper measurements of the tumours were made. However, the volumes of tumours less than 5 mm in dia. were only estimated.

Non-parametric techniques were used to test the significance of the observed effects since the data were not normally distributed. Probability values were obtained from the Mann–Whitney two-tailed *U*-test as described by Siegel [7].

RESULTS

Some effects of the hormonal treatments are summarized in Table 1. Oestriol was found to be toxic particularly at the 1 mg/kg/day dosage (group IV). Some animals died within 2–3 weeks after the DMBA-treatment with signs of general toxicity. The other treatments cannot be considered to increase mortality. (The early mortality from the DMBA-treatment in series of 50–100 animals under the present conditions is normally 5–10%.) Oestriol and also MER-25 definitely caused an early decrease in body weight increment. However, at day 105 of life body weights in all hormone-treated groups were equal to those of the controls. Testosterone, MER-25 and progesterone caused almost complete oestrogen antagonism (although not to the extent of castration). Free oestriol was somewhat less anti-oestrogenic. On the other hand, polyoestriol phosphate produced constant prooestrus–oestrus. It should be noted that the total administered amount of oestriol (based on free compound) in this group is equal to that in group IV (12 and 13 mg/kg respectively). Vaginal smears in the control group showed no marked deviations from normal cyclicity. About one month after the last hormone treatment essentially all surviving animals showed normal cycles. Two animals in group V were somewhat exceptional: they were cyclic but no real oestrus smears appeared.

The effect of hormonal treatment on DMBA-carcinogenesis is shown in Table 2. MER-25 and free oestriol significantly delayed tumour appearance. The total number of mammary cancers as well as the total tumour volume were also reduced by MER-25 and to a smaller extent by free oestriol. Polymeric oestriol had a small inhibitory effect on mammary carcinogenesis as measured by the number but not by the volume of the tumours. No other hormone affected mammary carcinogenesis significantly. Castration completely abolished cancer incidence thus confirming the finding by others [4].

Several animals treated with oestriol or MER-25 suffered from low initial increment in body weights or even loss in body weights (Table 1). A possible correlation between tumour incidence or tumour volume and body-weight changes in these groups was investigated but no evidence of such a correlation could be found.

DISCUSSION

The starting point of this investigation was the recent reports on a possible role of hormonal anomalies in the genesis of breast cancer in the woman [1–3]. The multifactorial etiology of the cancer diseases makes it desirable to study effects of hormone alterations separately, which only can be done experimentally in an animal model. It must be borne in mind, however, that in the animal the concentrations of peripheral steroids and their metabolism, clearance rates etc., may be quite different from those in man. Any extrapolation to man may be misleading.

Earlier studies and especially those of Dao (reviewed in Ref. 8) have shown that castration prior to DMBA treatment abolishes tumour incidence while ovary grafts to previously spayed animals 10, 25 or 30 days after DMBA, do not give rise to tumours. Talwalker *et al.* [9] showed that 1–10 μg of oestradiol-17β or 1 mg of somatotropin+1 mg of prolactin given to spayed rats 7 days before and 7 days after a single dose of DMBA restored the cancer incidence to 30–40% of that in intact animals. Thus, it is clear that oestrogens and the 2 pituitary hormones participate in mammary carcinogenesis.

Since oestrogens seem essential in the carcinogenesis, anti-oestrogenic substances could be expected to be inhibitory. Judging from the vaginal cytology all tested substances were anti-oestrogenic. However, oestriol was not at all anti-oestrogenic in the polymeric form. The vaginal smear scores following testosterone, progesterone or MER-25 treatments were equal (dioestrus only) while free oestriol produced less total antagonism. Only MER-25 and to a smaller extent oestriol, however, were significantly effective in delaying and reducing cancer incidence. It is well-known that MER-25 and oestriol are anti-oestrogens which block oestrogen binding to "receptors" in target tissues while testosterone and progesterone are not receptor blockers [10–12]. They still inhibit responses to oestrogens by some unknown mechanism. It thus appeared

that only anti-oestrogens which are receptor blockers were markedly effective inhibitors in DMBA-carcinogenesis under the present conditions. The crucial point in the induction of the lesion might therefore be the extent of occupation of the oestrogen receptors rather than the functional state as such of the mammary tree. On the other hand, established tumours are known to behave as other target tissues for oestrogens, i.e. regress after treatment with both types of anti-oestrogen [13–15]. In this connection it is interesting to note that Hierowski and Madon [16] report that the carcinogenic hydrocarbon 3-methylcholanthrene and oestradiol-17β interact for the same receptors in the uterine cytosol. Even if these results are not consistent with the known high structural selectivity of the oestrogen receptors it is tempting to believe that mammary carcinogens and oestrogens do attack similar structures in the mammary gland.

Although the total doses of oestriol in groups IV and VI were equal, only the treatment schedule of group IV produced anti-oestrogenicity while most animals in group VI which received the polymer were in constant pro-oestrus–oestrus. This is probably related to the fact that oestriol is very loosely bound to the receptors [17]. Infrequent injections (as in groups IV and V) give short periods of receptor occupation and produce little oestrogenic stimulation and antagonism while continuous supply, e.g. from a polymer, produces protracted receptor occupation and consequently a full oestrogenic response. It is therefore questionable whether or not oestriol under physiological conditions with continuous supply acts as an oestrogen antagonist.

From these data in the animal model there is thus no support for a protective role of testosterone or its metabolites in early phases of mammary carcinogenesis. There is neither convincing support for such a role of oestriol under physiologic conditions.

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SUMMARY

Mammary tumours (the Huggins tumour) were induced in rats by repeated intravenous injections of dimethylbenz(a)anthracene. During a period of time slightly overlapping the induction period, different groups of rats were treated with the following anti-oestrogens: progesterone, testosterone, MER-25 or oestriol, all at doses giving strong oestrogen antagonism (vaginal dioestrus).

It was found that only MER-25 or oestriol treatment delayed and reduced tumour incidence. No cancer of the breast arose in castrated rats.

MER-25 and oestriol are anti-oestrogenic by blockage of oestrogen receptors while testosterone and progesterone act by other mechanisms. It therefore seems possible that the presence of oestrogens on the receptor sites is more essential in the induction of the lesion than the functional state as such of the breast structure.

REFERENCES

1. S. C. Sommers, Endocrine abnormalities in women with breast cancer. *Lab. Invest.* **4,** 160 (1955).
2. R. D. Bulbrook and J. L. Hayward, Abnormal urinary steroid excretion and subsequent breast cancer. *Lancet* 519 (1967).
3. H. M. Lemon, Endocrine influences on human mammary cancer formation. A critique. *Cancer (Philad.)* **23,** 781 (1969).
4. T. L. Dao, The role of ovarian hormones in initiating the induction of mammary cancer in rats by polynuclear hydrocarbons. *Cancer Res.* **22,** 973 (1962).
5. G. Briziarelli, Effects of dosage and time of administration of testosterone propionate on 7,12-dimethylbenzanthracene mammary carcinogenesis in the rat. *Z. Krebsforsch.* **66,** 517 (1965).
6. C. Huggins, L. Grand and R. Fukunishi, Aromatic influences on the yields of mammary cancers following administration of 7,12-dimethylbenz(a)anthracene. *Proc. nat. Acad. Sci. (Wash.)* **51,** 737 (1964).
7. S. Siegel, *Nonparametric Statistics for the Behavioral Sciences.* McGraw-Hill, New York (1956).

8. T. L. Dao, Studies on mechanisms of carcinogenesis in the mammary gland. *Progr. exp. Tumor Res.* (*Basel*) **11,** 235 (1969).
9. P. K. Talwalker, J. Meites and H. Mizuno, Mammary tumor induction by estrogen or anterior pituitary hormones in ovariectomized rats given 7,12 dimethyl-1,2-benzanthracene. *Proc. Soc. exp. Biol.* (*N.Y.*) **116,** 531 (1964).
10. W. D. Noteboom and J. Gorski, Stereospecific binding of estrogens in the rat uterus. *Arch. Biochem.* **111,** 559 (1965).
11. A. J. Eisenfeld and J. Axelrod, Effect of steroid hormones, ovariectomy, estrogen pretreatment, sex and immaturity on the distribution of ^{3}H-estradiol. *Endocrinology* **79,** 38 (1966).
12. L. Terenius, The effect of steroidal hormones on the binding of 17β-estradiol by the mouse uterus and vagina. *Steroids* **13,** 311 (1969).
13. D. P. Griswold, H. E. Skipper, W. R. Laster, Jr., W. S. Wilcox and F. M. Schabel, Jr., Induced mammary carcinoma in the female rat as a drug evaluation system. *Cancer Res.* **26,** 2169 (1966).
14. M. B. Shimkin, L. Gropper, D. Thatcher and M. Gruenstein, Hormonal treatment of mammary tumours in rats induced by 3-methylcholanthrene (NSC-21970) and 7,12-dimethylbenz(a)anthracene (NSC-408823). *Cancer Res.* **27** (part 2), 1284 (1967).
15. K.-D. Schulz, B. Haselmeier and F. Hölzel, The influence of clomid and its isomers upon dimethylbenzanthracene-induced rat mammary tumours. *Acta endocr.* (*Kbh.*) *Suppl.* **138,** 236 (1969).
16. M. Hierowski and J. Madon, The influence of 20-methyl-cholanthrene on 17β-estradiol binding to 9·5-S receptor molecule from rat uterus. *Biochim. biophys. Acta* (*Amst.*) **170,** 425 (1968).
17. L. Terenius, Structure–activity relationships of anti-oestrogens with respect to interaction with 17β-oestradiol in mouse uterus and vagina. *Acta endocr.* (*Kbh.*). Submitted for publication.

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Hormone-Induced Functional Response in Mammary Glands and Mammary Tumours

E. HEISE, M. GÖRLICH, Ch. KLEITKE and G. BACIGALUPO
German Academy of Sciences, Robert-Rössle-Clinic, Berlin-Buch (GDR)

MANY investigations carried out in the past decades have clearly demonstrated the strong hormonal control of development and functional performance of the mammary gland [1]. Tumours in this organ may develop following a disturbance of the physiological control of growth and function and are often accompanied by a loss of the functional capacity of the normal gland. As convincingly demonstrated by Huggins [2], Dao [3], Furth [4] and others, tumour growth can be successfully influenced by alterations of the hormonal conditions in the host organism, demonstrating a hormone-dependence of this tumour proliferation. The aim of the present paper is to investigate the inducibility by hormones of a functional response in the normal and abnormal mammary gland.

MATERIAL AND METHODS

The appearance of lactose in the mammary tissues after changing the hormonal environment in the host organism was taken as a functional response of the organ and investigated in mammary glands of pregnant rats, in experimentally-induced mammary carcinomata and fibroadenomata and in transplanted fibroadenomata of Sprague–Dawley rats. Lactose has been estimated in human mammary carcinomata and fibroadenomata. The human mammary carcinomas were received from the surgical department of our clinic immediately after radical mastectomy. To obtain mammary glands of pregnant rats, female Sprague–Dawley rats were allowed to mate and the appearance of a vaginal plug was considered to be the first day of pregnancy. At the 20th day of pregnancy the rats were killed and the mammary glands removed for the biochemical investigations. Mammary tumours were induced by feeding 20 mg of dimethyl-benzanthracene (DMBA) in 1 ml of sesame oil to 50–54 day old female Sprague–Dawley rats. The incidence and growth of tumours were estimated by palpation of the animals 3 times a week when tumours reached 2–4 cm in diameter. The tumour-bearing animal was then subjected to hormonal treatment (described below) and further tissue samples were taken. This procedure permitted a direct comparison of the results obtained on the same tumour. The schemes of the hormonal treatments given to the animals are indicated in the Tables 1 and 3.

Lactose was identified by paper chromatography [5]. For this 1 g of tissue was homogenized in 2 ml of trichloroacetic acid (20%) and centrifuged 30 min at 15,000 *g*. Two parallel runs of the clear supernatant and a control run containing 20 μg of lactose were subjected to paper chromatography (Schleicher and Schüll 2043 b). The sugar was characterized by comparison of the *Rf*-values of samples and controls on the same sheet of paper. The solvent mixture for the ascending chromatography consisted of butanol–acetic acid–water (4 : 1 : 5). Colour development of the control run and the first run of the sample was performed by the method of Buchan and Savage [6]. If lactose could be detected, the undeveloped spot of the parallel sample was eluted

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Table 1. *Effect of testosterone on the lactose synthesis in the mammary gland of pregnant rats*

Treatment	Number of animals investigated		Lactose content	
	Lactose negative	Lactose positive	Mean±SD	Range
Controls (untreated animals)	5	0	—	—
Animals treated with testosterone				
14th–20th day of pregnancy	4	0	—	—
15th–20th day of pregnancy	2	0	—	—
16th–20th day of pregnancy	2	1	75·8	75·8
17th–20th day of pregnancy	0	3	72·5±19·7	51·3–90·0
18th–20th day of pregnancy	0	2	144·0	68·6–219·4

with distilled water overnight and the sugar content determined by the method of Dimler [7]. This method will detect as little as 5 μg per 0·1 ml of the supernatant. Only lactose content exceeding this limit has been considered as "lactose present". In the present paper the lactose content was expressed as μg per 100 mg of tissue.

RESULTS

Table 1 shows that the non-lactating glands of normal pregnant rats did not accumulate detectable quantities of lactose before parturition. Appearance of milk sugar, however, could be evoked by testosterone administration to the pregnant rats towards the end of pregnancy. The gland did not respond to the androgen by lactose accumulation before the 16th day of pregnancy. Previous studies have shown that lactation, combined with an appearance of milk sugar, can be induced by ovariectomy of pregnant rats at the 2nd or 3rd day before parturition.

The appearance of lactose in experimentally induced carcinomata and fibroadenomata is shown by the results in Table 2. Mammary carcinomata and transplanted fibroadenomata in animals which did not receive hormonal treatments (controls) did not contain detectable quantities of lactose. The sugar could be detected in 13% of the experimentally-induced fibroadenomata studied. Hormone administration to the tumour-bearing animals changed this sugar-content pattern in the individual tumours as shown in Table 3. The various hormonal pretreatments of the animals did not alter the appearance of lactose in the mammary carcinomata and transplanted fibroadenomata significantly. Lactose could be found after testosterone administration only in 1 mammary carcinoma. Moreover the growth of this carcinoma regressed following androgen therapy indicating hormone-dependence of its proliferation. There were, however, other carcinomata in this group which responded to testosterone application by decreased growth rates but did not contain any quantities of milk sugar.

Hormonal pretreatments of animals bearing experimentally-induced fibroadenomata, however, frequently caused the appearance of lactose in the tumour tissue. This inducibility of a functional response in the tumour was independent of dosage and duration of the hormone

Table 2. *Lactose incidence in mammary tumours*

Tissue	Number of animals investigated		% positives of all cases	Lactose content	
	Lactose negative	Lactose positive		Mean±SD	Range
DMBA-induced mammary carcinomata	24	2	7·7	109·5	79/140
Transplanted fibroadenomata	3	0	0	—	—
DMBA-induced fibroadenomata	33	5	13·2	104·9±68·0	8·6–167·8

Table 3. Lactose incidence in mammary tumours following hormonal pretreatments.

Tissue	Number of animals investigated		% positive of all cases	Lactose content	
	Lactose negative	Lactose positive		Mean±SD	Range
DMBA-induced mammary carcinomata					
testosterone-treated (a)	8	1	12·5	57·7	57·7
oestradiol-treated (b)	9	0	0	—	—
oestradiol plus progesterone-treated (c)	5	0	0	—	—
Transplanted fibroadenomata					
testosterone-treated (a)	3	0	0	—	—
oestradiol-treated (b)	2	1	33·3	252·0	252·0
DMBA-induced fibroadenomata					
testosterone-treated (a)	8	5	38·5	90·2±47·0	43·0–153·0
oestradiol treated (b)	9	6	40·0	253·0±20·0	89·4–667·0
oestradiol plus progesterone-treated (c)	1	2	66·6	95·2	39·0/151·5

(a) Dosages of testosterone injected 3 times a week: 1 or 2 mg per animal i.m. Duration: 4–12 injections.
(b) Dosages of oestradiol injected daily: 0·2, 1·0 or 10·0 µg per animal i.m. Duration: 2–23 injections.
(c) Dosages of oestradiol plus progesterone injected daily: 5·0 µg of oestradiol plus 1·0 mg of progesterone per animal i.m. Duration: 12–26 injections.

administration. Using the procedure of partial removal of the tumour and subsequent hormonal treatment of the recurrent tumour it could be shown that there is a true inducibility of milk-sugar synthesis in DMBA-induced fibroadenomata by the various hormonal treatments of which three examples are shown in Table 4. The hormone-induced appearance of lactose was independent of the sequence of the hormonal manipulations performed on the animals.

Table 5 summarizes the results obtained on human mammary carcinomata and fibroadenomata. Mammary carcinomata of untreated patients did not contain milk sugar, whereas among the fibroadenomata studied lactose has been found only in one case (62·4 µg per 100 mg tissue). Further the table contains data on the average age of the patients who suffered from mammary tumours. In contrast to the incidence of mammary tumours in rats the average age of patients who suffered from fibroadenomata was lower than that of mammary carcinomata-bearing patients.

DISCUSSION

The development of the mammary gland during pregnancy leads to a morphological differentiation reflected in alterations in the ultrastructure of the secretory epithelial cells. Before parturition neither lactation nor accumulation of milk components such as lactose or casein can be observed. As demonstrated by many authors [8, 9] this inhibited milk secretion is caused by a suppressing effect of ovarian hormones on the production of prolactin as the milk-promoting agent of the pituitary. This feedback mechanism acts up to the time of parturition, at which time the enhanced secretion of oestradiol and progesterone ceases.

Table 4. Pretreatments and lactose incidence in DMBA-induced fibroadenomata. Repeated operations to remove tumours partially

Treatment	First operation lactose	Growth rate of the tumour	Treatment	Second operation lactose	Growth rate of the tumour	Treatment	Third operation lactose	Growth rate of the tumour	Treatment	Fourth operation lactose	Growth rate of the tumour
Untreated	0	↑	5×1 mg of testosterone	+	↓	—	—	—	—	—	—
Untreated	0	↑	16×1 mg of testosterone	+	↑	19×10 micrograms estradiol	+	↑	—	—	—
12×2 mg of testosterone	+	↑	untreated (6 weeks)	0	↑	12×5 micrograms estradiol plus 1 mg progesterone	0	↓	19×10 micrograms estradiol	0	↑

0: Lactose undetectable;
+: Lactose detectable;
↑: Tumours increased;
↓: Tumours decreased.

Table 5. *Lactose incidence in human mammary tumours*

Tissue	Lactose incidence (a)	(b)	%	Average age of the patients (yr)
Untreated mammary carcinomata	0	15	0	64·9
Untreated fibroadenomata	1	9	11	36·6

(a) Incidence of lactose.
(b) Number of patients investigated.

Therefore, ovariectomy, a few days before parturition, removes the inhibition of prolactin secretion, and lactation, combined with the appearance of lactose, occurs. Also androgens possess the capacity for inducing lactose synthesis as a functional response of the gland (Table 1). As yet the mechanism of this testosterone action is unknown. Possibly, the androgen antagonizes the actions of the female sex hormones on the pituitary, abolishing the inhibited prolactin production and thus enabling the gland to secrete. The capacity of testosterone to induce lactose synthesis presumes that the structure of the mammary gland is already highly differentiated while testosterone has no action before the 16th day of pregnancy. Also, in DMBA-induced fibroadenomata it was possible to induce lactose synthesis by administration of testosterone and ovarian hormones. These tumours originate by proliferation of both connective tissue and the mammary epithelial cells. The stimulated proliferation of the epithelial cells is apparently sensitive enough for a lactose-producing effect of sex hormones. On the other hand carcinomata and transplanted fibroadenomata did not respond to estrogenic or androgenic hormones by production of milk sugar. The malignant proliferation of epithelial cells is supposed to be accompanied by a concomitant loss of structural elements which are necessary for the functional responsiveness of the normal mammary gland to hormones. In contrast to the hormonal responsiveness of the tumour proliferation often observed in mammary cancers [10] the process of functional dedifferentiation is not reversible by sex hormones. In this connection interesting results are obtained by Hilf *et al.* [11, 12] who could induce a functional response and a concomitant growth inhibition of transplanted mammary carcinomata (R 3230 AC) by continuous treatment of the animals with estradiol. These authors suggested that the tumours had only a limited metabolic capacity preventing a parallel development of both proliferation and functional performance in the tissue. Similar investigations have been performed by McGuire [13] who showed a synthesizing capacity of the mammary-gland enzyme lactose synthetase in the transplantable rat mammary carcinoma R 3230 AC.

Possibly the failure of the transplanted fibroadenomata to respond to sex hormones by milk-sugar production can be explained by an advanced process of dedifferentiation which does not permit a functional response of this tissue to hormones. The quantitative determinations of lactose in the various tissues investigated revealed great variations in the lactose content independent of the hormonal treatments carried out on the animals.

From our experiments it can be concluded that there exist remarkable differences in the kind of dedifferentiation between fibroadenomata and carcinomata, indicating the more autonomous malignant proliferation of the latter. Further experiments, especially histological examinations should be done to clarify more exactly the connection between growth and function of partially or completely dedifferentiated mammary tumours and their responsiveness to sex hormones.

SUMMARY

Mammary glands of pregnant rats, transplanted fibroadenomata and human mammary carcinomata do not contain lactose. In a few cases this sugar could be detected in dimethylbenzanthracene-induced mammary carcinomata (7·7%) *and fibroadenomata* (13·2%) *of rats. The lactose incidence could be elevated in induced fibroadenomata by treatment of the animals with testosterone, oestradiol or oestradiol plus progesterone to* 38·5, 40·0 *and* 66·6% *respectively. Also lactose was inducible in the normal mammary gland of pregnant rats by testosterone application or ovariectomy of the animals before parturition. In induced mammary carcinomata and transplanted fibroadenomata of rats hormonal treatment was unsuccessful in inducing the synthesis of milk sugar.*

REFERENCES

1. S. K. Kon and A. T. Cowie (Editors), *Milk: The Mammary Gland and its Secretion*, Vol. 1. Academic Press, New York (1961).
2. Ch. Huggins, G. Briziarelli and H. Sutton, Rapid induction of mammary carcinoma in the rat and the influence of hormones on the tumours. *J. exp. Med.* **109,** 25 (1959).
3. T. L. Dao, Carcinogenesis of mammary gland in rats. In *Progress of Experimental Tumor Research* (Edited by S. Karger), Vol. 5, p. 157. S. Karger, Basel (1964).
4. J. Furth, Vistas in the etiology and pathogenesis of tumors. *Fed. Proc.* **20,** 865 (1961).
5. I. M. Hais and K. Macek, *Handbuch der Chromatographie*, Vol. 1, p. 281. VEB Gustav Fischer Verlag, Jena (1958).
6. J. L. Buchan and R. I. Savage, Paper chromatography of some starch conversion products. *Analyst* **77,** 401 (1952).
7. R. J. Dimler, W. C. Schaefer, C. S. Wise and C. E. Rist, Quantitative paper chromatography of D-glucose and its oligosaccharides. *Analyst. Chem.* **24,** 1411 (1952).
8. A. T. Cowie, The hormonal control of milk secretion. In *Milk: The Mammary Gland and its Secretion* (Edited by S. K. Kon and A. T. Cowie), Vol. 1, p. 163. Academic Press, New York (1961).
9. W. R. Lyons, C. H. Li and R. E. Johnson, The hormonal control of mammary growth and lactation. In *Recent Progress in Hormone Research* (Edited by G. Pincus), Vol. 14, p. 219. Academic Press, New York (1958).
10. U. Kim, J. Furth and K. Yannopoulos, Observations on hormonal control of mammary cancer. *J. nat. Cancer Inst.* **31,** 233 (1963).
11. R. Hilf, J. Michel, C. Bell and M. J. Carrington, Influence of endocrine organ ablation on the growth and biochemical response of the R 3230 AC mammary tumor to hormonal treatment. *Cancer Res.* **26,** 1365 (1966).
12. R. Hilf, C. Bell and J. Michel, Influence of mammotropic tumor MtTF4 on the growth and biochemistry of the R 3230 AC mammary carcinoma and mammary glands. *Cancer Res.* **27,** 482 (1967).
13. W. L. McGuire, Hormonal stimulation of lactose synthetase in mammary carcinoma. *Science* **165,** 1013 (1969).

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Cytogenetic Studies in Sprague–Dawley Rats during the Administration of a Carcinogenic Nitroso Compound—Diethylnitrosamine

SHOBHA GROVER* and PATRICIA FISCHER†
Institute of Cancer Research of the University of Vienna, Borschkegasse 8a, A-1090 Vienna, Austria

INTRODUCTION

VERY LITTLE work has been done on the cytogenetic effects of chemical carcinogens in relation to the development of hepatomas in experimental animals. Grundmann and Sieburg [1] have made a detailed study of the histogenesis and cytogenesis of liver carcinoma in rats given diethylnitrosamine daily in their drinking water, but no chromosomal analysis of their material was undertaken. There have been a few reports on the chromosomal constitution of established chemically induced transplantable hepatomas in rats. Yoshida [2] has described cytological characteristics among 50 different cell lines of rat ascites hepatoma; Ishidate [3] has reported on cytological differences in 12 cell lines of ascites hepatoma derived from isolated nodules occurring in the liver of a single Donryu rat and the chromosomal constitution of 42 transplantable "Morris" hepatomas maintained in Buffalo and ACI rats have been analysed in detail by Nowell *et al* [4].

In a comprehensive review of the action of the nitroso compounds, Magee and Barnes [5] have shown diethylnitrosamine (DEN) to be actively carcinogenic while relatively low in toxicity. This drug was used by Zbar *et al* [6] to induce hepatomas in strain-2 Guinea pigs. The antigenic characteristics, histology, growth and chromosomal constitution of six tumor lines were examined. Their report was, however, confined to a comparison of six established hepatomas and there appears to be no literature on cytogenetic studies in the progressive stages of tumor induction. In this paper the results of cytogenetic analyses of the liver and spleen, undertaken at regular intervals, during the administration of DEN to newborn Sprague–Dawley rats are presented. These have been compared to the chromosomal constitution of a hepatocellular carcinoma induced in an adult animal of the same strain. Studies on the ploidy rates and DNA synthesis in the liver parenchymal cells are presented in a separate report [7].

MATERIAL AND METHODS

One litter (6–8 animals) of newborn rats of the Sprague–Dawley strain was used for each experiment. Half of these were injected subcutaneously with DEN, daily, in a dose of 10 mg/kg of body weight and the remaining half were kept as controls. The litters were sacrificed after the 1st, 2nd, 3rd, 7th, 10th and 12th day of treatment and thereafter at intervals of approximately 10 days up to a period of 48 days. In all experiments the livers and spleens of experimental and control animals were pooled to have sufficient material for cytogenetic analysis. Material from each animal was also taken for histology.

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*Research Fellow of the Austrian Government Scholarship Program. Present address: Department of Pathology, Medical College, Nagpur, India.
†To whom all requests for reprints should be addressed.

Table 1. Details of metaphases counted and karyotype analyses in the liver and spleen of experimental and control animals

No. of days treated	Organ		Total cells counted	No. of chromosomes (37)	(38)	(39)	(40)	(41)	(42)	(43)	(3n–6n)	Karyotype
1	Liver	Expt.	28	—	—	—	1	1	25	—	1	Normal
		Control	25	—	—	1	1	—	22	—	1	
2	Liver	Expt.	26	—	—	—	2	2	21	—	1	Normal
		Control	23	—	—	—	1	1	20	—	1	
3	Liver	Expt.	126	—	5	7	8	38	63	4	1	Loss of B5 or A7 (47 metaphases)
		Control	42	—	—	—	1	2	38	—	1	38 with 41 chromosomes 9 with 42 chromosomes
7	Liver	Expt.	75	—	1	—	1	28	42	2	1	Loss of B5 or A7 (34 metaphases)
		Control	36	—	—	—	1	1	34	—	0	28 with 41 chromosomes 6 with 42 chromosomes
10	Liver	Expt.	62	—	1	1	2	19	34	3	2	Loss of B5 or A7 (22 metaphases)
		Control	26	—	1	—	—	1	23	1	—	19 with 41 chromosomes 3 with 42 chromosomes
12–36	Liver	Expt.					No mitosis					
		Control										
48	Liver	Expt.	10	—	—	—	2	1	6	1	—	Loss of A7, extra C(2 Meta×42.) Extra C (1 meta×43) ? one B4 satellited in 2 metaphases
		Control					No mitosis					
Adult DEN 15 mg/kg for 60 days	Hepato-cellular carcinoma		172	2	8	8	16	19	62	18	39	Normal: 16 metaphases B5 or A7 loss: 31 metaphases B2 loss: 28 metaphases Markers M1: 7 metaphases, M2: 33 metaphases Satellited B4: 25 metaphases
Total expt. animals Spleen:			481	1	13	15	18	55	347	2	30	Stickiness of chromosomes after day 3—increase in chromatid breaks and gaps
Total control animals Spleen:			282	1	3	9	7	10	233	—	20	

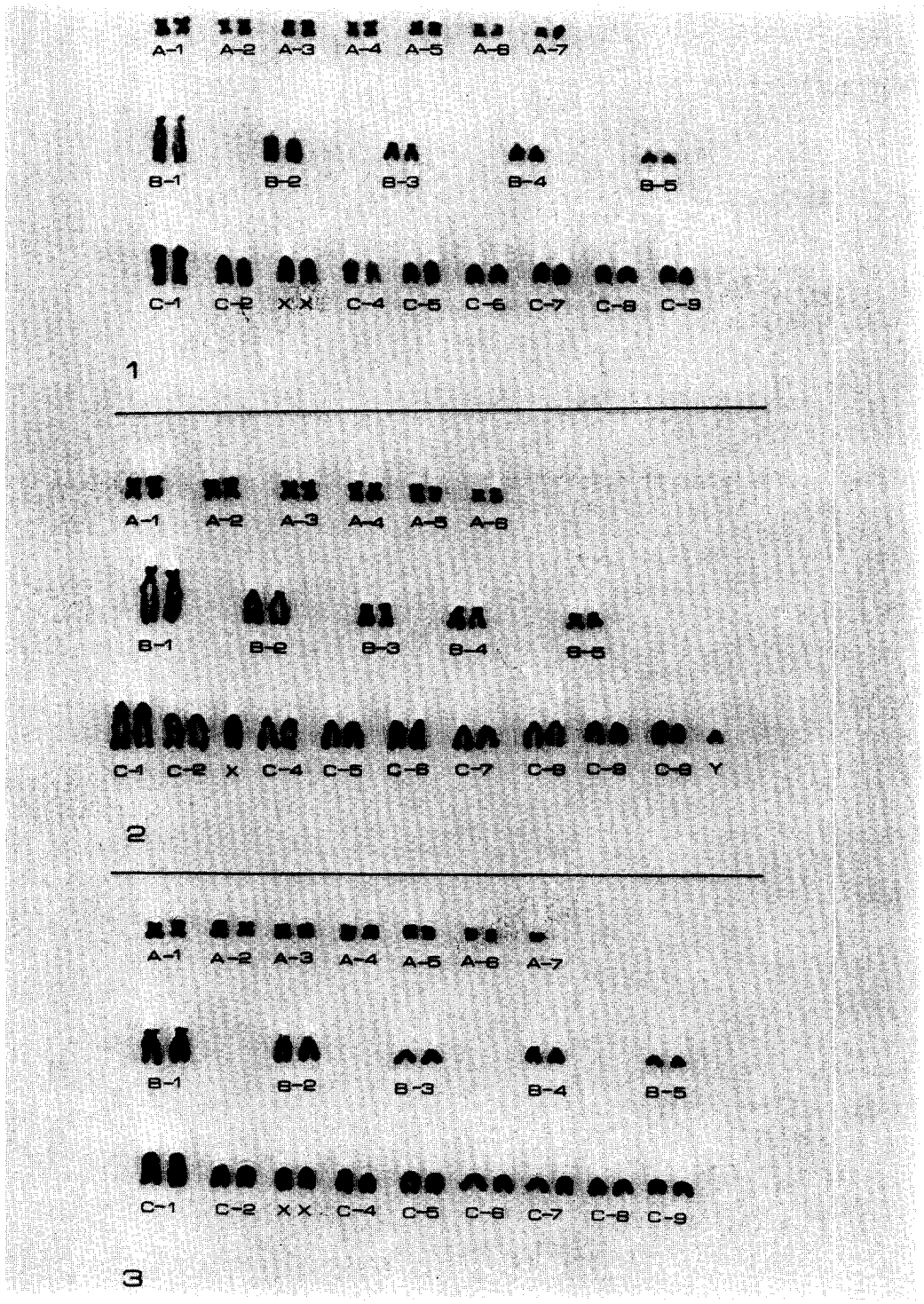


Fig. 1. *Normal karyotype of Sprague–Dawley rat* ($2n = 42$).

Fig. 2. *Karyotype from liver of experimental animal injected for* 3 *days. Pseudodiploid metaphase with loss of A7 chromosomes and two extra small acrocentric chromosomes.*

Fig. 3. *Karyotype from liver of Sprague–Dawley rat injected with* DEN *for* 3 *days. Chromosome number:* 41 *with monosomy of A7.*

(to face p. 78)

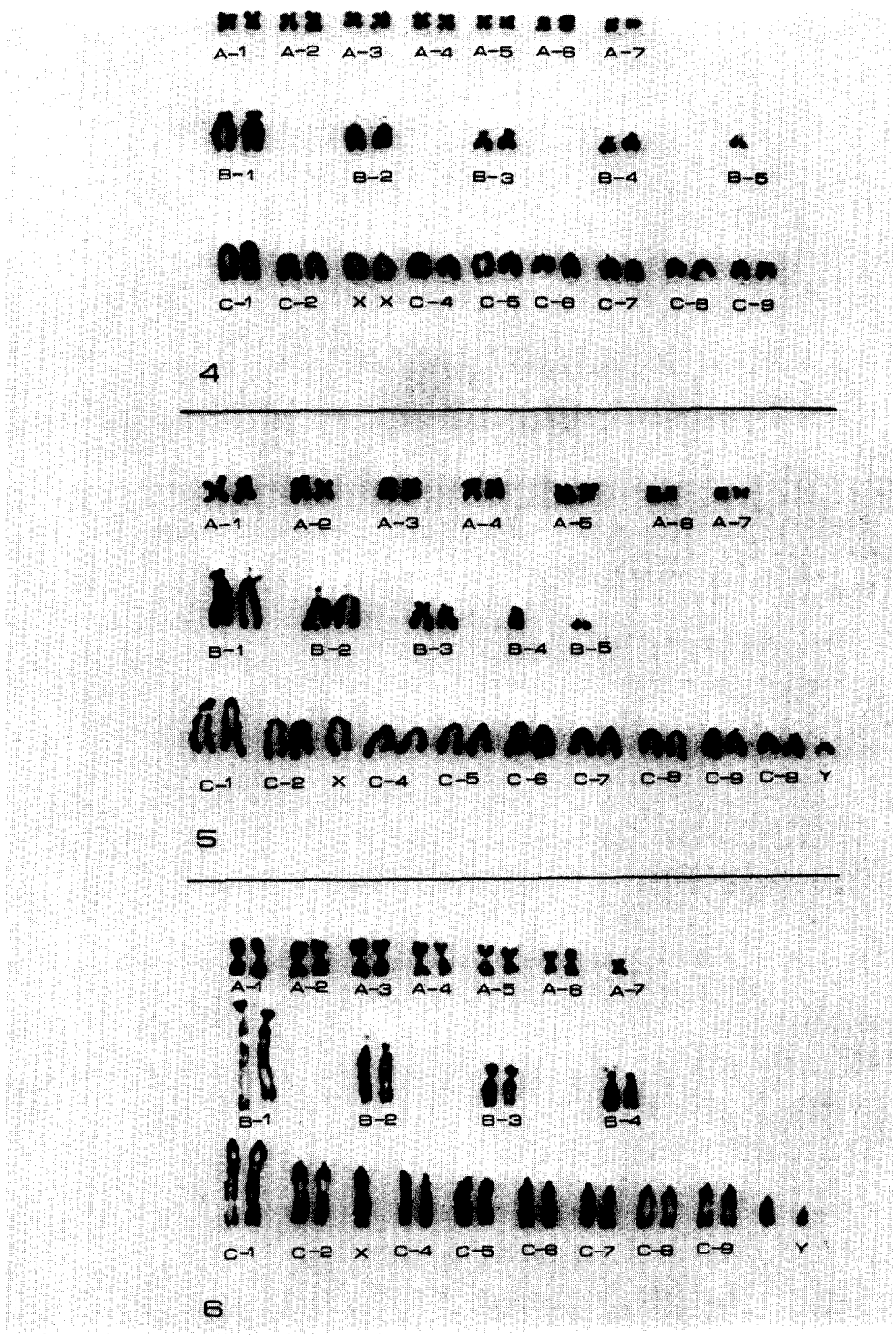


Fig. 4. *Karyotype from liver of experimental animal injected for* 7 *days. Chromosome number* 41 *with monosomy of* B5.

Fig. 5. *Karyotype from liver of experimental animal injected for* 10 *days. Pseudodiploid with monosomy of* B4 *and* B5 *and extra small acrocentrics.*

Fig. 6. *Karyotype from hepatocellular carcinoma. Chromosome number* 40 *with monosomy of* A7, *loss of* B5 *chromosomes, and satellites on one* B4.

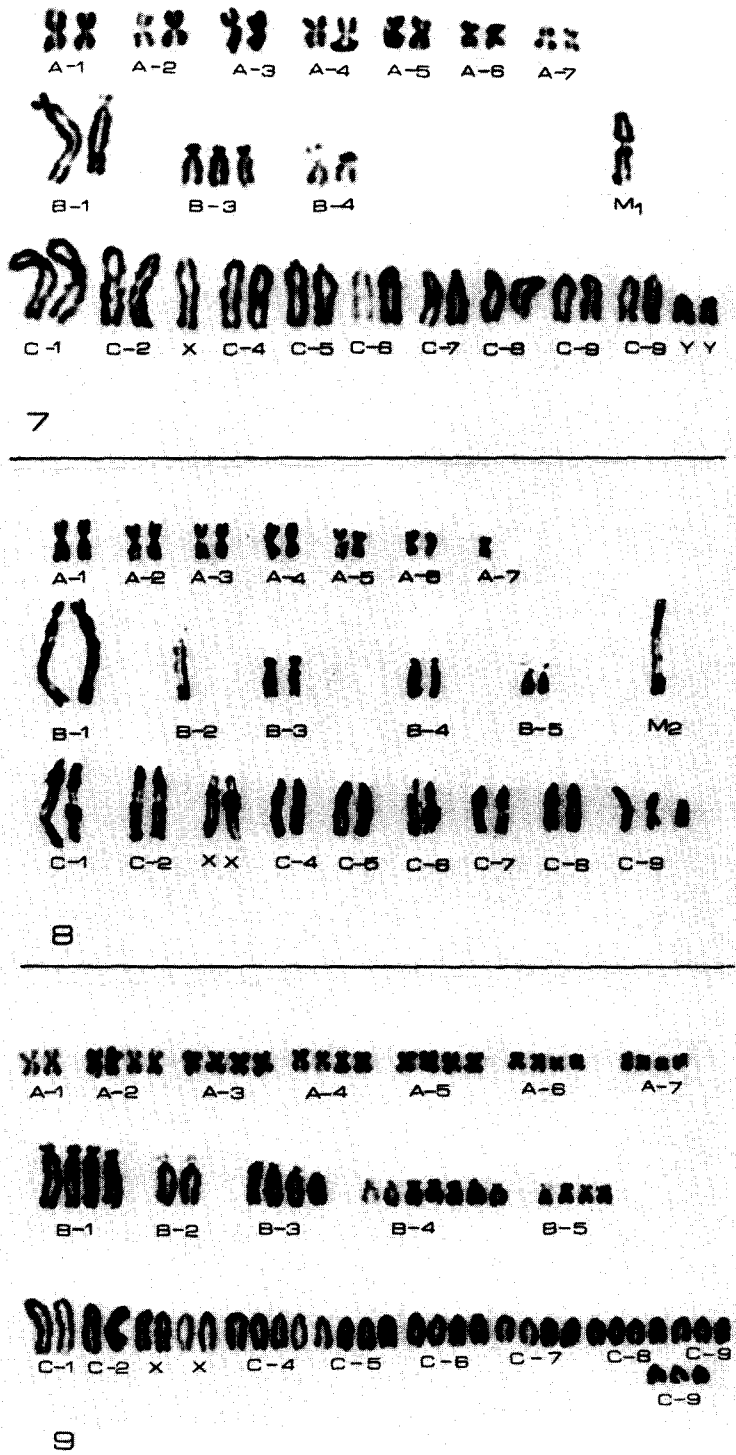


Fig. 7. *Karyotype from hepatocellular carcinoma. Chromosome number* 43 *with loss of* B5, *an extra* B3 *and acrocentrics, marker* M1 *and satellites on one* B4.

Fig. 8. *Karyotype from hepatocellular carcinoma. Pseudodiploid with* B2 *monosomy and presence of* M2 *marker.*

Fig. 9. *Karyotype from polyploid cell of hepatocelluar carcinoma. Chromosome number* 81.

In addition, four adult rats (60 days old) of the same strain were given DEN in a daily dose of 15 mg/kg body weight in drinking water. These animals were sacrificed at the age of 120 days. Bone marrow, spleen, and a nodule from each liver which appeared malignant on gross examination was processed for chromosomal analysis.

The technique used for chromosomal preparation was a modification of the method of Nowell *et al* [4]. The tissue was gently minced and a cellular suspension made in Earles solution with colcemid (0·2 μg/ml). The suspension was kept at 37°C for 1 hr and then centrifuged. The cells were then resuspended in hypotonic saline (1 part physiological saline and 4 parts aqua dest.) and kept at room temperature, for 30 min. The suspension was again spun down and the cell button fixed in acetic acid and methanol (1: 1). The fixation was repeated three times at intervals of 1 hr and then stored at 4°C overnight. This procedure was repeated three times on the following day. Slides were prepared by dropping the final suspension on clean slides kept in aqua dest. at 4°C. Spreading by air drying or ignition was controlled by test preparations examined under phase contrast. Slides were stained with aceto-orcein and examined under phase microscopy.

RESULTS

A normal karyotype of the Sprague–Dawley strain was prepared from the analysis of liver and spleen of control animals (Fig. 1). The chromosomes were classified according to the method of Kurita *et al* [8] into three groups: A1–A7 metacentric, B1–B5 submetacentric or subtelocentric, C1–C9 acrocentric or telocentric. Satellites were sometimes present on chromosomes B2 and B5 but were never observed on B4. Details of cells counted and karyotype analyses in the various experiments are presented in Table 1.

1st and 2nd day. In the livers and spleens obtained from animals which had received up to two injections, no abnormality of the karyotype was observed.

3rd day. In the experimental livers, 126 metaphases were counted. Sixty-three had the diploid number (2n = 42) but, on analysis of the karyotype, 9 were pseudodiploid with a loss of one small metacentric or submetacentric chromosome (A7 or B5) and an increase in the number of small acrocentrics (Fig. 2). Thirty-eight metaphases had 41 chromosomes with monosomy of either A7 or B5 (Fig. 3), 4 metaphases had 43 chromosomes with an increase of a small acrocentric. In the control livers, 2 out of 42 metaphases showed loss of an A7 or B5 chromosome. Both experimental and control spleens had a normal karyotype.

7th day. In the experimental livers, 75 metaphases were counted, 36 showed a normal diploid karyotype, and 6 were pseudodiploid with loss of either an A7 or B5 and an increase of the small acrocentrics. Twenty-eight metaphases had a count of 41 chromosomes with monosomy of either A7 or B5 (Fig. 4). The spleens showed a normal karyotype in both experimental and control animals.

10th day. In the experimental livers, 31 metaphases of a total of 62 showed a normal diploid karyotype, and 3 were pseudodiploid with monosomy of A7 or B5 (Fig. 5). Nineteen metaphases with 41 chromosomes lacked a small metacentric or submetacentric (A7 or B5). The chromosomes were sticky and separation poor. The spleen showed a normal karyotype both in the experimental and control animals.

12th–34th day. Liver: In spite of repeating the experiments using different age groups within this period, no metaphases were obtained in either the experimental or control animals. The experimental spleens showed an increased stickiness of chromosomes and it became technically difficult to get very good spreads. The chromosomes showed fuzziness and beading but the karyotype was normal. Control spleens had a normal count and karyotype.

48th day. Experimental livers: Chromosomes in these preparations were sticky and elongated with a tendency to non-disjunction and fusion. Only 10 metaphases could be counted, and 4 karyotyped. Two with 42 chromosomes lacked one A7, and had an extra small acrocentric and one with 43 chromosomes had an extra member in the C group. In two metaphases one B4 was possibly satellited. No dividing cells were present in the control livers. Metaphases were normal in both experimental and control spleens.

Adult animals

These were sacrificed after 60 days of treatment. On gross examination, the livers in the experimental animals showed coarse surface granularity and several nodules were present in each liver. These were processed separately. One nodule, about 1 cm in diameter, which on microscopic examination was a hepatocellular carcinoma, yielded analysable material. One hundred and seventy-two metaphases were counted from 35 slides prepared. The chromosomes showed a tendency to stickiness except in a few metaphases where the number of

chromosomes and karyotype was normal. There were 16 metaphases which had a normal diploid karyotype out of 62 with a diploid number. Loss of a small metacentric or submetacentric chromosome (B5 or A7) was noted in 31 metaphases, and of the larger submetacentric (B2) in 28 metaphases. Two marker chromosomes could be distinguished. One, a large metacentric (M1), was present in 7 metaphases, and a large submetacentric (M2) in 33. A prominent satellite was frequently observed on one B4 autosome. Thirty-nine metaphases were polyploid, and exhibited various rearrangements of the karyotype (Figs. 6–9).

DISCUSSION

This study appears to be the first to attempt an analysis of cytogenetic changes occurring in the liver and spleen of rats during the administration of a chemical carcinogen.

Newborn animals were chosen for two reasons. Firstly, dividing cells are not present in sufficient numbers in normal adult liver unless tissue regeneration is stimulated by partial hepatectomy. Examination of normal neonatal liver before the start of the experiment established that a sufficient number of dividing cells for cytogenetic analysis are available until about the tenth day of life. In order to obtain a maximal effect within this period the drug was administered daily. Under this regimen, however, it was not possible to maintain the rats until the development of frank hepatomas, and the longest survival period was 48 days, at which time the two surviving experimental animals were sacrificed. Isolated nodules were not present but histologic examination of the livers revealed early malignant changes in the center of the lobules.

A second reason for using newborn animals was based on Potter's suggestion [9] that the immature hepatocyte might be a more logical norm for comparison with the hepatoma cell than adult liver tissue which, in the rat, has a tetraploid rate of 30–40%. Furthermore, the enzyme spectrum in the neonatal liver differs markedly from that in the adult, and undergoes rapid changes during the first few days of life. A carcinogen introduced during this period of heightened biological activity could therefore be expected to exercise a maximal effect. This heightened susceptibility to the action of chemical carcinogens in newborn animals is well documented in the mouse, and a greater susceptibility to induction of both liver and renal tumors has been demonstrated in newborn Wistar Parton rats given a single subcutaneous injection of a nitroso compound [10]. As anticipated, the low mitotic rate yielded no analysable mitoses in the control animals after the 10th day. Our failure to obtain mitoses in the experimental animals after this time, until the histologic appearance of nuclear changes indicative of early malignancy, is in agreement with the observation of Grundmann and Sieburg [1] that there is no increase in the mitotic index in the liver during cancerogenesis until the development of nests of malignant cells.

A suppression of DNA synthesis in adult Wistar rats given DEN after partial hepatectomy is reported by Rabes *et al* [11]. Wiest has found no change in DNA synthesis in our group of neonatal animals but an early and marked increase in the ploidy rate, the number of polyploid cells reading 70% by the 20th day of treatment as compared to 14% in the controls. She attributes this mainly to a progressive endomitosis which reaches its peak in the third week with ploidy values of up to 64 n. This high ploidy component is then gradually replaced by 2 and 4n cells, the diploid–tetraploid ratio at 48 days being 40–60 as against 30: 70 in the control animals. This coincides with the appearance histologically of nests of small basophilic "malignant" cells also described by Grundmann and Sieburg, an increase in the mitotic rate, and the presence of aneuploid metaphases.

Although the frequency of chromatid and chromosome aberrations were not noticeably increased in the metaphases of experimental animals, karyotypic analysis demonstrates an early and direct action of the drug on the chromosomes of liver cells. After the third day of treatment, a small chromosome (B5 or A7) had been lost from 47 of 126 metaphases, and in cells with 42 chromosomes this loss was associated with an increase in the number of small acrocentrics (group C). A similar pattern was again present in 2 of 4 analysable metaphases after 48 days of treatment, when early malignant changes were present histologically, and in 31 of 172 metaphases from the hepatoma. A wide range of chromosome number, additional karyotypic rearrangements and gross morphological changes were present in the hepatoma, indicative of the instability associated with the progressive evolution of the malignant genome. A large sub-metacentric chromosome, the marker most commonly seen, could have arisen by translocation or fusion involving the B2 chromosome which was absent in 28 of 33 metaphases, and the metacentric marker by centric fusion of two

chromosomes in the "C.' group. These two markers were commonly present in the group of hepatomas with major structural changes studied by Nowell *et al* [4].

Of greater interest, however, is the presence of one satellited chromosome (B4) (B12 in Nowell's terminology) in the hepato cellular carcinoma, and its possible presence in the 48-day liver. Nowell has noted this satellited chromosome in 5 of 6 "minimal deviation" hepatomas — and in 7 of 16 hepatomas in which the chromosome number was abnormal but no major structural change was present. The technical quality of metaphases in the liver at 48 days was unfortunately poor, but analysis under the microscope suggests that a satellited B4 chromosome is also present at this stage. Our findings of a satellited B4 associated with early malignant changes in a rat species other than the Buffalo and ACI strains studied by Nowell, strengthens the case for a causal relationship between this abnormality and malignancy in the rat.

The effect of DEN on the spleen of the neonatal rat, and the spleen and bone marrow of the adult animals was minimal. There was no difference in the frequency of mitoses between control and experimental animals, and dividing cells were present throughout the period of the experiment. In the later stages there was an increased stickiness of chromosomes in the experimental animals, but karyotypes were normal.

The selective action of DEN is well illustrated in this study. The early effect on the genome of liver cells, and the persistence of similar karyotypic patterns into malignancy suggests a common mechanism for the mutagenic and carcinogenic actions of this compound.

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SUMMARY

Newborn rats of the Sprague–Dawley strain were injected subcutaneously with diethylnitrosamine in a dose of 10 *mg/kg body weight daily. This regimen could be maintained for* 48 *days.*

Cytogenetic studies on fresh tissue from the liver and spleen of experimental and control animals were made at regular intervals. A change in the chromosomal pattern in the liver cells of the experimental animals occurred after the third day of treatment.

Similar patterns were present at 48 *days and in a malignant nodule induced in an adult rat. Additional gross morphological changes were present in the hepatoma.*

Normal diploid metaphases were abundant in splenic tissue of both control and experimental groups in the neonatal animals at all stages of the investigation, and in the spleen and bone marrow of the adult animals.

REFERENCES

1. E. Grundmann and H. Sieburg, Die Histogenese und Cytogenese des Lebercarcinoms der Ratte durch Diäthylnitrosamin im lichtmikroskopischen Bild. *Beitr. Pathol. Anat. Allgem. Pathol.* **126,** 57 (1962).
2. T. Yoshida, Studien über das Ascites Hepatoma zugleich ein Beitrag zum Begriff der cellulären Autonomie der malignen Geschwulst. *Arch. Path. Anat. Physiol.* **330,** 85 (1967).
3. M. Ishidate, Cytological individuality of hepatoma cell lines derived from a single Donryu rat. *Gann.* **59,** 341 (1968).
4. P. C. Nowell, H. P. Morris and V. R. Potter, Chromosomes of "Minimal deviation" hepatomas and some other transplantable rat tumors. *Cancer Res.* **27,** 1565 (1967).
5. P. N. Magee and J. M. Barnes, Carcinogenic nitroso compounds. In *Advances in Cancer Research,* (Edited by A. Haddow and S. Weinhouse), Vol. 10, p. 163, Academic Press, New York (1967).
6. B. Zbar, H. T. Wepsic, H. J. Rapp, J. Whang Peng and T. Borsos, Transplantable hepatomas induced in strain-2 Guinea Pigs by diethylnitrosamine. Characterization by histology, growth and chromosomes. *J. nat. Cancer Inst.* **43,** 821 (1969).
7. L. Wiest, The effect of diethylnitrosamine on ploidy distribution in liver cells of newborn rats. In press.
8. Y. Kurita, T. Sugiyama and N. Yasuaki, Cytogenetic analysis of cell population in rat-leukemia induced by pulse doses of 7,12 dimethylbenz (α) anthrazene. *Gann.* **60,** 529 (1969).

9. V. R. Potter, Symposium on the developmental biology of neoplasia. *Cancer Res.* **28,** 1901 (1968).
10. G. Della Pista and B. Terracini (Milan), Chemical carcinogenesis in infant animals. *Progress in Exptl. Tumor Res.* **11,** 334 (1969).
11. H. Rabes, R. Hartenstein und P. Schlotze, Hemmung der Proliferationsaktivität partiell resezierter Leber durch Diäthylnitrosamin. *Z. Krebsforsch.* **73,** 239 (1970).

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Chromosome Study in a Case of Granulocytic Leukaemia with 'Pelgerisation' 7 Years after Benzene Pancytopenia*

M. SELLYEI and E. KELEMEN

Department of Pathology, Róbert-Károly Hospital, Budapest, XIII and 1st Medical Clinic, Semmelweis Medical University, Budapest, VIII, Hungary

INTRODUCTION

SEVERAL works have been published on the chromosome damage as a consequence of benzene intoxication [1–4]. Recently, it was shown by Tough and her co-workers [5] that individuals who had been exposed to benzene, even without haematological disorders, might have an elevated percentage of unstable chromosome aberrations in the lymphocytes cultured from their peripheral blood.

Some cases of benzene-induced haemopathy can evolve into leukaemias mostly of acute myelogenous type [6–8]. About one half of acute leukaemias do not reveal any chromosomal changes [9]. The leukaemic cells, however, in two published cases of benzene leukaemia had new cell lines differing from each other [6, 10]. Development of a new cell line was observed by us in a case of haemoblastosis, preceded 7 years before by pancytopenia which followed benzene exposition.

CASE REPORT

V.G., male, age 35. The patient, being 27, had been working for 18 months in a car repairing factory. He dissolved paint with benzene. The concentration of benzene in the atmosphere of the working place was of 200–1640 mg/m³. The allowed maximal concentration in this country is 20 mg/m³. In July 1962 he was admitted to the National Institute of Professional Health (O.M.I.), Budapest, with severe anaemia (5·1 g Hb/100 ml of blood), neutropenia (1400/mm³), and thrombocytopenia (40,000/mm³). Revision of his bone marrow specimens in 1970 did not reveal either blast cells or Pelger-type anomaly. The patient recovered after treatment with transfusions and corticosteroids. Thereafter, he worked as a truck driver till March 1969, when he started complaining of weakness. In June 1969 he was admitted to the 1st Medical Clinic of the Semmelweis University, Budapest, with cutaneous-subcutaneous and submucous haemorrhages. At admission the patient was pale and he had a slight hepatosplenomegaly. His blood findings were: Hb 7·6 g/100 ml, reticulocytes 3000/mm³; WBC 23,000/mm³ with blast cells 8%, promyelocytes 10%, myelocytes 33%, metamyelocytes 39%, bands 5%, lymphocytes 5%; platelets 5000/mm³. More than half of the granulocytic precursor cells and almost 100% of the 'mature' granulocytes had Pelger-type anomaly. The granulocyte alkaline phosphatase was positive in 83% of the peripheral granulocytes and the 'score' was 178 (Fig. 1).

Bone marrow was obtained on 16 June 1969 by trepanation of sternum. The smears contained granulocyte precursors with Pelger-

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type as well as other nuclear anomalies (Fig. 1), but cellularity was good and the myeloid-erythroid ratio appeared to be nearly normal. There were 5–10% of blasts in the smears. The patient was treated by transfusions and corticosteroids, but his thrombocytopenia could not be influenced. He had further cutaneous and visceral haemorrhages, developed up to 38·5°C fever along with Hb value of 5·5/100 ml of blood and up to 70,000 WBC with 10% of myeloblasts, otherwise as above. He died on 21 August 1969. At the autopsy the liver weighed 2100 g and the spleen 600 g. Microscopically they were infiltrated by many haemopoietic precursor cells. These cells formed also solid masses on the dura (Fig. 2).

Chromosome study was performed on direct preparations of bone marrow specimen obtained by trepanation. The cells were treated with Colcemid-CIBA for 1 hr at 37°C, thereafter with hypotonic solution and fixed with methanol-acetic acid (3: 1). Twenty-seven metaphases were photographed and analysed (Table 1). All of them had supernumerary D-group chromosome(s). Sixteen cells lacked one or more G-group chromosomes and 12 had one or two supernumerary C-group chromosomes (Fig. 3).

At the same time a 48-hr peripheral blood culture was set up with PHA added. The specimens were very poor in mitoses. Four cells had normal male karyotype (46,XY) and in one metaphase plate a supernumerary D group chromosome was found.

DISCUSSION

In benzene-induced haemopathies other than leukaemia chromosomal changes were revealed both in cultured peripheral lymphocytes [2, 3, 6, 11] and in bone marrow cells [1, 3]. According to Forni and Moreo [6], in a case of benzene-induced haemopathy bone marrow mitoses showed chromosomal breaks and other random aberrations before the development of leukaemia. However, after the onset of acute leukaemia C-trisomy was the typical change for the leukaemic cells. In another case of benzene-induced erythroleukaemia the leukaemic cells were characterized by two small marker chromosomes and by the lack of two E-group chromosomes [10]. In our own case the leukaemic cells revealed a rather uniform chromosomal rearrangement: all mitoses had supernumerary D-group chromosomes (Table 1). The benzene-induced chromosomal imbalance presumably predisposes to leukaemia with chromosomal rearrangement. It is important in the view that in 50% of acute leukaemias there are not any visible chromosome anomalies [9].

Vigliani and Saita [8] found among the workers dealing with benzene a leukaemia occurrence twenty times greater than that of the general population. Although a leukaemoid response could be suggested in our case as an alternative diagnosis, the presence of 'Pelgerisation' as well as of stable chromosome changes suggested us to speak of haemoblastosis, i.e. subacute granulocytic leukaemia. DeGowin

Table 1. *Chromosome aberrations of* 27 *metaphases from direct preparation of bone marrow*

No. of cells	No. of chromosomes	A	B	C	D	E	F	G	Fr.*
1	45			+1	+1			−3	
2	46				+1			−1	
6	47				+1				
4	47			+1	+1			−1	
2	47				+1			−1	+1
1	47				+1	+1		−1	
1	47			+2	+1			−2	
1	47				+2			−1	
1	47	−1	dic	+2	+1	+1		−3	+1
1	48				+1		+1		
1	48		+dic		+1			−1	+1
1	48			+1	+1				
1	48			+2	+1			−1	
1	48				+1				+1
1	49			+1	+1	+1			
1	49	−1		+2†	+1	+1			
1	49			+2	+2			−2	+ring
Total 27									

*Small chromosomal fragments.
†Two subacrocentric chromosomes of the size of C-group chromosomes.

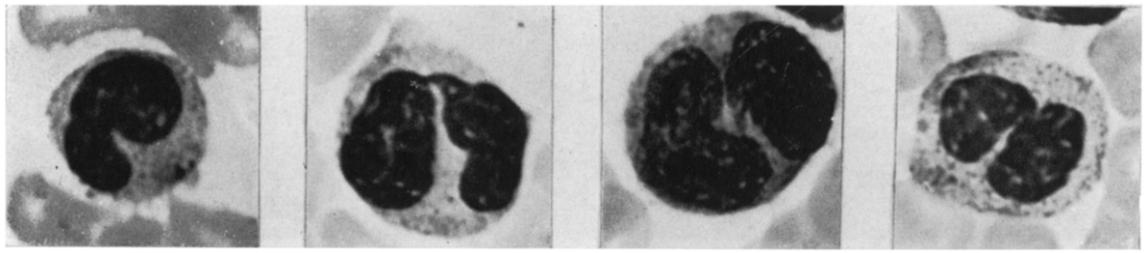


Fig. 1. *Leukaemic cells from bone marrow smears with hyposegmentation of the nuclei, corresponding to acquired Pelger anomaly.*

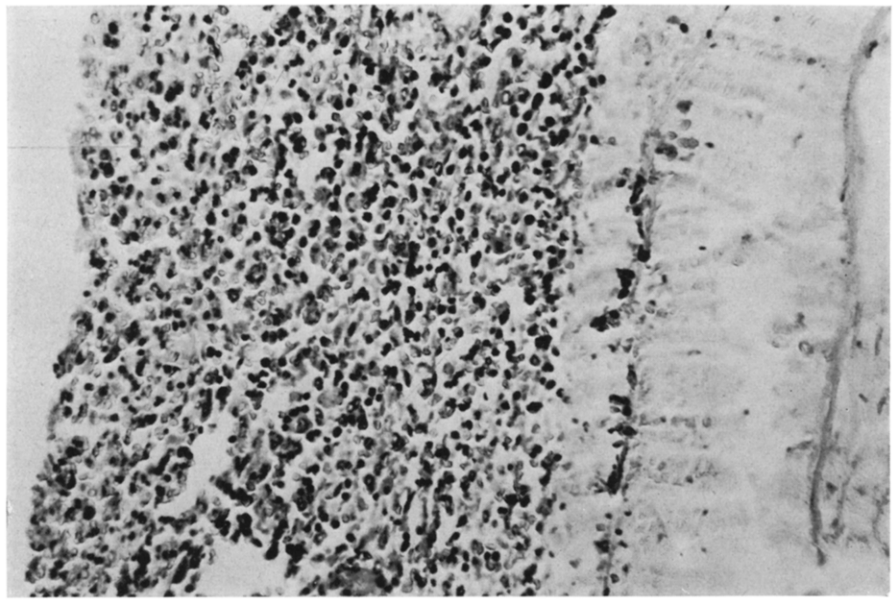


Fig. 2. *Crowded leukaemic cells on the inner surface of the dura* (*H.&E.* × 180).

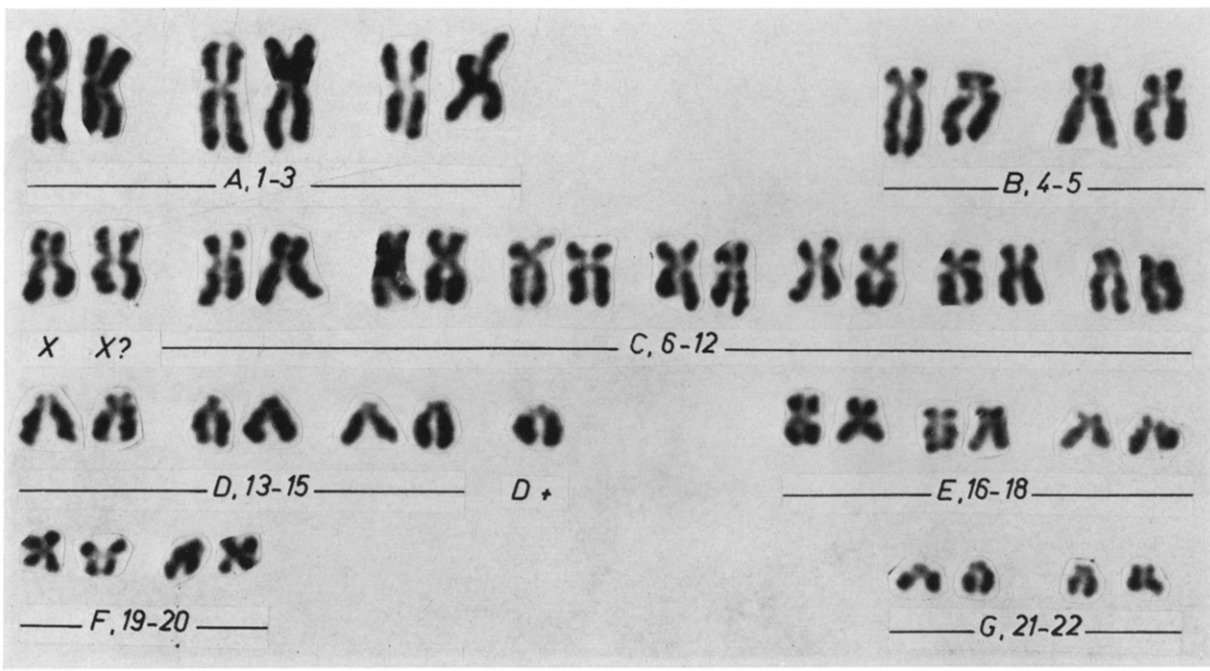


Fig. 3. *Karyotype from bone marrow direct preparation with* 47 *chromosomes. Note supernumerary D-group chromosome. It was present in all analysed bone marrow mitoses. In male patient some leukaemic cells were of female type due to supernumerary C-group chromosome and absence of one G-group chromosome.*

(to face p. 84)

[12] observed the onset of leukaemia 15 years after benzene exposition. In spite of this, a direct connection between the apparently healed benzene pancytopenia and the lethal haematological syndrome 7 years thereafter is a tempting possibility but it cannot be either proved or refuted.

The acquired Pelger anomaly [13] in the present case corresponded morphologically to the heterozygous type. To our knowledge no case of pseudo-Pelger anomaly associated with leukaemia of a probable benzene aetiology has, as yet, been published. The segmentation disturbance of the nuclei may or may not be related to D-trisomy of the leukaemic cells. Interestingly, Patau's syndrome (congenital D-trisomy) is also accompanied by segmentation disturbance (nuclear protrusions) of the granulocytes [14].

Our present project is to investigate the karyotype of other patients, just after apparently healed benzene cytopenia, from year to year, before any clinical sign of haematological malignancy.

SUMMARY

Report of a case in which 7 years after the healing of a benzene-induced pancytopenia a subacute haemoblastosis developed along with Pelgerisation of the granulocytes. At the same time direct bone marrow specimens revealed the emergence of a new cell line characterized by supernumerary D-group chromosomes. Other chromosomal anomalies were also present. Subacute granulocytic leukaemia has been diagnosed and confirmed by autopsy. The problem of the causal relationship between benzene poisoning and the haematological malignancy has been discussed briefly.

REFERENCES

1. G. Pollini and R. Colombi, Il danno cromosomico midollare nell'anemia aplastica benzolica. *Med. Lavoro* **55,** 241 (1964).
2. G. Pollini and R. Colombi, Il danno cromosomico dei linfociti nell'emopatia benzenica. *Med. Lavoro* **55,** 641 (1964).
3. G. Pollini, E. Stroselli and R. Colombi, Sui rapporti fra alterazioni cromosomiche delle cellule emiche e gravita' dell'emopatia benzenica. *Med. Lavoro* **55,** 735 (1964).
4. A. Forni, Chromosome changes due to chronic exposure to benzene. *Proc. XV Int. Congr. Occupational Health* II/1, Vienna, Verlag der Wiener Medizinischen Akademie, p. 437 (1966).
5. I. M. Tough, P. G. Smith, W. M. Court Brown and D. G. Harnden, Chromosome studies on workers exposed to atmospheric benzene. *Europ. J. Cancer* **6,** 49 (1970).
6. A. Forni and L. Moreo, Cytogenetic studies in a case of benzene leukaemia. *Europ. J. Cancer* **3,** 251 (1967).
7. A. Goguel, A. Cavigneaux and J. Bernard, Les leucémies benzéniques de la région parisienne entre 1950 et 1965. *Nouv. Rev. franç. Hémat.* **7,** 465 (1967).
8. E. C. Vigliani and G. Saita, Benzene and leukemia. *New. Engl. J. Med.* **271,** 872 (1964).
9. A. A. Sandberg, The chromosomes and causation of human cancer and leukemia. *Cancer Res.* **26/1,** 2064 (1966).
10. A. Forni and L. Moreo, Chromosome studies in a case of benzene-induced erythroleukaemia. *Europ. J. Cancer* **5,** 459 (1969).
11. G. Hartwich, G. Schwanitz and J. Becker, Chromosomenaberrationen bei einer Benzol-Leukämie. *Dtsch. Med. Wschr.* **94,** 1228 (1969).
12. R. L. Degowin, Benzene exposure and aplastic anemia and leukemia 15 years later. *J. Am. med. Ass.* **185,** 748 (1963).
13. A. D. Dorr and W. C. Moloney, Acquired pseudo-Pelger anomaly of granulocytic leukocytes. *New Engl. J. Med.* **261,** 742 (1959).
14. E. R. Huehns, M. Lutzner and F. Hecht, Nuclear abnormalities of the neutrophils in D_1 (13–15)-trisomy syndrome. *Lancet* **i,** 589 (1964).

Europ. J. Cancer Vol. 7, pp. 87–94. Pergamon Press 1971. Printed in Great Britain

Evaluation of Extent of Metastatic Liver Disease: A Proposed Discriminant

MAGDA M. STILMANT*, GEORGES M. VAMECQ, WILLY F. PIESSENS, and ROBERT R. BADJOU

Service of Pathology, Department of Statistics, and Service of Medicine and Clinical Investigation, Institut Jules Bordet, Centre des Tumeurs de l'Université Libre de Bruxelles, 1, rue Héger-Bordet, 1000 Bruxelles, Belgium

INTRODUCTION

QUANTITATIVE estimation of the extent of metastatic liver disease during life should be of interest in the assessment of the prognosis and may even be necessary since some therapeutic procedures are ineffective and even hazardous when massive liver involvement is present. This communication proposes a discriminant function, based on the results of certain liver function tests, to predict the degree of metastatic liver involvement.

MATERIAL AND METHODS

Clinical data

A set of liver tests† carried out less than two months before death was available in 40 patients, 18 males and 22 females aged between 30 and 90 years, recently admitted to the Institut Jules Bordet.

Liver function tests were performed according to standard techniques (Table 1). The activity of lactic dehydrogenase isoenzymes was estimated as suggested by Ressler [2] after agar gel electrophoresis according to Wieme [3].

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*Present address: Mallory Institute of Pathology, Boston City Hospital, Boston, Mass. 02118, U.S.A.

†Lactate dehydrogenase (LDH), and its isoenzymes (LDH_1 to LDH_5); sulfobromophtalein excretion (BSP); alkaline phosphatase (Alk P'ase); serum bilirubin (Bil); glutamic pyruvic transaminase (GPT); glutamic oxalacetic transaminase (GOT); isocitric dehydrogenase (ICD); Aldolase (Ald); serum protein electrophoresis; prothrombin time; cephalin flocculation; thymol turbidity.

All cases were submitted to a complete autopsy. Careful dissection of the biliary tract, cranial bones, vertebrae, sternum, ribs and other osseous localisations suspected of metastasis by X-ray examination was always performed. The liver was sectioned into slices of 1 cm thickness. The metastatic nodules were counted and measured. This led to a rough estimation of the amount of metastases relative to the total volume of the liver. The values ranged from 5 to 90%. None was comprised between 30 and 50%. Therefore the cases were classified into two anatomical groups:

Group I: (22 cases): one or several small nodules amounting to less than 30% of the total parenchyme.
Group II: (18 cases): massive involvement by numerous nodules, amounting to more than 50% of the total parenchyme.

The origins of the metastatic tumors are given in Table 2. Histological sections were routinely stained by hematoxylin-eosin.

Mathematical analysis

Two mathematical models were tentatively used to predict the degree of liver involvement on the basis of the liver function tests available: a multiple regression equation, and a discriminant function.

I. Linear multiple regression. In this model the predicted liver involvement y is related to a set of p predictive variables (the liver tests) by an equation of the type:

$$\bar{y} = b_0 + b_1 x_1 + \ldots + b_p x_p$$

Table 1. *Liver function tests used in this study*

Tests and abbreviations		Upper limit of normal values	References
Lactate dehydrogenase (LDH)		680 Units/ml	Wroblewski *et al.* [1]
LDH electrophoresis:	LDH1	39·7%	Ressler [2] and Wieme [3]
	LDH2	52%	
	LDH3	25%	
	LDH4	8·7%	
	LDH5	4·7%	
Sulfobromophtalein excretion (BSP)		5% retention at 45 min	Mateer *et al.* [4]
Alkaline phosphatase (Alk. P'ase)		4·5 Bodansky units	Autoanalyzer modification of Kind *et al.* [5]
Bilirubin (Bil.)		1·1 mg/100 ml	Malloy [6]
Glutamic pyruvic transaminase (GPT)		30 Karmen units	Wroblewski *et al.* [7]
Glutamic oxalacetic transaminase (GOT)		50 Karmen units	Wroblewski *et al.* [8]
Isocitric dehydrogenase (ICD)		264 units (m) m units/ml 224 units (f) m units/ml	Wolfson *et al.* [9]
Aldolase (Ald.)		10 Wroblewski units	Boehringer modification of Biesenherz [10]
Serum protein electrophoresis			
	Albumin	65%	
	α1—glob	7%	
	α2—glob	11%	
	β—glob	13%	
	γ—glob	20%	
Thymol turbidity		4 units	MacLagan [11]
Cephalin flocculation		2+ at 48 hours	Hanger [12]
Prothrombin time (PTT)		80% (lower limit)	Quick [13]

Table 2. *Origin of liver metastases*

Breast	13
Lung	8
Gastro-intestinal tract	4
Others*	15
Total	40

*The other primary sites included oral mucosa, uterus, ovary, urinary bladder, pancreas, skin, adrenal gland, prostate, lymph node.

where the 'x's refer to the test values and the 'b's are regression coefficients to be estimated. The actual involvement y differs from the predicted one by an error term:

$$e = y - \bar{y}$$

which is assumed to be a normally distributed random variate with zero mean and constant standard deviation σ whatever the 'x's values are. σ is a measure of the reliability of the prediction: the smaller it is, the better the prediction.

The determination of the regression coefficients requires the knowledge of the population from which the patients under study have been sampled out. The only thing that can be done is to estimate them from the sample at hand. The least squares method was used for this purpose. The estimates are among all the possible values of the 'b's those that render the sum of the squared errors

$$\Sigma e^2$$

as small as possible. The least squares estimates yield in some sense the closest fit to the observed data of the linear regression model. This method also yields an estimation of σ which will be referred to as 's'.

Before proceeding to the exposition of the computational method a general point must be made concerning the selection of the tests which are to be included in the regression. Some of the true regression coefficients (the adjective 'true' refers to the population from which the sample is drawn), can possibly be zero, in which case the corresponding variables have no predictive value. But even then the estimates of their coefficients will in general differ from zero because of sampling variations. Therefore it is common practice to exclude from the regression any variable whose estimated coefficient should not be significantly different from zero.

On the other hand, the most reliable regression is the one that yields the smallest value of s (the smallest σ in fact, but this is unknown). However, if several regression equations provide approximately the same value of s, the one

that should be retained is, for purpose of convenience, the one that uses the smallest number of tests.

The set of tests to be included in the regression was determined in a stepwise manner following a method described by Efroymsen [14].

The variables are introduced in the model one at a time to give successive regression equations of the type:

$$\bar{y} = b_0 + b_1 x_1$$
$$\bar{y} = b'_0 + b'_1 x_1 + b'_2 x_2$$
$$\bar{y} = b''_0 + b''_1 x_1 + b''_2 x_2 + b''_3 x_3$$
$$\cdot$$
$$\cdot$$
$$\cdot$$

The variable in the first equation is the one that yields the smallest value of s. At each of the following steps the next variable included is the one that provides the largest reduction of s, provided that its coefficient is significant. Furthermore, a variable whose coefficient should become not significant after other variables have been added, is removed from the equation before adding new ones.

The procedure is terminated when no more variables can be added. The final equation comprises of course only variables whose coefficients are significant.

II. Discriminant analysis. A discriminant was used to allot any individual to one of the two anatomical groups defined above, in function of the results of the test values. The underlying assumptions of this model are multinormality of the 'x's and equality of their dispersion matrices in the two groups.

The discriminant is a function of the form:

$$Z = k_1 x_1 + k_2 x_2 \ldots + k_p x_p$$

where 'x's represent the actual test values and the 'k's are coefficients calculated in such a way as to provide maximum separation between the two groups. If Z is less than a constant c, the patient is assigned to group I; if Z exceeds this constant, to group II. Two kinds of error are possible in applying such a rule: a patient of group I may be assigned to group II, and inversely a patient of group II to group I. c was fixed so as to equalize the risks of misclassification in both directions.

The method followed to determine the subset of tests which appear in the final equation is essentially the same as in linear regression. The criterion to select a new variable is based on the Mahalanobis generalized distance D^2 which is a measure of the difference between the group means [15]. A new variable was accepted only when it yielded a significant increase of D^2. A full discussion of the theory of discrimination is available in [16].

RESULTS

The mean results of single liver tests and the incidence of abnormal results are given in Table 3. Some usual tests are not represented in this table either because of the low frequency of abnormal results in both groups (e.g. cephalin flocculation: group I: 1/22, group II: 0/18; thymol turbidity: group I: 1/22, group II: 4/18; abnormalities in total serum proteins and/or electrophoresis of serum proteins: group I: 2/22, group II: 2/18) or because current treatment interfered with proper evaluation of the test (e.g. anticoagulant therapy and PTT). It should also be noted that nine patients with autopsy-proven bone and liver metastases had normal values for Alk P'ase. All these patients belonged to the first group and showed no preferential primary tumor site.

Statistical analysis

The results for some combinations of variables are listed in Tables 4 and 5.

The adjusted line of regression revealed a marked lack of linearity. Indeed, the degree of liver involvement was overestimated in most cases with small anatomical involvement, and underestimated in the cases with massive involvement. Therefore, several transformations of variables were carried out in order to improve linearity (Table 4). It was found that logarithmic transformation of the 'x's improved only slightly the linearity and that logarithmic transformation of the liver involvement had no effect on the linearity, although improved the multiple correlation coefficient*.

When discriminant function was studied the variables were also replaced by their logarithms in order to improve normality. The best results are given in Table 5.

The probabilities of misclassification as given in Table 5 were calculated using a formula developed by Lachenbruch [17], and take into account the fact that the coefficients in the discriminant function have been estimated from samples drawn from the two groups. The practical probability of misstatement is probably different from the calculated one because the underlying assumptions of discrimination theory are possibly not

*The multiple correlation coefficient is an ordinary correlation coefficient between the predicted and the actual liver involvement. The nearer it is to 1, the more reliable the adjustment.

Table 3. Mean values of liver tests for both groups and incidence of abnormal results

Variable	Actual values						Logarithmic transformation			
	Group I (22 cases)			Group II (18 cases)			Group I		Group II	
	Mean	s	No. cases abnormal results	Mean	s*	No. cases abnormal results	Mean	s*	Mean	s*
LDH	667	461	8	1377	1973	10	2·76	0·23	2·94	0·35
LDH4	5·05	4·27	3	8·64	6·72	7	cannot be performed because			
LDH5	6·50	8·42	9	13·16	15·11	13	zero values present			
BSP	8·14	5·32	8	9·72	6·76	12	0·84	0·24	0·93	0·23
Bil.	0·73	1·41	1	4·16	4·60	11	0·34	0·31	0·30	0·59
GOT	59·7	47·9	11	218	271	12	1·70	0·25	2·07	0·50
GPT	23·8	23·6	5	35·4	35·0	5	1·24	0·32	1·41	0·33
ICD	233	183	5	423	384	10	2·25	0·32	2·50	0·34
Ald.	13·3	10·8	9	23·6	17·3	13	1·04	0·25	1·19	0·48
Alk. P'ase	5·32	2·78	9	14·5	9·69	18	0·68	0·21	1·08	0·27

*Standard deviation

Table 4. Best results of regression analysis

Independent variable	Combination of test values after logarithm transformation	Multiple correlation coefficient	Estimation of standard error
Liver involvement	Alk. P'ase (0·0005), Bil. (0·01)	0·71	0·23
	Alk. P'ase (0·0005), GOT (0·005)	0·72	0·23
	Alk. P'ase (0·0005), ICD (0·025)	0·71	0·23
Logarithm of liver involvement	Alk. P'ase (0·0005), Bil. (0·001)	0·73	0·47
	Alk. P'ase (0·0005), Bil. (0·025) ICD (0·025), BSP (0·025)	0·80	0·42

Number in parentheses refers to significance of coefficient b for the corresponding variable.

Table 5. Best results of discriminant analysis

Combinations of test values after logarithmic transformation	Mahalanobis distance D^2	Probability of misclassification (Lachenbruch's estimation)	Number of misclassified cases from: Group I	Group II
Alk. P'ase (0·0005), Bil. (0·0005), Ald. (nearly 0·05), BSP (nearly 0·10)	6·25	0·16	0	2
Alk. P'ase (0·0005), Bil. (0·005)	4·62	0·17	2	3
Alk. P'ase (0·0005), Bil. (0·001), Ald. (nearly 0·05)	5·39	0·17	4	1
Alk. P'ase (0·0005), GOT (0·05)	3·64	0·20	3	5
Alk. P'ase (0·0005), ICD (0·10)	3·48	0·21	3	4

Number in parentheses refer to significance of the additional difference between groups provided by the inclusion of the corresponding variable.
Mahalanobis D^2 is highly significant for all combinations of variables.

strictly satisfied. Moreover, as pointed out by Armitage [18], selection of the most powerful combination amongst many variables could have increased the risk of misclassification, and this may only be assessed by applying the discriminant rule to further data. Nevertheless, the retained variables happen to be classically known as good indicators of metastatic liver involvement.

The best combination of variables, the one for which D^2 is maximum, includes Alk. P'ase, Bil., Ald. and BSP. When considered separately neither Ald. nor BSP increases significantly the distance between the groups; their simultaneous inclusion does, however*.

At the present stage of the study two discriminant functions may possibly be retained. The first includes only Alk. P'ase and Bil. The other involves Alk. P'ase, Bil., Ald. and BSP.

Further data have permitted us to judge whether the addition of the two supplementary variables Ald. and BSP is really useful. Indeed, when it became evident that, at the most, four classical liver function tests were necessary to calculate the discriminant function, an attempt was made to classify 21 additional patients, 10 of group I and 11 of Group II, discarded for the original calculations because of lack of LDH isoenzyme determinations; these patients fulfilled all other criteria listed under methods.

Both discriminant functions classified only two patients wrongly, one of each anatomical group.

Thereafter the two discriminant functions were recalculated using all 61 patients. As can be seen in Table 6, the contribution of BSP and Ald. is now totally insignificant. Thus, at present, the retained discriminant function which permits allotment of our patients into the two anatomical groups is a linear combination of two variables:

$$Z = 2 \log (\text{Alk. P'ase}) + \log \text{BSP}$$

and the rule of classification is:

If $Z < 1 \cdot 8$ assign the patient to group I; otherwise, assign him to group II.

*The level of significance is 5%.

Table 6. Final results of discriminant analysis

Combination of test values after logarithmic transformation	Mahalanobis distance D^2	Probability of misclassification (Lachenbruch's estimation)	Number of misclassified cases	
			Group I	Group II
Alk. P'ase (0·0005), Bil. (0·005)				
Ald. (>0·25), BSP (>0·5)	4·68	0·16	1	4
Alk. P'ase (0·0005), Bil. (0·001)	4·49	0·16	2	5

Numbers in parentheses refer to significance of the additional difference between groups provided by the inclusion of the corresponding variable.
Mahalanobis D^2 is highly significant for all combinations of variables.

or equivalently compute the quantity

$$u = (\text{Alk. P'ase})^2 \times \text{BSP}$$

and assign the patient to group I if $u < 62$; otherwise, assign him to group II.

Using this rule, only two patients of group I were erroneously attributed to group II and five patients of group II were wrongly attributed to group I. The other fifty-four patients were correctly allotted.

In Fig. 1, each point represents one patient, his Alk. P'ase being read on the horizontal scale

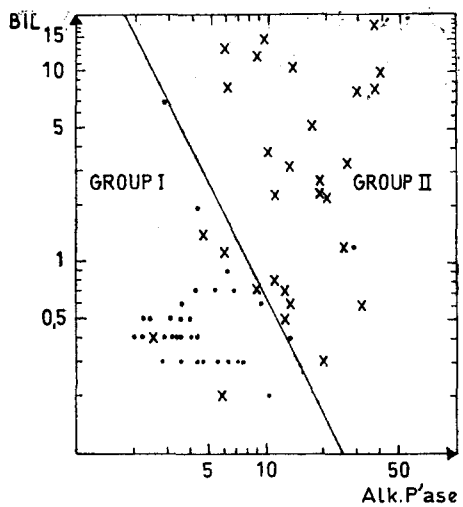


Fig. 1. Graphical representation of the discriminant function. The black dots ● represent the patients of Group I. *The crosses* X *designate the patients of Group* II. *The line represents the discriminant function. A patient is alloted to Group* I *or to Group* II *according to the position of his representative point below or above the line.*

and his BSP on the vertical scale. The two scales are logarithmic. The discriminant function is represented by a straight line dividing the plane in two regions. The patients whose representative points fall below the line are allotted to group I, the others to group II.

Clearly, the assumptions of discriminant analysis are not perfectly satisfied. The dispersion of the points is smaller in group I than in group II. The classifying rule appears, nevertheless, to be a reasonably good one.

In order to gain more insight into the actual differentiating power of the discriminant, each of the 61 cases was one by one excluded for the calculation of the discriminant function, (thus, this function was calculated each time with the 60 remaining patients), and each dropped case was thereafter reassessed by calculating his proper $\mathcal{Z}$ value. This situation artificially reproduces the real situation in the future, when a new individual will have to be classified in function of past experience. By this procedure only three patients of group I and five of group II were attributed to the wrong anatomical group. The exact interpretation of these error rates may be found in ref. [19].

DISCUSSION

Since the introduction of more aggressive surgical and chemical therapy for generalized cancer disease, a precise estimation of the extent of the disease becomes necessary. Indeed, the toxic side effects of several carcinostatic agents such as hormones and methotrexate are enhanced by the scarcity of metabolically active liver tissue [20]. On the other hand, only metabolic derivatives of cyclophosphamide are cytotoxic, and require functional liver parenchyme to become activated *in vivo* [21].

In addition, it has been clearly shown that adrenalectomy fails to induce remissions in breast cancer and even may be dangerous if metastatic liver disease exceeds 30% [22]. To our knowledge no test is available at present to evaluate this percentage. Regression analysis possibly could have been useful to determine this percentage, but the persistent lack of linearity reveals that the linear regression model is not likely to be appropriate for fitting the present data. Perhaps further research would

be worthwhile, trying, for example, to adjust the data with a non-linear model. Such an attempt has not been made in the present study.

Thus this model was discarded in favor of discriminant analysis. This method has the disadvantage over regression that it gives less information about the extent of liver involvement since it enables one only to allot patients to one of the two anatomical groups.

The proper redistribution of 53 out of 61 patients indicates, however, that this method is a valuable tool for the prediction of the degree of metastatic liver involvement. The variables that appear in the final discriminant function are based on the results of two well-known classical liver function tests easily performed in almost every laboratory. It should be pointed out, however, that this study is based on data from patients deceased less than two months after the predictive variables were obtained, and it cannot be ascertained that the results can be extrapolated to patients whose life expectancy is much longer. A prospective study on this subject is currently in progress.

SUMMARY

An attempt was made to predict the degree of metastatic liver involvement with the values of liver function tests obtained less than two months before death in 61 *patients.*

Two mathematical models were tentatively used. The linear regression appeared to be unsuitable because lack of linearity was found after having adjusted the model to data. A discriminant function was calculated allowing proper redistribution into two anatomical groups (<30% *involvement and* > 50% *involvement*) *of* 53 *out of* 61 *patients. Implications for treatment and prognosis are briefly discussed.*

REFERENCES

1. F. Wroblewski and J. S. Ladue, Lactic dehydrogenase activity in blood. *Proc. Soc. exp. Biol. (N.Y.)* **90,** 10 (1955).
2. N. Ressler and R. Joseph, Simple method for electrophoretic analysis of serum lactic dehydrogenase. *J. Lab. clin. Med.* **60,** 349 (1962).
3. R. J. Wieme, *Agar Gel Electrophoresis.* Elseviers, Amsterdam (1965).
4. J. G. Mateer, J. I. Baltz, D. F. Marion and J. M. Macmillan, Liver function tests and appraisal of comparative sensitivity and reliability of newer tests, with particular emphasis on cephalincholesterol flocculation test, intravenous hippuric acid test and improved bromsulphalein test with new normal standard. *J. Am. med. Ass.* **121,** 723 (1943).
5. P. R. N. Kind and E. J. King, Serum alkaline phosphatase: autoanalyzer modification. *J. clin. Path.* **7,** 322 (1954).
6. H. T. Malloy and K. A. Evelyn, Determination of bilirubin with photoelectric colorimeter. *J. biol. Chem.* **119,** 481 (1937).
7. F. Wroblewski and J. S. Ladue, Serum glutamic pyruvic transaminase (SGP-T) in hepatic disease; preliminary report. *Ann. Intern. Med.* **45,** 801 (1956).
8. F. Wroblewski, G. Jervis and J. S. Ladue, Diagnostic, prognostic and epidemiologic significance of serum glutamic oxaloacetic transaminase (SGO-T) alterations in acute hepatitis. *Ann. Intern. Med.* **45,** 782 (1956).
9. S. K. Wolfson and H. G. Williams-Ashman, Isocitric and 6-phosphogluconic dehydrogenase in human blood serum. *Proc. Soc. exp. Biol. Med.* **96,** 231 (1957).
10. G. Biesenherz, H. J. Boltze, T. Bücher, R. Czok, K. H. Garbade, E. Meyer-Arendt and G. Pfleiderer, Die Phosphofructose Aldolase, Phosphoglyceral Dehydrogenase Milchsaure, Dehydrogenase Glycerophosphate, Dehydrogenase und Pyruvate-Kinase aus Kaninchen Muskulatur in einem Arbeitsgang. *Z. Naturforschung,* **8B,** 555 (1953).
11. N. F. Maclagan, Thymol turbidity test; new indicator of liver disfunction. *Brit. J. exp. Path.* **25,** 234 (1944).
12. F. M. Hanger, Serological differentiation of obstructive from hematogenous jaundice by flocculation of cephalin-cholesterol emulsions. *J. clin. Invest.* **18,** 261 (1939).
13. A. J. Quick, Determination of prothrombin. *Amer. J. med. Sci.* **190,** 501 (1935).

14. M. A. EFROYMSEN, Multiple regression analysis. In *Mathematical Methods for Digital Computers*, (Edited by A. RALSTON and H. S. WILF), Part V, p. 191, John Wiley, New York (1960).
15. C. R. RAO, *Advanced Statistical Methods in Biometric Research*, p. 252. John Wiley, New York (1952).
16. T. W. ANDERSON, *An Introduction to Multivariate Statistical Analysis*, p. 126, John Wiley, New York (1958).
17. P. A. LACHENBRUCH, On expected probabilities of misclassification in discriminant analysis, necessary sample size and a relation with the multiple correlation coefficient. *Biometrics* **24,** 823 (1968).
18. P. ARMITAGE and C. K. MCPHERSON, Statistical studies of prognosis in advanced breast cancer. *J. chron. Dis.* **22,** 343 (1969).
19. M. HILLS, Allocation Rules and their Error Rates. *J. R. Statist. Soc. Series B*, **28,** 1 (1966).
20. Y. KENIS, Personal communication.
21. N. BROCK and H. J. HOHORST, Metabolism of cyclophosphamide. *Cancer* **20,** 900 (1967).
22. T. NEMOTO and T. L. DAO, Significance of liver metastasis in women with disseminated breast cancer undergoing endocrine ablative surgery. *Cancer* **19,** 421 (1966).

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Announcement

REPORT ON THE MEETING IN BOLOGNA OF THE WORKING-GROUP ON HUMAN CARCINOGENESIS UNDER THE EUROPEAN CO-ORDINATING COMMITTEE FOR HUMAN TUMOUR INVESTIGATIONS

THE WORKING group on human carcinogenesis of the Co-ordinating Committee for Human Tumour Investigations met at the Istituto di Oncologia "F. Addari" in Bologna on 11th and 12th September, 1970. The following members were present: Dr. H. Barlebo, København, Dr. J. Clemmesen København (Moderator), Professor L. A. Elson, London, Dr. Franco, Bologna, Professor C. Maltoni, Bologna, Professor G. Moberger, Stockholm, Dr. K. E. Sjølin, København, Dr. F. de Waard, Utrecht.

The advantages to epidemiology, as well as to diagnosis, treatment, and prognosis, offered by cytological grading of tumours of the urinary tract appeared clearly from demonstrations by Professor Moberger, Dr. Barlebo and Professor Maltoni of specimens from voided urine. The merits of various criteria were discussed on the basis of statistics.

The need for laboratory-methods in delineating risk factors in cancer epidemiology was further emphasized by Dr. de Waard's review of results from hormonal screenings of normal populations, and groups with regard to the risk of mammary and endometrial cancer.

As a recent observation, which may become useful, Dr. Sjølin presented results from analysis for beta-amino-iso-butyric acid of urine from patients with tumours of the urinary tract. Dr. Sjølin suggested that this compound was derived from the decomposition of thymine and that its occurrence in the urine may indicate an increased thymine production in cancer patients.

Professor Elson reported on work in collaboration with Professor Passey and Mr. Betts on the higher smoking risks of flue cured cigarette tobacco in relation to cigar or pipe smoking and on results from experimental examination of the effect of smoking of tobacco of various types on the metabolism and growth of animals.

It appeared from Professor Elson's, as well as from Dr. de Waard's results, that cancer registries should not restrict their statistical publications to incidence rates, but should supplement such information with local data on pertinent factors such as fertility, average height and weight, tobacco consumption, industrial exposure etc., as far as possible.

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Fundamental and Practical Aspects of the Application of Fast Neutrons in Clinical Radiotherapy

Meeting held at the Radiobiological Institute TNO, Rijswijk Z.H., The Netherlands June 22–24, 1970

Introduction to the Proceedings

G. W. BARENDSEN, J. J. BROERSE, L. M. VAN PUTTEN
(Guest Editors)

DURING the past years, interest in the application of fast neutrons in clinical radiotherapy has increased considerably as a result of the accumulation of new radiobiological knowledge providing insights in the importance of various factors which can cause differences in responses of normal tissues and tumours to fractionated irradiations. In addition, technical advances have provided possibilities to produce more intense beams of fast neutrons using various types of accelerators and nuclear reactions while the neutron-emitting nuclide ^{252}Cf has become available for intracavitary treatments.

Because of these developments it is expected that in the next few years a number of radiotherapy centres will plan and start clinical applications of fast neutrons. However, rapid progress in the evaluation of possible advantages of fast neutrons will be hampered not only by the as yet incomplete radiobiological knowledge, but also by the fact that in individual centres only a limited number of cases suitable for treatment with neutrons will become available each year. Consequently a long-time period would be required to obtain statistically significant differences. Close co-operation between the centres involved could alleviate these difficulties.

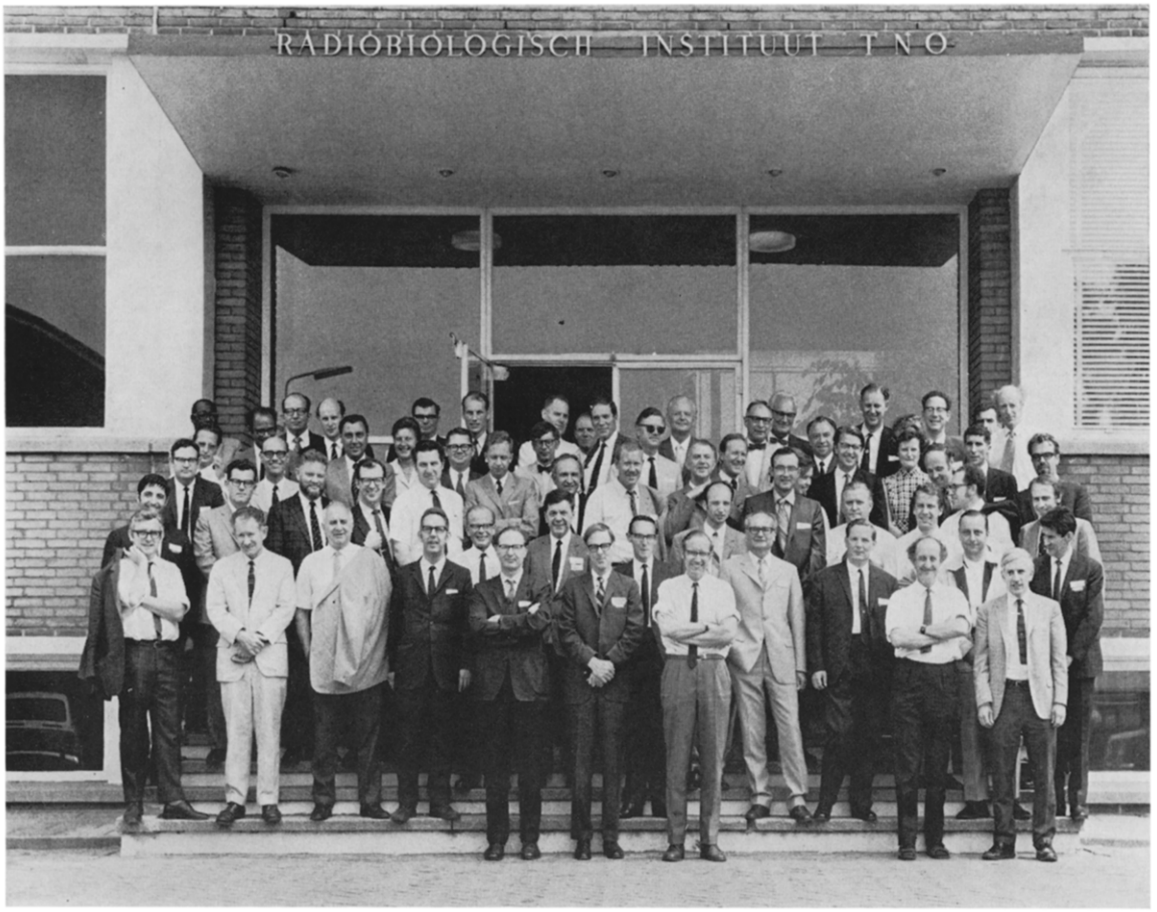
The meeting was organized to review available knowledge with respect to production and properties of fast neutron beams, their relative biological effectiveness and the limited clinical experience with fast neutrons from the cyclotron at Hammersmith Hospital, London. In addition the aim was to discuss the types of tumours to be selected for initial trials and the methods of evaluating the results in order to facilitate co-ordination of efforts in all radiotherapy centres concerned. All speakers for the first two days of formal presentations were invited. In addition, in preparation for a general discussion during the third day, a group of experts consisting of physicists, radiobiologists and radiotherapists, was asked to review a preliminary set of statements and recommendations on various aspects. The discussions after the formal papers have not been printed as recorded, but have been summarized by discussion secretaries for each session. The discussions of the third day have

been summarized by the chairmen of the three sessions on technical, radiobiological and radiotherapeutical aspects. Concluding remarks by the organizers of the meeting complete the proceedings of this meeting. Thanks are due to all who helped in the preparation of the reports and summaries.

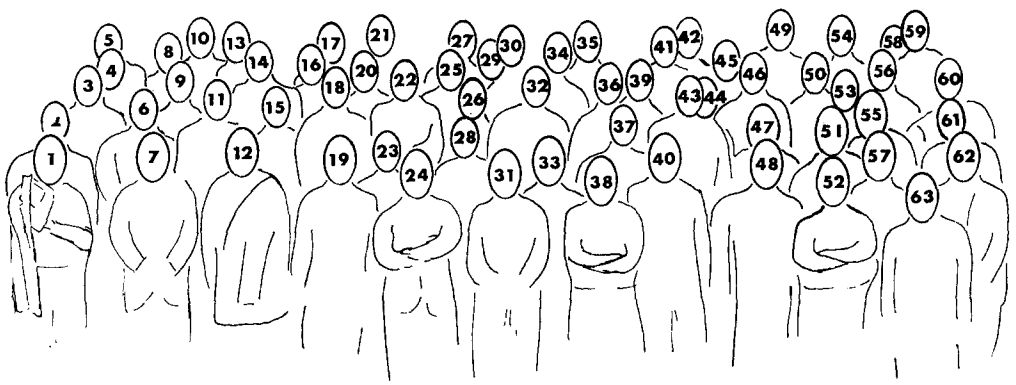
The generous support of the Organization for Health Research TNO and of the Ministry of Education and Sciences of the Netherlands made it possible to solve financial problems.

Finally our thanks are due to Prof. H. J. Tagnon, editor of the *European Journal of Cancer* for making the pages of this journal available for publication of these proceedings.

We hope that the publication of these papers, which present reviews of available knowledge as well as new experimental data and interpretations of radiotherapeutical results, will serve as a useful source of information and reference for all those interested in the application of fast neutrons in radiotherapy and that it will contribute to the development of a rational and judicious approach to the clinical use of this type of radiation.



(a)



(b)

1. *J. v. d. Geijn* 2. *P. R. Almond* 3. *G. Griggs* 4. *J. Booz* 5. *A. C. Ford* 6. *B. Djordjevic* 7. *M. M. Elkind* 8. *S. K. Lahiri* 9. *L. M. van Putten* 10. *M. R. Cleland* 11. *P. Wootton* 12. *H. H. Rossi* 13. *W. Duncan* 14. *A. Rodriguez-Antunez* 15. *R. G. Parker* 16. *J. C. Klein* 17. *J. T. Brennan* 18. *J. Nelson* 19. *J. J. Broerse* 20. *T. Phillips* 21. *H. B. Kal* 22. *A. van Dijk* 23. *M. Ebert* 24. *K. E. Halnan* 25. *J. A. Belli* 26. *J. Bataini* 27. *H. S. Reinhold* 28. *R. Morrison* 29. *C. F. Hollander* 30. *M. M. Kligerman* 31. *D. Herring* 32. *B. Offermann* 33. *R. C. Lawson* 34. *P. Thomas* 35. *V. P. Bond* 36. *H. Franke* 37. *J. Colditz* 38. *G. W. Barendsen* 39. *K. Breur* 40. *O. Reifenschweiler* 41. *H. S. Kaplan* 42. *H. O. Wyckoff* 43. *C. A. Kelsey* 44. *J. Denekamp* 45. *J. Dutreix* 46. *R. H. Thomlinson* 47. *R. H. Schittenhelm* 48. *D. A. Lundberg* 49. *D. W. Downton* 50. *M. Catterall* 51. *F. Stähler* 52. *J. F. Fowler* 53. *E. J. Hall* 54. *R. J. Berry* 55. *S. B. Field* 56. *E. Vernin* 57. *W. Porschen* 58. *A. C. Engels* 59. *D. K. Bewley* 60. *P. Lelieveld* 61. *H. R. Withers* 62. *D. Greene* 63. *S. B. Curtis.*

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Physical Characteristics of Fast Neutron Beams

D. K. BEWLEY

M.R.C. Cyclotron Unit, Hammersmith Hospital, Ducane Road, London W12, England

Abstract — *The physical properties of fast neutrons which are important to understand the biological effects are discussed and a comparison is made with the analogous properties of photons. Changes in radiation quality with neutron energy and with the establishment of secondary charged-particle equilibrium are more important than in the case of photons.*

Penetration of fast neutrons in tissue depends on the neutron energy. To match the penetration of ^{60}Co γ-rays it is necessary to use 14 MeV neutrons at 1 m or more, or to use neutrons generated by at least 30 MeV deuterons on beryllium. Scattering of neutrons by tissue is of smaller importance than scattering of orthovoltage X-rays. Gamma-radiation is produced by interactions between neutrons and tissue and complicates the dosimetry. A biological dosimeter is a useful way of checking that depth-dose curves give a correct representation of biological effect.

Fast neutrons give some degree of skin-sparing due to lack of secondary charged particle equilibrium on the surface. To match the degree of skin-sparing given by ^{60}Co γ-rays it is necessary to use at least 50 MeV deuterons on beryllium. Energy absorption depends on hydrogen concentration and is a minimum in bone and a maximum in fat. The variation of biologically effective dose between different tissues and across the build-up zone is not necessarily proportional to the observed ionization.

COMPARISON WITH X- AND GAMMA-RAYS

WHEN CONSIDERING the physical properties of fast neutrons in relation to their biological effects, it is convenient to compare fast neutrons with X- or γ-rays whose properties are more familiar to radiotherapists and radiobiologists.

In such a comparison the neutron has certain analogies with the photon. Both are uncharged and are therefore indirectly ionizing particles, unlike electrons, protons and α-particles. On the other hand the photon has a rest mass of zero while the mass of the neutron is almost the same as the mass of the proton.

In the context of biological effect the vital difference lies in their modes of interaction with matter. Absorption of photons gives rise to recoil electrons which are responsible for the biological effect. Fast neutrons, on the other hand, interact only with atomic nuclei, and energy is transferred to matter via heavy particles. In biological material these are mostly recoil protons, but nuclear reactions also give rise to alpha-particles and other products. Some energy is dissipated by recoil nuclei of C N and O. Gamma-radiation is also produced which complicates the dosimetry but usually has comparatively little biological effect.

RADIATION QUALITY

The heavy secondary charged particles are more densely ionizing than electrons, and give a higher value of Linear Energy Transfer (LET) in tissue. The difference in biological effects of neutrons are usually put down to this increased ionization density relative to X-rays. LET spectra of four beams of fast neutrons

have been calculated by Bewley [1]. They cover a wide range of LET, extending from 30 to 8000 MeV $cm^2 g^{-1}$ (3–800 keV/μ in unit density tissue). The LET spectrum of electrons, on the other hand, lies mostly below 10 MeV $cm^2 g^{-1}$ and does not exceed 300 MeV $cm^2 g^{-1}$ [2]. Table 1 gives a summary of the LET of the various secondary charged particles.

Table 1. *LET of Various Charged Particles*

Particle	Range of LET (MeV $cm^2 g^{-1}$)
Electrons (X, γ)	2– 300
Protons	50–1000
Products of nuclear reactions (mainly α-particles)	400–2500
Heavy recoils	10^3–10^4

Up to 300 MeV $cm^2 g^{-1}$ the response of mammalian cells shows little change with LET while changes became rapid at higher LET values [3, 4] (see Fig. 1). The response to X-rays and electrons therefore changes only slightly with the spectrum of the radiation. With neutrons on the other hand spectral changes can be important. Recoil protons deposit an important fraction of their energy below 300 MeV $cm^2 g^{-1}$ and so behave to some extent like low LET radiation (i.e. X-rays). Recoil protons are also responsible for the major part of the energy absorption by tissue from neutrons, about 90% at low neutron energies falling to 70% at 14 MeV [5]. Attainment of secondary charged-particle equilibrium with fast neutrons is therefore accompanied by a change in RBE. A full explanation of the effects of fast neutrons on mammalian cells in terms of the effects of the associated charged particles has not so far been possible [6].

PENETRATION IN TISSUE

From the point of view of radiation beam therapy the most important physical property of the beam is its penetration in tissue. Measurement of depth doses is complicated by a varying proportion of γ-radiation and of thermal and epithermal neutrons. However, both theoretical calculations [7] and measurements [8] have shown that the dose from these low energy neutrons is always less than 1% of the incident neutron dose. Gamma-radiation is therefore the main problem. One way of measuring neutron depth doses is to measure the total dose with a tissue-equivalent chamber and to subtract the dose due to γ-rays measured by another method such as thermoluminescent dosimetry (TLD). Bewley and Parnell [8] preferred to use a special ionization chamber in which the effect of γ-radiation is eliminated by internal compensation. Gamma-rays were measured separately with films or TLD (CaF_2), and the depth doses were then checked using a biological dosimeter [9].

The ionization chamber is shown in Fig. 2. It consists of three coaxial chambers with a common central electrode. All chambers are air-filled. The central part has walls made of graphited polythene and has a high sensitivity to fast neutrons plus a slightly smaller sensitivity (in terms of charge per rad) to γ-rays. The two outer parts have walls containing no hydrogen and have a sharply reduced sensitivity to neutrons while still responding to γ-rays. The polarizing voltages on the central and outer parts are of opposite polarity. The chamber can be "balanced" to give zero response to X- and γ-rays by adjusting the volume of the section at the end, and it then responds only to the fast neutron component of a mixed neutron and γ field.

Only a few measurements of neutron depth

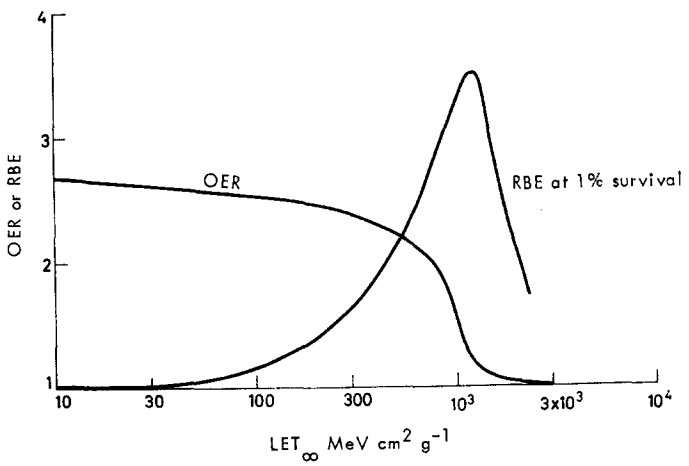


Fig. 1. *OER and RBE of human cells* in vitro *as a function of LET, from Barendsen* et al. [4].

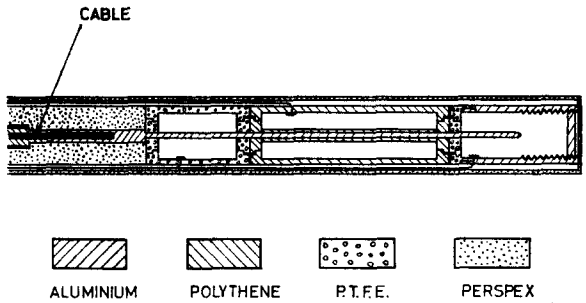


Fig. 2. Compensated ionization chamber for measuring fast neutron dose in the presence of γ-radiation.

doses for limited field areas have been published at the present time. The most relevant ones are shown in Fig. 3 together with curves for 250 kV X-rays and ^{60}Co γ-rays. Neutrons of mean energy 8 MeV and those of 14 MeV at 50 cm fsd give depth dose curves intermediate between 250 kV X-rays and ^{60}Co γ-rays. Lower energy neutrons are less penetrating and would not be suitable for deep therapy. To obtain penetration as good as that from ^{60}Co it is necessary to use 14 MeV neutrons at 100 cm fsd or more, or deuterons of at least 30 MeV on a beryllium target. It is also possible that lower energy deuterons on a deuterium target may give a high mean neutron energy and good penetration if used at 100 cm fsd or more [12].

Fast neutrons are scattered less in tissue than 250 kV X-rays. The effect of field size on percentage depth dose is therefore not very great. Figure 4 shows central axis depth-dose curves for several field sizes for neutrons of mean energy 8 MeV from the MRC cyclotron. They were measured with the compensated chamber and refer to neutron dose only. Scattering outside the beam is also less important [8]. Back-scatter on the incident surface is also rather small, varying from 4% to 10% over a range of field sizes from 20 to 400 cm^2, for neutrons of mean energy 8 MeV [8].

GAMMA RADIATION AND TOTAL BIOLOGICAL EFFECT

The variation with depth of dose due to γ-radiation has been measured for the neutrons of mean energy 8 MeV [8, 9]. The gamma-dose as a percentage of the local neutron dose is shown in Fig. 5. The percentage rises steadily

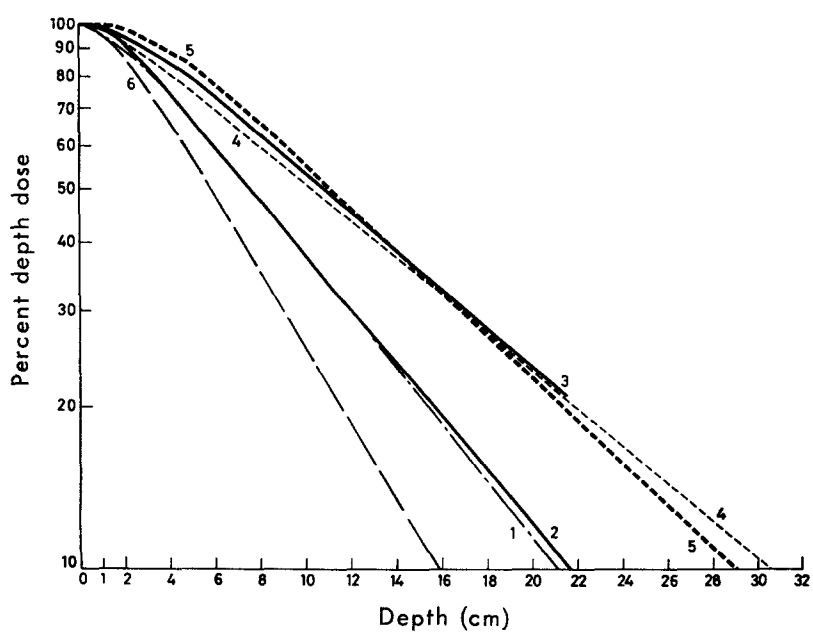


Fig. 3. Central axis neutron depth-dose curves for a 5 × 5 cm field.

(1) $\bar{E}_n$=8 *MeV*, 120 *cm fsd* [8]
(2) E_n=14 *MeV*, 50 *cm fsd* [10]
(3) E_n=14 *MeV*, 100 *cm fsd* (10 × 10 *cm field*) [10]
(4) ^{60}*Co γ-rays*, 80 *cm fsd*
(5) *Fast neutrons from* 30 *MeV deuterons on thin Be*, 120 *cm fsd* [11]
(6) 250 *kV X-rays*, 50 *cm fsd.*

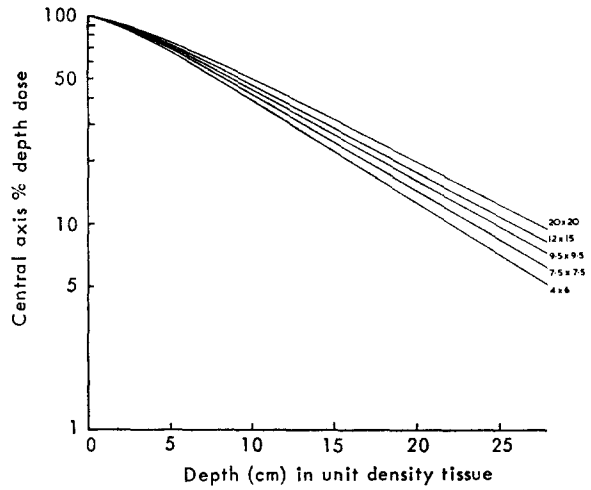


Fig. 4. Variation of central axis percentage depth dose in unit density tissue with field size, at 125 cm fsd, for neutrons of $\bar{E}$=8 MeV.

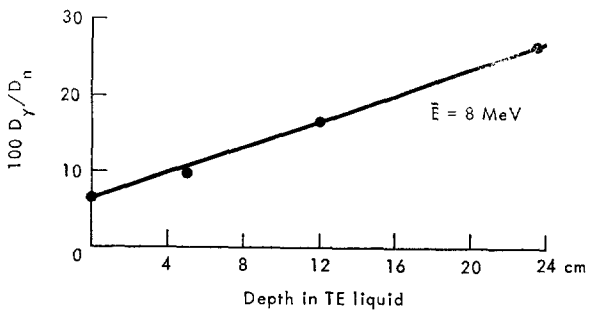


Fig. 5. Dose due to γ-rays as a percentage of local neutron dose, for a 14×14 cm field at 75 cm fsd, $\bar{E}$=8 MeV.

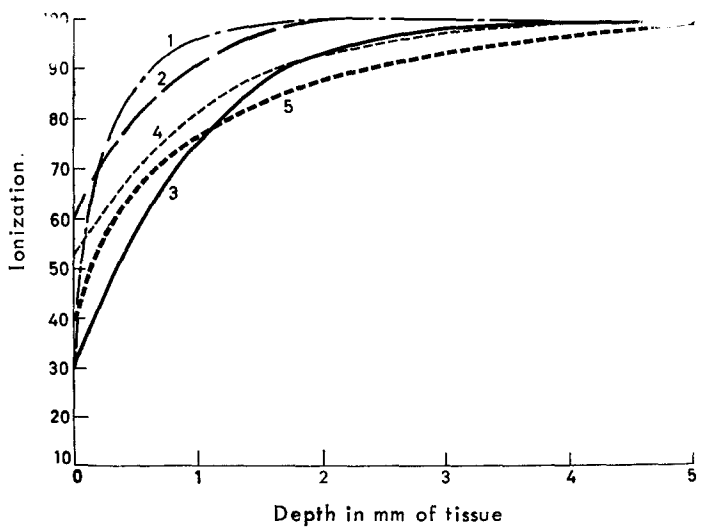


Fig. 6. Dose build-up from neutrons.

(1) $\bar{E}_n$= 8 *MeV* [8]
(2) E_n=14 *MeV* [10]
(3) *Neutrons from* 50 *MeV d on thin Be* [14]
(4) ^{60}*Co γ-rays*
(5) *Neutrons from* 30 *MeV d on thin Be* [11]

with depth. The RBE of γ-radiation relative to neutrons is about one-third at the dose levels used in clinical practice; thus 15% of γ-dose at 10 cm depth represents about 5% of the biologically effective dose. Exit doses with neutrons of this energy are small and a correction for γ-radiation is not important, but γ-radiation may be a more important factor in skin dosage with more penetrating beams especially for large field sizes.

McNally and Bewley [9] used mammalian cells as a biological dosimeter to check whether depth doses measured with the compensated chamber, plus an allowance for γ-radiation, correctly represented the biologically effective dose. The results confirmed that this was so, showing that changes in neutron spectrum did not affect the relevance of the physical measurements. This result agrees with the work of Field and Parnell [13] who were unable to detect any significant change in neutron spectrum with depth. With mono-energetic neutron sources however, such as those producing 14 MeV neutrons, some reduction in mean energy must be expected at a depth, and it will be important to find out if this affects physical measurements or causes a change in RBE.

SKIN-SPARING

A finite thickness of tissue is required to give secondary charged particle equilibrium. This effect is particularly marked at the skin where the neutron beam is incident on the patient, since recoil protons are emitted in the forward direction.

Figure 6 shows the measured build-up of ionization below the surface for various beams of neutrons and for ^{60}Co γ-rays.

At the same kinetic energy the range of protons in tissue is at least 30 times less than the range of electrons and the thickness of the build-up zone is correspondingly reduced.

A further difference between photons and neutrons is the fact, noted above, that in the build-up zone biological effect is not necessarily proportional to ionization. Charged particle equilibrium for heavy recoils and the products of nuclear reactions is attained in a very short distance, comparable to the dimensions of a single cell. Consequently the radiation near the incident surface has a higher effective LET than the radiation under full charged-particle equilibrium. Broerse *et al.* [15], using 15 MeV neutrons, found that near the surface the radiation had a higher RBE and a lower OER than under conditions of charged-particle equilibrium.

ENERGY ABSORPTION

Relative energy absorption, in units of rads/kerma (analogous to rads/roentgen), is nearly proportional to the hydrogen content of tissue. Thus bone experiences the lowest absorption of energy and fat the highest, the reverse of the situation with orthovoltage X-rays. In Table 1 the calculated values of rads/kerma for 14 MeV neutrons and neutrons of mean energy 8 MeV is given [12]. At bone-tissue interfaces a finite distance is required for attainment of charged particle equilibrium [12]. One should note that in bone the situation is similar to that in the build-up zone, namely the mean LET will be higher than in tissue. The variation in biologically effective dose between tissues will therefore be smaller than the variation in the calculated values of rads/kerma.

REFERENCES

1. D. K. Bewley, Calculated LET distributions of fast neutrons. *Radiat. Res.* **34,** 437 (1968).
2. A. Cole, Absorption of 20-eV to 50,000-eV electron beams in air and plastic. *Radiat. Res.* **38,** 7 (1969).
3. G. W. Barendsen, H. M. D. Walter, J. F. Fowler and D. K. Bewley, Effects of different ionizing radiations on human cells in tissue culture. *Radiat. Res.* **18,** 106 (1963).
4. G. W. Barendsen, C. J. Koot, G. R. van Kersen, D. K. Bewley, S. B. Field and C. J. Parnell, The effect of oxygen on impairment of the proliferative capacity of human cells in culture by ionizing radiations of different LET. *Int. J. Radiat. Biol.* **10,** 317 (1966).
5. R. L. Bach and R. S. Caswell, Energy transfer to matter by neutrons. *Radiat. Res.* **35,** 1 (1968).
6. D. K. Bewley, A comparison of the response of mammalian cells to fast neutrons and charged particle beams. *Radiat. Res.* **34,** 446 (1968).
7. J. A. Auxier, W. S. Snyder and T. D. Jones, Neutron interactions and penetration in tissue. In *Radiation Dosimetry* (Edited by F. H. Attix and W. C. Roesch), Vol. 1, p. 275. (1968). Academic Press, New York.

8. D. K. Bewley and C. J. Parnell, The fast neutron beam from the M.R.C. cyclotron. *Brit. J. Radiol.* **42,** 281 (1969).
9. N. J. McNally and D. K. Bewley, A biological dosimeter using mammalian cells in tissue culture and its use in obtaining neutron depth dose curves. *Brit. J. Radiol.* **42,** 289 (1969).
10. D. Greene and R. L. Thomas, An experimental unit for fast neutron radiotherapy. *Brit. J. Radiol.* **41,** 455 (1968).
11. L. J. Goodman, S. A. Marino and J. T. Brennan, Minimum cyclotron size for radiation therapy. Conference on cyclotrons in chemistry, metallurgy and biology, p. 209. Oxford (1969).
12. D. K. Bewley, Fast neutron beams for radiotherapy. In *Current Topics in Radiation Research* (Edited by M. Ebert and A. Howard), Vol. 5, p. 251. North-Holland, Amsterdam (1970).
13. S. B. Field and C. J. Parnell, The use of threshold detectors to determine changes in a fast neutron energy spectrum with depth in a phantom. *Brit. J. Radiol.* **38,** 618 (1969).
14. A. C. Lucas and W. M. Quam, Personal communication (1970).
15. J. J. Broerse, G. W. Barendsen and G. R. van Kersen, Survival of cultured human cells after irradiation with fast neutrons of different energies in hypoxic and oxygenated conditions. *Int. J. Radiat. Biol.* **13,** 559 (1968).

Europ. J. Cancer Vol. 7, pp. 105–114. Pergamon Press 1971. Printed in Great Britain

Production and Shielding of 15 MeV Neutrons

J. J. BROERSE, A. C. ENGELS and J. S. GROEN
Radiobiological Institute TNO, 151 Lange Kleiweg, Rijswijk Z.H., The Netherlands

Abstract — *The characteristics of various types of D–T neutron generators are described and the current status of the development of fast neutron therapy machines is reviewed. For clinical applications a minimum average yield of* 4×10^{12} *neutrons/sec is required. However, it is concluded that at present no* 15 *MeV neutron radiotherapy system can produce this output for a reasonable period of time, e.g.* 50 *hr. Further developments of all systems are urgently required.*

The properties of tritium targets are discussed with respect to the target life time and the radiation hazards. Experimental data are presented on the neutron yield as a function of the energy of the bombarding deuterons and target age. The tritium contents of the oil of the accelerator vacuum pumps and the tritium contents of the gas released at the exhaust opening of the rotary pump have been measured. It is concluded that only a small fraction of the total amount of tritium originally present in the target is expelled through the forepump exhaust. With regard to the shielding of 15 *MeV neutrons, a summary has been given of experimental attenuation data for various materials.*

INTRODUCTION

SINCE it has been demonstrated [1] that the neutron energy has little influence on the oxygen enhancement ratio in the range between 1 and 15 MeV, the final choice of the most suitable neutron beam for radiotherapy is determined by the depth dose characteristics of the neutrons and the intensity which can be produced by neutron generators. As far as penetration is concerned, 15 MeV neutrons produced by the reaction between deuterium and tritium have to be preferred. However, in the past commercially available generators could not provide the required neutron yield in excess of 2×10^{12} neutrons/sec. Only recently substantial progress has been made in the development of high yield D–T neutron generators. It can be expected that, within one or two years, low energy accelerators with neutron yields between 2×10^{12} and 5×10^{12} neutrons/sec will become available.

In the present contribution the production of 15 MeV neutrons will be discussed. Characteristics of various types of neutron generators will be described and the current status of the development of fast neutron therapy machines will be reviewed. Three major health physics problems are encountered in connection with the operation of 15 MeV neutron generators namely the activation of materials around the neutron generator, the tritium release from the target and the shielding of the neutrons. The first problem will not be discussed in this paper. It should be realized, however, that considerable activities can be induced especially in the parts of the machine close to the target and in the steel collimators to be used for clinical applications. It has been calculated [2] that dose rates of 60–260 mR/hr will be found due to activation of these components for a generator at 3×10^{12} neutrons/sec. In the present paper the characteristics of tritium targets will be discussed separately with respect to the target life time and the radiation hazards. Finally a review will be given of available shielding data of 15 MeV neutrons.

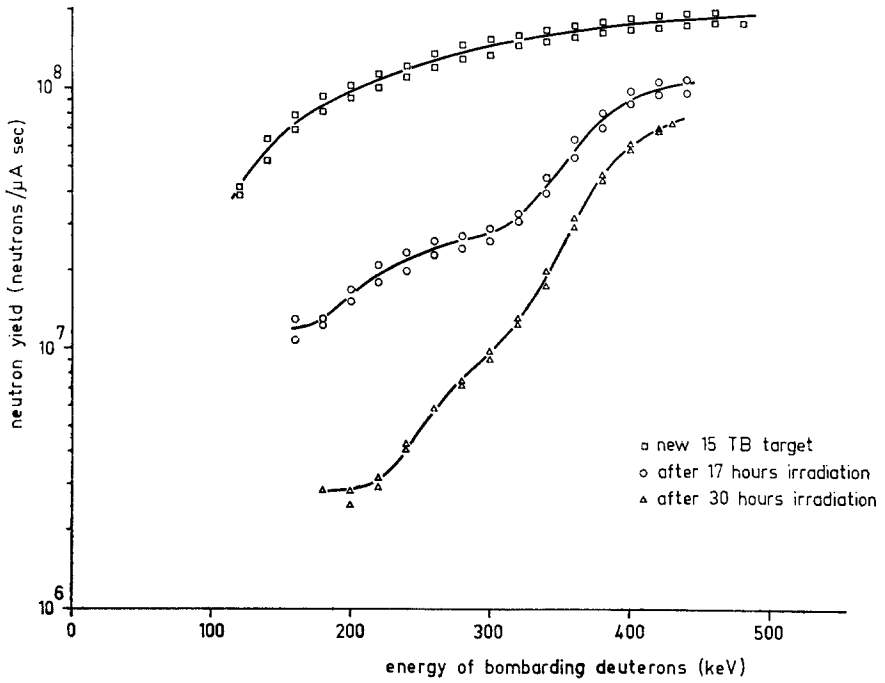


Fig. 1. Yield of 15 MeV neutrons from the D–T reaction as a function of the energy of bombarding deuterons for different target age.

Table 1. Yields of fast neutrons produced by the $^3H(d,n)^4He$ reaction per mA ion current

Accelerating voltage (kV)	Yield (10^{10} n/mA.sec)	Dose rate at 1 m from target (rads/min)
A. Pumped accelerators with RF—or duoplasmatron ion sources and thick ^{3}H–Ti targets		
100	3	0·09
150	7	0·21
200	10	0·31
250	12	0·39
300	15	0·47
350	16·5	0·53
400	18	0·57
450	18·5	0·59
500	19	0·61
B. Sealed tube generators		
115	0·6	0·02
125	1	0·03
150	2	0·06
175	2·5	0·08
250	3·5	0·12

CHARACTERISTICS OF THE D–T REACTION

With positive ions of a few hundred keV energy, which can be produced with relatively small and inexpensive machines, only the ^{3}H (d,n)^{4}He reaction (often referred to as D–T reaction) yields sufficiently large flux densities at the ion currents presently attainable. The neutron emission from the D–T reaction produced by low energy deuterons is approximately isotropic. The neutron energy is dependent on the angle between the directions of the incident deuteron beam and the emerging neutrons and on the energy of the deuterons. For a 400 keV deuteron beam a neutron energy of 15·6 MeV is obtained in the 0° direction, while in the 90° direction the energy is equal to 14·2 MeV [3]. The neutron yield is a function of the energy of the bombarding deuterons and the target age. In Fig. 1 the measured neutron yield is given for a tritium–titanium target with a diameter of 1 in. containing 12 Ci of

tritium, immediately after insertion, and after 17 and 30 hr of exposure to a 400 keV deuteron beam at a current of 250 μA. On the basis of the curve measured for a new target, the D–T neutron yield can be calculated in dependence on the accelerating voltage (see Table 1). Furthermore in Table 1 the soft tissue dose rate is given at a distance of 1 metre from the target. For the latter calculation a conversion factor of $6{\cdot}7\times10^{-7}$ ergs/g of tissue per neutron/cm^2 has been employed, according to the calculations of Bach and Caswell for a neutron energy of 15·5 MeV [4]. Previously we have employed a slightly larger conversion factor, based on the calculations of Randolph [5, 6]. Finally in Table 1 yield values are given for neutron generators with sealed tubes in which mixed beams of deuterium and tritium ions are accelerated [7]. The comparison of pumped accelerators and sealed tubes, given in Table 1, shows that for equal neutron yields the required ion currents in sealed tubes are a factor of about 3 larger as compared with pumped accelerators operated at equal voltage. It must be noted that not with all generators the optimal yields are obtained [10].

With regard to the sealed tubes it should be realized, however, that the neutron output can be maintained at a constant level, e.g. for the 10^{11} neutrons/sec tube, 500 hr operation with less than 10% change in neutron output are specified [7]. Although it is not known whether the proportionality between yield and current will hold for higher ion currents, the table might be used for a first estimation of the required accelerating voltage and ion current to obtain neutron yields in the range of 10^{12} neutrons/sec.

D–T NEUTRON GENERATORS

For therapeutic applications, minimum levels of dose rate and corresponding neutron yields have to be assessed. The requirements for source strength are determined by a number of parameters namely

1. *Sufficient distance between source and patient*

Adequate collimation has to be achieved and the influence of the inverse square law on the depth dose characteristics has to be sufficiently diminished. On the basis of absorption characteristics of various materials, it can be concluded that a source to surface distance (SSD) equal to 50 cm is minimally required, if the treatment is restricted to superficial lesions. A larger SSD is required for deep-seated tumours.

2. *Dose administered per fraction*

Although obviously dependent on the fractionation schedule to be employed and on the RBE of the neutrons for effects on normal human tissues, it can be estimated that for most treatments a dose per fraction of 200 rads should probably not be exceeded, because with an RBE of 2·5, this would be equivalent to 500 rads of X-rays.

3. *Maximum time permissible for the administration of an individual dose fraction*

On the basis of practical considerations concerning the time interval during which a patient can remain immobile and concerning the requirement of treating a minimum number of between 10 and 20 patients per day, a treatment time of 20 min is considered to be the maximum.

4. *Location of the tumour*

Attenuation of fast neutron radiation in tissues overlying a deep-seated tumour increases the requirements for the neutron yield of the accelerator.

By consideration of these four factors it is possible to derive estimates of source strength requirements for D–T neutron generators. At present the production of adequate flux densities at the site of the tumour presents problems, which are mainly concerned with target technology. As a consequence with some generators the limited source strength may impose limitations with respect to the location and size of tumours which can be adequately treated. For instance with a superficial lesion of less than 2 cm thickness it would be possible to employ an SSD of 50 cm. For a dose fraction of 200 rads to be administered in 20 min, i.e. in order to attain a dose rate of 10 rads/min, a source strength of 8×10^{11} neutrons/sec would be required.

For a large and deep-seated tumour the SSD should be larger, in order to diminish the influence of the inverse square law. Furthermore multiple fields must be used. Larger SSD values and absorption of the neutrons in overlying tissues increase the required source strength. For instance, for a tumour of 5 cm diameter at 10 cm depth treated at an SSD of 100 cm, a source strength of $6{\cdot}4\times10^{12}$ neutrons/sec is required to deliver a dose of 200 rads within 20 min.

Finally it is necessary to take into account the fact that for generators with evacuated acceleration tubes, the yields of targets containing ^{3}H adsorbed at titanium will presumably decrease by a factor of 2 within 10–50 hr. The yield for a new target should be a factor of 2 larger than is minimally required. Consequently, an adequate machine should deliver about 10^{13} neutrons/sec with a new target, in

order to provide an average yield of 6×10^{12} neutrons/sec over a period of about 20 hr.

In a program for development of a prototype neutron source to be used in radiotherapy [8], three alternate approaches have been considered, namely:

(*a*) an accelerated beam of pure deuterium ions in a pumped accelerator,
(*b*) a sealed tube with a mixed deuterium and tritium beam,
(*c*) a mixed beam of deuterium and tritium in a pumped accelerator.

(*a*) The main problem with the pumped D–T neutron source using an accelerated beam of deuterons is expected to be the short target life time at the neutron intensity required. As a consequence, complicated target design, cooling problems and potentially hazardous target replacement requirements will have to be faced. The problem of the fast decrease of the neutron output, has been solved partly by using rotating targets. Most satisfying results have been obtained with a target rotating at a high speed of 1100 rev/min, constructed by Booth [9]. With this target, half lives of about 400 mA hr have been measured. It can be concluded from Table 1 that for accelerating voltages between 400 kV and 500 kV and beam currents between 10 and 15 mA, maximum neutron yields of 3×10^{12} neutrons/sec can be obtained. Several machines with this neutron output are available at present although they are not used for radiotherapy [10].

(*b*) Various groups of investigators are actively engaged in the development of a sealed tube with a mixed deuterium and tritium beam [11–14]. However, at present only limited neutron yields have been produced by these sealed tubes. A maximum yield of 3×10^{11} neutrons/sec has been obtained for a sealed tube operating at 180 kV and 12 mA [15], whereas Reifenschweiler [12] stated an output of $3{\cdot}5 \times 10^{11}$ neutrons/sec for an experimental tube operating at 200 kV and 5 mA. It can be inferred that a neutron yield of 10^{12} neutrons/sec will be obtained with a 50% deuterium and 50% tritium beam operating at 250 kV and 30 mA ion current. At this output the life time of the target is limited by erosion of the titanium layer due to sputtering. Experience with the present targets has shown that the sealed tube might produce the required 10^{12} neutrons/sec, however, no data are available on target life time. In addition, it has to be realized that the 10^{12} neutrons/sec output is a factor of 4 below that considered adequate for neutron therapy [8]. It is unknown whether the neutron yield of this system can be increased significantly without changes in target life time and target diameter.

(*c*) The approach of a mixed beam in a pumped accelerator can in principle avoid the target life time problem, but not the target cooling problem of the pure deuterium beam system. A single stage mixed beam accelerator has been proposed to operate at a beam energy of about 175 keV and incorporates a modified

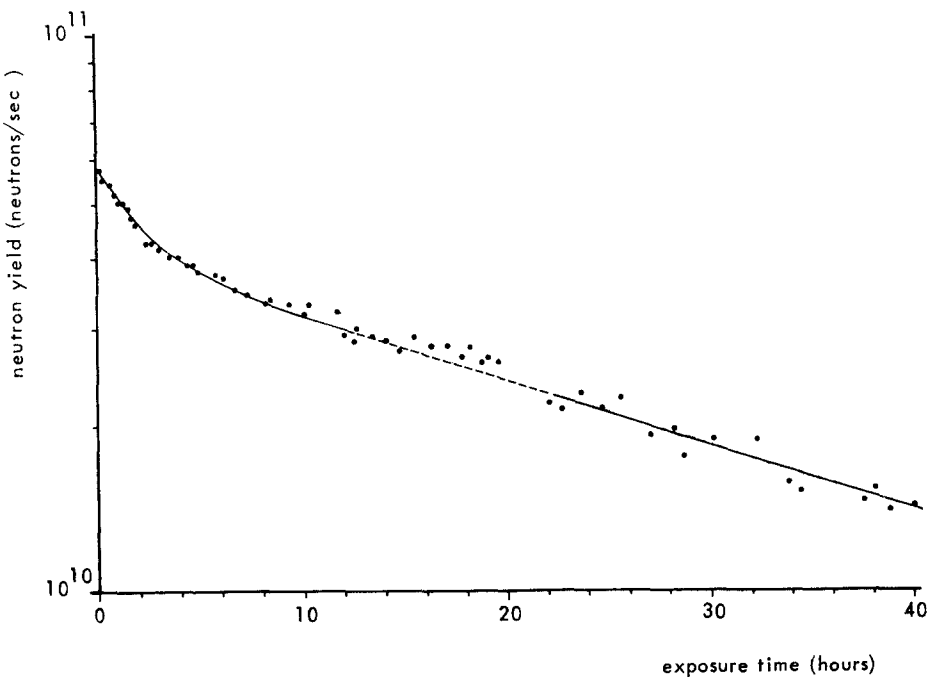


Fig. 2. Deterioration of a tritium target as a function of time during bombardment with a 400 keV deuteron beam at a current of 220 μA.

version of a pumped closed loop tritium handling system [8]. Unfortunately the research and development program of this machine has not been completed. At present only experiments are performed with a deuterium beam directed on a thick copper target. For the D–D reaction a neutron yield of 8×10^8 neutrons/sec was measured for a beam current of 18 mA and an accelerating voltage of 120 kV. A D–T neutron yield of $1{\cdot}2 \times 10^{12}$ neutrons/sec is expected for a 95 mA mixed deuterium and tritium beam accelerated to 175 keV. It has to be realized, however, that the construction of this fast neutron therapy system still involves major technological problems.

It can be concluded that at present no fast neutron radiotherapy system is available for which an output of 4×10^{12} neutrons/sec has been demonstrated for a reasonable period of e.g. 50 hr and further developments of all systems are urgently required.

PROPERTIES OF TRITIUM TARGETS

It is a well-known phenomenon that the neutron yield decreases continuously during the bombardment of a tritiated titanium target by an incident deuteron beam. An example of the target deterioration has been given in Fig. 2 for the bombardment of a tritium target with a deuteron beam current of about 220 μA at an accelerating voltage of 400 kV and a beam spot diameter of about 1 cm. After an initial fast decrease over a period of 8 hr, the neutron yield decreases exponentially with total charge. For this fixed target containing 12 Ci of tritium a half life of 6 mA.hr can be calculated. The curves of Fig. 1 indicate that under bombardment of a deuteron beam, tritium disappears from the region where the D–T reaction takes place. The curve for the new target shows that the tritium is uniformly distributed throughout the effective depth of the target. The yield curve after 17 hr of exposure indicates the depletion of tritium in the target and shows that more tritium has disappeared from the front layers of the target than from the deeper seated layers. A similar yield curve was measured after another 13 hr of operation. Further depletion of the tritium in the target is apparent from these data.

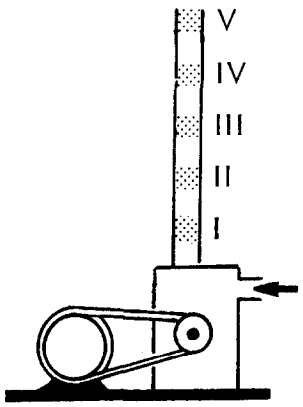
The mechanism of target deterioration has been investigated especially in relation to the history of the tritium which might be lost by the target [16, 17]. Nellis and co-workers [16] concluded that during bombardment of tritium targets each incident deuteron dislodges one tritium atom, and that most of the tritium is in a gaseous form when it leaves the target. This conclusion is incorrect since it can easily be calculated that maximum life times of the target of 2·5 mA.hr will be obtained under these conditions, contrary to the experimental findings obtained for our targets [18]. The studies of Nellis and co-workers [16] suggested further that a relatively small amount of the tritium released from the target actually remains within the accelerator itself. According to their conclusions most of the tritium lost by the bombarded target would be trapped in the elements of an ion pump or released through the vacuum system exhaust.

In order to verify the experimental data of Nellis and co-workers we have measured the tritium content of the oil of the accelerator vacuum pumps and the tritium content of the gas released at the exhaust opening of the rotary pump. Since it is well-known at present that tritium is rapidly absorbed in organic materials, a trap filled with polyethylene strips or with polystyrene granules has been mounted on the exit of the forepump. Tritium measurements of the oil were carried out by adding a small volume of the oil to a scintillation solution (one litre toluene, 4 g PPO and 50 mg POPOP). The polyethylene strips were suspended in the scintillation liquid and tightened in the caps of the counting vials. Although the appropriate quenching corrections are applied, the efficiency for these measurements is not known accurately, due to the poor geometry. The use of a tritium absorber which could be dissolved in the scintillation liquid would be preferred. The polystyrene granules were found to dissolve in the toluene-base solution. All samples

Table 2. *Tritium content of oil in accelerator vacuum pumps*

Experiment	Conditions	Activity in μCi of tritium/ml oil
1	Forepump	0·3
2	Forepump	1·3 1·2 1·2
3	Oil diffusion pump	7

Table 3. Tritium activity in polyethylene strips and polystyrene granules mounted on exit of vacuum pumps



Position	Average activity of five polyethylene strips (nCi)		Average activity of polystyrene granules (nCi/g)
V	0·8	4·2	119
IV	—	—	304
III	12·0	21·2	433
II	—	—	522
I	14·8	14·0	538
	The area of strips counted was 6 cm², the total polyethylene area was equal to 3600 cm²		Each compartment contained 40 g of polystyrene granules

were counted in a Mark 1 Liquid Scintillation Counter (Nuclear Chicago). The counting results of the oil samples are given in Table 2. The values are in agreement with our earlier measurements [18] and the analysis of a great number of oil samples from various neutron generator facilities [19]. The oil in the vacuum pumps was employed over a period of two years in which a total number of about ten targets had been used. The oil diffusion pump contained a total volume of 100 ml, while the forepump has a total oil volume of 500 ml. In consequence in the oil of the vacuum pumps, maximum tritium contents of 700 μCi and 650 μCi have been found respectively. The tritium activities measured in the strips and in the granules are given in Table 3. The polyethylene strips and the polystyrene granules were kept at the exhaust of the vacuum pump for periods of two months. In general a decrease in activity has been observed for higher positions in the trap nearer to the outlet. For the polyethylene strips it could be calculated that total tritium activities of 17 μCi and 25 μCi were absorbed, whereas 77 μCi was absorbed in the polystyrene granules. Finally it is of interest to report some measurements of tritium activity in the released gas at the exhaust opening and in the accelerator room. The tritium activity was measured with a tritium gas monitor (Tritium Monitor Model TSM 91-B, Atomic Accessories). In previous experiments the exhaust opening was connected by means of a plastic tube to the monitor. The rather low activities measured might be attributed to absorption of the tritium in the tube. However, regular measurements of the air activity in the acceleration room have always yielded values below 10^{-4} μCi/ml.

Nellis and co-workers [16] concluded that a large fraction of the tritium released from the target, is slowly expelled through the forepump exhaust. Our quantitative data on the tritium activity in the released gas and on the activity found in the absorbers at the exit of the vacuum pumps are not in agreement with their results. Nevertheless, the presence of tritium at the forepump exhaust is clearly demonstrated. The experiments reported in this section pertain only to fixed targets with activities of 12 Ci each. Much higher amounts of released tritium might be expected in the case of rotating targets employed in pumped accelerators. With regard to the health physics precautions to be taken during operation of D–T neutron generators, the use of polyethylene or polystyrene filters at the exhaust of the forepump has to be recommended. Furthermore is might be advisable to connect the exit of the pumps with a metallized tube to a separate exhaust ventilator.

With regard to the mechanism of target deterioration a model has been developed assuming that each deuteron which enters the target has a cross section for causing the removal of a tritium atom [8]. The calculations suggest that small angle scattering of the incoming deuteron occurs, in which the recoil tritium atom receives sufficient energy to move from its original position in the titanium lattice so that the probability of being recaptured in that position is small. After removal from this position, the tritium atom will be free to diffuse through the titanium layer in the target. The experimental data presented in this section do not support the conclusion that all of the tritium lost from the interaction region of the target, leaves the target in gaseous form.

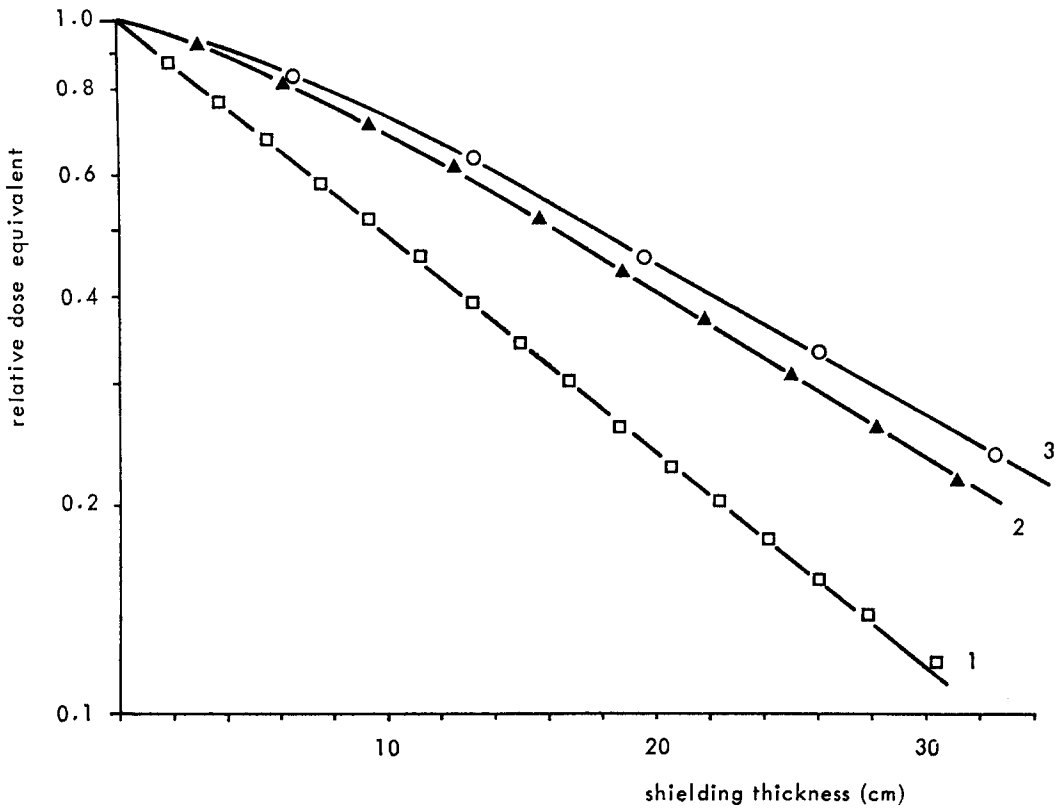


Fig. 3. *Relative dose equivalent values for D–T neutrons as a function of shielding thickness for steel (curve* 1*), paraffin (curve* 2*) and concrete (curve* 3*).*

If the triggering mechanism as described by Herring [8] is correct, then the tritium must diffuse to deeper layers in the target. It has to be concluded that more experimental and theoretical information has to be obtained for an appropriate explanation of the target deterioration processes.

EXPERIMENTAL SHIELDING DATA

In contrast to the large amount of experimental and theoretical attenuation data for fission neutrons, only scanty information about the shielding of 15 MeV monoenergetic neutrons has been published. In experiments reported earlier, the attenuation of the neutron dose equivalent and of the neutron absorbed dose were investigated with a neutron rem counter and a set of ionization chambers [20–22]. The shielding characteristics of the following materials were examined: concrete, paraffin, laminated wood, water, steel and sand. In general, the attenuation curves show an expo-

Table 4. *Shielding parameters for the attenuation of the dose equivalent measured for* 15 *MeV neutron beams*

Material	Density (g/cm³)	Relaxation length (cm)	Correction factor C	Range of shielding thicknesses (cm)
Concrete	2·3	19·7	1·2	0 – 32·6
Concrete	2·3	17·0	2·3	63·5– 96·1
Laminated wood	1·15	20·5	1·35	0 – 50
Paraffin	0·9	17·5	1·3	0 – 31·4
Steel	7·8	14·0	1·0	0 – 30·4
Water	1·0	16·9	1·3	0 – 38·5
Concrete+paraffin*	—	15·0	1·5	0 – 60·7
Paraffin+concrete*	—	18·7	1·3	0 – 64
Water+paraffin+ Concrete*	—	18·3	1·0	0 –141

*Materials mentioned first are located on the side of the incident neutrons.

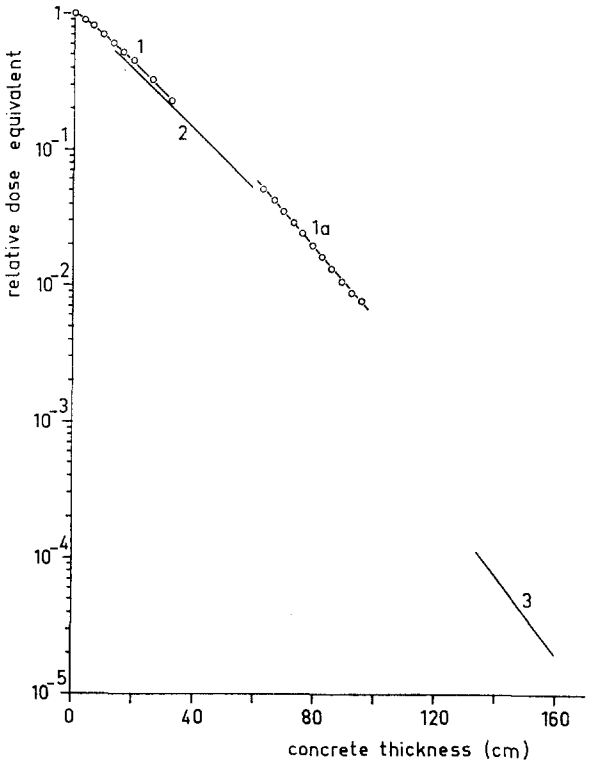


Fig. 4. Relative dose equivalent values for various concrete thicknesses up to 160 cm as measured by various authors. Curves 1 and 1a have been obtained by Broerse [22], curve 2 by Sauermann and Schäfer [23] and curve 3 by Hacke [24].

nential decrease beyond a thickness of 15 cm. The composite curve results from different processes and can be represented by the relation

$$DE_x = DE_0.C.e^{-x/\lambda} \text{ for } x > 15 \text{ cm}$$

where DE_0 and DE_x are the dose equivalent values measured without and behind a shield respectively, λ is the relaxation length and the correction factor C represents the contribution of the scattering effects. In Fig. 3 the attenuation curves are given for steel, paraffin and concrete. The shielding parameters, derived from measured attenuation curves, are summarized in Table 4.

Recently additional information has become available about the shielding properties of concrete for D–T neutrons. Sauermann and Schäfer [23] measured the attenuation of 14·2 MeV neutrons in ordinary concrete (density 2·3 g/cm³) with a rem counter and a proton recoil counter. These authors obtained an exponential attenuation curve which can be described by a relaxation length of 19·7 cm. Hacke [24, 25] reported dose equivalent transmission factors for concrete shielding thicknesses in the range from 130 to 160 cm. The attenuation curves reported by Hacke all show an exponential shape; from his measurements a relaxation length of 15 cm for concrete (density 2·4 g/cm³) can be derived. The attenuation curves in normal concrete obtained by the various authors are summarized in Fig. 4. It can be seen that the results of Sauermann and Schäfer are in good agreement with our results. The attenuation curve measured by Hacke shows a clear discrepancy with the other curves. A detailed discussion of this difference can be found elsewhere [22]. The special geometry and the properties of his detector presumably are responsible for the discrepancy. According to our present knowledge, it seems advisable to employ a relaxation length of 17 cm and a correction factor of 2·3 for shielding calculations of normal concrete with a density of 2·3–2·4 g/cm³.

CONCLUSION

At present D–T neutron sources seem to be more economical and flexible to be used as neutron therapy machines than cyclotrons. The physics and engineering technology of intense neutron sources is sufficiently advanced to initiate clinical applications on a limited scale [26]. As discussed in this paper for full scale clinical trials the D–T neutron therapy sources should provide neutron yields of about 10^{13} neutrons/sec with a new target. Brennan [27] has adopted a minimum average yield of 4×10^{12} neutrons/sec as a source strength specification. According to the current status of

development this high yield will probably first be obtained with continuously evacuated generators with accelerated beams of pure deuterium ions. Generators with mixed beams are preferable however, since it might be expected that with these machines the target problems will be greatly reduced. At present the sealed tube development has reached the stage where outputs of 10^{12} neutrons/sec have been achieved for short periods with a beam current of 28 mA at 250 kV [7]. However, no information is available about the long-term performance at this yield level.

In the second part of this paper two health physics problems have been discussed notably the tritium release from the targets and the shielding of the neutrons. Tritium contents of the oil of the accelerator pumps and the tritium contents of the gas released at the exhaust opening of the forepump have been measured. The presence of tritium in the oil and the gas have clearly been demonstrated, but the amounts cannot explain the mechanism of target deterioration. With respect to radiation hazards some precautionary measures are recommended. In the last part of this paper a review has been given of shielding characteristics of various materials for 15 MeV neutrons. Recommendations are presented for the relaxation lengths and correction factors to be employed in shielding calculations.

REFERENCES

1. G. W. Barendsen and J. J. Broerse, Dependence of the oxygen effect on the energy of fast neutrons. *Nature Lond.* **212,** 722 (1966).
2. D. A. Lundberg, The design of a practical fast neutron therapy equipment for routine clinical use. *Proc. 12th Int. Congress of Radiology*, Tokyo (1969), in press.
3. J. L. Fowler and J. E. Brolley Jr, Monoenergetic neutron techniques in the 10–30 MeV range. *Rev. mod. Phys.* **28,** 103 (1956).
4. R. L. Bach and R. S. Caswell, Energy transfer to matter by neutrons. *Radiat. Res.* **35,** 1 (1968).
5. J. J. Broerse, Dosimetry for fast-neutron irradiations of cultured cells and intact animals. 2. Comparison between activation and ionization methods. *Int. J. Radiat. Biol.* **10,** 429 (1966).
6. M. L. Randolph, Energy deposition in tissue and similar materials by 14·1 MeV neutrons. *Radiat. Res.* **7,** 47 (1957).
7. J. D. L. H. Wood, Private communication (1970).
8. D. F. Herring, C. D. Pruett, R. M. Potenza, J. Dobson and D. M. J. Compton, Review of the program for development of a prototype fast neutron therapy system for the University of Pennsylvania. *Enviro-Med. Report* No. ESD-211 (1970).
9. R. Booth, Rotating neutron target system. *IEEE Trans. Nuclear Science* 938 (1967).
10. M. R. Cleland, Machine problems in generating neutrons for tumor therapy. *Proc. Symp. on Applications of Small Accelerators*, New York (1969).
11. O. Reifenschweiler, Sealed-off neutron tube: the underlying research work. *Philips Res. Reports* **16,** 401 (1961).
12. O. Reifenschweiler, A high output sealed-off neutron tube with high reliability and long life. *Proc. Modern Trends in Activation Analysis*, p. 905. Washington (1968).
13. J. E. Bounden, P. D. Lomer and J. D. L. H. Wood, A neutron tube with constant output (10^{10} n/sec) for activation analysis and reactor applications. *Nucl. Instr. Methods* **33,** 283 (1965).
14. J. R. Andrews and H. Hollister, Fast neutron beam radiotherapy. *Am. J. Roentgenol.* **99,** 954 (1967).
15. D. Greene and D. Major, Private communication (1970).
16. D. O. Nellis, E. L. Hudspeth, I. L. Morgan, P. S. Buchanan and R. F. Boggs, Tritium contamination in particle accelerator operation. *U.S. Dept. of Health, Education and Welfare, Public Health Service Publ.* No. 999-RH-29 (1967).
17. J. D. Berger, E. E. Watson and R. J. Cloutier, Health physics aspects of low-energy accelerators. *Proc. Conf. Use of Small Accelerators for Teaching and Research*, p. 112. Tennessee (1968).
18. J. J. Broerse, Effects of energy dissipation by monoenergetic neutrons in mammalian cells and tissues. Thesis, Amsterdam (1966).

19. E. E. Watson, R. J. Cloutier and J. D. Berger, Tritium concentrations in neutron generator pump oil. *Hlth. Phys.* **17,** 739 (1969).
20. J. J. Broerse and F. J. van Werven, Shielding measurements for 3 MeV and 15 MeV neutrons. *Hlth. Phys.* **12,** 83 (1966).
21. J. J. Broerse, Penetration parameters for 15 MeV neutron beams. *Kerntechnik* **9,** 446 (1967).
22. J. J. Broerse, Shielding properties of concrete for D–T neutrons. *Int. J. Appl. Radiat. Isot.* **19,** 74 (1968).
23. P. F. Sauermann and W. Schäfer, Experimental studies of fast neutron shielding. *Kerntechnik* **11,** 197 (1969).
24. J. Hacke, Dosimetrie und Abschirmung bei einem 14 MeV Neutronengenerator. *Int. J. Appl. Radiat. Isot.* **18,** 33 (1967).
25. J. Hacke, Shielding of 15 MeV neutrons. *Kerntechnik* **11,** 193 (1969).
26. D. F. Herring, Neutron sources for radiotherapy. *Enviro-Med. Report* No. ESD-209 (1970).
27. J. T. Brennan, Fast neutrons for radiation therapy. *Rad. Clin. North Amer.* **VII,** 365 (1969).

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Dosimetry in Neutron Radiotherapy

H. H. ROSSI

Dept. of Radiology, Radiological Research Laboratories,
630 *West* 168*th Street, New York, N.Y.* 10032, *U.S.A.*

Abstract—*The meaningful specification of patient dosage in neutron therapy requires in addition to a statement of the absorbed dose of neutrons, an accurate assessment of the absorbed dose of gamma-radiation, including both capture gamma-radiation produced in a patient and any contaminant gamma-radiation that accompanies the neutrons. In addition the dependence of* RBE *on neutron energy requires specification of radiation quality.*

Various methods of measuring absorbed doses of neutrons and gamma-rays are considered and it is concluded that in the present state of the art it should be possible to determine these quantities with an accuracy that is adequate for the purposes of neutron therapy. The specification of radiation quality is a more difficult problem but it is suggested that for the time being the least ambiguous characterization is in terms of the incident neutron spectrum.

ALTHOUGH absorbed dose is the principal physical quantity in radiotherapy, the relation between its magnitude and the number of cells inactivated may not be linear and depend furthermore on a variety of other factors. Therapists employing X- or gamma-radiation are familiar with the fact that cell killing increases faster than linearly with the absorbed dose and that a number of other physical and physiological factors such as dose rate, oxygenation, etc., have an important influence on treatment results. On the other hand, the effect of quantum energy is comparatively minor. When the X-ray energy is increased from 100 to 1000 kV the dose has to be adjusted by perhaps 15% in order to achieve equal effect. In the next one or two decades of energy this dependence, if any, is certainly much less. Consequently, there is no need for concern over spectrum changes caused by absorption or scattering, and for the same absorbed dose one may expect the same effect in those portions of the skin where the beam enters as in those where it leaves the body.

The situation in the case of neutrons is different. At neutron energies of the order of a few hundred keV there is a simple exponential relation between dose and cell inactivation and this is little affected by changes in dose rate. On the other hand, there is a much more pronounced dependence of biological effect on radiation energy. As the neutron energy increases from 100 to 1000 keV the biological effectiveness is likely to be reduced by a factor of two or more. Neutrons of this energy band have only very limited penetration in tissue and they are unsuitable for teletherapy although they might be employed in brachytherapy. A substantial fraction of the neutrons emitted by ^{252}Cf fall into this category.

As the neutron energy is raised still further the biological effectiveness decreases more slowly, but the complicating features characteristic of X- and gamma-radiation also appear to some extent. Survival curves have shoulders, recovery becomes appreciable and hypoxia increases survival markedly. It would appear that neutrons in the energy range of greatest interest to radiotherapy, that is, between perhaps 5 and 15 MeV, possess to some degree each of the complicating features mentioned above. However, spectral changes even in tissue masses as large as the human body are comparatively minor and one need not be seriously concerned

about RBE changes in the irradiated volume. On the other hand, extensive attenuation and scattering in collimators or filters might at least in some cases introduce appreciable changes in biological effectiveness.

These considerations indicate that dosage specification for neutrons must include some sort of characterization of radiation quality as well as of absorbed dose. Various such characterizations will be discussed later.

It must be emphasized that neutron RBE does not only depend on neutron energy. Since the dose-effect curves for neutrons usually have a shape that differs from that for X- or γ-radiation, it is evident that *the* RBE must also be a function of dose. It may be convenient to talk of RBE of neutrons in relation to their energy with the implication that equal levels of effect are being considered. It must, however, be recognized that the notion of *the* RBE of neutrons of a given energy is at best an approximation that might be crudely applied at high doses only. At worst it has, in the past, led to severe errors in patient dosage and it may do so again in the future unless the limitations of this concept are thoroughly appreciated.

The basic principles of neutron dosimetry differ little from those employed in X- and gamma-ray dosimetry. However, there are important practical differences with regard to the choice of materials and in particular the atomic composition of the sensing element. The interaction between neutrons and matter depends greatly and seemingly erratically on atomic number, isotope, and neutron energy. Of course, for the energies employed in neutron therapy, elastic collision with hydrogen is by far the most important interaction. But other elements particularly oxygen and carbon absorb appreciable amounts of energy. Fortunately, the differences between these two elements are rather slight provided one is not dealing with very narrow bands of neutron energies that are located near resonances in the cross-section curves for these elements. Since this is quite unlikely in practical situations, oxygen may be replaced by carbon, and one can compound various plastics that can be considered to be tissue equivalent within a few %, over essentially the entire range of neutron energies [1]. Commonly, these plastics are made with a polyethylene base. Admixture of carbon does not only result in the correct hydrogen content but also provides quite adequate conductivity for ionization chamber work. Addition of nylon introduces the requir d amount of nitrogen with considerable improvement in mechanical and particularly molding characteristics [2].

Tissue equivalent ionization chambers are made from such plastics and filled with gas of substantially the same atomic composition [3]. It can be shown that such chambers can be made of any size and that they may be utilized for the determination of absorbed dose of any ionizing radiation with substantially only two comparatively minor uncertainties [4]. One of these is the dependence of W (the average energy expended in the production of an ion pair) on the nature and energy of the charged particles that deliver the dose. According to the best available information it is possible to choose a value of this quantity which is accurate to within 2% for the electrons, protons and α-particles encountered in practical dosimetry [5]. The error for heavy recoils is considerably larger, particularly when they have energies of less than about 100 keV. However, heavy particles of such energies carry only a few percentage of the total energy absorbed in tissue no matter what the primary neutron energy is. The other uncertainty concerns the polarization effect which results in different energy loss by charged particles in solid and gases [5]. It amounts to certainly less than 1% for the neutron energies considered here.

Although tissue-equivalent ionization chambers suitable for therapy dosimetry are appa-

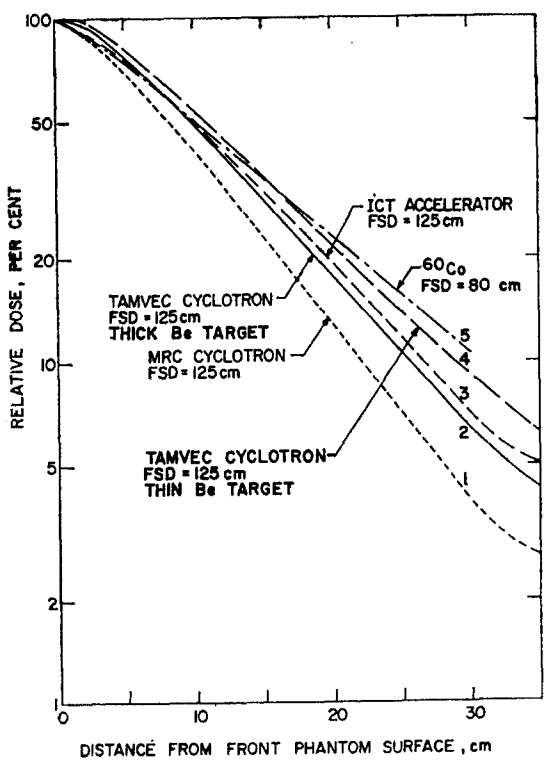


Fig. 3. Central axis depth dose curves for cobalt 60 and a variety of neutron beams. For identification of neutron sources see Fig. 4.

a

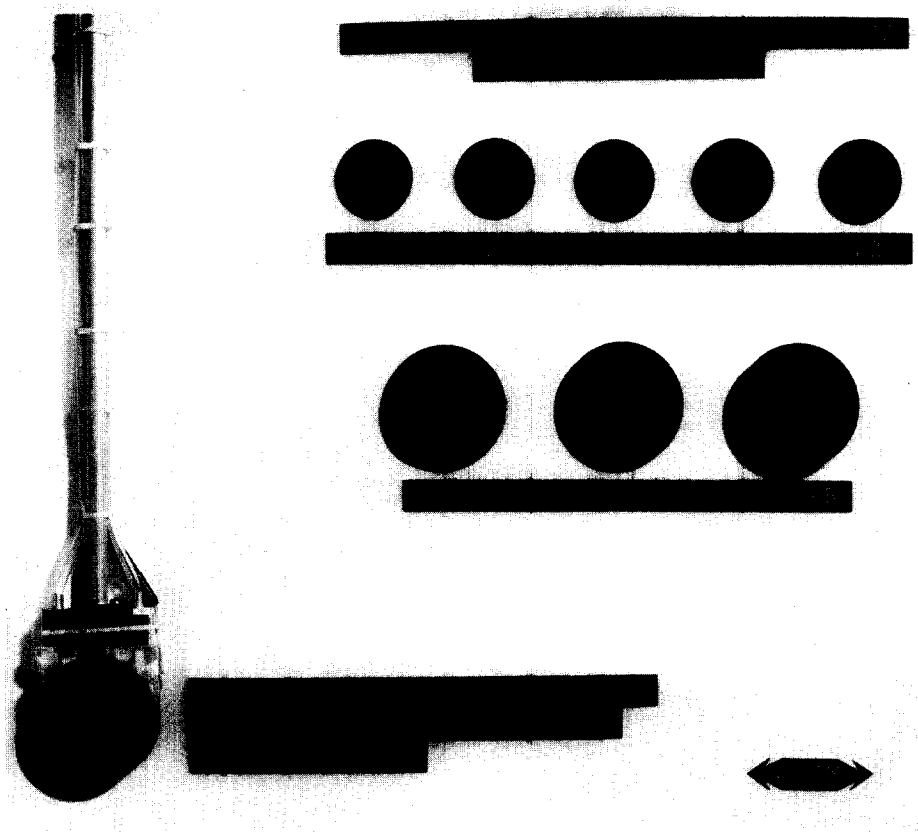


Figure 1(a) is an external view of the chamber and of a set of tissue-equivalent discs utilized to study build-up of secondaries.

b

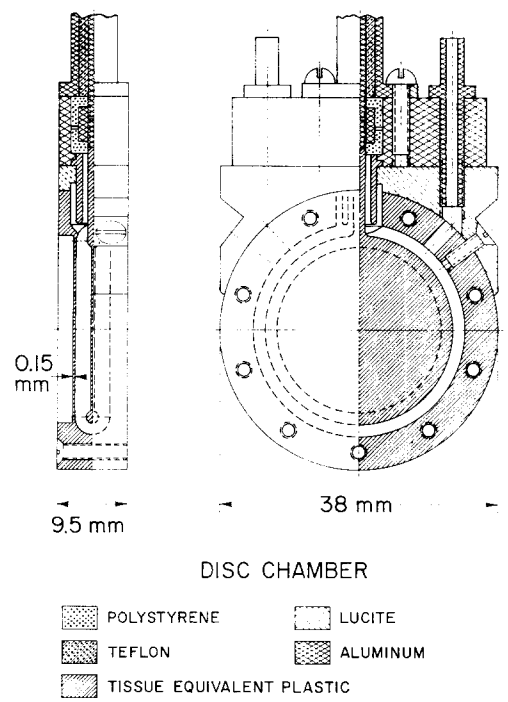


Figure 1(b) shows some construction details of the chamber.

Fig. 1. Tissue-equivalent ionization chamber suitable for depth dose measurements.

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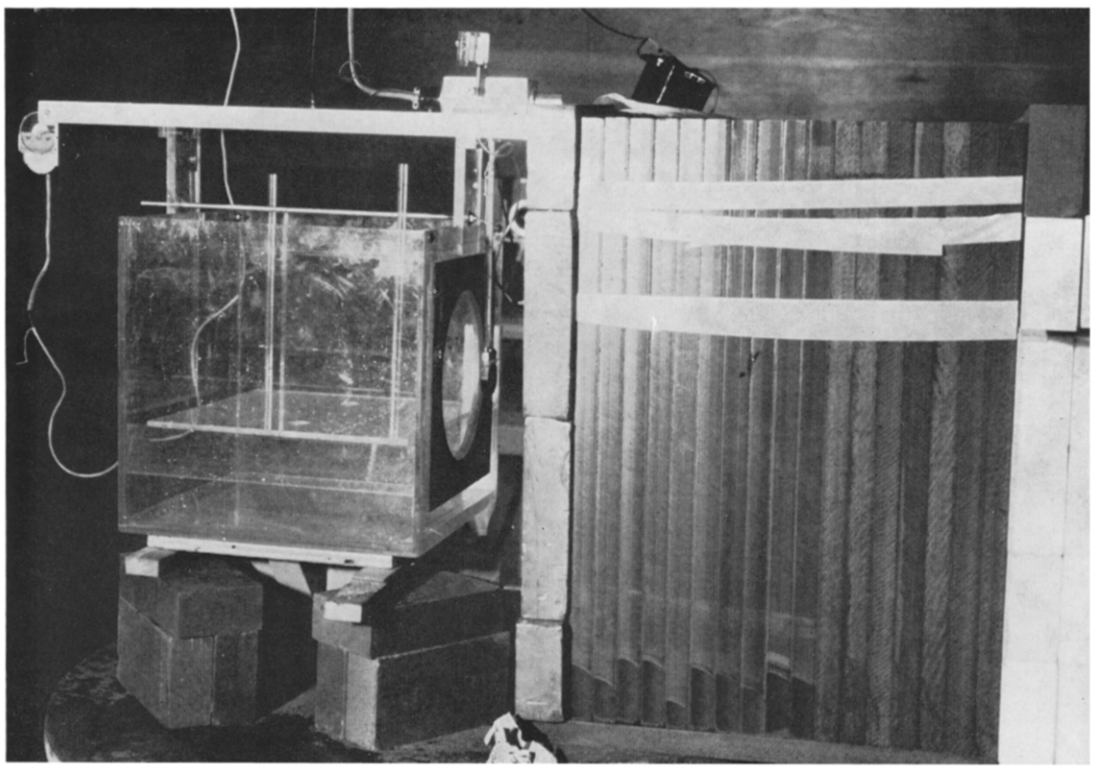


Fig. 2. Arrangement for depth dose studies. The chamber is shown outside the phantom. The horizontal rail contains a transport mechanism.

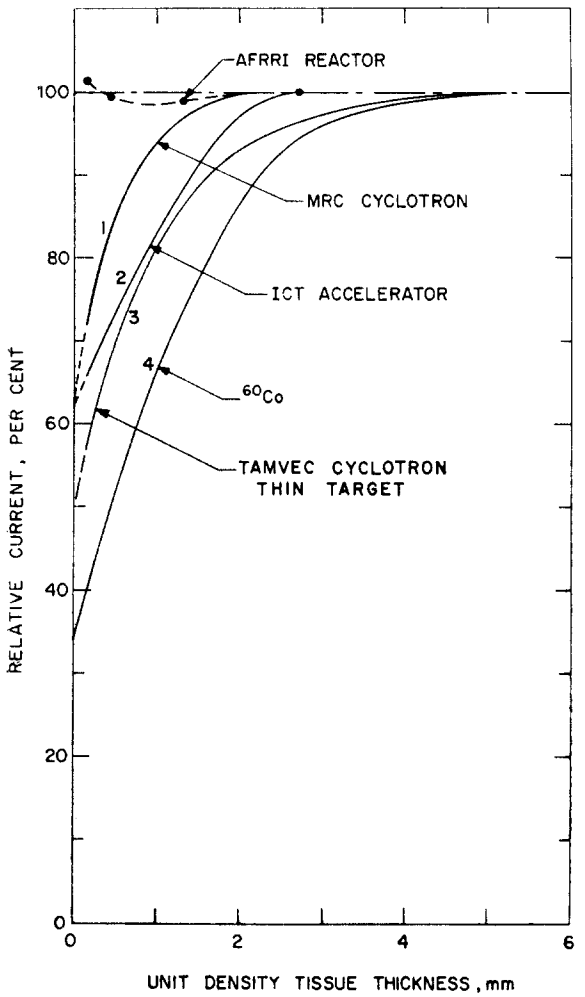


Fig. 4. Charged particle build-up for cobalt-60 and a variety of neutron beams. The AFFRI *reactor is a source of fission neutrons. The* ICT *accelerator produces 15 MeV neutrons. The* MRC *cyclotron produces a broad neutron spectrum having a modal energy of about 6 MeV. The Tamvec cyclotron also produces a broad neutron spectrum having a mean energy in the neighbourhood of 11 MeV. This is raised somewhat by the employment of a thin target.*

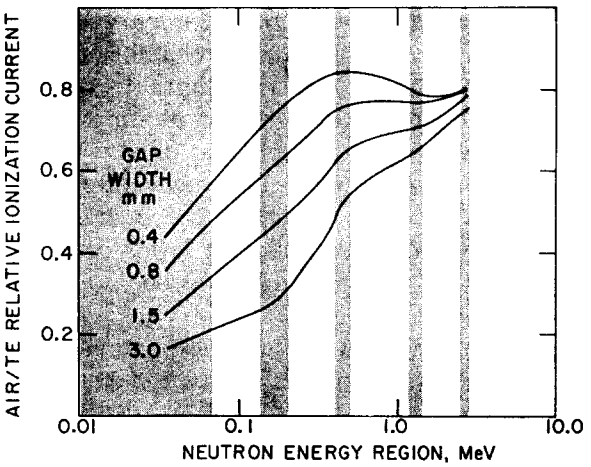


Fig. 5. Relative ionization currents obtained when an ionization chamber formed by two parallel plates of tissue-equivalent plastic is filled with air and tissue-equivalent gas. The response is shown as a function of both plate separation and neutron energy.

rently not available commercially, they are comparatively easy to construct and convenient to use. Figure 1 shows a model that was employed by Goodman and his co-workers in depth dose studies of various neutron beams [6–8]. The tissue-equivalent discs shown in this figure have varying thickness and can be utilized to investigate the manner in which secondary particle equilibrium is established.

Figure 2 shows this chamber and a phantom filled with tissue-equivalent liquid as used in central axis depth dose studies. Other papers at this meeting are concerned with depth dose characteristics, and therefore only one example has been given of the results obtained by Goodman and his colleagues (Fig. 3). Figure 4 shows the variation of absorbed dose near the surface of the phantom (i.e. the radiation build-up)*.

The special gas filling required for tissue-equivalent ionization chambers is a distinct inconvenience. Either chambers have to be operated with a steady flow of tissue-equivalent gas (such as the chamber shown in Fig. 1) or they are operated in a sealed-off mode. In the latter case large chambers designed for radiation protection may be operated for at least several months with acceptable error. On the other hand, in dosimetry for radiotherapy, it is not only necessary to achieve much higher accuracy but also to employ rather small chambers having a large surface-to-volume ratio. Under these conditions acceptable variations (particularly due to absorption of the gas in the plastic) are likely to be exceeded within days or, at most, weeks.

It would obviously be preferable if neutron dosimetry could be carried out with ionization chambers that are constructed of tissue-equivalent plastic but filled with air at atmospheric pressure. Such inhomogeneous ionization chambers are Bragg-Gray cavities of the "first kind" which means that the linear dimensions of the gas volume must be small compared with the range of secondary particles. This requirement is difficult to fulfill, particularly at lower neutron energies. Figure 5 shows the results of some studies we have

*In a document prepared prior to this meeting Barendsen and Broerse have pointed out that the secondaries having high LET and RBE build up more rapidly than the energetic protons of low LET and RBE [21]. This should be kept in mind when applications of these data are considered.

recently carried out [9]. The curves were obtained with a parallel plate chamber having tissue-equivalent plates of adjustable spacing and they represent the readings at atmospheric pressure when the chamber was filled with air, relative to those when tissue-equivalent gas was present. Because of differences in stopping power the ionization collected in air could be expected to be approximately 80% of that collected in T.E. gas. However, this ratio depends somewhat on neutron energy. In addition there is a marked decrease of sensitivity at low neutron energies. This is particularly pronounced for the larger gaps. In the case of the smallest separation it would seem that an air gap might be acceptable for neutrons having energies in excess of perhaps 1/2 MeV. If gaps are so narrow, ionization currents are rather low. However it is possible to obtain a considerable increase by internal gas multiplication, even in air [10, 11]. Further development work on this method may well be useful.

Perhaps the most serious deficiency in the employment of tissue-equivalent chambers for neutron dosimetry is their essentially identical response to gamma-radiation. Usually the first characterization of radiation quality in a mixed neutron-gamma field is a separate assessment of the doses due to electrons and due to atomic nuclei (this is what is usually meant by the "gamma dose" and the "neutron dose"). It is possible to construct ionization chambers that respond in essentially tissue-equivalent manner to gamma-radiation but have a low response to neutrons. The employment of such chambers in conjunction with tissue-equivalent ionization chambers represents an entirely adequate system in radiation protection, but in radiotherapy it is suitable only when the gamma contamination is low [12]. This is, of course, desirable from the viewpoint of the therapist, but gamma contamination will always be present. Part of the gamma contamination can arise from the target of the particle accelerator and it can be suppressed with various degrees of success. However, another component which appears to be quite unavoidable, arises from thermalization of neutrons in the irradiated tissues with subsequent capture by hydrogen which, in turn, is followed by the emission of a 2·2 MeV γ-ray. This process typically contributes 10% or more of the absorbed dose in the tumour volume. It would seem that the employment of ionization chamber pairs in the manner discussed above results in marginal accuracy, particularly when the neutron energy is high and the neutron response of an aluminium chamber filled with argon approaches 15%. A thorough error analysis has yet to be carried out, but it might be estimated that in practical uses the uncertainties may be as high as 5–10% for the neutron dose and as much as 30% in the gamma dose.

A separate measurement of the gamma dose would obviously be quite useful. Unfortunately many conventional gamma dosimeters have appreciable neutron response and sometimes a very high response to thermal neutrons. This is particularly true for lithium fluoride which is otherwise well suited for phantom measurements. Powders with fully enriched lithium-7, which are said to have a negligible neutron response, are available at present. Other solid state devices, such as photographic emulsions, scintillation counters, etc. might also be useful although their employment in phantoms irradiated by neutrons has been scant.

An alternate approach is, of course, a separate measurement of the neutron dose. Since neutrons are the principal radiation involved and since their RBE is higher the accuracy requirements are greater. Various devices used in health physics, such as threshold detectors, shielded scintillators and semiconductor detectors are probably not accurate enough. Proportional counter instruments, especially if they are small, may well be adequate [13]. When these counters are filled with gas at low pressure, one may in fact obtain more information than a mere separation of neutron and gamma doses [14]. However, this aspect relates more specifically to the question of radiation quality which will be discussed below.

In summary it might be stated that the principal instrument for the measurement of neutron dose in radiotherapy should be the ionization chamber, and in particular the tissue-equivalent ionization chamber. Subsidiary measurements designed to evaluate the γ-ray or the neutron contribution to the total dose should employ other instruments including various ionization chambers, proportional counters or solid state devices. The choice of these instruments should be largely dictated by the energy of the neutrons and the type of accelerator employed. It probably should also be influenced by personal expertise and available facilities. In any case, it appears that presently available techniques are probably adequate to perform dose measurements within the accuracy required for radiotherapy if this is assumed to be set somewhere between 5 and 10%. The precision of such measurements (i.e. the reproducibility) should be at least twice as good.

Since, as already mentioned, the biological

effectiveness of neutrons depends to a considerable extent on their energy; an unambiguous statement of dosage requires information on radiation quality. The term "quality" is here employed to denote the property of the radiation which causes differences in degree of biological effect for equal absorbed doses. It is generally agreed that this is dependent on the microscopic distribution of absorbed energy. The quantities of microdosimetry and in particular the lineal energy, y, (closely related to the former event size Y) and the specific energy, z, (formerly local energy density, Z) specify this directly. The more widely used linear energy transfer (LET) is a similar quantity which is, however, not entirely unambiguously related to the microdosimetric quantities. On the other hand, the spectrum of the incident neutrons unambiguously determines both microdosimetric quantities and LET and may therefore be used at least as an implicit characterization of radiation quality. The microdosimetric quantities can be experimentally determined, utilizing tissue-equivalent proportional counters [15], and a neutron spectrum can be determined utilizing a variety of techniques [16].

It is frequently stressed that the distribution of these quantities must be given and that averages are of little value because the method of averaging may be inappropriate [17]. The energy average of a microdosimetric quantity is usually quite different from the probability average. The same is true for the dose and track averages of LET or the fluence and kerma averages of neutrons. However, if one of the underlying distributions is specified, the others and all types of averages can always be derived quite simply.

It is important to realize that this insistence on provision of a complete distribution is, in fact, an admission of ignorance. If we knew enough about the action of radiation on biological systems, we could almost certainly specify an averaging procedure, complex as it might be, that could serve to meaningfully characterize radiation quality in terms of pertinent averages of one or perhaps a few, physical quantities. We are still a long way from being able to do so. Although it is assumed that a dose average of LET is appropriate for radiation protection [18], it appears impossible to account for the action of a radiation producing a range of LET values in terms of the action of radiations having discrete LETs [19]. Seemingly minor variations of neutron energy distribution can apparently also cause considerable differences in RBE [20].

It is to be hoped that a better understanding of fundamental physical processes will permit the formulation of more suitable physical quantities or averages. Until this point is reached, it has to be concluded that the best and most unambiguous quality specification is in terms of the neutron spectrum employed in radiotherapy.

REFERENCES

1. H. H. Rossi and G. Failla, Tissue-equivalent ionization chambers. *Nucleonics*, **14**, 32 (1956).
2. F. R. Shonka, J. E. Rose and G. Failla, Conducting plastic equivalent to tissue, air and polystyrene. *Prog. in Nuclear Energy*, Series XII, Vol. 1. Geneva Conference Paper (1958).
3. H. H. Rossi, Neutrons and mixed radiations. In *Radiation Dosimetry* (Edited by G. J. Hine and G. L. Brownell), p. 667. Academic Press, New York (1956).
4. H. H. Rossi and G. Failla. Neutrons: Dosimetry. In *Medical Phys.* (Edited by O. Glasser), Vol. II, p. 605. Year Book, Chicago (1950).
5. International Commission on Radiological Units and Measurements (ICRU), Physical aspects of irradiation. Report 10b (1962). *National Bureau of Standards Handbook* **85** (1964).
6. U.S. Atomic Energy Commission, Annual report on research project. Report No. NYO-2740-1, 26 (1964).
7. L. J. Goodman and U. Koch, Dosimetry studies for a fast neutron therapy beam. U.S. AEC Annual report on research project, Report No. NYO-2740-6, 32 (1969).
8. J. T. Brennan, Fast Neutrons for radiation therapy. *Radiologic Clinics of North America* **VII**, 2 (1969).
9. R. D. Colvett, Neutron energy response of an air-filled parallel plate cavity. *Annual Report on Research Project*, U.S. AEC Report No. NYO-2740-7 p. 35. (1970).

10. H. H. Rossi and M. Lubert, Dosimetry with ionization chambers employing internal gas multiplication. *Hlth. Phys.* **1,** 46 (1958).
11. L. G. Bengtsson, L. J. Goodman and H. H. Rossi, Gas multiplication in air-filled ionization chambers. *Hlth. Phys.* **16,** 756 (1969).
12. L. J. Goodman and H. H. Rossi, The measurement of dose equivalent using paired ionization chambers. *Hlth. Phys.* **14,** 168 (1968).
13. G. S. Hurst and R. H. Ritchie, Fast neutron dosimetry. *Radiology* **60,** 864 (1953).
14. H. H. Rossi and W. Rosenzweig, A device for the measurement of dose as a function of specific ionization. *Radiology* **64,** 404 (1955).
15. H. H. Rossi, Microscopic energy distribution in irradiated matter. In *Radiation Dosimetry* (Edited by F. H. Attix and W. C. Roesch), Vol. 1, p. 43. Academic Press, New York (1968).
16. International Commission on Radiation Units and Measurements, Neutron fluence, neutron spectra and kerma. ICRU Report 13 (1969).
17. International Commission on Radiation Units and Measurements, Linear energy transfer. ICRU Report 16 (1970).
18. International Commissions on Radiological Protection and on Radiological Units and Measurements, Report of the RBE Committee to the International Commissions on Radiological Protection and on Radiological Units and Measurements. *Health Physics* **9,** 357 (1963).
19. D. K. Bewley, Fast Neutrons — LET distributions and the response of mammalian cells. In *Biophysical Aspects of Radiation Quality*, Second Panel Report. p. 65. IAEA, Vienna (1968).
20. H. H. Rossi, Linear energy transfer and microdosimetry. *Proc. Symp. on Neutrons in Radiobiology*, p. 129. Oak Ridge (1969).
21. G. W. Barendsen and J. J. Broerse, Personal communication (1970).

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Collimation of 14 MeV Neutron Beams

D. GREENE and D. MAJOR
Christie Hospital and Holt Radium Institute, Manchester, England

Abstract — *Some measurements on 14 MeV neutron beams defined by a number of different collimator designs are discussed. It is shown that the differences produced in the beam definition, under scattering conditions, are fairly small, and limited in their effect by the presence of a large component of scattered radiation outside the main beam, in a phantom. A high proportion of the scattered radiation is gamma-rays.*

INTRODUCTION

THE DIFFERENTIALS between the attenuation characteristics of the different elements are much smaller for fast neutrons than they are for photons or charged particles, and this is the primary difficulty in designing shielding and collimators for neutron sources. A further difficulty arises because the composition of the neutron beam (i.e. the neutron spectrum, and the accompanying gamma-ray contamination) varies markedly with depth in the shield. The process of attenuation of neutrons also involves the production of radioactivity. To a large extent, the main bulk of a neutron shield is self-shielding for the radiation generated by this process, but this is not so in the region of the collimator.

In earlier studies it was shown that to achieve minimum transmission of radiation from 14 MeV neutrons, through a given thickness of material, it was desirable to use a layer of iron nearest the neutron source, serving mainly as an attenuator for primary neutrons, followed by a layer of hydrogenous material to attenuate secondary neutrons coming from the steel, and a further layer of high density material to attenuate gamma-radiation generated by the low energy neutrons in the preceding layer [1]. These results have been confirmed quantitatively by computer calculations carried out by Lundberg [2] and by further experimental work by Marshall [3].

THE NEUTRON SHIELD

The neutron shield used in the present work is shown in section in Fig. 1. It consists of layers of steel, polythene and lead, to a total thickness of 50 cm in the direction of the beam. In three dimensions the structure is almost cubical. The neutron source, a Services Electronic Research Laboratory Q-Tube [4], is contained in a cylindrical canister, and is entered into the shield as shown in Fig. 1. The target T is accurately centred on the square section hole S. In terms of minimizing the overall weight of the shield, it would be more efficient to use a shield whose front half is hemispherical about the neutron source, but the cubical form was chosen in the present set-up for ease of fabrication. The rectangle marked M in Fig. 1 represents an ionization chamber used to monitor the neutron output.

Two sets of numbers are given in Fig. 1. These show the measured transmission through the shield, at the points marked, when the hole S is blocked. The measurements were made with a polythene–ethylene ionization chamber, and give the total radiation dose (neutrons+gammas). These results are expressed as a percentage of the dose on the centre line of the hole S at 50 cm from the neutron source. The transmission measurements were made at a distance of 8 cm from the surface of the shield. The first set of numbers was measured before the outer lead layer was placed in position, while the second set in parentheses was measured after the lead was added. The measured transmission data are in reasonable agreement with our previous attenuation data [1] on which the shield design was based. In this design it was

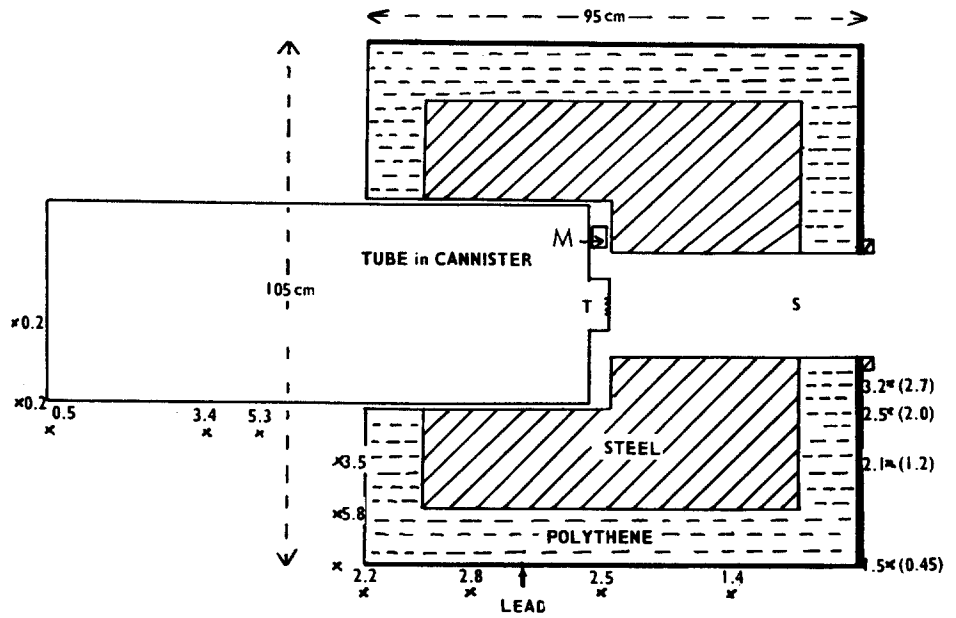


Fig. 1. Section through experimental neutron shield, with neutron generator in position. The numbers give the percentage transmission through the shield without outer lead layers, while the numbers in parentheses refer to the shielding arrangement with lead layer.

assumed that the tube canister (which is mainly filled with insulating oil) would be self-shielding at the end remote from the target, and the data shown justify this assumption. There is a weakness in the shield in the neighbourhood of the junction of the outsides of the canister and shield but this was not considered important as this region is remote from the position of a patient, whose long axis would be parallel to the opposite side, or front, of the structure.

THE EXPERIMENTAL COLLIMATORS

Figure 2 shows a detail of the collimator which is formed of 35 cm of steel, 12 cm of polythene and 1 cm of lead. The outside edge of the hole is framed with a 3-cm thickness of steel.

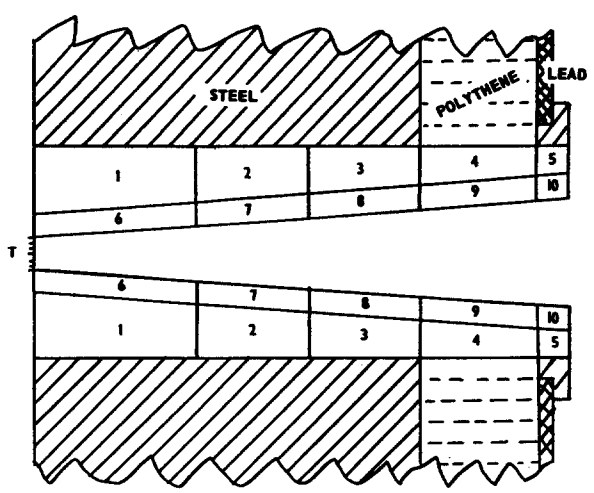


Fig. 2. Section showing details of shield and collimator. The collimator components labelled 1–10 were available in a number of different materials.

The items marked 1–10 are rectangular in the section normal to that shown and together form a collimator whose inside surfaces define a 10×10 cm field at 50 cm from the target and which converge down to a $2 \cdot 5$ cm $\times$ $2 \cdot 5$ cm hole at the target end. This does not make full use of the circular target, but this form was used for simplicity in construction. The components 1–10 of the collimator were available in steel and polythene, except component 4, which was available in polythene only; components 6 and 7 were also available in lead. The "liners" 6–10 were $2 \cdot 5$ cm thick. It was thus possible to construct a collimator entirely of polythene, one of steel and polythene of the same section as the main shield, and a number of variations in between. It was also possible to have sections all of one material, in the plane of the diagram, or to have collimators completely lined with steel, lead or polythene. It is easy to design a set of structures to allow a large number of variations on collimator design in this way, but this does lead to an alarming number of experiments for their evaluation.

MEASUREMENTS AND RESULTS

The main parameter in evaluating the usefulness of the various collimators is the dose distribution perpendicular to the central axis of the radiation beam and this has been measured in air and in water.

Figure 3 shows results of some measurements in air along a line perpendicular to the beam axis at 55 cm SSD, i.e. at 5 cm from the end of

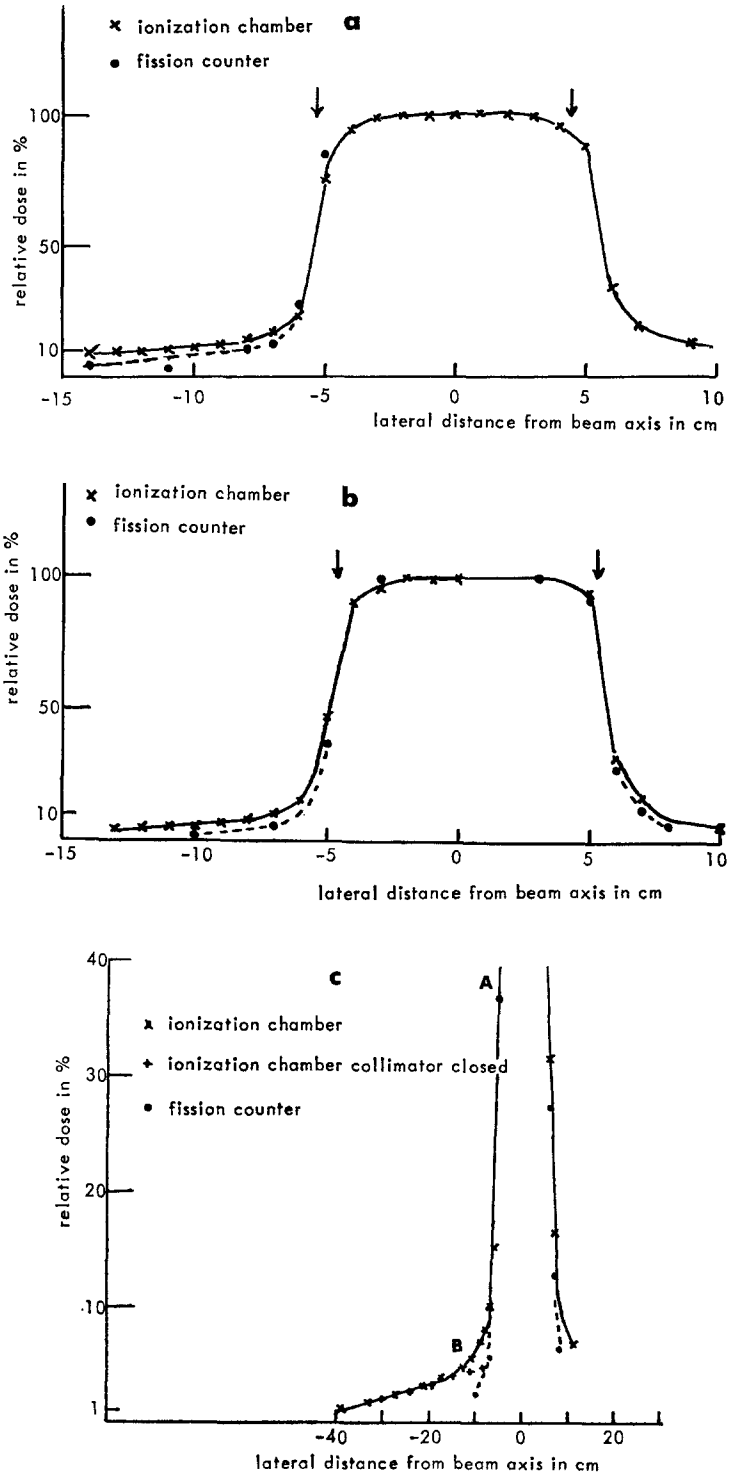


Fig. 3. *Dose distributions along a line perpendicular to the beam axis, at 5 cm from the end of the collimator, in air.*

(a) Using a collimator of 47 cm polythene and 3 cm steel.

(b) Using a collimator of 35 cm steel, 12 cm polythene and 3 cm steel.

(c) Details of dose distribution outside beam, collimator as in (b).

the collimator. Figure 3a gives the results for the collimator made of a thickness of 47 cm of polythene and 3 cm steel (components 5 and 10 of steel, in Fig. 2, all other components of polythene). The crosses indicate the data measured with a polythene walled, ethylene filled ionization chamber and the circles give data measured with a pulse fission counter. Each of these probes was 6 mm in diameter and therefore small enough to resolve the dose

distribution at the beam edge. The ionization chamber measured the total radiation dose, i.e. neutrons+gammas, while the pulse fission counter measured the neutron fluence only, with an energy threshold of about 1 MeV. The fissionable material in the chamber was Th-232. The results in these and in all the diagrams have been normalized to 100% at the beam centre.

A similar set of measurements for a collimator consisting of 35 cm of steel, 12 cm of polythene and 3 cm of steel (items 1–3, 6–8 and 10, item 4 and 9 of polythene in Fig. 2) are shown in Fig. 3b.

With both these collimators, the ionization chamber and pulse fission counter results were in good relative agreement inside the beam, indicating that the proportion of fast neutrons in the total radiation dose was constant. Outside the beam, it can be seen that approximately half the radiation consists of fast neutrons. All the other measurements, in air, with the various collimators lay inside the limits defined by Figs. 3a and 3b, and for this reason it is not necessary to quote them in detail. The use of either steel or lead liners did not appreciably change the beam profile.

Figure 3c shows further detail of the dose distribution, in air, given by the 35 cm steel, 12 cm polythene and 3 cm steel collimator. In this case the measurements outside the beam have been extended and compared with those obtained when the collimator is filled with absorber. It should be noted that the scales in Fig. 3c are very different from those in Figs. 3a and 3b. It can be seen that the dose distribution in the first 5 cm outside the beam edge is appreciably affected by scatter from the collimator, but beyond this, the contribution of the collimated beam to the local dose is negligible.

Figure 4 shows some cross field dose distributions in water at depths of 5 and 10 cm. It has been previously shown [5] that water and tissue equivalent solution give similar depth dose data for 14 MeV neutrons. These were all measured with the 6 mm diameter ionization chamber. Figure 4a gives the results measured with the 47 cm polythene and 3 cm steel collimator, and Fig. 4b was obtained using the 35 cm steel, 12 cm polythene, and 3 cm steel collimator.

Figure 5 shows further details of the dose

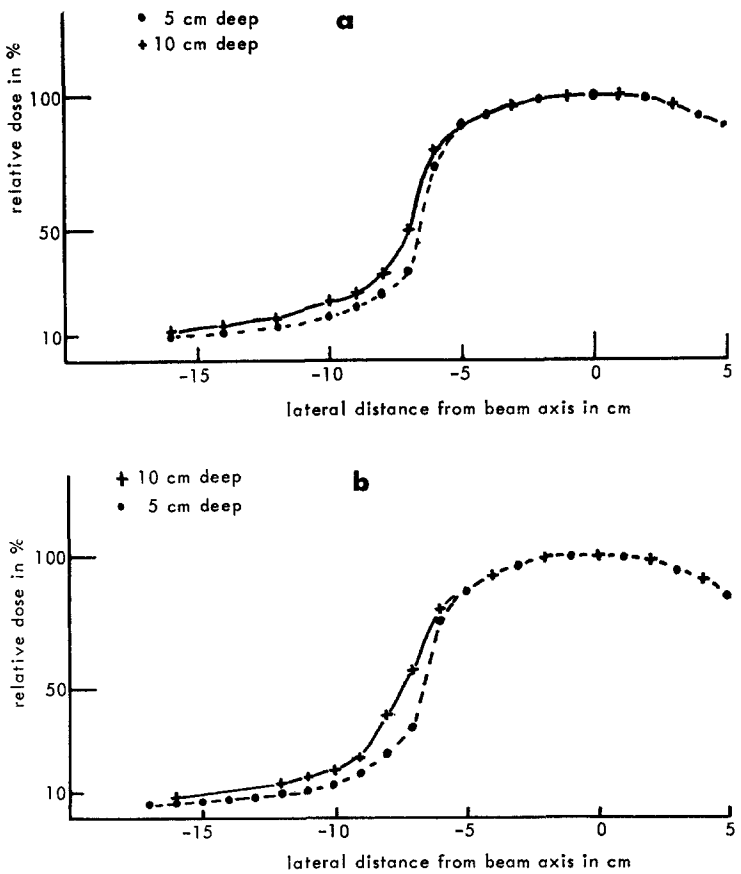


Fig. 4. Dose distributions across the beam, in water, at depths of 5 and 10 cm.
(a) With a 47 cm polythene and 3 cm steel collimator.
(b) With a 35 cm steel, 12 cm polythene and 3 cm steel collimator.

distribution outside the beam edge, in water, obtained with the two collimators. Figure 5a shows the results measured with the 47 cm polythene and 3 cm steel collimator at 5 and 10 cm depth in water, and 5b was obtained using the 35 cm steel, 12 cm polythene and 3 cm steel variation. Figure 5b also shows some of the "in air" points from Fig. 3c which were measured along the same line.

DISCUSSION OF FIELD MEASUREMENTS

It was obvious at the start of these experiments that a collimator made mainly of steel would give rather better beam definition, in air, than one made mainly of polythene, but the difference would be fairly marginal. However, polythene collimators would have great practical attractions, because they are lighter than steel ones, and also because they would not become radioactive. The experiments reported up to this point were performed to answer the following questions:

1. Assuming that the overall length of a collimator is limited to 50 cm, what is the difference between the beams defined in air by a predominantly polythene collimator, and by one consisting mainly of steel?
2. Is there any compromise between these limits which would give an acceptable beam while minimizing the overall weight of the collimator?
3. Is the dose distribution at the beam edge, e.g. along the line A B of Fig. 3c improved by the use of collimator liners?
4. What proportion of the dose outside the beam edge is due to scatter in the phantom from the collimated beam and what proportion is due to radiation transmitted through the shield?

These are all to some extent interdependent questions. In particular, if the answer to question 4 was that the dose distribution outside the beam was dominated by scatter, then it might be worthwhile to accept quite a large difference between the fields defined in air by the collimators mentioned in question 1, in order to minimize collimator handling problems. Figure 5b provides an answer to question 4. The differences between the "in air" and "in water" measurements outside the beam edge are experimentally significant, and the dose level outside the beam at a depth of 10 cm is made up of about equal proportions of scattered radiation and radiation penetrating the collimator. This means that the relative difference between the dose levels outside the beam demonstrated in Figs. 3a and b are reduced by the addition of scattered radiation, as demonstrated in Figs. 5a and 5b.

The answer to question 1 is given by Fig. 3a and Fig. 3b. The slopes of what might loosely be called the penumbra regions of these beams

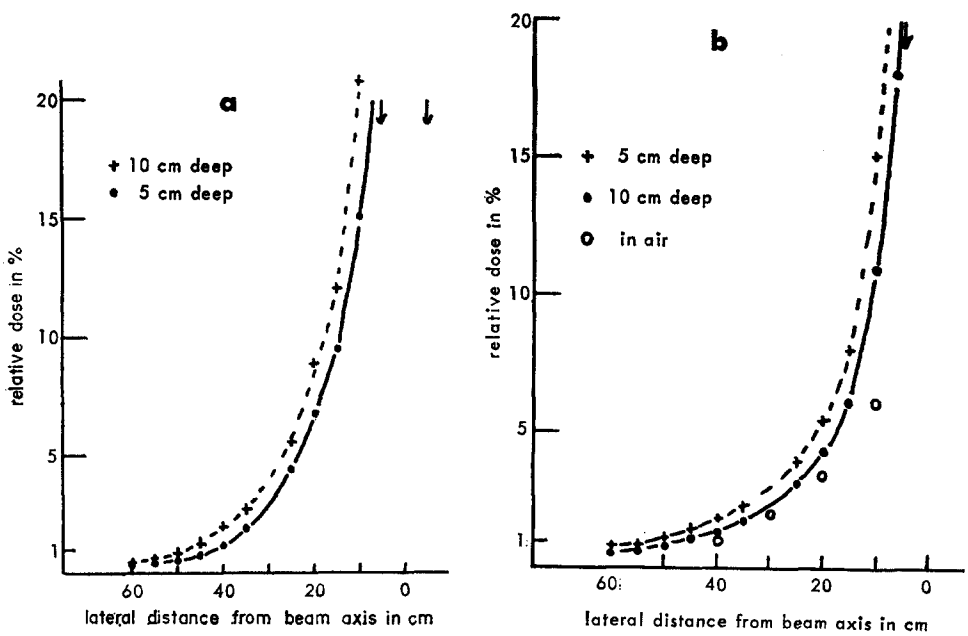


Fig. 5. *Details of dose distribution outside the beam, in water, at depths of* 5 *and* 10 *cm.*

(*a*) *With a* 47 *cm polythene and* 3 *cm steel collimator.*

(*b*) *With a* 35 *cm steel,* 12 *cm polythene, and* 3 *cm steel collimator. Some "in air" points are given for comparison.*

are not significantly different. The difference between the dose levels outside this region is experimentally significant, but possibly not of major practical consequence because of the comments above about the effects of scatter. The answer to question 2 is that the difference between the two limiting designs is so marginal that intermediate designs are not very interesting. The experimental answer to question 3 is not given in detail because the measured curves are so like those given in Figs. 3a and 3b. The use of liners would only be expected to affect the slope of the region of the beam at A B in Fig. 3c, and this is both acceptable with the simpler collimators and not improved by the use of liners.

From a family of curves such as Fig. 4b and a depth dose curve it is possible to construct an isodose chart for the field defined by the 35 cm steel, 12 cm polythene and 3 cm steel collimators, at 55 cm SSD which is shown in Fig. 6.

ACTIVATION MEASUREMENTS

The dose rate at the end of each collimator, after the neutron beam was switched off, was measured with a gamma-ray survey meter. At the beginning of a working day, the dose rate at the end of the collimator was measured before switching on the generator. The generator was then run for 1 hr, and a known dose delivered, and the dose rate at the end of the collimator was remeasured 30 sec after the end of the exposure. In general the dose rate before the exposure was negligible compared to that after exposure.

The dominant activity producing the radiation measured was from Mn-56 with a half life of 2·6 hr. From the measured dose rates, the rate to be expected after an exposure of one half life of this activity, for a generator operating at an output of 10 rads/min at 75 cm SSD was estimated, and these figures are given in Table 1. These dose rates might be regarded as an average, during a working day, for a generator giving this output. The activity measured in these experiments is produced partly in the generator and partly in the collimator.

For the predominantly polythene collimator (line 1 of the Table) all the activity measured is in the tube itself. When a steel section is placed in the collimator, immediately adjacent to the target, the dose rate falls significantly (line 2 of the Table). In other words the shielding provided by the steel of the collimator against the activity in the target end of the tube is more important in determining the dose rate, than the additional activity generated in the steel itself. The main idea in trying out collimators with lead liners was to see if these offered any significant protection from the activity generated in both tube and collimator. It can be seen from line 3 of the Table that the reduction of dose rate is not very great. Lines 4 and 5 of the Table show that the use of longer

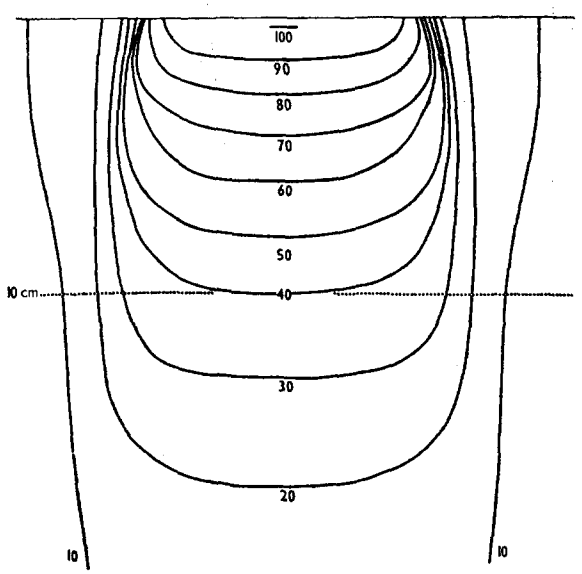


Fig. 6. *Isodose curve given by steel–polythene–steel collimator at* 55 *cm SSD.*

Table 1. *Average dose rate levels due to activation for a neutron generator yielding* 10 *rads/min at* 75 *cm SSD*

Collimator	Dose rate at end of collimator (mR/hr)
47 cm polythene 3 cm steel	640
15 cm steel 32 cm polythene 3 cm steel	500
15 cm steel (with lead liner) 32 cm polythene 3 cm steel	432
25 cm steel 22 cm polythene 3 cm steel	460
35 cm steel 12 cm polythene 3 cm steel	460
35 cm steel 12 cm polythene 3 cm steel 2 in. thick lead plug at target end of collimator	200

steel sections in the collimator do not change the dose rate due to activation. The last two lines of the Table compare the γ-ray dose rates from the longest steel section, with and without a 2 in. thick lead plug inserted at the target end after the irradiation. This reduces the dose rate by a factor of about 2.

CONCLUSIONS

It will be clear that the experiments described do not yet provide complete information about the design of an optimal collimator. None of the collimators described can be regarded as satisfactory, although the paper by Duncan *et al.* [6] shows that they at least give beams which are acceptable for initial clinical experiments.

The quality of the scattered radiation outside the main beam, in water, has been investigated, using the Lithium Fluoride technique [7]. Preliminary measurements show that the gamma-ray component at the 10% dose level, in water, is about 50% of the total dose and increases with distance from the beam centre, approaching 100% at the extreme of the measured field.

In so far as the dose levels outside the beam in a scattering medium contain approximately equal quantities of scattered radiation and radiation penetrating the collimator, possible improvements in collimator design are limited in their effect. It would also seem that any substantial improvements in beam definition can only be achieved by accepting the use of collimators longer than 50 cm. The degree to which the marginal differences between the collimators described is important depends ultimately on clinical observations.

REFERENCES

1. D. Greene and R. L. Thomas, The attenuation of 14 MeV neutrons in steel and polythene. *Phys. Med. Biol.* **14,** 45 (1969).
2. D. Lundberg, private communication (1969).
3. T. O. Marshall, private communication (1969).
4. M. Hillier, P. D. Lomer, D. Stark and J. D. L. H. Wood, A 14 MeV neutron source for radiotherapy. *Proc. 12th Congress of Radiology*, Tokyo, in press.
5. D. Greene and R. L. Thomas, An experimental unit for fast neutron radiotherapy. *Brit. J. Radiol.* **41,** 455 (1968).
6. W. Duncan, D. Greene and D. Major, Radiotherapeutic requirements of 14 MeV fast neutron beams with respect to depth dose and collimation. *Europ. J. Cancer* **7,** 121 (1971).
7. G. P. Naylor, The application of thermoluminescent phosphors in dosimetry problems in radiotherapy and radiobiology. *Brit. J. Radiol.* **40,** 170 (1967).

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Radiotherapeutic Requirements of 14 MeV Fast Neutron Beams with Respect to Depth–Dose and Collimation

W. DUNCAN, D. GREENE and D. MAJOR
Christie Hospital and Holt Radium Institute, Manchester, England

Abstract — *The shielding of 14 MeV neutron generators cannot be as efficient as that of X-ray machines. A method of estimating the level of stray radiation outside the geometric beam is suggested as compared with the stray radiation from telecobalt units and 250 kV X-ray machines. The level of whole body neutron radiation which will have to be tolerated by patients is considered to be within acceptable limits.*

THE MOST important requirement of fast neutron beams for radiotherapy is that they should have sufficiently good penetration to treat deep-seated tumours. It has been suggested that at a depth of 10 cm the dose should be at least 50% of the surface dose. A more practical criterion is that at least 30% should be obtained at 15 cm depth as this would allow most deep-seated tumours to be treated with an arrangement of three fields, whereby the overlying tissues each receive a dose which will not be larger than the total dose received by the tumour. Fig. 1 illustrates the depth–dose curve of 14 MeV neutrons for a 10×10 cm field at 75 cm SSD and shows that adequate penetration is achieved. Some improvement in depth–dose is obtained by increasing the source to skin distance but unless larger neutron yields can be attained little advantage (Fig. 2) as regards depth–dose is to be gained by going beyond about 75 cm because the reduction in dose rate with distance from a D–T generator quickly increases treatment times to an unacceptable extent.

The isodose curves of 14 MeV neutrons measured by Greene and Major [1] using a 55 cm thick steel and polythene collimator (Fig. 3) are similar to those for 250 kV X-rays, but do show much better flatness of the beam. Recent preliminary results of Nias and Greene [2] indicate that these isodose curves are in fact iso-effect curves and that the quality of the beam does not significantly change with attenuation. In profile (Fig. 4) the neutron beam is flat and well defined and compares well with a 250 kV X-ray beam at depth.

A serious problem is encountered beyond the edge of the beam where the stray radiation continues at a much higher level than we are accustomed to with megavoltage X-rays. The stray radiation (Fig. 5) at 10 cm depth in a phantom has been measured [3] outside beams of X-rays, gamma-rays and fast neutrons. The upper two curves in Fig. 5 show the stray radiation outside collimated beams of 14 MeV neutrons and are seen to be much less satisfactory. The lower of these two curves was obtained by Marshall at the Radiological Protection Service, Sutton, using a 75 cm collimator and measuring the fast neutron dose with a nuclear emulsion film. The highest curve was measured by Greene and Major [1] for a 55 cm collimator, using an ionisation chamber.

Shielding of fast neutron sources can never achieve the standards attained with megavoltage X-ray apparatus. The specification of the shielding and collimators of these machines permits a dose level of no more than 0·1% in air outside the penumbra. The practical

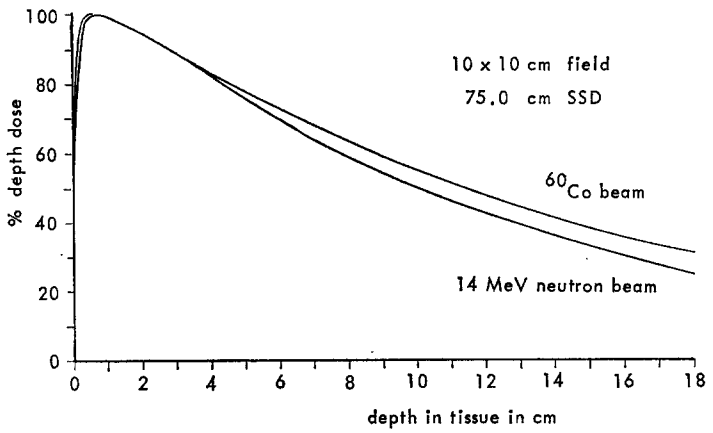


Fig. 1. Depth–dose curve for a 10 × 10 cm collimated beam of 14 MeV neutrons compared with a telecobalt gamma-ray beam.

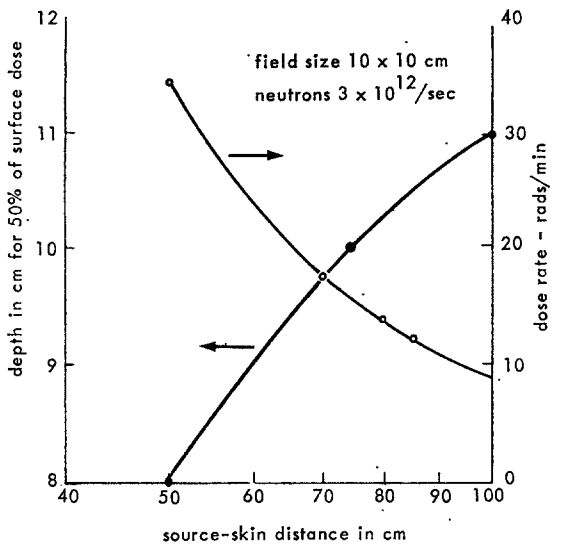


Fig. 2. Relation of the penetration qualities of a 14 MeV neutron beam to dose rates obtained at the incident skin surface for different source to skin distances for a generator with an output of 3 × 10^{12} neutrons/sec.

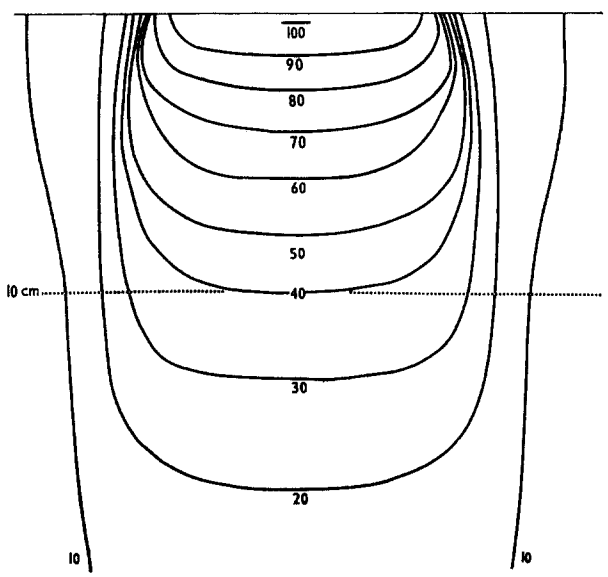


Fig. 3. Isodose curves for a 10 × 10 cm collimated beam of 14 MeV neutrons at 55 cm source to skin distance [1].

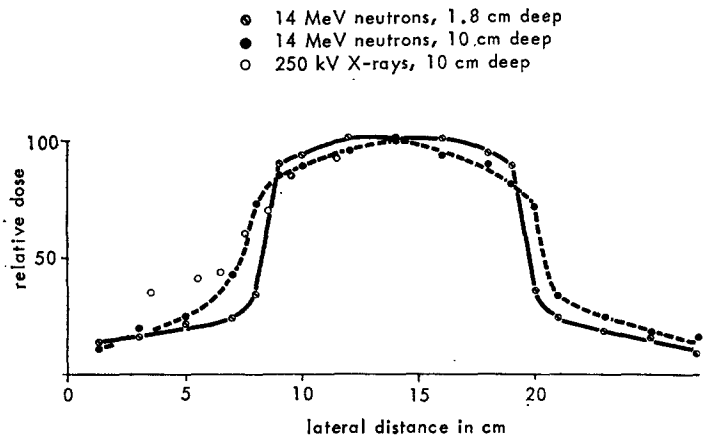


Fig. 4. Profile of a 10 × 10 cm collimated beam of 14 MeV neutrons at 1·8 cm and 10 cm depth and a 250 kV X-ray beam at 10 cm depth.

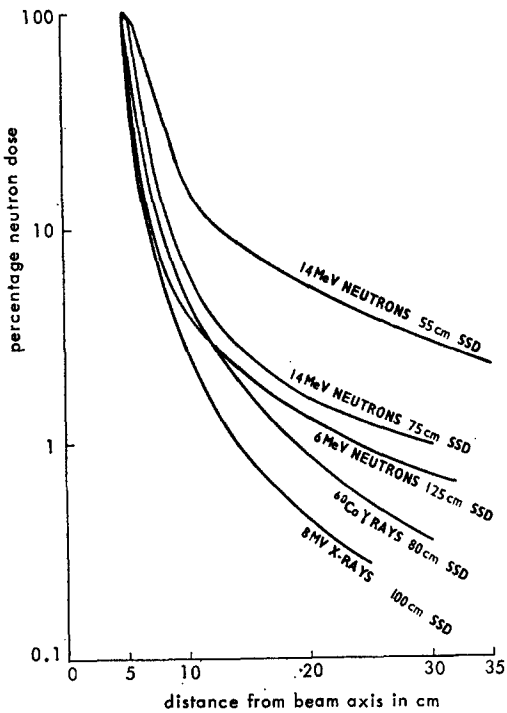


Fig. 5. The stray radiation from 10 × 10 cm beams of X-rays, gamma-rays and fast neutrons at 10 cm depth in a phantom.

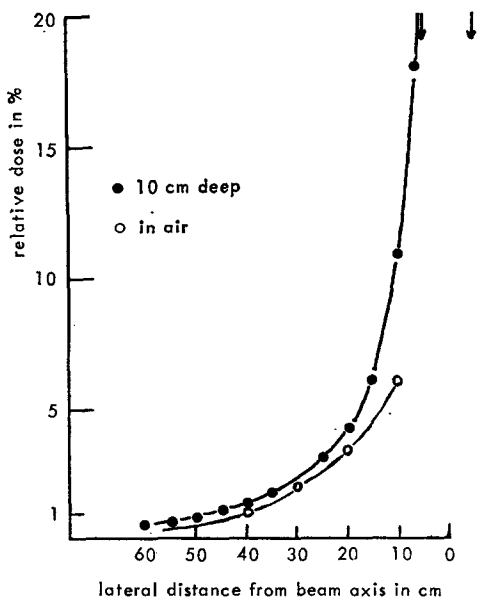


Fig. 6. Profile of a 10 × 10 cm collimated beam of 14 MeV neutrons measured in air and 10 cm deep in a phantom [1]. *The contribution of stray radiation may be approximated by subtraction.*

difficulties in terms of thickness of material required in designing collimators for 14 MeV neutrons implies that the comparable curve of stray radiation has to be much greater. It is important to note, however, that the level of 0·1%, specified for X-ray machines, has not been determined primarily on any clinical consideration but as a reasonable level which can be readily obtained by good engineering design. With fast neutron beam therapy the average whole body dose outside the useful beam will be much greater than with X-rays and one must consider what level of dose is tolerable and acceptable clinically and how one may estimate the whole body dose delivered by stray radiation.

The level of whole-body radiation received by a patient treated with X-rays is determined predominantly by scattered radiation from the primary beam, rather than by leakage radiation through the collimator, while the latter is an important factor with fast neutrons from D–T neutron generators. The comparison of the fields of stray radiation, obtained with different types of neutron collimators and X-ray collimators, has to be made in relation to the total dose distribution produced in a phantom, extending over a region comparable to the trunk of a patient, taken as 50 cm in length. The following discussion is based on the clinical situation of treating a patient with a tumour at a depth of 10 cm and expressing the whole-body dose as a percentage of the tumour dose at that depth. The dose gradient from the central axis of a 10×10 cm field to a distance of 50 cm was plotted on a linear scale for 250 kV X-rays, cobalt gamma-rays and 14 MeV neutrons and the average determined. It is necessary to find a figure which will give a meaningful average whole-body dose for the different qualities of radiation. Three ways of arriving at such a figure have been considered (see Table 1). The first method is to estimate the mean dose from the beam centre to the 50-cm point. This is unsatisfactory because the estimate of whole-body dose is largely determined by the dose in the main beam and we know in clinical practice that this is only valid when field sizes greater than about 25 cm in length are used.

A more relevant approach is to find a reference point somewhere in the penumbra region. With single field treatments the tumour dose is commonly considered to extend to the 80% isodose line and in a sense all radiation outside this is unwanted. The average dose is now taken from the 80% isodose line to a point 50 cm from the tumour centre axis of the beam. For this reference point it is clear that the telecobalt collimation is very much better than the others and that the measure of stray radiation from 250 kV X-rays is similar to that obtained from fast neutrons using the experimental collimators.

However, it may be thought that this figure is unduly weighted by the radiation in the penumbra and that a more reasonable figure may be obtained by averaging the dose from the edge of the penumbra. Looking at the 14 MeV neutron beam (Fig. 4) the penumbra ends at 8 cm from the central axis. This point corresponds to the 30% isodose line for fast neutrons, 40% for X-rays and 10% for cobalt gamma-rays. Although one cannot be certain about the most appropriate method of estimating the stray radiation level, it would appear that the most meaningful figure is given by this last method.

From Fig. 6 which shows beam collimation of 14 MeV neutrons in air and in a phantom it is possible by subtracting one curve from the other to approximate to the situation pertaining to an "ideal" collimator which allows negligible transmission of neutron radiation. This situation is shown in the last column of the Table and it does make clear that about 40% of the average whole-body dose as defined here is derived from processes of beam scattering and cannot be improved by collimator design. The percentages in this Table refer to the total absorbed dose and it is essential to determine the qualities of radiation contributing to the total dose. When fast neutrons are attenuated in

Table 1. *Average whole-body dose*

Beam reference	Percentage of tumour dose at 10 cm depth				
	^{60}Co gamma-rays	250 kV X-rays	14 MeV neutrons		
			collimator 1	collimator 2	ideal
Central axis	14	12	18	19	15
80% dose level	2·5	7·8	7·8	8·7	3·8
8 cm from central axis	1	4	5·3	6·6	2·3

tissue, slowed-down neutrons and gamma-rays are produced and the biological effectiveness of the absorbed radiation will depend on the proportion of each component. These spectra may be different outside and inside the main beam. We are also aware that the RBE for the very small doses of fast neutrons scattered outside the beam will be much higher than the RBE of the high dose delivered to the tumour volume [4].

Recent measurements made in Manchester indicate that in the penumbra region gamma-rays contribute almost half the total dose. Their contribution quickly increases with distance and is 100% at the 50 cm point. Bewley and Parnell [5] have also shown that at 10 cm from the central axis of the 6 MeV neutron beam under similar conditions the gamma-radiation dose exceeds the neutron dose which then very quickly becomes negligible beyond this point.

From the clinical point of view this finding is of great importance. The calculated whole-body dose is mainly due to gamma-radiation and the neutron dose is likely to be not greater than 30% of the total dose. If an RBE for the level of stray radiation is taken as 5 for tolerance of the blood forming organs, as suggested by Field [4] then the effective whole body dose shown in the Table will be no more than twice the calculated value — about 10% in terms of gamma-radiation. However, RBE values for haemopoietic stem cells have been found to be much closer to unity [6–10]. Assuming for example that a tumour dose of 1600 rads of 14 MeV neutrons is given in 8 exposures in 2–3 weeks, the estimated 'average whole-body dose' would be effectively 10% or equivalent to 160 rads of gamma-rays. There is evidence that this is certainly acceptable in clinical practice, although a drop in the total white blood cell count (WBC) might be expected.

Whole-body radiation was given, in the past, to patients suffering from polycythaemia [11]. Two hundred and fifty kV X-rays were used and commonly a whole body dose of 10 rads was given each day for 3–4 weeks, resulting in a total dose of 150–175 rads. It was felt that the total dose should not exceed 250 rads. Consideration of wide field X-ray techniques for abdominal and chest baths and treatment of the whole cerebrospinal axis in patients with medulloblastoma, gives additional evidence about tolerance to whole body doses. In these patients very large volumes of the bone marrow are included in the high dose region, receiving about 2500 rads given in 15 exposures in three weeks. The whole body dose calculated for regions outside the 80% isodose line will deliver at least another 100 rads to the remainder of the bone marrow.

A whole body dose of 200 rads is also accepted as tolerable in the treatment of patients with thyroid cancer using iodine-131. Indeed some patients with metastatic thyroid cancer have been treated on a regime which, after an ablative dose of radio-iodine, gives 50 mCi of radio-iodine twice weekly for 6–8 weeks [12]. A fair estimate of the whole body dose in these patients would be 15–20 rads for each treatment dose resulting in a total dose of 250 rads.

In radiotherapeutic applications of 14 MeV fast neutrons much higher levels of stray radiation outside the treatment beam will have to be accepted than with high energy X-ray beam therapy and this is a matter of concern. Examined in the way suggested here, it seems however that the hazards should be, at least, not greater than accepted with certain other forms of radiation therapy.

REFERENCES

1. D. Greene and D. Major, Collimation of 14 MeV neutron beams. *Europ J. Cancer* **7,** 121 (1971).
2. A. H. W. Nias and D. Greene, Changes in the biological parameters for mammalian cells as a function of position in a 14 MeV neutron field. Proc. IVth Int. Congress of Radiation Research, *Biology and Medicine*, Vol. 1, Gordon and Breach (1970).
3. D. K. Bewley, Fast neutron beams for therapy. In *Current Topics in Radiation Research* (Edited by M. Ebert and A. Howard), Vol. VI, p.249. North-Holland, Amsterdam, (1970).
4. S. B. Field, The relative biological effectiveness of fast neutrons for mammalian tissues. *Radiology* **93,** 915 (1969).
5. D. K. Bewley and C. J. Parnell, The fast neutron beam from the M.R.C. cyclotron. *Brit. J. Radiol.* **42,** 281 (1969).
6. S. Sawada and H. Yoshinaga, The relative biological effectiveness of X-ray, Co^{60} gamma-ray, and 14–1 MeV fast neutron for acute death in mice. *Nippon Acta Radiol.* **23,** 1080 (1963).

7. M. L. Davis, E. B. Darden and G. E. Cosgrove, Early hematologic effects of whole-body 14 MeV neutron irradiation in mice. *Acta Radiol.* **3,** 87 (1965).
8. J. J. Broerse, Dose-mortality studies for mice irradiated with X-rays, gamma-rays and 15 MeV neutrons. *Int. J. Radiat. Biol.* **15,** 115 (1969).
9. W. Duncan, D. Greene, A. Howard and J. B. Massey, The RBE of 14 MeV neutrons. Observations on colony-forming units in mouse bone-marrow. *Int. J. Radiat. Biol.* **15,** 397 (1969).
10. J. J. Broerse, W. Duncan, A. C. Engels, C. W. Gilbert, D. Greene, J. H. Hendry, A. Howard, P. Lelieveld, J. B. Massey and L. M. van Putten, The survival of colony-forming units in mouse bone-narrow after *in vivo* irradiation with D–T neutrons, X- and gamma-radiation. To be published in *Int. J. Radiat. Biol.*
11. R. Paterson, *The Treatment of Malignant Diseases by Radium and X-rays*, p. 454. Arnold, London (1949).
12. K. E. Halnan, Techniques and dosage in treatment of thyroid cancer. *Proc. Roy. Soc. Med.* **56,** 723 (1963).

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Summary of Discussions in the Session on Physical Characteristics of Fast Neutron Beams

H. B. KAL

Radiobiological Institute TNO, 151 Lange Kleiweg, Rijswijk Z.H., The Netherlands

THE DISCUSSION on the papers in this session centered around a number of topics, namely alternative neutron sources, characteristics of tritium targets, collimator characteristics and neutron dosimetry.

ALTERNATIVE NEUTRON SOURCES

In answer to a question by Fowler, Bewley pointed out that even if the target thickness is reduced to an optimal value, the penetration of neutrons produced by the reaction of 16 MeV deuterons on Be is still smaller than that of 14 MeV monoenergetic neutrons, assuming that 14 MeV neutrons are produced with a sufficiently large yield to allow comparison at the same SSD of 120 cm used with the cyclotron neutrons. A reduction of the Be-target thickness would cause a reduction of the present dose rate of about 50 rads/min by a factor of about 2. Catterall considered the increase of the depth dose at the cost of a factor of 2 in dose rate to be undesirable from the clinical point of view.

In order to obtain penetration similar to that of 14 MeV neutrons from the D–T reaction and dose rates similar to that obtained at the Hammersmith cyclotron, cyclotrons capable of accelerating deuterons to energies between 30 and 40 MeV would be required. The broad energy spectrum of the neutrons produced by these deuterons would have a mean energy of about 14 MeV.

Kelsey reported experimental studies on a D–T neutron generator with a gas target. The accelerator tube has a length of 150 cm, the entrance hole to the gas target has a diameter of 2 mm, the length of the gas target is 30 cm. A yield of 10^{13} neutrons/sec is expected for a 40 mA mixed beam of deuterium and tritium accelerated to 250 keV. The apparatus is still in the stage of development, the preliminary results seem promising.

CHARACTERISTICS OF TRITIUM TARGETS

Cleland mentioned experimental data of Kobisk (Isotope Development Group, Oak Ridge), indicating that the initial neutron yield per mA increases with increasing tritium–titanium thickness until the target thickness is about 4–5 times greater than the range of the bombarding deuterons. These findings suggest that the presence of a tritium reservoir beyond the range of the beam has an important influence on the yield. It was discussed that this effect might be caused by the method of target preparation. The titanium targets are heated in the presence of tritium gas. The tritium will diffuse into the target. During cooling the tritium might move in the opposite direction, causing a higher tritium concentration in the surface layer if the target is thicker. Porschen had the experience that, after removal of the surface layer of the target with sand paper, the neutron output increased because the deeper seated tritium layers can then be reached by the incident deuterons. The curve on target deterioration as a function of time, as presented by Broerse, showed two components: a rapid decrease over the first six

hours and subsequently a slower decrease. Rossi suggested that the initial fast decrease in neutron yield might be produced by contamination of the target by carbon deposits originating from the oil of the vacuum pumps. This layer will cut down the penetration of the deuterons in the target itself. These problems will not be present if ion getter vacuum pumps are used.

It was discussed whether the proportionality between neutron yield and ion current will hold for higher beam currents. Herring stressed that the most important parameter in the description of the target characteristics is the diffusion coefficient, which is highly dependent on the temperature. Neutron yield values at high currents cannot be extrapolated from the values at lower currents unless one has a constant power density or a constant temperature in the target.

Reifenschweiler stated that one of the advantages of sealed tubes with respect to continuously pumped accelerators is that there will be no loss of tritium from the tubes. This would become important when one would enter the period of routine application of fast neutron radiotherapy.

COLLIMATOR CHARACTERISTICS

In answer to several questions Bewley stated that with the neutron beam from the Hammersmith cyclotron the effect of the source size on the penumbra is not known yet, because of the fixed size of the targets which he used. His collimators are very long (50–60 cm). Neutrons which are scattered by the inner wall of the collimator will produce an additional penumbra. With regard to the contribution of gamma-radiation in and outside the penumbra, Bewley reported measurements with CaF dosimeters which show that the gamma dose is a steadily increasing proportion of the total dose as one moves laterally out from the beam central axis. About 20 cm outside the beam at 10 cm depth for a 10 × 10 cm field, the neutron component becomes negligible and the radiation field consists only of gamma-rays. It is technically difficult to use a biological dosimeter to determine whether the biological significance of the penumbra will be less than indicated by the physical measurements, because the dose rate in and outside the penumbra changes rapidly with position. Bewley found no variation in RBE of the cyclotron neutrons with depth in a phantom. Morrison asked whether a difference in the RBE might be expected for positions in the beam and in the penumbra area. It was mentioned that in the penumbra the proportion of scattered neutrons will be more important, in consequence the mean neutron energy will decrease and it might be expected that the RBE will increase. However, experiments of Nias [1] with D–T neutrons have shown that there are no significant variations of the RBE and OER in air, at depth on the central axis and at the 50% level in the penumbra.

For the collimators to be used at Manchester, Duncan and Greene reported beam profile measurements of a 10 × 10 cm collimated beam of 14 MeV neutrons in air and at depth in a phantom. At distances of 10 to 15 cm from the central axis they found total dose of 10 to 6% of the central axis dose, whereas a relative dose of 4% was measured at a 20 cm distance. Fowler considered the total dose received by the trunk in these conditions, to be alarmingly large. Greene answered that the high relative doses are due to scattering processes and that no large improvements can be expected by better collimator design. Phillips stated that in conventional therapy often a dose of 35 rads or more has been given to a large part of the body. Since the radiation field outside the beam consists mostly of gamma radiation, a 5% relative dose outside the field in a 1400 rads neutron course might be acceptable from the radiotherapist's point of view.

Several participants stressed the danger of leukaemia induction and other carcinogenic effects if inadequate collimation will be used. Fowler mentioned the results of Vogel [2] who found RBE values of 20 to 30 for induction of mammary carcinoma in rats after fission neutron doses of the order of 5 rads. Duncan answered that Darden and co-workers [3] found an RBE of only 2 for malignancy induction in mice using 14 MeV neutrons and no increase in RBE for leukaemogenesis and induction of aplastic anaemia. The sharpness of the beam edge is important because of possible overdosage of critical organs such as the spinal cord. Brennan recommended two measures to reduce the exposure to the whole body and to tissues in the penumbra, namely an increase of the SSD and the use of additional shielding over the untreated portion of the patient's body.

The application of other collimator materials to reduce the size of the collimator or to make a given thickness more effective was extensively discussed. Greene said that, at present, no materials are known to give any dramatic improvement over the results obtained with polythene and iron. Broerse and Brennan suggested that the use of depleted uranium might be favourable. Borated steel as colli-

mator material as suggested by Offermann is not significantly better with respect to the effect on the transmitted radiation than steel. It will only reduce the gamma-ray component and is not important for the neutron radiation. Further investigations about the applicability of other materials for collimator design are still required.

NEUTRON DOSIMETRY

With reference to Bewley's paper Herring asked whether the skin sparing effect is large enough to be of clinical significance. Barendsen reported experiments on the survival of cultured cells after 15 MeV neutron irradiation under conditions where secondary particle equilibrium was provided or deliberately avoided [4]. In this way a distinction could be made between the low-LET component of the neutron radiation, due to recoil protons, and the high-LET component due to interactions with carbon, oxygen and nitrogen nuclei. For exposures without secondary-charged particle equilibrium, the dose delivered to the cells was decreased to about 40% of the value under normal conditions. However, the remaining component consists mainly of radiation with a high LET and has an RBE which is approximately twice as high as the RBE found for the low-LET component of the neutron dose. These experiments indicate that the extent of skin sparing effect may be smaller than expected on the basis of the physical dose only.

Rossi suggested, in his paper, that the most practical way of measuring neutron dose is to measure the total dose and to subtract the gamma dose. Bewley stated that it would be better to measure the neutron dose separately rather than by a differential method. An error in the estimate of the gamma dose may not be very important as far as the gamma dose itself is concerned, owing to the low RBE, but it will be more important when it is reflected in the uncertainty of the calculated neutron dose. Rossi replied that the gamma dose is usually smaller than the neutron dose and in addition neutron insensitive gamma-ray detectors seem more effective than gamma insensitive neutron detectors. The use of the Rossi spherical proportional counter was suggested to measure gamma and neutron dose separately, by changing the bias to accept low or high LET events. However, this counter is a very complex device and at present commercially not available. Almond said that, for the treatment of patients with ^{252}Cf, the radiotherapist wants to know the neutron dose and gamma dose separately. The neutron dose will have to be multiplied by the appropriate RBE to arrive at an equivalent dose for the complex radiation field. Rossi stressed that the RBE is not a constant factor, but that it will depend on dose and dose rate. Withers stated that the RBE of ^{252}Cf neutrons relative to gamma-rays at low dose rates (of about 40 rads/hour) will not vary with dose because it seems probable that the survival curves for both ^{252}Cf neutrons and low-dose rate gamma-rays extrapolate to 1. Rossi said that he was inclined to doubt whether the extrapolation number for the gamma-rays at low dose rates would be 1.

In reply to a question of Phillips, Rossi commented that solid state semi-conductors are highly energy dependent. Even if relative values are required, they can only be used when minimal changes in neutron spectrum will occur (e.g. in the vicinity of ^{252}Cf sources). Semi-conductor detectors will be quite unsuitable for external beam dosimetry.

Using tissue-equivalent ionization chambers, Almond found no significant differences between the neutron doses measured with chambers filled with air and with tissue-equivalent gas for neutrons produced by the reaction of 30 MeV and 50 MeV deuterons on a beryllium target. The use of the air-filled chamber is much more convenient and is found to be acceptable for these high neutron energies.

Booz reported studies on the differences observed with various tissue-equivalent plastics and gases for microdosimetry. Two effects can cause significant differences between tissue-equivalent material and ICRU tissue. The first is due to resonances of the oxygen cross section. For monoenergetic neutrons of 1 MeV he observed a difference of about 15%. This effect might become insignificant in the case of broad neutron spectra. The second effect was that the contribution of the energy transfer, due to high-LET recoils, was in general larger in the case of tissue-equivalent material, due to its higher carbon content. A difference of about 20% was found for recoils with LET values in excess of 100 keV/μ, produced by 6 MeV neutrons. This effect is not very much affected by changes in the neutron spectrum.

Rossi noted that the compensated chamber of Bewley has no response to gamma-rays. He expected that part of the neutron dose would not be measured with this device and asked to what extent the neutron dose is underestimated. Bewley answered that this chamber was only used in a relative manner. With regard to the absolute dosimetry he had applied a 7% correction in order to agree with the

neutron dosimetry at Harwell. Berry stated that on the basis of biological experiments the agreement between the neutron dosimetry of Brennan's group and that of Bewley was within 5%. After a suggestion of Broerse it was concluded that it is highly desirable to constitute a committee which will recommend neutron dosimetry methods and initiate an intercomparison of neutron dosimetry between various treatment centres.

REFERENCES

1. A. H. W. Nias and D. Greene, Changes in the biological parameters for mammalian cells as a function of position in a 14 MeV neutron field. *Proc. IVth Int. Congress of Radiation Research*, Evian, 1970, in press.
2. H. H. Vogel, Mammary gland neoplasms after fission neutron irradiation. *Nature (Lond.)* **222,** 1279 (1969).
3. E. B. Darden, Jr., G. E. Cosgrove, A. C. Upton, K. W. Christenberry, J. W. Conklin and M. L. Davies, Late somatic effects in female RF/Un mice irradiated with single doses of 14 MeV fast neutrons. *Int. J. Radiat. Biol.* **12,** 435 (1967).
4. J. J. Broerse, G. W. Barendsen and G. R. van Kersen, Survival of cultured human cells after irradiation with fast neutrons of different energies in hypoxic and oxygenated conditions. *Int. J. Radiat. Biol.* **13,** 559 (1968).

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The Oxygen Effect and Radiotherapy with Fast Neutrons

R. H. THOMLINSON

Experimental Radiopathology Research Unit, Medical Research Council, Hammersmith Hospital, Ducane Road, London W.12, England

Abstract — *Grounds for supposing that neutron therapy may be advantageous in the treatment of cancer are largely based on the effect of hypoxic cells in tumours. This should be considered in relation to the development of hypoxia before treatment is given; to the effect of the size of each dose; to reoxygenation between doses and to the situation prevailing when the last dose is given. These effects are governed by the changing balance of demand for oxygen and its supply before and during treatment. The consequence of some of these changes is illustrated by the experimental fibrosarcoma RIB_5. This tumour contains 17% hypoxic cells before irradiation. This population rises immediately after a dose of radiation to an extent dependent on the dose, falls within 18 hr of a large dose, rises again to a second peak at 48 hr, then falls again. These changes will affect the RBE which will consequently vary with time after irradiation. The mechanism of these changes is discussed.*

WE MAY now accept that fast neutrons from the Medical Research Council's cyclotron at Hammersmith Hospital are a not specially bad form of radiotherapy [1], but it is unlikely, in general, that they will be dramatically better than X- or γ-rays. Yet there are grounds for believing that in certain circumstances their use may greatly increase the chance of curing a patient.

One such circumstance may exist in relation to the last dose of a fractionated course of treatment. This dose is unique in that in giving it, we are solely concerned, in relation to the tumour, with the number of neoplastic clonogenic cells which are killed by it. Every other dose, in addition to killing a fraction of the cells, alters the state of a tumour in a way which may affect the response to doses which are given subsequently and may be regarded as preparing for the last dose.

The object of the last dose is to ensure the greatest probability of cure, by reducing the number of neoplastic clonogenic cells below some threshold value. Because of the variability of tumour response we might take, for example, a hypothetical goal — the reduction of the number of cells to one tenth of this threshold. The presence of hypoxic clonogenic cells in the population at this time might reduce the chance of achieving this. We can calculate the upper limit of the proportion of cells which would just not have this effect (Fig. 1). This fraction is related to the oxygen enhancement ratio of the system. Were the proportion of hypoxic cells present greater than this limit, the chance of cure could be increased either by reducing their numbers with hyperbaric oxygen or by using radiations of higher LET with a lower oxygen enhancement ratio, or a combination of the two. For example, if the OER were 3 with X-rays, 1% of hypoxic cells would be the upper limit, whereas if fast neutrons, with an OER of 1·7 were used, the limit would be 6%. If the proportion of hypoxic cells were between 1% and 6%, the use of neutrons could make a dramatic difference to the chance of cure; were

the proportion below the limit, there would be no advantage in using neutrons at this stage of therapy.

There is evidence from clinical trials of hyperbaric oxygen and from experimental tumours in animals that, at the start of treatment, many types of tumour do contain cells protected from injury by X-rays because they are hypoxic and figures of around 12%–20% have been reported in a number of different tumours in animals [2–7].

The object of irradiation is to destroy the maximum number of clonogenic tumour cells for the minimum damage to normal tissue and so we may think of the efficiency of any form of radiation in terms of doing this. The presence of a higher proportion of hypoxic cells in a tumour than in the limiting normal tissue is bound to reduce the efficiency, but, because of the lower oxygen enhancement ratio for fast neutrons, the impairment will be less for them than for X-rays.

The presence of hypoxic cells in a tumour not only reduces the overall efficiency but causes a degree of inefficiency which increases with the size of dose. This is solely because the proportion of an insensitive component of a population is bound to be higher amongst the survivors of a dose and any further increment of dose is therefore given to a more hypoxic situation. It is possible to calculate the rise in this proportion after a given dose from a cell-survival curve and the oxygen enhancement ratio of any type of cell. For example, with the Ehrlich ascites tumour [8] the rise can be shown, starting from an assumed initial value of 1% (Fig. 2). It is also possible to calculate this rise in the proportion of anoxic cells from the gross response of sarcoma RIB_5, by comparing the effect of second doses of radiation, under conditions of complete anoxia of the tumour and of normal air-breathing respectively, given 5 min after the doses of varying size which are being investigated. The curve derived from the Ehrlich ascites tumour cells can, in this instance with fortuitous accuracy, be fitted to the points derived from the tumour response by simple displacement along the abscissa. No more should be concluded from this than that doses given to a tumour become progressively less efficient in a manner that would be expected in a partly hypoxic cell-population and that a lesser degree of inefficiency might be predicted for neutrons were they used to irradiate the tumour. This has been borne out by experi-

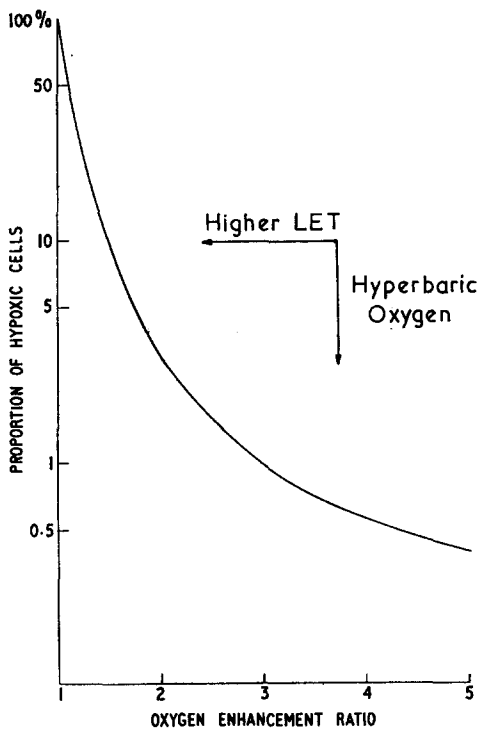


Fig. 1. A proportion of hypoxic cells in a tumour would reduce the chance of cure if it exceeded an upper limit. This limit is the proportion which would be reduced to the same level of survival by a given dose of radiation as would the whole of a well oxygenated population. It also depends on the oxygen enhancement ratio of the system and the relationship can be calculated for any chosen fraction of surviving cells. The chance of cure when the proportion is higher than the limit may be increased by hyperbaric oxygen and radiation of higher LET. The exact shape of the calculated curve depends on the level of survival chosen; in this example, 1/1000 in a well oxygenated population.

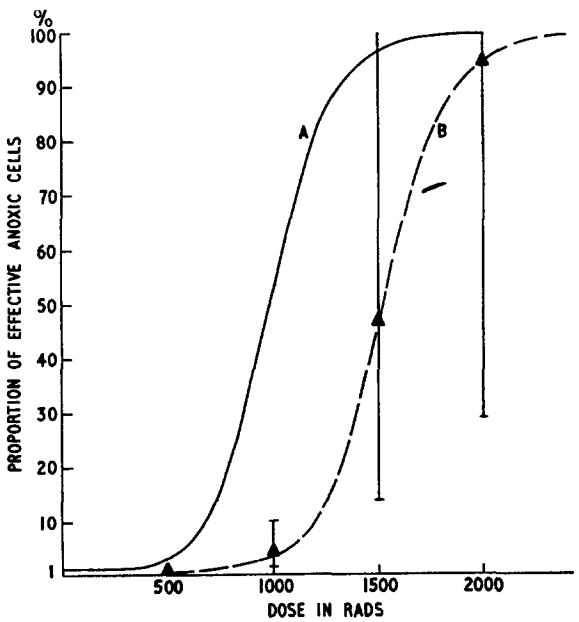


Fig. 2. In a partly hypoxic population of cells, the hypoxic proportion amongst survivors rises with the dose of radiation given. This proportion has been calculated from survival curve data of the Ehrlich ascites tumour [8] *assuming an initial value of* 1% *(curve A). This curve has been fitted to anoxic proportions calculated from the gross response of tumour* RIB_5 *(curve B).*

mental results [9] where, for comparable damage to normal tissue, the response of the tumour increases more with dose when neutrons rather than X-rays are used. In either case, however, a smaller dose must be more efficient than a larger and therefore, were this the only factor, two doses would be better than one of the same total size if reoxygenation were to restore the original proportion of hypoxic cells during the interval between them.

The term "reoxygenation" implies the raising of oxygen tension in and around certain cells restoring their radiosensitivity to a level from which it had previously fallen because of hypoxia. By this definition "complete reoxygenation" must mean the elimination of all hypoxic protection of cells, not merely the restitution of the proportion which existed before treatment was given. Reoxygenation entails the reversal of the processes which caused the hypoxia and these are concerned with the balance between demand and supply of oxygen.

The rate at which the demand for oxygen by a tumour increases must be related to the rate of increase of metabolizing cells within it. This is also a balance between the birth-rate and the death-rate of all cells within the tumour and varies from one type of tumour to another. The rate at which the supply of oxygen to the tissue increases depends on two principal factors, the rate at which the number of functioning "gas-exchange" vessels grows and the rate at which the quantity of blood available for distribution amongst them can be increased. This may depend on the rate of hypertrophy of the arteries supplying the tumour and the degree of obstruction of the veins caused by the growth of the tumour. Arteries supplying tumours unquestionably enlarge, but there seems little information about the rate at which this happens. If it were lower than the rate at which new capillary pathways were formed, there would, on average, be a reduction in the quantity of blood-flow per capillary.

The oxygenation of clonogenic cells, in addition, depends on their geometrical relation to particular capillaries, the flow of blood through them and the conditions for transport and consumption of oxygen between the capillaries and the cells. These factors must vary from one type of tumour to another and can be quite different in some adenocarcinomas, where clonogenic cells lie in perhaps a single layer between capillaries and the lumen of a gland and some squamous carcinomas in which metabolizing cells adhere to one another in clumps and cords, separating capillaries by many layers of cells. Even in squamous carcinomas there may be great differences between the well differentiated type in which clonogenic cells form a basal layer as in skin and undifferentiated types where the clonogenic cells are distributed through the masses of metabolizing cells.

However, one might expect the more slowly growing tumours to contain a smaller proportion of hypoxic clonogenic cells than the fast growers. An indication of this, based on not entirely satisfactory data, can be seen from the ratio of doses given to six different experimental tumours in rats breathing air or hyperbaric oxygen. The proportion of hypoxic cells is related to this ratio, which decreases as the time taken for each type of tumour to grow from one chosen size to another lengthens (Fig. 3) [10]. In more rapidly growing tumours, an upper limit of the proportion of hypoxic cells may be imposed because the processes which lead to hypoxia cause their death; those present at any one moment being a transient population. To the list of experimental tumours already reported containing between 12% and 21% hypoxic cells can now be added sarcoma RIB_5 found by McNally to have 17% [11]. In this tumour, at the standard size which we use for experiments, all cells lying in a position in which they might be hypoxic, die within three days when no treatment is given. The time is probably much less than this because necrosis encroaches on viable tissue at a rate of about 1 μ/min during this period. The gross response to doses of radiation given to the tumour in rats breathing air, but anaesthetised with sodium amytal, is more consistent with an anoxic proportion of 1% than 17% [12]. This implies that some 16 out of every 17 cells, which survive the radiation injury because they were hypoxic, die of nutritional failure in the post-irradiation period, and that 1% are rescued from death as an indirect consequence of the radiation. This must mean an improvement of their nutritional status and probably of their condition of oxygenation.

In sarcoma RIB_5 we have been able to calculate the fluctuations of the proportion of hypoxic cells with time after an initial dose of 1500 rads given with air-breathing (Fig. 4). This has been done by comparing the difference between the gross response to second doses of radiation given to the tumour either made wholly anoxic or with a free circulation in the rat breathing air. Following the initial rise of the proportion of hypoxic cells, there is a fall within the first 18 hr and then a rise again to a

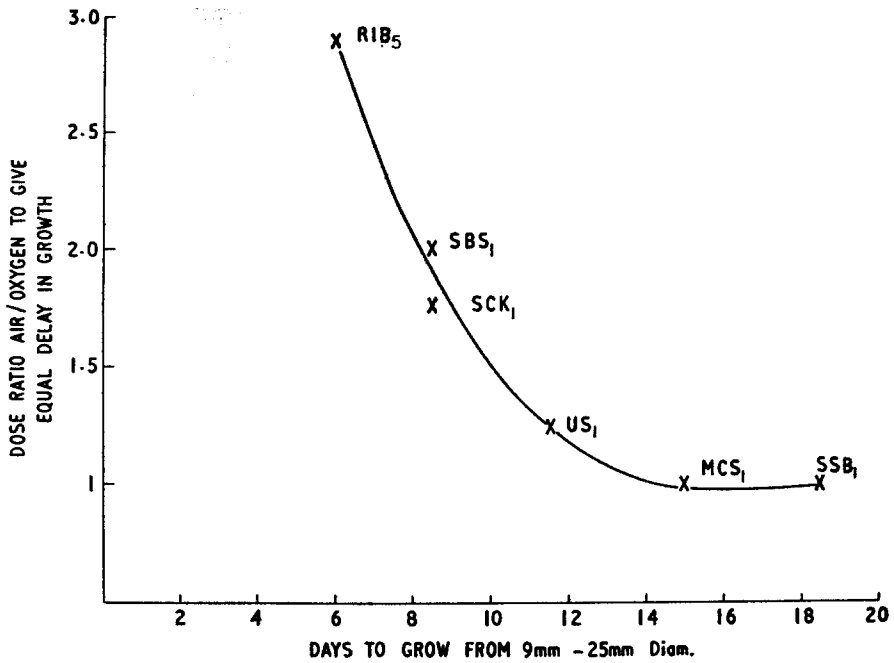


Fig. 3. The ratio of doses which produce the same response in six different types of experimental tumour when given to rats breathing hyperbaric oxygen or air is related to the rate at which these tumours grow from the diameter of 9 mm, at which they were irradiated, to 25 mm dia. This ratio is related to the proportion of hypoxic cells. (RIB_5 is fibrosarcoma induced with benzpyrene in 1945; SCK_1 is a spontaneous transitional cell carcinoma of the kidney; SBS_1 is a spontaneous sarcoma associated with a congenital spina bifida; US_1 is a spontaneous fibrosarcoma of the body of the uterus; MCS_1 is a fibrosarcoma of the breast induced with methylcholanthrene and SSB_1.)

peak at 48 hr after treatment. This corresponds to the time at which the tumour reaches its maximum volume before shrinking. During the next two days the tumour gets smaller and in this period there is a second fall in the proportion of hypoxic cells. Subsequently the growth of the tumour is resumed and the proportion of hypoxic cells rises again.

The relevance of all these investigations to therapy is that the RBE of neutrons compared with X-rays must fluctuate in the same sort of way as the proportion of hypoxic cells; neutrons appearing to be a more effective form of treatment when the proportion is high, although the change of hypoxic proportion may be different after doses of neutrons. The mechanism of these changes is under active investigation but meanwhile it seems worth considering how they might come about.

One of the first effects of a dose of radiation must be to reduce the growth rate of the tumour, partly because of the temporary inhibition of mitosis and partly because of the killing of clonogenic cells. The lower birth rate of cells in the tumour does not inevitably prevent an increase of tumour volume, but may slow down the rate of increase of demand for oxygen without a corresponding deceleration of the blood supply and so tend toward a state of better oxygenation, as in the more slowly growing tumours. This process is independent of reoxygenation.

The balance between demand and supply of oxygen may be further improved by an absolute diminution of demand as cells dying after the irradiation cease to metabolize. In sarcoma RIB_5 the oxygen consumption falls to about 75% and 65% of its pre-irradiation value 18 and 24 hr respectively after a dose of 2000 rads [13]. Cells die in tumours from a number of different causes; congenital defects, maturation and ageing, nutritional failure, immune phenomena and the direct consequences of treatment. The first two of these processes, which are probably occurring all the time, will continue after irradiation. Their extent may vary greatly from tumour to tumour. The nutritional failure may be diminished, but, at least in RIB_5, it causes death of many of the hypoxic survivors of the dose. The death of cells as a consequence of radiation reduces the demand for oxygen still further. Where the tumour contains a large proportion of hypoxic cells more of these will be killed by neutrons than by X-rays for a given amount of cell-killing in well-oxygenated normal tissues. This difference will be most marked not only when the proportion of hypoxic cells is high, but when their absolute number is at its highest, that is, at the beginning of treatment. As the

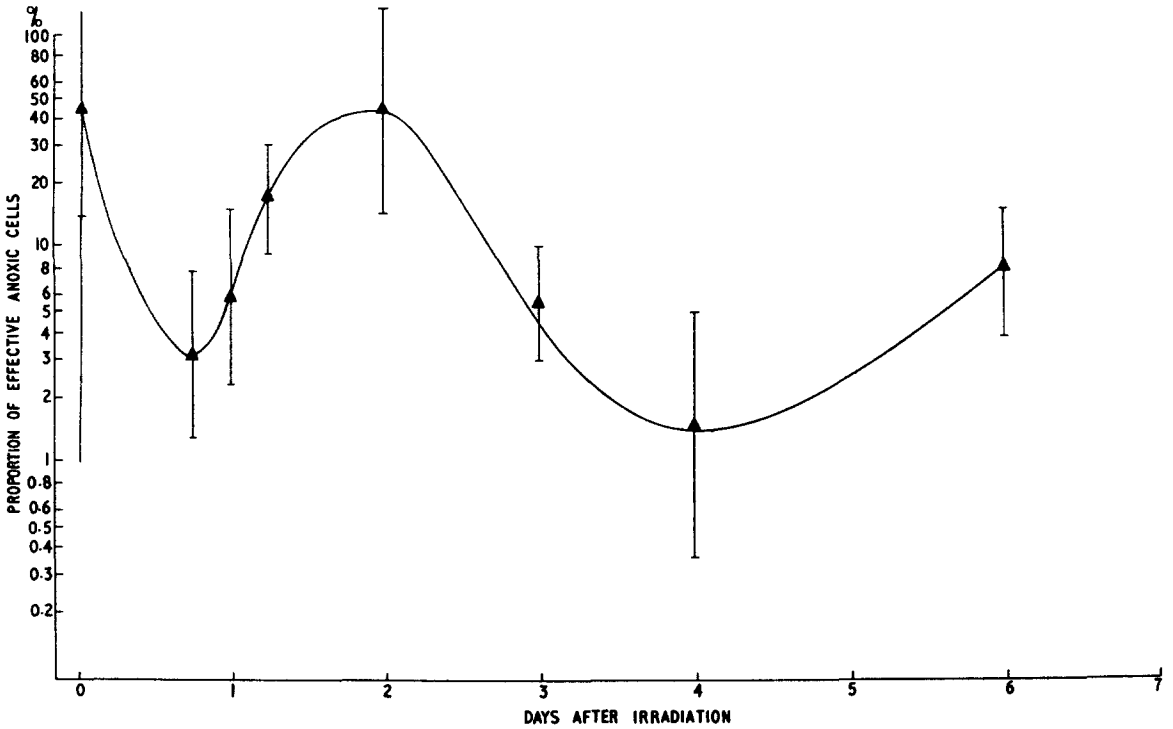


Fig. 4. The proportion of hypoxic cells varies with time after irradiation. The anoxic proportion in tumour RIB$_5$ has been calculated from differences of gross responses to second doses of X-rays given either to tumours in rats with normal air-breathing or to anoxic tumours at various times after a single dose of 1500 *rads.*

number of metabolizing cells in the tumour falls, differences in this respect between neutrons and X-rays will probably get less. However, a first dose of neutrons given to a hypoxic tumour might have great value in starting the processes of reoxygenation. Cell-death from any cause may lead to greater availability of oxygen to surviving cells, independent of changes of supply.

Changes in cells that occur after their death depend both on their type and on their relation to the circulation. Cells in some normal tissues autolyse and disappear, in others they coagulate and their breakdown products remain for considerable periods, eventually being removed by the processes of phagocytosis, growth of new capillaries and fibrous tissue which pathologists call "organization". There are the same differences in tumour tissues. In sarcoma SSB$_1$, few dead cells are to be seen, but the density of intact cells falls toward the centre, whereas in RIB$_5$, there are coagulated masses of necrotic cells in this position. The changes of volume which follow cell death are also variable. The space occupied by cells which autolyse and disappear may be filled by extravascular liquid or dilated blood vessels, presumably depending largely on the mechanical properties of the tumour. The volume occupied by cells which coagulate on death may increase as it does in infarcts in the spleen and kidney and this swelling may interfere with the flow of blood through neighbouring capillaries as it does in the infarcts.

As a consequence of this type of swelling, as well as of the formation of giant-cells and of some cellular proliferation, sarcoma RIB$_5$ continues to enlarge for two days irrespective of the dose of radiation. During this period the blood flow through the existing capillary channels probably deteriorates because of pressure effects and the diminishing supply of oxygen off-sets the reoxygenation resulting from the lesser demand.

With shrinkage of the tumour it seems likely that the blood-flow improves again. This has already been shown by Reinhold in his "mini-tumours" [14]. It is also probable that any impairment of blood-flow caused by venous obstruction would be relieved by the shrinkage, that the total capacity of the capillary bed relative to the arteries which fill it would be reduced, thus increasing the mean flow of blood through capillaries, and that the distribution of flow through the different capillary pathways would become more even. All these processes could lead to reoxygenation.

In sarcoma RIB$_5$, it appears that after a large single dose of X-rays, the two groups of processes leading to reoxygenation, by diminishing the demand and improving the supply, do so at different times and so cause the fluctua-

tions of the hypoxic proportion which are seen in this tumour. In other tumours, notably the mammary carcinoma in mice reported by Howes [7], shrinkage of the tumour occurs at an early stage after large doses of X-rays. Here the two groups of processes probably operate simultaneously and lead to reoxygenation which is more nearly complete within the first few days. Van Putten will discuss his tumour in which a pattern of poor reoxygenation may be seen [15].

These different patterns of reoxygenation, all seen in tumours starting with similar proportions of hypoxic cells, indicate that the value of neutrons for therapy will vary from one type of tumour to another. Used at the beginning of a course of treatment they may well be more efficient than X-rays not only by killing more of the anoxic cells but by inducing a more effective process of reoxygenation. As the number of clonogenic cells decreases during fractionated treatment, reoxygenation will diminish the value of neutrons relative to X-rays, until, were it complete, their use would have no advantage in this respect. However, if the reoxygenation of a tumour were never complete, the use of neutrons might greatly increase the chance of curing the patient.

Our task is now to unravel the principles controlling reoxygenation in different types of tumour so that we can predict the situations in which hypoxic cells reduce the efficiency of treatment. We must also design the clinical trials of fast neutrons, bearing these factors in mind, in a manner which demonstrates their worth.

REFERENCES

1. M. Catterall, Clinical experience with fast neutrons from the Medical Research Council's cyclotron at Hammersmith Hospital. *Europ. J. Cancer* **7,** 227 (1971).
2. K. K. Clifton, R. C. Briggs and H. B. Stone, Quantitative radiosensitivity studies of solid carcinomas *in vivo*: methodology and effect of anoxia. *J. nat. Cancer Inst.* **36,** 965 (1966).
3. H. S. Reinhold, Quantitative evaluation of the radiosensitivity of cells of a transplantable rhabdomyosarcoma in the rat. *Europ. J. Cancer* **2,** 33 (1966).
4. L. M. van Putten and R. F. Kallman, Oxygenation status of a transplantable tumour during fractionated radiation therapy. *J. nat. Cancer Inst.* **40,** 441 (1968).
5. H. D. Suit and M. M. Maeda, Oxygen effect factor and tumour volume in the C_3H mouse mammary carcinomas. *Am. J. Roentgenol.* **96,** 177 (1966).
6. H. B. Hewitt, D. P. S. Chan and E. R. Blake, Survival curves for clonogenic cells of murine keratinizing squamous carcinoma irradiated *in vivo* or under hypoxic conditions. *Int. J. Radiat. Biol.* **12,** 535 (1967).
7. A. E. Howes, An estimation of changes in the proportions and absolute numbers of hypoxic cells after irradiation of transplanted C_3H mouse mammary tumours. *Brit. J. Radiol.* **42,** 441 (1969).
8. S. Hornsey and G. Silini, Studies on cell-survival of irradiated Ehrlich ascites tumour. II. Dose-effect curves for X-ray and neutron irradiations. *Int. J. Radiat. Biol.* **4,** 135 (1961).
9. S. B. Field, T. Jones and R. H. Thomlinson, The relative effects of fast neutrons and X-rays on tumour and normal tissue in the rat. Part II Fractionation: recovery and reoxygenation. *Brit. J. Radiol.* **41,** 597 (1968).
10. R. H. Thomlinson, B. Dixon and T. Zebro, In preparation.
11. N. J. McNally, Personal communication.
12. R. H. Thomlinson and E. A. Craddock, The gross response of an experimental tumour to single doses of X-rays. *Brit. J. Cancer* **21,** 108 (1967).
13. N. T. S. Evans, Personal communication.
14. H. S. Reinhold, Improved microcirculation in irradiated tumours. *Europ. J. Cancer*, in press.
15. L. M. van Putten, P. Lelieveld and J. J. Broerse, Responses of a poorly reoxygenating mouse osteosarcoma to X-rays and fast neutrons. *Europ. J. Cancer* **7,** 153 (1971).

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Hypoxic Protection Against Fast Neutrons of Different Energies— A Review

R. J. BERRY

M.R.C. External Scientific Staff, Radiobiology Laboratory, Churchill Hospital, Oxford, England

Abstract — *The differences between the dependence of Oxygen Enhancement Ratio (OER) on fast neutron energy in P-388 murine leukaemia cells, and the lack of effect of neutron energy upon OER reported in human kidney-derived T-1 cells correlate well (perhaps fortuitously) with differences in responses of the same cells to irradiation with monoenergetic track-segments of beams of deuterons and alpha-particles whose ionization densities (LET) are known. However, irradiation of the P-388 tumour cells with collimated beams of fast neutrons at 10 cm depth in a tissue-equivalent phantom yields OER values lower than predicted by consideration of average LET and relatively independent of neutron energy.*

For irradiation from a planar array of neutron-emitting ^{252}Cf sources in a tissue-equivalent holder simulating a brachytherapy implant, the OER is disappointingly high (2·0), although still significantly lower than for irradiation with ^{226}Ra gamma rays in the same geometry.

IT HAS been firmly established that hypoxia offers less protection against lethal effects of irradiation with fast neutrons, than against irradiation with X- or gamma rays — whether one measures lethality by the decrease in reproductive capacity of mammalian cells *in vitro* or by the death of the irradiated whole animal. Many authors have compared, for a single or several biological end-points, the magnitude of hypoxic protection (conversely measured as the *Oxygen Enhancement Ratio*, OER) for X- or gamma rays and *one* energy of fast neutrons, but only two groups of workers have systematically studied the effects of neutrons of several different energies — with conflicting conclusions! Both of these groups have used as their biological end-point a measure of the survival of reproductive capacity of individual mammalian cells. As the majority of the effects of ionizing radiations upon tissues which are of interest to the radiotherapist are the consequence of loss of reproductive capacity of cells in those tissues, this review will be limited to a discussion in depth of the differences between the results of these two groups. Other reviewers have cast their nets wider; Broerse [1], Barendsen [2] and Bewley [3] have each covered admirably the data available at the time of their writing, the latter presents the data in a particularly concise tabular form.

1. THE FIRST REPORTS — A VARIATION IN OER WITH FAST NEUTRON ENERGY

In 1963, Berry and Andrews [4] and later Berry *et al.* [5] presented data from a series of experiments in which the survival of reproductive capacity of murine P-388 leukaemia cells was assayed *in vivo* after irradiation with fast

neutrons of three different energies. The irradiation was delivered to this ascitic tumour *in situ* in a donor mouse, 7 days after intraperitoneal inoculation of the donor with 10^6 tumour cells. The volume of the ascitic tumour was such that the cells behaved as a hypoxic population i.e. their survival was not increased by killing the tumour-bearing donor 20 minutes prior to irradiation. The tumour cells could be oxygenated briefly, however, by the intraperitoneal inoculation of hydrogen peroxide. Without damaging the tumour cells, the action of tumour catalase upon the H_2O_2 released free oxygen in the ascitic fluid which maintained the tumour cells in a well-oxygenated state for 8–10 minutes, until this oxygen supply had been respired away. This limited the aerobic experiments to relatively short radiation exposures and necessitated high neutron dose-rates. Despite this, however, there was a clear difference in the response of the hypoxic tumour cells to the neutrons of the three different energies which led to widely differing OER values from 1·1 for fission-spectrum neutrons to a calculated value equal to about 1·7 for monoenergetic 14 MeV neutrons from the D–T reaction, compared with an OER of 2·3–2·5 for irradiation with X-rays of different energies and ^{60}Co gamma rays.

These data were corroborated by experiments of Andrews *et al.* [6] in which irradiation of the tumour cells under 'aerated' conditions was achieved by irradiation of donor animals only 1–2 days after they had been inoculated with their tumour, and harvesting the cells by peritoneal washing. Although their data were limited, they clearly showed different values of OER for irradiation with fission-spectrum and with 14 MeV neutrons, respectively.

2. ANOTHER REPORT — NO VARIATION IN OER WITH FAST NEUTRON ENERGY

In 1966, Barendsen and Broerse [7] reported data for clonal survival *in vitro* of T-1 cells derived from human kidney. Initially studying fast neutron beams of four energies, they found no significant variation in OER (1·5–1·6) over the same neutron energy range studied by Berry *et al.* Subsequent studies with other neutron beams by Broerse and Barendsen [8] have also yielded similar OER values.

3. RECENT FAST NEUTRON EXPERIMENTS WITH THE P-388 LEUKAEMIA SYSTEM

From 1968–70, Professor J. T. Brennan assembled an *ad hoc* working group* to attempt to answer the question "what fast neutron source which is available today gives the best combination of physical and biological parameters for a new clinical trial of fast neutron radiotherapy?". The geometry of irradiation with beams of limited cross sectional area (5×5 and 15×15 cm) at a long distance from the neutron source (125 cm), using a large phantom, filled with a tissue-equivalent glycerol-urea-water mixture, faithfully reflected the clinical practice of the radiotherapist. The biological test system was the P-388 murine leukaemia, irradiated either in air (to simulate a superficial tumour) or at a depth of 10 cm in the tissue-equivalent phantom (to simulate a deep-seated tumour). For operational reasons connected with the difficulties of carrying out such experiments at remote sites in two countries, irradiation under 'aerated' conditions was delivered to tumour cells held *in vitro* while irradiation under 'hypoxic' conditions was delivered to tumour cells *in situ* in the 7-day tumour-bearing mouse. Berry had earlier shown that the tumour cell radiosensitivity did not alter during a 24-hour holding period *in vitro*, and that the OER for irradiation with 250 kVp X-rays was the same when aerobic and hypoxic irradiations were carried out *in vitro* as when irradiations were carried out *in situ* in the 7-day tumour-bearing mouse, with or without hydrogen peroxide [9]. In all cases, however, survival of reproductive capacity of the cells was assayed *in vivo* by injection of serial dilutions of tumour cells into recipient mice of the same highly-inbred DBA/2J strain from which the tumour arose. Five neutron energies, from fission-spectrum (*circa* 1 MeV) to *circa* 25 MeV were studied. The survival of tumour cell reproductive capacity was measured down to levels of 10^{-4}–10^{-7} in every case, and the survival curves fitted to the data by the method of Pike and Alper [10] indicated that in each case, oxygen was acting

*The members of the *ad hoc* working group were:
Dr. R. J. Berry, M.R.C. External Scientific Staff, Radiobiology Laboratory, Churchill Hospital, Oxford, U.K.
Professor J. T. Brennan, Department of Radiology, Univ. of Pennsylvania Hospital, Philadelphia, Penna., U.S.A.
L. J. Goodman, Radiological Research Accelerator, Facility, Columbia University, Upton, Long Island, N.Y., U.S.A.
Dr. D. F. Herring, Enviro-Med Inc., La Jolla, California, U.S.A.
A. C. Lucas, Edgerton, Germeshausen and Grier, Inc., Goleta, California, U.S.A.
W. M. Quam, E. G. & G. Inc., Goleta, Calif., U.S.A.

as a true-dose-modifying factor, i.e. for irradiation under aerobic *and* hypoxic conditions, the data could be fitted no less well by a pair of curves having a common extrapolation number. For irradiation 'in air' with these collimated fast neutron beams, the OER varied inversely with neutron energy, from 1·1 for fission-spectrum to 2·0 for the *circa* 25 MeV neutrons produced by bombarding a beryllium target with 50 MeV deuterons from the Texas A & M variable energy cyclotron. These data are summarised in Table 1, and are compared with the results obtained by Barendsen and Broerse with human T-1 cells in Fig. 1.

4. LET vs. OER—THE CONFLICT RESOLVED, OR A FORTUITOUS COINCIDENCE

Barendsen *et al.* [11] had studied the response of T-1 cells to irradiation with mono-energetic track-segments of beams of deuterons and alpha-particles of known ionization density (*Linear Energy Transfer* — LET) using the M.R.C. cyclotron at Hammersmith Hospital. In an attempt to explain the apparent differences between the response of T-1 cells and that of the P-388 murine leukaemia cells to neutrons of different energies, these latter cells were irradiated *in vitro* with track-segments of deuteron and alpha-particle beams using the same experimental set-up which Barendsen *et al.* had used for the T-1 cells [12]. Additional experiments were performed using the helium ion beam from the tandem Van de Graaff generator at A.E.R.E., Harwell. Survival curves were fitted to the data exactly as in the fast neutron experiments; the data are summarized in Table 2, and compared with the results of Barendsen *et al.* in Fig. 2. The greatest difference between the responses of the two cell types is that for the leukaemic cells the OER falls to near 1·0 at an LET value

Table 1. *Survival curve parameters for P-388 leukaemia cells irradiated with fast neutrons of different energies*

Neutron source	Mean neutron energy (MeV)	Dose-rate (rads/min)	Extrapolation number (n)	D_0 (rads) Aerobic	D_0 (rads) Hypoxic	OER	Therapeutic gain factor*
1. Fission-spectrum A.F.R.R.I., Bethesda 'TRIGA' reactor	~ 1	40–60	0·9 (0·3–1·7)	91	102	1·1 (0·9–1·4)	2·8
2. M.R.C. Cyclotron Hammersmith Hospital 16 MeV D–Be	~ 6	15–40	1·9 (0·4–9·2)	77	125	1·6 (1·4–1·8)	1·9
3. 14 MeV (D–T) Lawrence radiation lab. I.C.T.	14	10–15†	3·1 (1·0–9·9)	87	153	1·8 (1·6–2·0)	1·7
4. 14 MeV (D–T) neutrons A.W.R.E., Aldermaston I.C.T.	14	5–15†	0·8 (0·2–2·7)	76	139	1·8 (1·5–2·2)	1·7
5. Texas A. & M. variable energy cyclotron							
(a) 30 MeV D–Be	~15	10–15	2·2 (1·2–4·3)	94	168	1·8 (1·7–1·9)	1·7
(b) 50 MeV D–Be	~25	25	2·4 (1·0–6·0)	102	201	2·0 (1·8–2·2)	1·5

Figures in brackets represent 95% confidence intervals.
*Relative to OER=3·1 for 250 kVp X-rays (aerobic irradiation *in vitro*, hypoxic irradiation *in vivo*).
†Source-tumour distance *circa* 50 cm, not 125 cm as for all other irradiations.

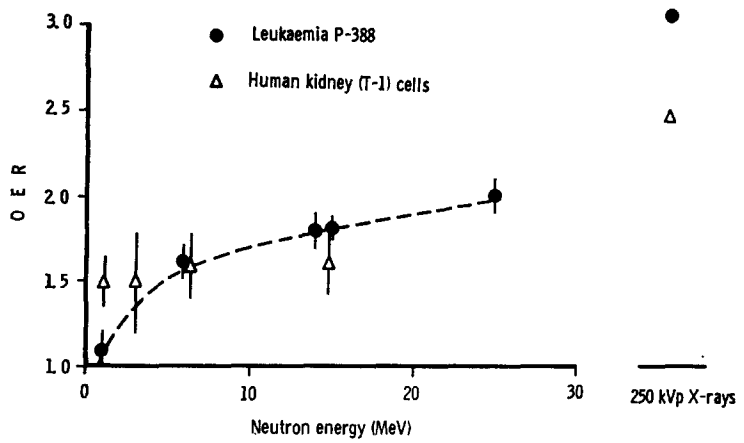


Fig. 1. Oxygen enhancement ratio for responses to external beams of fast neutrons of different energies. Vertical bars indicate standard errors. The data for human kidney (T-1) cells are from ref. 7, those for P-388 cells are from recent studies reported in this paper.

Table 2. Survival curve parameters for P-388 leukaemia cells exposed to irradiation with monoenergetic track-segments of beams of deuterons and alpha-particles

Radiation	LET (keV/μm)	Extrapolation number (n)	D_0 (rads) Air	D_0 (rads) Nitrogen	OER
Deuterons	6	13·7 (5·8–32·0)	123	260	2·1 (2·0–2·3)
	14	7·9 (1·0–61·0)	118	231	2·0 (1·6–2·4)
Alpha	26	1·6 (0·6– 4·1)	138	321	2·3 (1·9–2·9)
	45	2·1 (0·3–14·4)	93	232	2·5 (2·0–3·3)
	52*	1·0 (0·1– 6·1)	85	175	2·1 (1·6–3·1)
	62	0·9 (0·1– 5·7)	121	191	1·6 (1·0–2·2)
	85*	0·7 (0·1– 4·2)	87	97	1·1 (0·8–1·6)
	86	1·9 (0·7– 4·6)	80	86	1·1 (0·9–1·3)

Figures in brackets represent 95% confidence intervals.
*Irradiations using tandem Van de Graaff accelerator at A.E.R.E., Harwell, all other irradiations using M.R.C. Cyclotron, Hammersmith Hospital.

circa 80 keV/μm, whereas it does not reach this level for LET's below *circa* 160 keV/μm in the human kidney-derived T-1 cells. Bewley [13] has calculated that approximately one-third of the dose from fission-spectrum fast neutrons is accounted for by events having ionization densities of 80–150 keV/μm in tissue, and nearly another 30% by events of 50–80 keV/μm size. There would still be significant hypoxic protection against these events in the human kidney cells, whereas in the leukaemic cells the effects of these events would be largely independent of the presence or absence of oxygen. Thus, although the response to particulate radiations of known ionization densities is known to be an inadequate predictor for the response of mammalian cells to fast neutrons, differences seen between the responses to fast neutrons of different energies in these two mammalian cell lines are closely paralleled by differences in the responses of the same cells to monoenergetic charged particles.

5. OER FOR SURVIVAL AFTER IRRADIATION WITH FAST NEUTRONS AT A DEPTH IN A TISSUE-EQUIVALENT PHANTOM

In the recent experiments with the P-388 leukaemia system, the tumour cells were also irradiated with collimated beams of fast neutrons at 10 cm depth in the tissue-equivalent phantom, to attempt to simulate the geometry of a deep-seated human tumour treated by radiotherapy. The data are presented in Table 3, and should be compared with the results obtained irradiating the same tumour cells 'in air' with the same neutron beams, which are recorded in Table 1. Where possible, a higher dose-rate was used for irradiation of the cells at the 10 cm depth in the phantom, so that the overall exposure times were similar to those for irradiation of the tumour cells 'in air'. For all except the highest neutron energy studied, the OER was relatively invariant and was lower than expected. For fission-spectrum neutrons, the OER at 10 cm depth was higher than for irradiation with the same neutrons 'in air' due to the higher proportion of the total dose delivered by gamma rays. For all the other neutron energies, however, there was an apparent decrease in OER at 10 cm depth, compared with the OER 'in air', in spite of some increase in the gamma dose and in spite of the absence of any dramatic changes in the LET spectrum measured for each neutron energy at the two sites with a Rossi-type spherical proportional counter [14]. These data make one suspect that the correlation between responses of T-1 and P-388 cells to charged-particle irradiation of known LET and their response 'in air' to fast neutrons of different energies may have been fortuitous — unless the physical measurement of distribution of event sizes (LET distribution) is itself either too insensitive or inaccurate. If taken at face value,

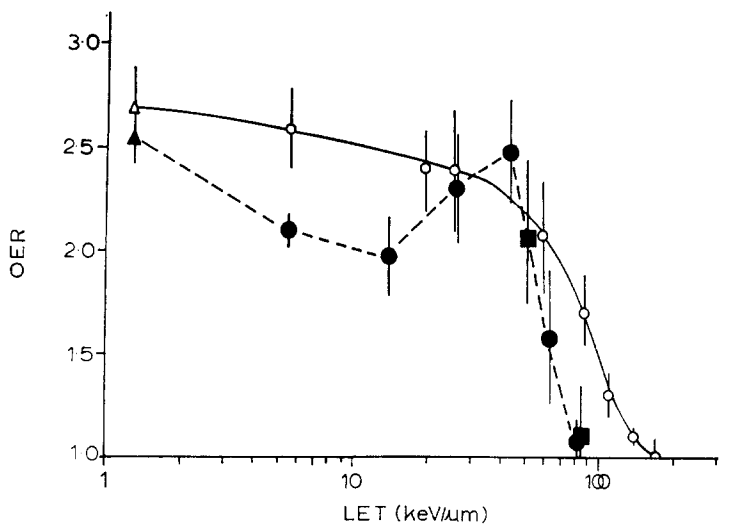


Fig. 2. Oxygen enhancement ratio (OER) for irradiation with monoenergetic charged heavy particles, as a function of their ionization density (LET). Open circles represent charged particle data for human kidney (T-1) cells from Barendsen et al. [2], *the open triangle represents data for human T-1 cells from Barendsen* et al. *for* 250 *kVp X-rays. The solid triangle represents* 250 *kVp X-ray data for P-*388 *leukaemia cells irradiated* in vitro [3]. *The closed circles represent data for P-*388 *leukaemia cells obtained using deuteron and alpha-particle beams from the M.R.C. Cyclotron, Hammersmith Hospital. The closed squares represent P-*388 *data obtained with the alpha-particle beam from the tandem Van de Graaff generator at A.E.R.E., Harwell. Vertical bars indicate standard errors.*

Table 3. Survival curve parameters for P-388 leukaemia cells irradiated at 10 cm depth in a tissue-equivalent phantom with fast neutrons of different energies

Neutron source	Extrapolation number (n)	D_0 (rads) Aerobic	Hypoxic	OER	Therapeutic gain factor*
1. Fission-spectrum A.F.R.R.I., Bethesda 'TRIGA' reactor	2·8 (1·5 – 5·5)	96	119	1·2 (1·2–1·3)	2·6
2. M.R.C. Cyclotron Hammersmith Hospital 16 MeV D–Be	0·8 (0·5 – 1·3)	99	127	1·3 (1·2–1·3)	2·4
3. 14 MeV (D–T) neutrons† Lawrence radiation lab. I.C.T.	1·2 (0·2 – 6·5)	115	146	1·3 (1·1–1·5)	2·4
4. 14 MeV (D–T) neutrons† A.W.R.E., Aldermaston I.C.T.	1·0 (0·05–21·0)	73	113	1·5 (0·5–4·3)	2·0
5. Texas A. & M. variable energy cyclotron					
(a) 30 MeV D–Be	0·8 (0·3 – 2·6)	139	154	1·1 (0·9–1·3)	2·8
(b) 50 MeV D–Be	3·5 (0·9 –14·0)	112	181	1·6 (1·4–1·9)	1·9

Figures in brackets represent 95% confidence intervals.
*Relative to OER=3·1 for 250 kVp X-rays (aerobic irradiation *in vitro*, hypoxic irradiation *in vivo*).
†Source-tumour distance *circa* 50 cm, not 125 cm as for all other irradiations.

Table 4. Preliminary survival curve parameters for P-388 leukaemia cells irradiated in a tissue-equivalent phantom 0·6 or 2·5 cm from a planar array of 226-radium or 252-californium needles

Distance	Radiation	Dose-rate (rads/hr)	D_0 (rads) Air	Nitrogen	OER	RBE Air	RBE Hypoxic	Therapeutic gain factor
0·5 cm	^{226}Ra	300–420	220	561	2·6	(1·0)	(1·0)	—
	^{252}Cf	200–260	80	161	2·0	2·7	3·5	1·3
2·5 cm	^{226}Ra	50	267	—	—	(1·0)	—	—
	^{252}Cf	35	112	—	—	2·4	—	—

the recent P-388 results suggest that for irradiation of a deep-seated human tumour with fast neutrons, even higher Therapeutic Gain Factors may be obtained than have been predicted from experiments using cellular and animal radiobiological systems whose dimensions are small. McNally and Bewley have, however, shown no significant decrease in OER at a depth of 8·7 cm in a tissue-equivalent phantom, using a 15 × 15 cm beam from the M.R.C. Cyclotron, Hammersmith Hospital with a different murine ascites tumour [15].

6. OER FOR MIXED NEUTRON-GAMMA IRRADIATION FROM A SIMULATED TISSUE IMPLANT WITH ^{252}CALIFORNIUM NEEDLE SOURCES

Neutron-emitting ^{252}Cf needles have recently become available* for clinical radiobiological evaluation as a possible alternative to gamma-emitting ^{226}Ra sources used in many radiotherapy departments for intracavitary, interstitial and surface mould brachytherapy. Atkins [16] has reported that for irradiation of HeLa cells *in vitro* at a dose-rate of 16 rads/hr, the OER was *circa* 1·3, although for technical reasons it was not possible to make the experimental set-up tissue-equivalent. With the help of Dr. G. D. Oliver, Jr., at the M. D. Anderson Hospital, Houston, Texas, we had the opportunity of irradiating P-388 leukaemia cells *in vitro* in a tissue-equivalent plastic holder at distances of 0·5 and 2·5 cm from arrays of ^{252}Cf or ^{226}Ra needles simulating planar implants used in clinical brachytherapy. After irradiation, the survival of reproductive capacity of the cells was measured *in vivo* by serial dilution-assay of the tumour cells in DBA/2J mice (the strain from which the tumour arose). The results are shown in Table 4; the Therapeutic Gain Factor was disappointingly low when compared with those for the same cells with collimated external fast neutron beams. In a geometrical arrangement which realistically represented that arrangement used in clinical brachytherapy, a high proportion of the total dose delivered was due to gamma rays, and this in turn resulted in a relatively high OER for survival after irradiation by the ^{252}Cf sources. The effect of this mixed neutron-gamma radiation was also surprisingly dependent upon dose-rate; the Relative Biological Effectiveness of the ^{252}Cf radiation in a geometrical arrangement simulating a tissue implant did not increase as the dose-rate decreased from *circa* 200 rads/hr to 35 rads/hr, due to the fact that the slopes of the survival curves for ^{252}Cf and ^{226}Ra-irradiated cells equally became shallower as the dose-rate decreased.

*U.S. Atomic Energy Commission, Savannah River Operations Office, Post Office Box A, Aiken, South Carolina 29801, U.S.A.

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REFERENCES

1. J. J. Broerse, Effects of energy dissipation by monoenergetic neutrons in mammalian cells and tissues. Thesis, Univ. Amsterdam. Published by the Radiobiological Institute TNO of the Organization for Health Research TNO, Rijswijk (Z.H.), The Netherlands (1966).
2. G. W. Barendsen, Responses of cultured cells, tumours and normal tissues to radiations of different linear energy transfer. In *Current Topics in Radiation Research* (Edited by M. Ebert and A. Howard) Vol. IV, pp. 293–356. North-Holland, Amsterdam (1968).
3. D. K. Bewley, Fast neutron beams for therapy. In *Current Topics in Radiation Research* (Edited by M. Ebert and A. Howard) Vol. V, pp. 249–292. North-Holland, Amsterdam (1970).
4. R. J. Berry and J. R. Andrews, The effect of radiation ionization density (LET) on the reproductive capacity of mammalian tumour cells irradiated and assayed *in vivo*. *Brit. J. Radiol.* **36,** 49 (1963).
5. R. J. Berry, D. K. Bewley and C. J. Parnell, Reproductive capacity of mammalian tumour cells irradiated *in vivo* with cyclotron-produced fast neutrons. *Brit. J. Radiol.* **38,** 613 (1965).

6. J. R. Andrews, J. N. Correa and J. A. Belli, Fast neutron irradiation, cell reproductive capacity, and the oxygen effect. In *Biological Effects of Neutron and Proton Irradiations*, Vol. II, pp. 283–291. STI/PUB/80, International Atomic Energy Agency, Vienna (1964).
7. G. W. Barendsen and J. J. Broerse, Dependence of the oxygen effect on the energy of fast neutrons. *Nature, Lond.* **212,** 722 (1966).
8. J. J. Broerse and G. W. Barendsen, Measurements of biological effects and physical parameters of neutrons from the $^{9}Be(^{3}He, n)^{11}C$ reaction; possibilities for fast neutron radiotherapy. *Int. J. Radiat. Biol.* **13,** 189 (1967).
9. R. J. Berry, Hypoxic protection and recovery in tumour cells irradiated at low dose-rates and assessed *in vivo*. *Brit. J. Radiol.* **41,** 921 (1968).
10. M. C. Pike and T. Alper, A method for determining dose-modification factors. *Brit. J. Radiol.* **37,** 458 (1964).
11. G. W. Barendsen, C. J. Koot, G. R. van Kersen, D. K. Bewley, S. B. Field and C. J. Parnell, The effect of oxygen on impairment of the proliferative capacity of human cells in culture by ionizing radiations of different LET. *Int. J. Radiat. Biol.* **10,** 317 (1966).
12. R. J. Berry, Survival of murine leukaemia cells *in vivo* after irradiation *in vitro* under aerobic and hypoxic conditions with monoenergetic accelerated charged particles. *Radiat. Res.* **44,** 237 (1970).
13. D. K. Bewley, Fast neutrons-L.E.T. distributions and the response of mammalian cells. In *Biophysical Aspects of Radiation Quality, Second Panel Report*, International Atomic Energy Agency, Vienna, pp. 65–86.
14. H. H. Rossi, Distribution of radiation energy in the cell. *Radiology* **78,** 530 (1962).
15. N. J. McNally and D. K. Bewley, A biological dosimeter using mammalian cells in tissue culture and its use in obtaining neutron depth dose curves. *Brit. J. Radiol.* **42,** 289 (1969).
16. H. Atkins, Personal communication (1969).

Europ. J. Cancer Vol. 7, pp. 153–160. Pergamon Press 1971. Printed in Great Britain

Response of a Poorly Reoxygenating Mouse Osteosarcoma to X-Rays and Fast Neutrons

L. M. VAN PUTTEN, P. LELIEVELD and J. J. BROERSE
Radiobiological Institute TNO, 151 Lange Kleiweg, Rijswijk Z.H., The Netherlands

Abstract — *A transplantable osteosarcoma, which had previously been shown to reoxygenate its anoxic cells very slowly after a single large dose of X-rays, was studied after X- and neutron-irradiation. It was shown to be relatively resistant to fractionated X-ray therapy, probably due to anoxia. Nevertheless it could be shown that a small degree of reoxygenation must also occur in this tumour. Its response to treatment with* 15 *MeV neutrons was unusual; RBE values for cell survival of* 10^{-3} *were for fractionated irradiation* 2·5, *for single dose* in vivo *irradiation* 1·8, *and for* in vitro *irradiation* 1·6. *The small difference between the RBE values for the well oxygenated osteosarcoma cells* in vitro *and for the partially hypoxic cells* in vivo *is associated with an unexpectedly high OER of* 2·0 *for neutron irradiation of these cells* in vitro *as compared with the OER for X-rays of* 2·4. *The gain factor for neutrons, that is the ratio of OER values for X-rays and neutrons, is only* 1·2, *indicating that although hypoxia may be a limiting factor for the cure of this tumour by fractionated X-ray theory, the use of neutrons does not offer a major advantage. The search for other models of radiotherapy-resistant tumours should be continued.*

INTRODUCTION

REOXYGENATION of tumours in the course of fractionated radiotherapy is a well known phenomenon. It has been found in all of the tumours tested for its occurrence with one exception; a transplantable mouse osteosarcoma (see Table 1). Since a tumour which reoxygenates slowly or not at all is of great interest for the application of fast neutrons, a number of further studies were devoted to this osteosarcoma. As described earlier [14], this tumour, which contains on the average between 10 and 20% anoxic cells, was found to reoxygenate slowly after a single dose of 1000 rads. After fractionated irradiation (4 daily doses of 250 rads) the mean anoxic cell fraction was 32%, a figure which permitted no conclusion on the occurrence of reoxygenation after these doses.

The present report is concerned with further studies, to learn whether reoxygenation occurs in this tumour during fractionated irradiation with daily doses of similar magnitude as used in clinical radiotherapy. In addition this report describes the effects of 15 MeV neutron irradiation of the osteosarcoma.

MATERIAL AND METHODS

Tumours

Osteosarcoma C22LR was used in serial transplantation passages 76–85 in (CBA/Rij × C57BL/Rij)F_1 hybrid mice, in which the tumour was originally induced [14].

Tumour cell survival was estimated by an endpoint dilution assay after suspending the tumour cells by the method of Reinhold [15]. In this estimate survival refers to reproductive

Table 1. *Evidence for tumour reoxygenation*

Host animal of original tumour	Tumour*		References
	Evidence for tumour reoxygenation		
	Mouse		
$C3H_{km}$	Sarcoma KHT	(L.T.)	van Putten and Kallman [1, 2]; Kallman *et al.* [3]
DBA	Mammary carcinoma	(E.T.)	Suit *et al.* [4]
C3H/J		(E.T.)	Suit and Maeda [5]
C3H/He		(E.T.)	Suit and Schiavone [6]
C3H/(Barts) $\times$ $(C3H \times O)F_1$ R.H.		(Prim.) and (E.T.)	Hawkes *et al.* [7]; Cheshire and Lindop [8]
C3H (Hammersmith)		(E.T.)	Howes [9]
C3H/He	Fibrosarcoma	(E.T.)	Suit and Suchato [10]
	Squamous cell carcinoma	(E.T.)	
	Rat		
John's strain	Sarcoma RIB_5	(L.T.)	Thomlinson [11, 12]
WAG/Rij	Rhabdomyosarcoma	(L.T.)	Barendsen and Broerse [13]
	No evidence for reoxygenation		
$(CBA/Rij \times C57BL/Rij)F_1$ mice	Osteosarcoma C22LR	(L.T.)	van Putten [14]

*L.T. = Late transplant passages.
E.T. = Early transplants.
Prim. = Primary tumours.

integrity of the cells as assessed by their capacity to give rise to a tumour within 100 days after inoculation in an isologous recipient. The quantitative suspension technique used for estimating the total number of reproductively intact cells per tumour was described earlier [16]. This technique was used for evaluating cell survival after fractionated radiotherapy.

Tumour volumes were estimated by caliper measurements of the tumour diameter in three dimensions; multiplication of these values and correction by a factor of 0·525 gave a dependable measure for tumour volume as verified by excision and volume measurements by submersion. For all volume measurements 5 to 10 tumours per treatment group were followed simultaneously in order to obtain a reproducible average.

Irradiations

X-rays were administered by a Philips-Müller machine operated under the following conditions: 300 kVp; 10 mA; HVL 3·0 mm Cu; dose rate 60 rads per min for *in vivo* irradiation and 130 rads per min for *in vitro* irradiation. The 15 MeV neutrons were produced through the D–T reaction using a Van de Graaff generator working at 400 kV and 250 μA. Due to the limited output of the neutron generator, the irradiations were carried out at small distances from the target. For the *in vitro* experiments the tumour cell suspensions were irradiated in culture dishes at a dose rate of 20 rads/min. The *in vivo* neutron irradiations were carried out at a dose rate of 15 rads/min. To compensate for the inhomogeneities in the neutron flux density distribution and to obtain multi-lateral exposure, the mice in centrifuge tubes were mounted on a styrofoam irradiation cylinder which was rotated in front of the target during the irradiations. The neutron dosimetry was carried out with a tissue equivalent ionization chamber and sulphur activation detectors [17, 18].

Cell oxygenation in vitro

The oxygenation status of the cells during *in vitro* irradiation was controlled by the following methods: tumour cells suspended in Hanks' balanced salt solution with 5% calf serum were irradiated in a volume of 4–7 ml in a shallow culture dish with a surface of 15 cm^2.

Before and during aerobic irradiation the cooled cell suspensions were agitated by a shaker and by a flow of humidified air of 600 ml per min, which was led over the surface of the suspension. For hypoxic irradiation the cell suspensions kept at room temperature were similarly agitated but gassed with pure nitrogen. A Hersch cell measured the oxygen content of

the outflow gas and after a 10 min equilibration period prior to irradiation an oxygen content below 20 ppm was reached. The culture dishes contained no materials which dissolve large quantities of oxygen; the walls were made of glass and the bottom of Melinex (polyethylene terephthalate).

High pressure oxygen treatment

Mice anaesthetized with pentobarbital were irradiated in a stainless steel container covered with a 1 cm thick glass plate. After flushing the container with oxygen the pressure was slowly raised to 3 atm gauge pressure (4 atm absolute). After irradiation the pressure was lowered over a period of 20 min. Control irradiations were performed in the same container on similarly anaesthetized mice breathing air.

RESULTS

Daily local X-irradiation of tumours located subcutaneously in the flank of anaesthetized mice was performed for 1, 2, 3 or 4 weeks with doses of 300 rads five times weekly. The tumour volume changes under these treatments are presented in Fig. 1. It is evident that this tumour is very resistant, it is for example much less affected than the rat rhabdomyosarcoma described by Barendsen and Broerse, which regresses after 20×300 rads to a volume of less than 10% of its volume at the start of treatment [13]. Although the radiosensitivity *in vitro* for the cells of the two tumours is rather similar [14, 19], the osteosarcoma does not decrease in size below the pre-irradiation volume, even after four weeks of treatment. This might be ascribed to persistent anoxia in the tumour associated with the poor reoxygenation observed after single high doses [14], but an alternative explanation for the poor response, might be found in rapid repopulation, associated with the high growth rate of the osteosarcoma. Its volume doubling time is approximately 1·8 days.

In an attempt to differentiate between these two possibilities a study was made of the comparative effect of 4 vs. 5 doses per week. If repopulation was important, the longer radiation free interval would cause a marked difference between these two regimens. If however, anoxia is an important factor in the radiation resistance, the last dose of each week would in this slowly reoxygenating tumour certainly be less effective than the earlier doses, which after the radiation-free weekend might find a frac-

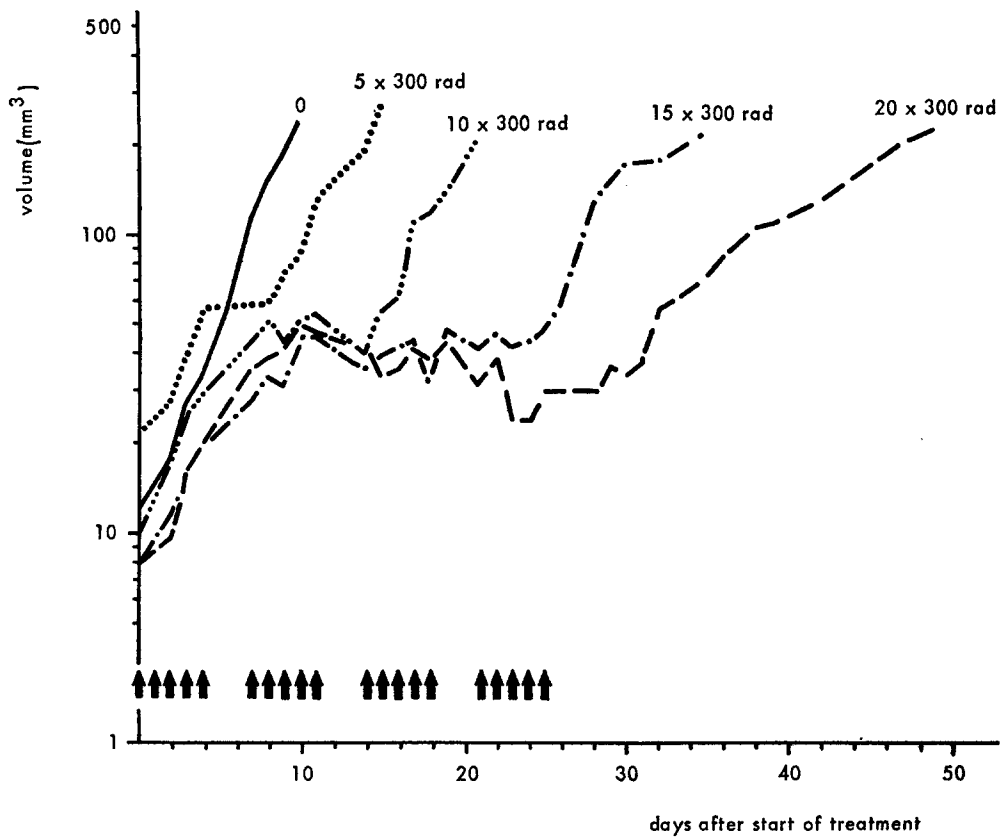


Fig. 1. The effect of fractionated radiotherapy with five times 300 *rads of* 300 *kV X-rays per week on the volume of osteosarcoma isotransplants (local irradiation under pentobarbital anaesthesia). Even after* 4 *weeks of treatment tumour volume does not fall below the pretreatment value.*

tion of well-oxygenated cells. The results of these studies are presented in Fig. 2; for comparison the results of chemotherapy treatment with 4 and 5 doses per week are also presented. In these studies the mice were anaesthetized for irradiation, but not for chemotherapy; there was no significant effect on body weight of the treatments. The difference between the two irradiation regimens is much smaller than between the chemotherapy groups, suggesting that indeed anoxia may be very important in the radiation resistance of this tumour.

Attempts to obtain more complete oxygenation during fractionated treatment of the tumour were performed by irradiation of tumours under high pressure oxygen. This was carried out as local irradiation of tumours located on the leg of mice under nembutal anaesthesia, both in mice breathing 4 atmospheres of oxygen and in control mice breathing air at ambient pressure. The results are presented in Fig. 3 (lines A and B). The tumours appear more sensitive under high pressure oxygen, but the most interesting results are seen in the different types of control mice. A comparison of the results in anaesthetized controls, line A, with line C, which shows the cell survival in tumours of total-body irradiated, non-anaesthetized control mice, indicates that anaesthesia caused a marked decrease in radiosensitivity, probably through anoxia. Some constriction of the mouse leg which carried the tumour, by the lead which shielded the rest of the body may have contributed to this anoxia, although such constriction was avoided as much as possible. Although the high pressure oxygen treatment was therefore not successful, this experiment demonstrated the complications caused by anaesthesia and this led us to study the effect of neutrons in non-anaesthetized mice. In Fig. 3 the effect of fractionated total body irradiation with fast neutrons is represented (line D). The RBE for 0·01% survival is 2·5, a disappointingly low value for an anoxic tumour. In order to analyse the reasons for this finding additional studies with fast neutrons were performed: the effect of single doses *in vivo* are presented in Fig. 4 and the results of *in vitro* irradiation of osteosarcoma cells with 15 MeV neutrons under different conditions of oxygenation are given in Fig. 5. Table 2 presents the parameters of the dose-effect curves for X-rays and neutrons and the derived values for RBE, OER, and gain factor.

The most striking result is the finding of an OER for fast neutrons of 2·0, which is very high, especially in comparison with the OER of 2·4 for X-rays. This may explain the relatively

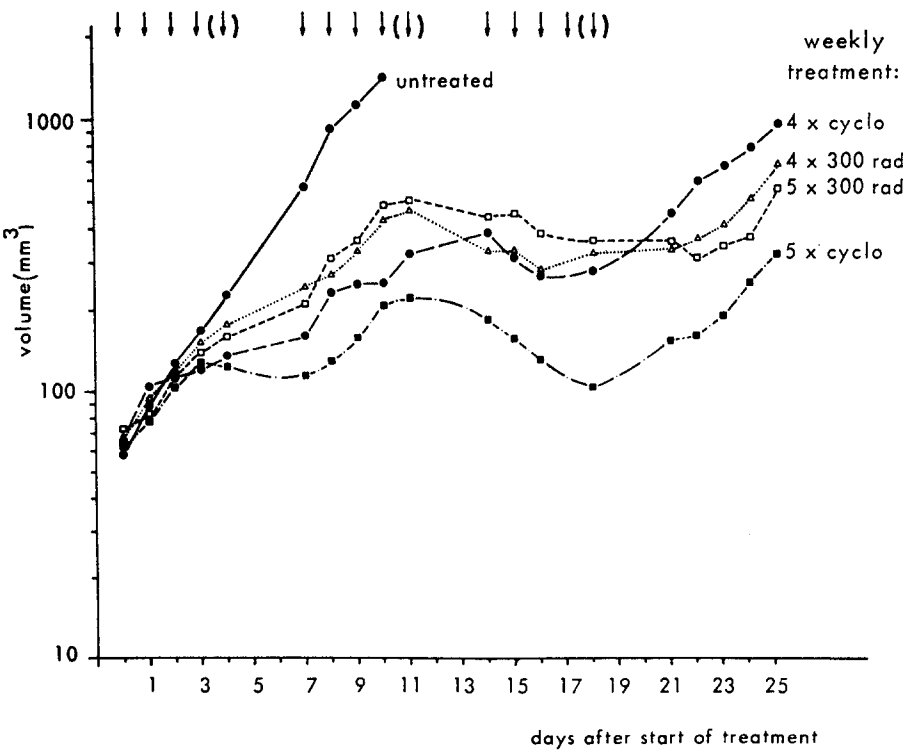


Fig. 2. A comparison of 4 and 5 weekly doses of 300 rads of X-rays with 4 and 5 weekly doses of cyclophosphamide (25 mg/kg per dose i.p.). The differences between 4 and 5 weekly doses is much more marked after chemotherapy than after irradiation.

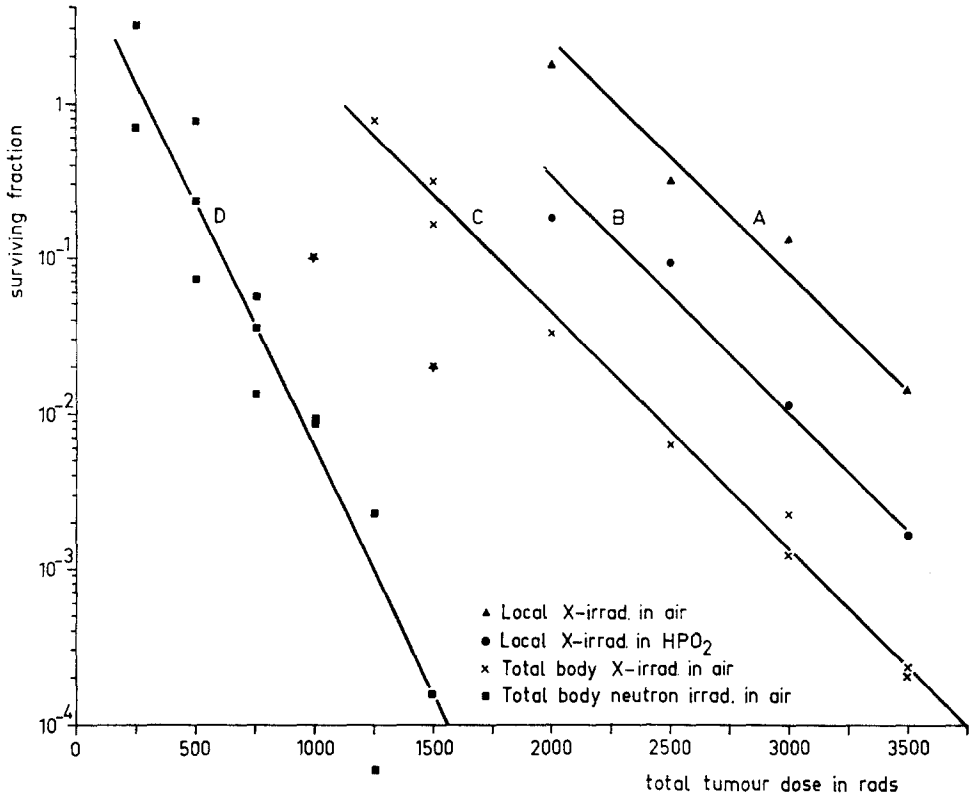


Fig. 3. The effect of 5 daily doses of irradiation on tumour-cell survival. Survival is plotted as the ratio of the number of reproductively intact tumour cells in treated tumours immediately after the last dose to the number in control animals at the time of the first treatment; in the treatment period this cell number increases in the control tumours by a factor of 5·4.

Line A: Localized tumour irradiation (X-rays) in anaesthetized mice.

Line B: Localized tumour irradiation (X-rays) in anaesthetized mice under hyperbaric oxygen.

Line C: Total body X-irradiation without anaesthesia.

Line D: Total body irradiation with fast neutrons without anaesthesia.

*The points * represent the effect of 5 daily doses of X-ray treatment on the rhabdomyosarcoma, described by Barendsen and Broerse* [13].

low RBE for fast neutrons on this poorly oxygenated tumour.

DISCUSSION

Three aspects of these studies deserve further discussion; the radioresistance of the tumour; its degree of oxygenation under fractionated radiotherapy and its response to fast neutrons.

The radioresistance of this tumour to fractionated X-irradiation was demonstrated for anaesthetized mice by a study of the tumour-volume response. The later finding that the anaesthesia may affect the tumour sensitivity seems to diminish the value of these observations. Nevertheless there remains a marked resistance to X-ray treatment even without anaesthesia; this is clear from a comparison in Fig. 3 of line C which represents osteosarcoma cell survival after five daily doses of total body irradiation, with the data from Barendsen and Broerse [13] on rhabdomyosarcoma cell survival after five daily doses of local irradiation which are also presented in this figure.

An approximately tenfold-higher cell survival in the osteosarcoma after five days treatment is observed even when the mice are not anaesthetized. It would seem likely that this resistance is due to hypoxia.

The study on the use of hyperbaric oxygen was not successful but we have obtained some information from the hypoxia caused by anaesthesia in this tumour. Many other transplanted tumours do not show this effect (e.g. [3, 9, 13]) and this may be a peculiar property of the osteosarcoma, possibly related to its poor reoxygenation after single large doses of treatment. An analysis of the effect of anaesthesia (compare lines A and C of Fig. 3) indicates that it is responsible for an approximately fiftyfold difference in cell survival, presumably through hypoxia.

If no reoxygenation took place in this tumour, at most a sixfold difference in survival could be ascribed to hypoxia, since initially about one-sixth of the cells are already anoxic. This suggests that at least an additional eight-

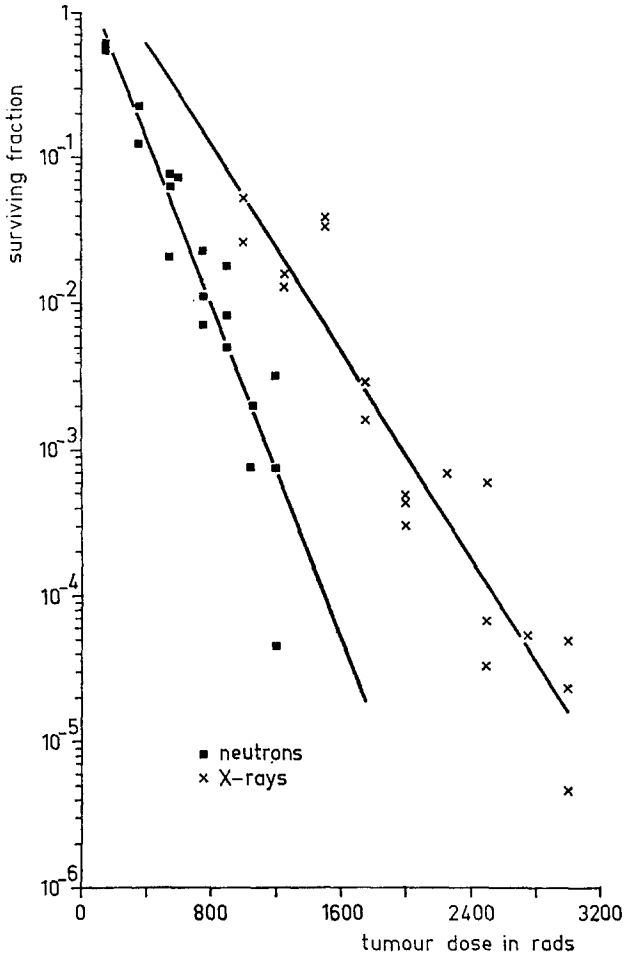


Fig. 4. The effects of X- and neutron irradiation on osteosarcoma cell survival. Immediately after irradiation in vivo, *the mice were killed and the fractions of reproductively intact cells were determined.*

fold ratio of survival must be explained by reoxygenation, and more if we assume that there are initially some oxygenated cells in the tumour in anaesthetized mice. It is thus for example possible during four of the five treatments, there were 60% (or a fraction of 1/1·7) well-oxygenated cells due to reoxygenation. In comparison with a completely anoxic tumour the survival ratios during the five treatment doses could thus have been $6\times1\cdot7\times1\cdot7\times1\cdot7\times1\cdot7=50$. This example indicates that an approximately fiftyfold difference in survival would still be compatible with a degree of reoxygenation that is sub-optimal in the sense that the effectiveness of the fracionated treatment might still be limited by the presence of anoxic cells. A tumour with this property fulfills our search for a model of radiation resistance in which hypoxia is limiting the probability of cure. Such a tumour in man would provide the rationale for treatment with hyperbaric oxygen or other treatment modalities which are less affected by the oxygenation status of the cells.

Unfortunately, the osteosarcoma did not respond to neutrons in the expected way; an OER as high as 2·0 implies that the neutrons are hardly more effective than X-rays in killing the anoxic cells of this tumour. The agreement between the results with regard to slope and RBE for anoxic cells *in vitro*, and terminal slope and RBE *in vivo* support the reality of this finding.

For most well reoxygenating tumours one would not expect much difference in RBE on comparing single and fractionated doses *in vivo* since the higher effectiveness of neutrons on the anoxic cells, which is important in the single dose studies, is compensated for by the higher effectiveness of neutrons in fractionated exposure, associated with the smaller shoulder. For the osteosarcoma there is little higher effectiveness on anoxic cells, so the absence of repair of sub-lethal damage, giving a higher effectiveness of neutrons in fractionated irradiation, dominates and is thus responsible for the increased RBE with fractionation. It will be clear that the small gain factor does not permit an evaluation of the degree of reoxygenation from these results and our high expectations of

finding an elevated RBE for this resistant tumour are not fulfilled, most likely as a consequence of the high OER for neutrons in this system.

This tumour thus demonstrates an unexpected behaviour in a number of ways: it reoxygenates poorly, it is anoxic under anaesthesia and it shows a high OER for neutron irradiation.

Although the first two properties may well be

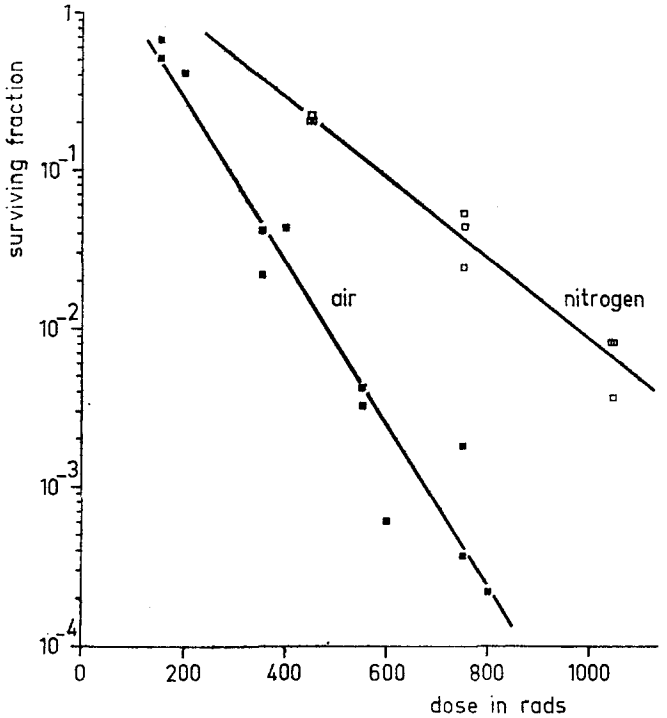


Fig. 5. *The effects of neutron irradiation on osteosarcoma cells* in vitro *for oxygenated and hypoxic conditions. Note the high Oxygen Enhancement Ratio for neutrons of about* 2·0.

Table 2.

	X-rays		Neutrons		RBE for surviving fraction			RBE slope ratio
	D_0 (rads)	n	D_0 (rads)	n	10^{-1}	10^{-2}	10^{-3}	
A. RBE of 15 MeV neutrons for survival of osteosarcoma cells								
In vitro								
Air	124	6·5	85	2·9	1·8	1·7	1·6	1·5
Nitrogen	282	9·3	171	3·0	2·2	2·0	1·9	1·7
In vivo								
Single doses	246	3·2	151	1·85	1·9	1·8	1·8	1·7
Five daily doses	287	45·6	137	8·8	2·9	2·6	2·5	2·1

B. OER values for osteosarcoma cells *in vitro*

	X-rays	Neutrons	Oxygen enhancement gain factor
For survival 10^{-1}	2·5	2·0	1·2
10^{-2}	2·4	2·0	1·2
10^{-3}	2·4	2·0	1·2
Slope ratio	2·3	2·0	1·1

related, we cannot see how the third one could be more than coincidental. A search among other experimental tumours for a suitable model for the radiotherapy-resistant clinical tumour should be continued in order to see whether other models show different patterns of response, for which neutron therapy might indeed provide a therapeutic gain.

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REFERENCES

1. L. M. van Putten and R. F. Kallman, Effect of pre-irradiation on the ratio of oxygenated and anoxic cells in a transplanted mouse tumor. Abstract no. 910, *Third Int. Congress of Radiation Research*, Cortina d'Ampezzo (1966).
2. L. M. van Putten and R. F. Kallman, Oxygenation status of a transplantable tumor during fractionated radiation therapy. *J. nat. Cancer Inst.* **40,** 441 (1968).
3. R. F. Kallman, L. J. Jardine and C. W. Johnson, The effects of different schedules of dose fractionation on the oxygenation status of a transplantable mouse sarcoma. *J. nat. Cancer Inst.* **44,** 369 (1970).
4. H. D. Suit, R. Lindberg, C. Suchato and A. Ozenne, Radiation dose fractionation and high pressure oxygen in radiotherapy of the DBA mouse mammary carcinoma. *Am. J. Roentgenol.* **99,** 895 (1967).
5. H. D. Suit and M. Maeda, Hyperbaric oxygen and radiobiology of a C3H mouse mammary carcinoma. *J. nat. Cancer Inst.* **39,** 639 (1967).
6. H. D. Suit and J. V. Schiavone, Effect of a single dose of radiation on proportion of hypoxic cells in a C3H mouse mammary carcinoma. *Radiology* **90,** 325 (1968).
7. M. J. Hawkes, R. P. Hill and P. J. Lindop, The response of C3H mammary tumours to irradiation in single and fractionated doses. *Brit. J. Radiol.* **41,** 134 (1968).
8. P. J. Cheshire and P. J. Lindop, The influence of intracellular recovery and hypoxic cells on the radiation response of mammary tumours and skin in C3H mice. *Brit. J. Radiol.* **42,** 215 (1969).
9. A. E. Howes, An estimation of changes in the proportions and absolute numbers of hypoxic cells after irradiation of transplanted C3H mouse mammary tumours. *Brit. J. Radiol.* **42,** 441 (1969).
10. H. D. Suit and C. Suchato, Hyperbaric oxygen and radiotherapy of a fibrosarcoma and of a squamous-cell carcinoma of C3H mice. *Radiology* **89,** 713 (1967).
11. R. H. Thomlinson, The effects of irradiation on the proportion of anoxic cells in tumours. *Third Int. Congress of Radiation Research*, Abstract no. 878, Cortina d'Ampezzo (1966).
12. R. H. Thomlinson, Changes of oxygenation in tumours in relation to irradiation. *Front. Radiat. Ther. Oncol.* **3,** 109 (1968).
13. G. W. Barendsen and J. J. Broerse, Experimental radiotherapy of a rat rhabdomyosarcoma with 15 MeV neutrons and 300 kV X-rays. II. Effects of fractionated treatments, applied five times a week for several weeks. *Europ. J. Cancer* **6,** 89 (1970).
14. L. M. van Putten, Tumour reoxygenation during fractionated radiotherapy; studies with a transplantable mouse osteosarcoma. *Europ. J. Cancer* **4,** 173 (1968).
15. H. S. Reinhold, A cell dispersion technique for use in quantitative transplantation studies with solid tumours. *Europ. J. Cancer* **1,** 67 (1965).
16. L. M. van Putten, Oxygenation and cell kinetics after irradiation in a transplantable osteosarcoma. In *Effects of Radiation on Cellular Proliferation and Differentiation*, p. 493. IAEA, Vienna (1968).
17. J. J. Broerse and H. van Ammers, Dosimetry for fast neutron irradiations of cultured cells and intact animals. I. Characteristics of tissue-equivalent ionization chambers. *Int. J. Radiat. Biol.* **10,** 417 (1966).
18. J. J. Broerse, Dosimetry for fast neutron irradiations of cultured cells and intact animals. II. Comparison between activiation and ionization methods. *Int. J. Radiat. Biol.* **10,** 429 (1966).
19. H. S. Reinhold, Quantitative evaluation of the radiosensitivity of cells of a transplantable rhabdomyosarcoma in the rat. *Europ. J. Cancer* **2,** 33 (1966).

Europ. J. Cancer Vol. 7, pp. 161–169. Pergamon Press 1971. Printed in Great Britain

RBE Values for Cyclotron Neutrons for Effects on Normal Tissues and Tumours as a Function of Dose and Dose Fractionation

S. B. FIELD and S. HORNSEY

*Medical Research Council, Cyclotron Unit, Hammersmith Hospital, London, W.*12, *and Medical Research Council, Experimental Radiopathology Unit, Hammersmith Hospital, London W.*12, *England*

Abstract — *For various normal tissue endpoints, the RBE is shown to vary with size of dose and with the differential amount and rate of repair of sublethal damage for each tissue. In particular, the RBE for late damage in skin and deformity, is shown to be the same as for early damage, for single and fractionated exposures. However, for all treatments, once above a certain dose level, a small increase in dose leads to a small increase in early reaction, but results in a very large increase in late damage. Neutrons are a more effective treatment than X-rays for those experimental tumours whose response is limited by hypoxic cells, but in some cases, the use of properly fractionated doses of X-rays may be equally efficient.*

THERE are three major differences between the dose–effect curves produced by exposing cells and tissues to X-rays and fast neutrons.

1. At all dose levels, the RBE (relative biological effectiveness) of fast neutrons, relative to X-rays is greater than unity. This fact alone would be of no advantage or disadvantage in radiotherapy.
2. Dose effect curves after neutron irradiation show evidence of a smaller sparing effect of fractionation, in relation to the dose needed to produce a given effect. A consequence of this difference is that the RBE for fast neutrons is not constant, but is largest for small doses and decreases with increasing level of injury.
3. The oxygen enhancement ratio for X-rays, now measured in many cells and tissue systems, is usually between 2 and 3. With fast neutrons this factor is consistently lower by a factor of about 1·6. Therefore, if a proportion of hypoxic cells in a tumour is a limiting factor in its response to X-rays, then treatment with neutrons would be more effective.

Before patients can be treated with any new form of radiation, the RBE, as a function of dose, must be accurately known for the normal tissues at risk. Since the absolute dosimetry of fast neutrons is not yet precise, and since the quality of beams produced at different centres may differ, the following analysis of RBE will include only tissues irradiated on the M.R.C. Cyclotron at Hammersmith Hospital. These neutrons are produced by 16 MeV deuterons on beryllium and have a mean energy of about 8 MeV.

Let us first consider repopulation after X-rays or fast neutrons. Denekamp and her co-workers [1] showed that the rise and fall in the skin reaction followed the same pattern after doses of either of the two radiations when matched to produce the same average effect. This is also true for rat skin [2, 3], pig skin [4]

and human skin [5, 6]. There is also little difference in the shapes of regrowth curves of tumour RIB_5 after either of the two radiations, as illustrated in Fig. 1 [2]. Thus it seems that repopulation rates after irradiation are independent of the radiation quality and will not affect the RBE for fractionated exposures. We therefore include data for single and fractionated irradiations when considering the RBE.

Figure 2 shows a plot of the RBE based on visual observations of skin damage as a function of the dose per fraction of fast neutrons. It is seen that the RBE is independent of species, and increases with decreasing dose as expected from the differences in shapes of dose effect curves after low or high LET irradiation. The small increase in RBE seen for doses greater than about 1500 rads of neutrons may be due to a small proportion of hypoxic cells in the skin.

Rossi [8] has suggested a method of plotting these results as the logarithm of the RBE against the logarithm of the dose of neutrons. The skin results are presented in this way in Fig. 3, together with the data for damage to the haemopoietic system, growing cartilage and the intestine. Within the experimental errors straight lines may be drawn through the points for each type of tissue.

The RBEs for the four tissues are clearly different [15] which may be explained by the amount and rate of intracellular recovery of the various systems. This sparing effect of fractionation may be expressed by the quantity D_2-D_1, where D_1 is the single dose and D_2 the dose in two fractions to produce the same effect. D_2-D_1 will depend on several factors, each of which may vary with time after the first dose. Some of these are:

(i) The degree of recovery from sub-lethal injury;
(ii) mitotic delay;
(iii) induction of partial synchrony in the cell population surviving the first dose, with subsequent changes in sensitivity with progression through the succeeding cell cycles;
(iv) the rate of repopulation;
(v) changes in the connective tissue, including the vascular and nervous elements, and

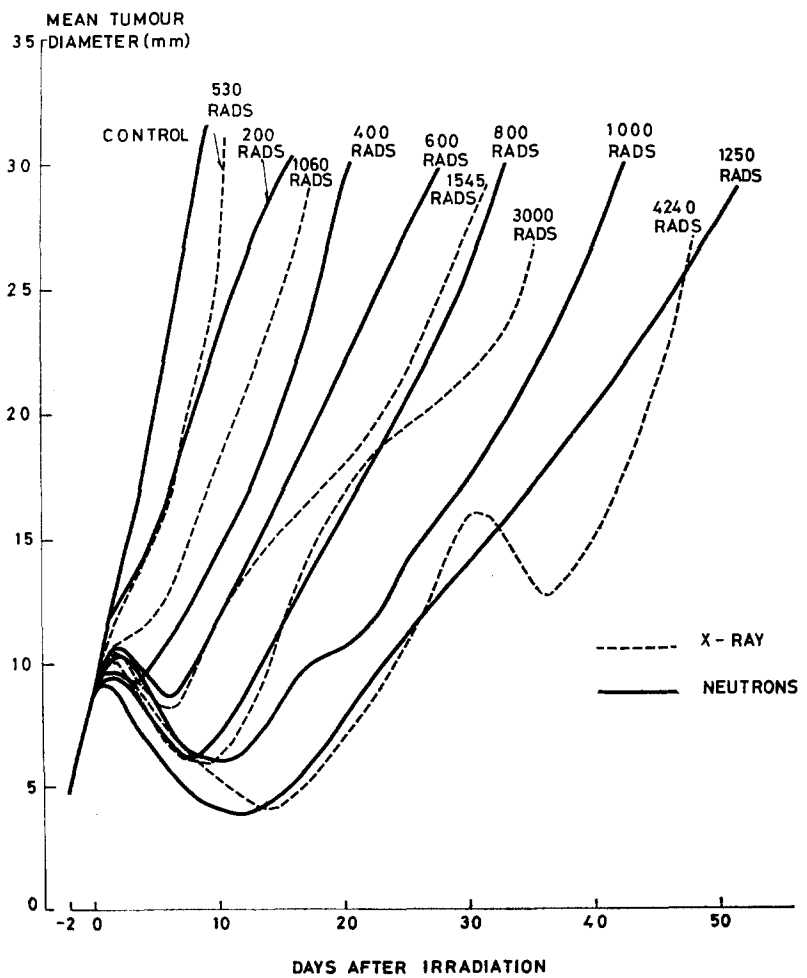


Fig. 1. Regrowth curves for tumour RIB_5 after irradiation with single doses of X-rays or fast neutrons.

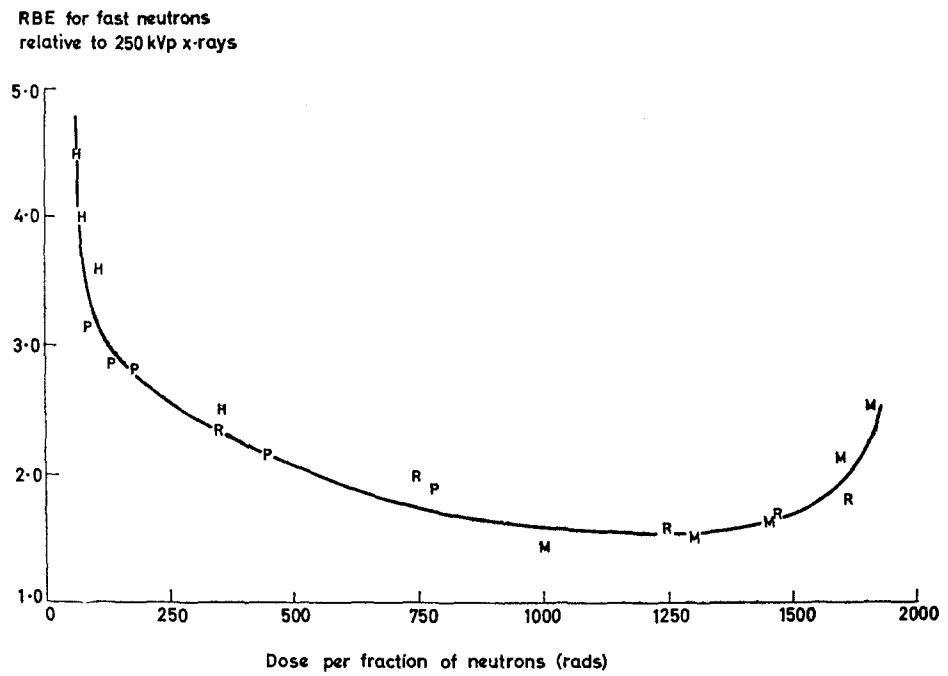


Fig. 2. The RBE for skin irradiated with fast neutrons from the Hammersmith cyclotron, as a function of the dose per fraction of neutrons. H—human skin [5]; *P—pig skin* [7]; *R—rat skin* [2, 3]; *M—mouse skin* [1].

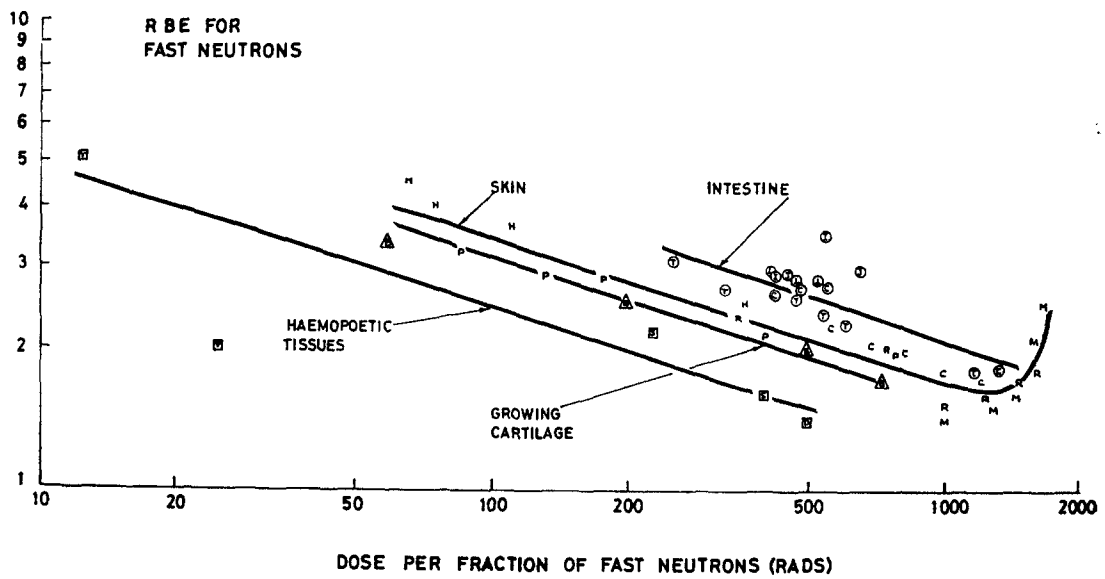


Fig. 3. RBE as a function of dose per fraction of neutrons for various tissues. Y—mouse lymphocytes [9]; *T—weight loss in mouse thymus* [10]; *S—mouse-haemopoetic cells, spleen colonies assay* [9]; *D—30-day death in mice* [9]; *B—stunting of the tail growth in 1-day old rats* [11]; *H—human skin reactions* [5]; *P—pig skin reactions* [7]; *R—rat skin reactions* [2, 3]; *M—mouse skin reactions* [1]; *C—clones in mouse skin* [12, 13]; *I—5-day death in TO mice* (1965) [14]; *T—5-day death in TO mice* (1969) [15]; *C—5-day death in C_3H mice* [15]; *T—survival of mouse colonies in the jejunum* [15]; *J—reduction of crypts in the jejunum* [15]; *⊥—reduction of crypts in the ileum* [9].

(vi) alteration in the proportion of the hypoxic cells.

The resulting D_2-D_1 may therefore be greatly influenced by the time between fractions, but it seems that with normal well oxygenated tissues at 24 hr D_2-D_1 primarily reflects the repair of intracellular sub-lethal damage to cells. In tumours, however, this is unlikely to be true, since changes in the proportion of hypoxic cells may greatly affect the issue. In fact, D_2-D_1 at 24 hr has been shown to be negative in some tumours [3, 16, 17], in which extensive reoxygenation takes place during this time. This complication is avoided if the single and split doses are delivered with the entire tumour hypoxic.

$(D_2-D_1)/D_1$ for neutrons, divided by the similar expression for X-rays, may be taken as a measure of the fraction of recovery from sub-lethal damage after irradiation with neutrons compared with X-rays. Values for various tissues are given in Table 1. The recovery after neutrons compared with X-rays is less for gut than for skin. However, a marked effect of dose rate on intestinal damage has been observed after low LET radiation [19, 20]. This is due to a rapid component of recovery from sub-lethal damage to the target cells and is not observed after neutron irradiation. The RBE shown in Fig. 3 are relative to 250 kV X-rays given at about 200 rads/min. At higher dose rates, X-rays would be more effective in damaging gut, and the RBEs would therefore be lower, and nearer to that of skin, where such a dose rate effect has not been observed [21]. However, at lower dose rates, equivalent, for example to that from radium implants, the RBE would be much higher. For damage to the haemopoietic tissues, there is much less recovery from sub-lethal damage after X-rays or fast neutrons than skin or gut, and the RBE values are correspondingly lower. However, Broerse [22] has suggested that the RBE for haemopoietic tissues might be slightly reduced because of increased absorption of X-rays and reduced absorption of neutrons in bone cavities. The same might also be true for damage to growing cartilage.

Extrapolation of the curves in Fig. 3 would give an RBE of less than 1 at high doses. For damage primarily caused by cell killing, the RBE would approach the ratio of D_0 for X-rays to that for neutrons. There have been many estimates of this ratio for cells *in vivo* and *in vitro*. Some of these, for Cyclotron-produced neutrons are: 2·2 for the Ehrlich ascites tumour [23], 1·1 for skin clones [13], 1·1 for gut clones [9], 2·1 for lymphocytic-leukaemia cells [24], 2·1 for clones in cartilage [18]. We know of no results giving a ratio of less than one, which demonstrates that the curves in Fig. 3 cannot be linearly extrapolated, but will all bend to constant values of RBE greater than 1.

In radiotherapy, it is the damage to normal tissues which determines the maximum dose which can be tolerated. With presently available neutron sources, it seems likely that skin damage will be the limiting factor, although the intestine will also be important where it is included in the irradiation field. In clinical terms the variation in RBE from tissue to tissue may be illustrated by the following comparison. A single treatment of 120 rads of neutrons is equivalent to 280 rads of X-rays to the haemopoetic tissue, 350 to growing cartilage, 390 to skin and about 470 to gut. (The estimate for gut requires an extrapolation to lower doses than have been investigated and is therefore less certain.)

Table 1. *Values of* (D_2-D_1) 24 *hr*

System	Reference	D_2-D_1 (rads)		$(D_9 \pm D_1)/D_1$ for neutrons
		X-rays	Neutrons	$(D_9 \pm D_1)/D_1$ for X-rays
Pig skin	[7]	600	120	0·6
Mouse skin	[1]	500	160	0·6
Skin clones in mice	[12, 13]	570	170	0·4
Rat skin (early reaction)	[3]	900	180	0·4
Rat skin (late reaction)	[3]	500	120	0·6
Mouse gut	[14]	400	40	0·2
Cartilage clones in rats	[18]	350	100	0·6
Rat tails	[11]	450	0	0
Sarcoma in rats (anoxic values deviated by 3)	[3]	350	15	0·2

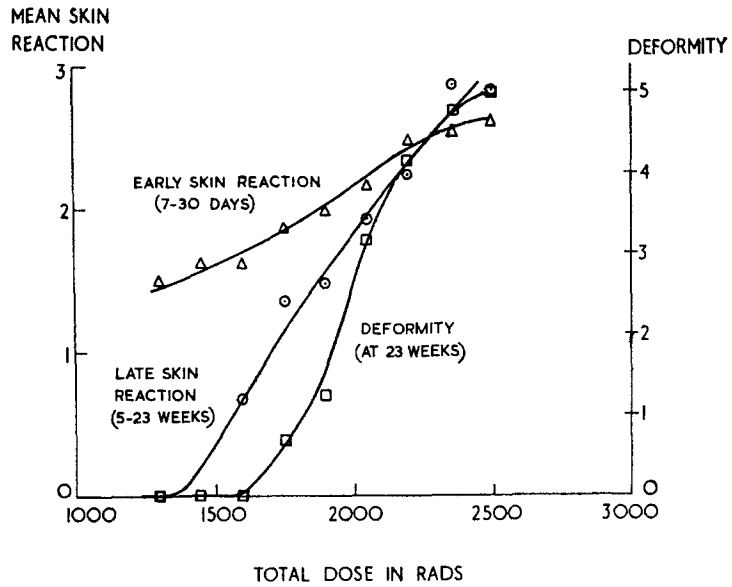


Fig. 4. Dose-response curves for early and late skin reactions and deformities after irradiation with five fractions of fast neutrons.

It was suggested by Stone [25], after the first trial of neutron therapy, that relative to early damage, the late damage after neutrons was more severe than after X-rays. At Hammersmith Hospital, pigs have been irradiated with single and fractionated doses of X-rays and fast neutrons, and some have been observed for as long as six years [7]. There was no sign of an increased or qualitatively different reaction after neutrons, i.e. the RBE for the late reactions was the same as for the early reactions. With rat skin similar observations were made of early and late damage on a more quantitative basis [26]. Figure 4 shows the dose-effect curves for the early damage, late damage, and deformity for one type of treatment. An advantage of this type of analysis is that at any dose level, the data points for each of the three curves came from the same group of animals. The deformity was scored on a different scale from the skin reactions, and consequently the curve has been normalised to cross at the same point as the other two. The scales of injury are, of course, not linear, but it is clear that the early reaction increases steadily, with no threshold. The corresponding late reactions are at first quite small, but with increasing dose suddenly increase very sharply. This is illustrated in another way in Fig. 5, where the deformity has been plotted as a function of the early skin reaction. If the dose is given to cause an early reaction of about 2 (a small to moderate degree of breakdown) the feet will later become very slightly deformed. If the dose is increased by about 25%, the early reaction becomes about 2·5 (which is the breakdown of about one-half the total area of the foot) but by six months most of the foot will have fallen off. A similar finding has been reported by Kember [27] who observed the incidence of clones of proliferating cells in the cartilage plate of rat legs. After a single dose of 1500 rads of X-rays, these cells were still able to differentiate and produce new cartilage. After 1800 rads, an increase of only 20%, most of them aborted at about 7 weeks after irradiation.

The mechanisms leading to the late skin reactions are not well understood, but it

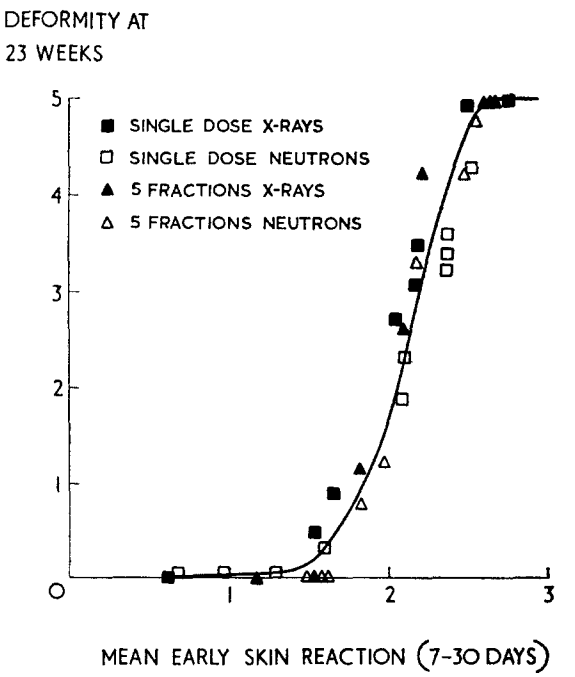


Fig. 5. Deformity as a function of early skin reaction after various treatments.

appears that different tissues play different roles in the reactions. In the clinical trial at Hammersmith it has been shown that there is a marked effect of skin sparing with fast neutrons for reactions where the skin breaks down, but not at lower levels of damage where the skin only develops an erythema. It has been suggested that desquamation is primarily a result of damage to cells in the basal layer of the epithelium. The skin sparing result then suggests that the early erythema is the result of direct damage to deeper structures. In the experiments on rat skin, at a given dose, an animal which developed a particularly severe early desquamation relative to the others, did not necessarily develop a severe late reaction, and vice versa. This suggests that the late reactions are not an indirect consequence of damage leading to moist desquamation, i.e. damage to the basal layer. It is possible that the late reactions might be a result of vascular injury, which might take some time to manifest itself. Failure in blood supply, with damage to the supporting tissues, could also lead to deformity.

The relationship between early and late reactions is identical for single doses or up to five daily doses of X-rays or fast neutrons. The RBE values for the various reactions in skin are given in Table 2, and there is clearly no increase in RBE for the late reactions.

Regarding Stone's hypothesis that the late reactions after neutrons were excessive, Sheline and his colleagues [28] have clearly shown that these severe necroses reported by Stone [25] were simply the result of overdoses of radiation.

It seems likely that many tumours contain a significant proportion of hypoxic cells. Therefore, for single doses, the RBE for damage to most tumours might be expected to be greater than that for normal tissues. This is illustrated in Fig. 6 where the data for single doses to some tumours is included with the normal tissue data from Fig. 3. Whether such a therapeutic advantage is maintained for a fractionated course of therapy will depend on many factors, but particularly on the kinetics of reoxygenation of the particular tumour. The therapeutic advantage of neutrons for tumours with hypoxic foci would not be expected in a tumour which reoxygenates rapidly and extensively. Whether such a situation exists in clinical practice can at present only be determined by a clinical trial, but some information is being obtained from experiments on animal tumours and normal tissues. One such experiment has been done by Field and his coworkers [2, 3] who have irradiated the sarcoma RIB_5, and the skin of rats with single, two and five fractions of X-rays and fast neutrons. The results of these experiments are summarized in Fig. 7, where the injury to the tumour is plotted as a function of the injury to the skin for each of the six types of treatment. Clearly the aim is to find the treatment giving the curve nearest to the upper left hand corner, i.e. as much damage to the tumour and as little to the normal tissue as possible. The least effective form of treatment was the single dose of X-rays. Two fractions of X-rays were more effective, probably resulting from improved oxygenation of the tumour between fractions. Five fractions of X-rays were hardly different in effectiveness from two, possibly due to less reoxygenation per fraction with smaller doses [30] or a considerable amount of proliferation of the tumour cells between fractions [31]. It is not possible to say whether a greater number of fractions would be more or less effective than 2 or 5. However, in this tumour, with the fractionation schemes employed, the hypoxic cells still appear to affect the overall response, because a single dose was more effective than any of the X-ray treatments. There was also an optimum treatment, in these experiments, of two fractions of neutrons. Thomlinson [30] has speculated that there may be more reoxygenation after neutron irradiation, because of more killing of hypoxic cells, but this rather unexpected result might also serve to suggest that the oxygen effect is only one of the factors involved. Other tumours will almost certainly respond differently, so that a great deal more knowledge of the processes underlying the radiation response of both normal and neo-

Table 2. *RBE values of "8 MeV" neutrons for reactions of rat skin*

	Early reaction	Late reaction	Deformity
Single dose	1·69	1·59	1·68
Two fractions	1·93	2·11	
Five fractions	2·41	2·38	2·32

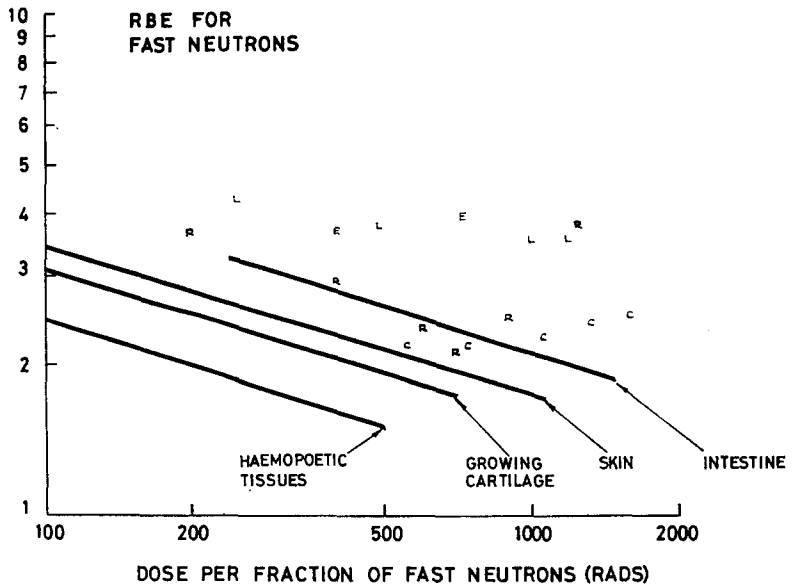


Fig. 6. *RBE as a function of dose per fraction for normal tissues (solid lines) and tumours: R—rat sarcoma RIB*$_5$ [2]; *E—Ehrlich ascites tumour* [23]; *L—lymphocytic leukaemia* [24]; *C—C*$_3$*H mammary carcinoma in oestrogen fed, male mice* [29].

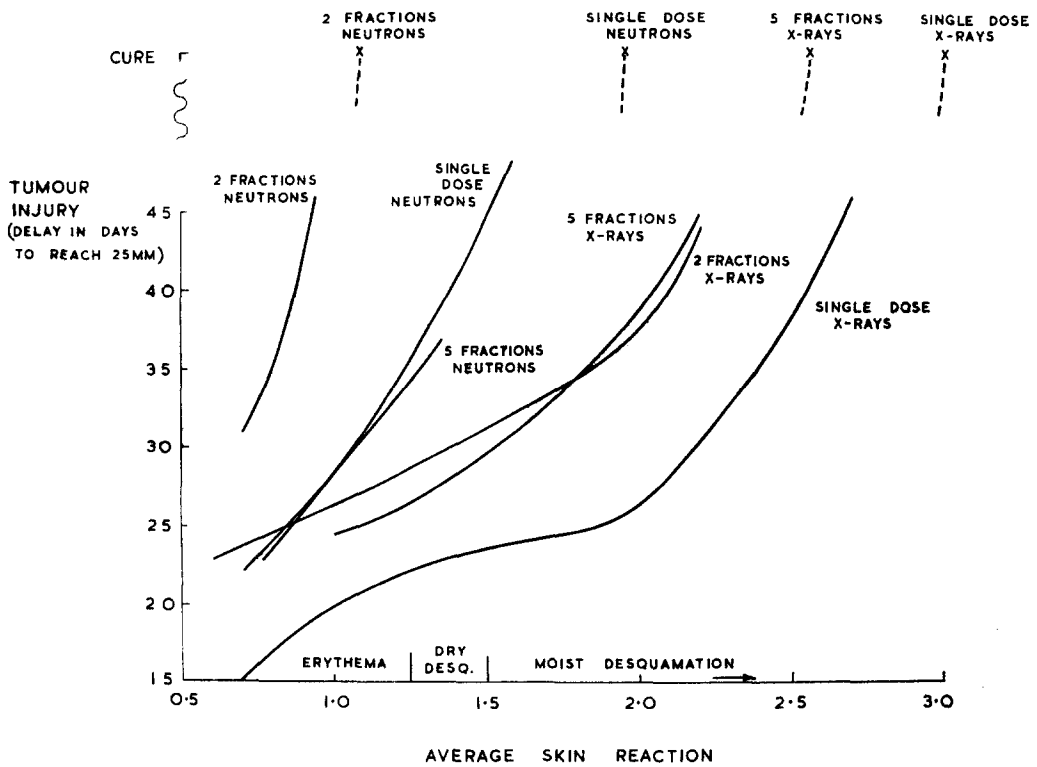


Fig. 7. *Injury to tumour RIB*$_5$ *as a function of skin injury for various types of treatment.*

plastic tissues is needed in order to optimize radiotherapy.

Thus the differences in relative response of tissues to fast neutrons and to X-rays are entirely consistent with the three basic principles stated, in terms of dose-effect curves at the beginning of this paper.

It was also shown that late damage in skin after neutrons is not more severe, relative to early damage, than after X-rays. However, once above a threshold reaction, the late damage increases very quickly with increasing dose with all treatment regimes.

In preparing to use new high LET machines in therapy, it would seem reasonable to carry out radiobiological experiments, of the kind illustrated by Fig. 3, and at first to use the results to estimate the RBE as a function of dose. Clinical experience can later point to more accurate dose relationships.

The relative response of a tumour and normal tissue to neutrons and X-rays depends on many factors, which interact in a complex and as yet poorly understood way.

At the present time, if results from animal experiments are to be applied to the human situation, it is most important to try to understand the mechanisms underlying the responses of normal and malignant tissues to radiation.

Acknowledgements — The following Figs. are reprinted by permission of author(s) and respective publishers:
Fig. 2. D. K. Bewley — published in *Current Topics in Radiation Research* Vol. 5, North Holland, Amsterdam.
Figs. 4 *and* 5. S. B. Field — published in *Radiology* ref. 26 in text.
Fig. 7. S. B. Field, T. Jones and R. H. Thomlinson — published in *British Journal of Radiology* ref. 3 in text.

REFERENCES

1. J. Denekamp, J. F. Fowler, K. Kragt, C. J. Parnell and S. B. Field, Recovery and repopulation in mouse skin after irradiation with cyclotron neutrons as compared with 250 kv X-rays or 15 MeV electrons. *Radiation Res.* **29,** 71 (1966).
2. S. B. Field, T. Jones and R. H. Thomlinson, The relative effects of fast neutrons and X-rays on tumour and normal tissue in the rat. Part I: Single doses. *Brit. J. Radiol.* **40,** 834 (1967).
3. S. B. Field, T. J. Jones and R. H. Thomlinson, The relative effects of fast neutrons and X-rays on tumour and normal tissue in the rat. Part II: Fractionation: Recovery and Reoxygenation. *Brit. J. Radiol.* **41,** 597 (1968).
4. J. F. Fowler, Kinetics of injury and repair to mammalian tissue by high-LET radiation. *Radiation Res. Suppl.* **7,** 276 (1967).
5. R. L. Morgan, Fast Neutron Therapy: clinical applications. In *Modern Trends in Radiotherapy*, (Edited by T. J. Deeley and C. A. P. Wood) p. 271, Butterworths, London (1967).
6. M. Catterall, Personal communication.
7. D. K. Bewley, S. B. Field, R. L. Morgan, B. C. Page and C. J. Parnell, The response of pig skin to fractionated treatments with fast neutrons and X-rays. *Brit. J. Radiol.* **40,** 765 (1967).
8. H. H. Rossi, The effects of small doses of ionizing radiation. *Phys. in Med. Biol.* **15,** 255 (1970).
9. S. Hornsey, Personal communication.
10. E. A. Wright, D. K. Bewley and C. J. Parnell, The effects of cyclotron neutrons on the intact mouse thymus under normal and hypoxic conditions. *Brit. J. Radiol.* **39,** 552 (1966).
11. B. Dixon, The effect of radiation on the growth of vertebrae in the tails of rats. III: The response to cyclotron neutrons. *Intern. J. Radiat. Biol.* **15,** 541 (1969).
12. E. W. Emery, J. Denekamp, M. M. Ball and S. B. Field, Survival of mouse skin epithelial cells following single and divided doses of X-rays. *Radiation Res.* **41,** 450 (1970).
13. J. Denekamp, E. W. Emery and S. B. Field, Response of mouse epidermal cells to single and divided doses of fast neutrons. *Radiation Res.* In press.
14. S. Hornsey, S. Vatistas, D. K. Bewley and C. J. Parnell, The effect of fractionation on four day survival of mice after whole-body neutron irradiation. *Brit. J. Radiol.* **38,** 878 (1965).
15. S. Hornsey, The relative biological effectiveness of fast neutrons for intestinal damage. *Radiology* **97,** 649 (1970).

16. K. J. HAWKES, R. J. HILL, P. J. LINDOP, R. E. ELLIS and J. ROTBLAT, The response of C_3H mammary tumours to irradiation in single and fractionated doses. *Brit. J. Radiol.* **41,** 134 (1968).
17. A. E. HOWES, An estimation of changes in the proportions and absolute numbers of hypoxic cells after irradiation of transplanted C_3H mammary tumours. *Brit. J. Radiol.* **42,** 441 (1969).
18. N. F. KEMBER, Radiobiological investigations with fast neutrons using the cartilage clone system. *Brit. J. Radiol.* **42,** 595 (1969).
19. S. HORNSEY and T. ALPER, Unexpected dose-rate effect in the killing of mice by radiation. *Nature Lond.* **210,** 212 (1966).
20. S. HORNSEY, Differences in survival of jejunal crypt cells after radiation delivered at different dose rates. *Brit. J. Radiol.* In press.
21. J. DENEKAMP and J. F. FOWLER, Further investigations of the response of irradiated mouse skin. *Int. J. Radiation Biol.* **10,** 435 (1966).
22. J. J. BROERSE, Effects of energy dissipation of monoenergetic neutrons in mammalian cells and tissues. Ph.D. Thesis, Univ. Amsterdam. Published by the Organisation for Health Research TNO, Rijswijk (ZH), Holland (1966).
23. S. HORNSEY and G. SILINI, Pre-Therapeutic experiments with the fast neutron beam from the Medical Research Council Cyclotron IV: Comparisons of the effects of X-rays and cyclotron neutrons on mouse ascites tumours, mouse testis and chick embryos. *Brit. J. Radiol.* **36,** 92 (1963).
24. R. J. BERRY, D. K. BEWLEY and C. J. PARNELL, Reproductive capacity of mammalian tumour cells irradiated *in vivo* with cyclotron-produced fast neutrons. *Brit. J. Radiol.* **38,** 613 (1965).
25. R. S. STONE, Neutron therapy and specific ionisation. *Am. J. Roentgenol.* **59,** 771 (1948).
26. S. B. FIELD, Early and late reactions in skin of rats following irradiation with X-rays or fast neutrons. *Radiology* **92,** 381 (1969).
27. N. F. KEMBER, Cell survival and radiation damage in growth cartilage. *Brit. J. Radiol.* **40,** 496 (1967).
28. G. E. SHELINE, T. L. PHILLIPS, S. B. FIELD, J. T. BRENNAN and A. RAVENTOS, Effects of fast neutrons on human skin. *Am. J. Roentgenol.* In press.
29. R. P. HILL, P. J. CHESHIRE, P. J. LINDOP and S. B. FIELD, A comparison of the response of tumour and normal tissue in the mouse exposed to single doses of fast neutrons. *Brit. J. Radiol.* **43,** 894 (1970).
30. R. H. THOMLINSON, The importance of the oxygen effect for radiotherapy with fast neutrons. *Europ. J. Cancer* **7,** 139 (1971).
31. A. F. HERMENS and G. W. BARENDSEN, Changes of cell proliferation characteristics in a rat rhabdomyosarcoma before and after X-irradiation. *Europ. J. Cancer,* **5,** 173 (1969).

Europ. J. Cancer Vol. 7, pp. 171–177. Pergamon Press 1971. Printed in Great Britain

RBE Values of 15 MeV Neutrons for Effects on Normal Tissues

J. J. BROERSE, G. W. BARENDSEN, G. FRERIKS and L. M. VAN PUTTEN

Radiobiological Institute TNO, 151 Lange Kleiweg, Rijswijk Z.H., The Netherlands

Abstract — *For the application of fast neutrons in radiotherapy, it is important to evaluate the responses of various types of normal tissues in comparison with responses of experimental tumours after fast neutron- and X-irradiation. RBE values of 15 MeV neutrons are reported for effects on normal rodent tissues, namely mouse bone marrow, mouse intestine and rat skin. In general it has been found that these values are lower than the RBE values for effects on animal tumours. However, more data have to be obtained for the responses of normal tissues after fractionated irradiation with 15 MeV neutrons and X-rays before a definitive evaluation with respect to a possible therapeutic advantage of 15 MeV neutrons can be given.*

INTRODUCTION

FOR AN assessment of the potential advantage of fast neutrons for radiotherapy, effects of neutron irradiations on tumours have to be compared with effects on normal tissues. Various responses of a rhabdomyosarcoma in the rat and an osteosarcoma in the mouse have been studied for exposures to 15 MeV neutrons and 300 kV X-rays [1–3]. In this paper results will be presented on the effects of 15 MeV neutrons on normal tissues in experimental animals, namely rat skin, mouse bone marrow and mouse intestine. Application of fast neutrons in radiotherapy will only provide an advantage if the RBE values for the tumour response are larger than the RBE values for effects on normal tissues. A comparison of the relevant RBE values will be given.

MATERIAL AND METHODS

Irradiation techniques and dosimetry

X-ray exposures were carried out with a General Electric Maxitron Generator (250 kVp, 30 mA, HVL 2·1 mm Cu) and a Philips-Müller X-ray generator (300 kV, 10 mA, HVL 3·0 mm Cu). 15 MeV neutrons were produced through the D–T reaction using a Van de Graaff generator (400 kV, 250 μA of deuterons).

In order to ensure an accurate positioning in the different exposure arrangements, rats were irradiated in nylon cylinders. These cylinders with the rats were turned 180° halfway the neutron irradiation in order to achieve a homogeneous dose distribution. Mice were irradiated in perforated Lusteroid centrifuge tubes. The mice in centrifuge tubes were mounted on a Styrofoam cylinder, which was rotated in front of the target during irradiations. This permitted compensation for any inhomogeneities in the neutron flux density distribution while the rotation of the mice caused multilateral exposure. The neutron dosimetry was carried out with a tissue equivalent ionization chamber and sulphur activation detectors [4, 5].

Rat skin

In order to evaluate differences between effects of various types of ionizing radiation on skin, without interference of unirradiated cells migrating from outside the irradiated area and of damage to blood vessels and connective tissue, a transplantation technique

was employed to study radiation-induced damage to the skin of rats. Circular pieces of skin, about 20 mm in dia., were cut from the backs of white WAG/Rij rats after whole body irradiation with different doses of 300 kV X-rays and 15 MeV neutrons. These pieces of skin were transplanted onto the backs of brown (WAG/Rij × BN/Bi)F_1 hybrid rats in which a graft bed was prepared by excision of similar pieces of skin. Due to the difference in colour the grafted skin can easily be distinguished from the host skin. Immunological complications are avoided by this choice of graft and host. The area of grafted skin remaining was measured over a three-months period after transplantation and the ratio of the treated and untreated areas has been used as a parameter for skin damage.

Mouse bone marrow and mouse intestine

Acute bone marrow death and acute intestinal death have been studied in (CBA/Rij × C57BL/Rij)F_1 hybrid mice after irradiation with X-rays and 15 MeV neutrons. $LD_{50/30\ d}$ and $LD_{50/5\ d}$ values have been determined for different exposure periods [6].

Survival of the haemopoietic stem cells in these hybrid mice was investigated with the spleen colony technique [7] after single and fractionated exposures. The donor cells were irradiated either *in vitro* or *in vivo*. For each *in vitro* experiment one single bone marrow suspension was irradiated in a culture dish. The *in vitro* irradiations were carried out under hypoxic and oxygenated conditions, by flushing the dish with nitrogen or air, 10 min prior to the irradiation and during exposure. At different radiation doses part of this cell suspension was removed and appropriate dilutions were made. In the *in vivo* experiments the mice were killed immediately after the irradiation. The bone marrow suspensions were obtained by washing the marrow from the femoral shafts with Tyrode's solution and passing the resulting suspension through fine mesh nylon gauze. Suitable dilutions of the suspensions were injected intravenously in the recipient animals irradiated with 750 rads of X-rays. The recipients were sacrificed on the ninth day after irradiation. Subsequently the spleens were fixed in Telleyesniczky's solution and the number of superficial colonies was counted. The effect of dose fractionation *in vivo* was studied by dividing the total dose in five equal fractions administered at 1-day intervals. The mice were sacrificed immediately after the last dose. The surviving fraction of colony-forming units (CFU) was calculated relatively to the number of spleen colonies found per femur in a group of simultaneously studied unirradiated donors.

The survival of intestinal crypt cells has been investigated with the microcolony method of Withers and Elkind [8]. About 3·5 days after total body irradiation of the F_1 hybrid mice the animals were killed. Segments of the jejunum were fixed in Telleyesniczky's fluid. After histological preparation, transverse sections of the jejunum were stained with H.E. (haematoxylin and eosin). The number of regenerating crypts per circumference was counted and the number of surviving crypt stem cells was calculated on the basis of Poisson statistics. In unirradiated control mice, an average number of 90 crypts was found per circumference.

RESULTS

Rat skin

During the first 14 days after transplantation of the skin graft, little difference between irradiated and non-irradiated skin is observed macroscopically and microscopically with respect to the healing of the graft. After about 14 days, however, the irradiated skin grafts start to decrease in area. The ratio of the remaining areas of the irradiated skin graft relative to the area of unirradiated grafts approaches a constant value after about 8 weeks. This ratio depends on the dose and can be used as a measure of the skin damage produced by the irradiation. In Fig. 1 relations between the area of remaining skin and the radiation dose have been presented for single doses and two daily fractions of 15 MeV neutrons and 300 kV X-rays. The X-ray as well as the neutron curve show large shoulders and consequently at low doses of up to 600 rads an RBE value cannot be estimated. At single doses in excess of about 1200 rads of 15 MeV neutrons and in excess of 1600 rads of 300 kV X-rays, very small areas of grafted skin remain, which cannot be measured accurately. An estimate of the RBE of 15 MeV neutrons can only be made with sufficient accuracy for the dose region where a reduction of the skin area to a fraction between 60% and 20% is obtained. For this region the RBE of 15 MeV neutrons after single irradiations can be calculated to be equal to $1{\cdot}45 \pm 0{\cdot}2$. For two daily fractions, an RBE of $1{\cdot}65 \pm 0{\cdot}2$ has been obtained whereas preliminary results for five daily fractions indicate an RBE in excess of 2·0.

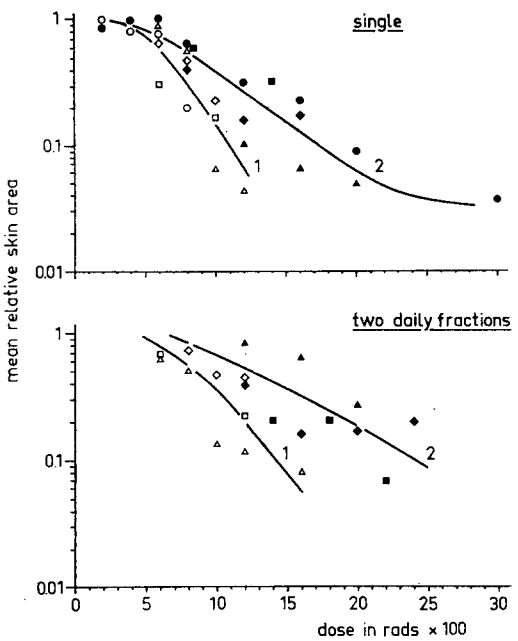


Fig. 1. *Dose-effect relations for reduction of the area of skin grafts from irradiated WAG/Rij rats, transplanted on the backs of unirradiated (WAG/Rij × BN/Bi)F1 hybrid rats, measured 4–6 months after transplantation for single doses and two daily fractions of* 15 *MeV neutrons* (*curves* 1) *and* 300 *kV X-rays* (*curves* 2).

Mouse bone marrow

The relative mortality after X- and neutron irradiation has been investigated at different time intervals after irradiation as a function of the dose. For X-ray exposures in the lowest dose interval a distinct mortality peak is found on the eleventh day after irradiation. A similar peak is not present for the neutron exposures, but in the lowest lethal dose range a considerable proportion of animals survive more than 7 days after irradiation. On the basis of the $LD_{50/30\ d}$ values for 2-hr exposures an RBE of $1{\cdot}12 \pm 0{\cdot}02$ was obtained for the haemopoietic syndrome after 15 MeV neutron irradiation [6].

The effects on intact animals have been compared with the effects at the cellular level. Survival data of the haemopoietic stem cells have been obtained after neutron- and X-irradiations. The survival curve parameters are presented in Table 1. The survival curves for *in vitro* irradiation with X-rays and neutrons under oxygenated conditions show a similar slope, but a significant difference in extrapolation number. This results in a decrease of the RBE with decreasing surviving fractions. For a CFU survival of 0·1%, an RBE of 1·10 has been obtained. The results for single dose irradiations of bone marrow stem cells *in vivo* indicate a slightly higher D_0 for both radiations as compared with *in vitro* irradiation whereas the extrapolation numbers for neutrons and X-rays again differ significantly from each other. The RBE of the 15 MeV neutrons appears to be slightly lower as compared with *in vitro* irradiation, being 1·03 at a surviving fraction of 0·1%.

For a valid comparison of the effects of neutron- and X-irradiation on the intact animal and the effects on the haemopoietic stem cells comparable survival levels should be considered. Doses in the $LD_{50/30\ d}$ range correspond to surviving fractions between 10^{-3} and 10^{-4}. It should be emphasized that the RBE values for CFU survival at these levels are based on extrapolation estimates which have a low accuracy. Taking this into account it can be concluded that the RBE for the $LD_{50/30\ d}$ is in good agreement with the RBE values for the CFU survival at a surviving fraction of 0·1%.

If the mice are subjected to five daily doses

Table 1. *Survival curve parameters for haemopoietic stem cells irradiated with neutrons or X-rays*

	CFU				RBE for cell survival of:		
	X-rays		neutrons				
	D_0 (rads)	n	D_0 (rads)	n	10^{-1}	10^{-2}	10^{-3}
In vitro							
Air	62·2	4·03	65·6	1·25	1·39	1·18	1·10
Nitrogen	177·9	2·68	123·6	1·21	1·90	1·68	1·60
In vivo							
Single dose	72·8	2·53	78·0	1·19	1·22	1·08	1·03
Single dose delayed	69·2	1·16	76·8	1·19	0·89	0·90	0·90
Five daily doses	92·8	0·94	90·1	1·67	0·82	0·91	0·96

of radiation and the number of CFU per femur is assayed immediately after the last dose, the dose–effect curve for fast neutrons is not significantly different from that for X-rays, as illustrated in Fig. 2. However, in this case the X-rays seem slightly more effective especially at low dose levels. This result is the more surprising if one considers the small shoulder in the survival curve for fast neutrons and the smaller repair which is usually observed in split dose neutron irradiations in comparison with X-rays. A partial explanation for this finding is obtained from another series of experiments on the effects of single doses of X-rays and fast neutrons *in vivo*, in which a delay of 24 hours was allowed between irradiation and sacrifice of the donors for assay. The delayed assay causes a marked reduction of the recovery of colony forming cells from the bone marrow of X-irradiated mice. For the neutron exposures however, this loss of CFU has not been observed. Due to these phenomena, the RBE values for immediate assay are higher than the RBE for delayed assay and the RBE for fractionated exposures. As indicated in Table 1 for the assay after a 24-hr interval, an RBE of 0·9 has been obtained independent of the dose. The loss of CFU after X-irradiation has been described by a number of investigators and presently it seems most likely that it is caused by differentiation of colony-forming cells to a cell type which has lost the capacity for proliferation to the size of a visible spleen colony [9]. However, at present there is no explanation for the absence of this phenomenon after neutron irradiation.

In addition to the *in vitro* irradiation of the haemopoietic stem cells under oxygenated conditions, the response of the CFU was studied under anoxic conditions. At a 0·1% survival level, OER values of 2·7 and 1·9 have been obtained for X- and 15 MeV neutron irradiations, respectively.

Mouse intestine

A mean survival time of 4·5 days was found for death due to the intestinal syndrome. On the basis of the $LD_{50/50\ d}$ values for 2-hr exposures an RBE of $1{\cdot}42 \pm 0{\cdot}02$ can be calculated for the intestinal syndrome for 15 MeV neutrons with respect to X-rays [6].

The survival curves for jejunal-crypt stem cells in mice after irradiation with 15 MeV neutrons and X-rays are presented in Fig. 3. D_0 values of about 140 rads are obtained for both survival curves. An RBE value of 1·4 can be calculated at radiation doses in the lethal range for these mice. This value is in good agreement with the RBE for the $LD_{50/5\ d}$.

DISCUSSION

A good agreement has been found between the RBE values observed with fast neutrons for the haemopoietic and intestinal syndromes and the RBE values for the effects on bone marrow stem cells and intestinal crypt cells respectively.

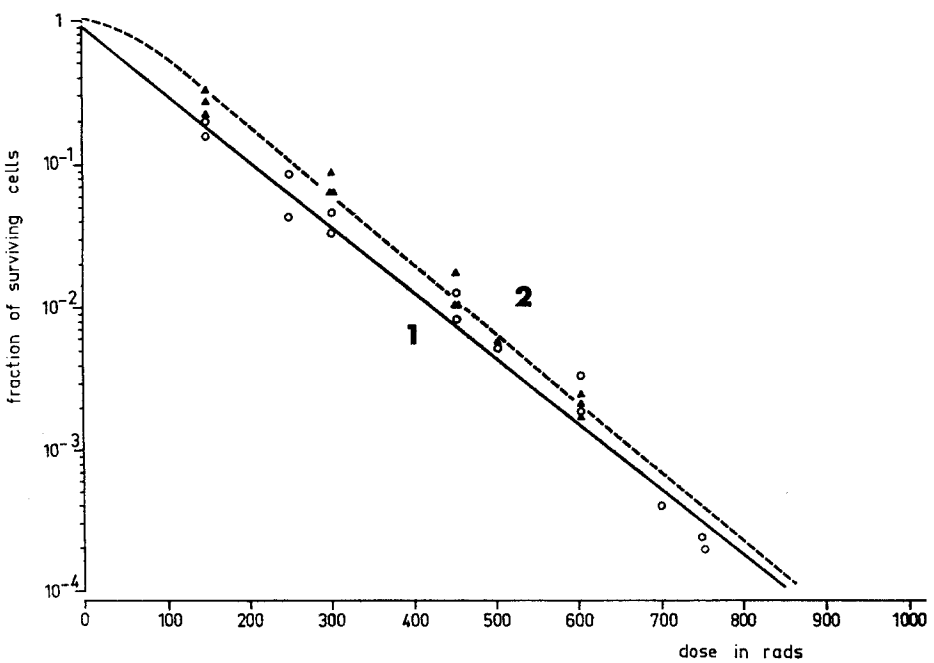


Fig. 2. Survival of haemopoietic stem cells in the femora of mice after in vivo *irradiation with five equal daily doses of* 300 *kV X-rays (curve* 1*) and* 15 *MeV neutrons (curve* 2*). The animals were sacrificed for spleen colony assay immediately after the last dose.*

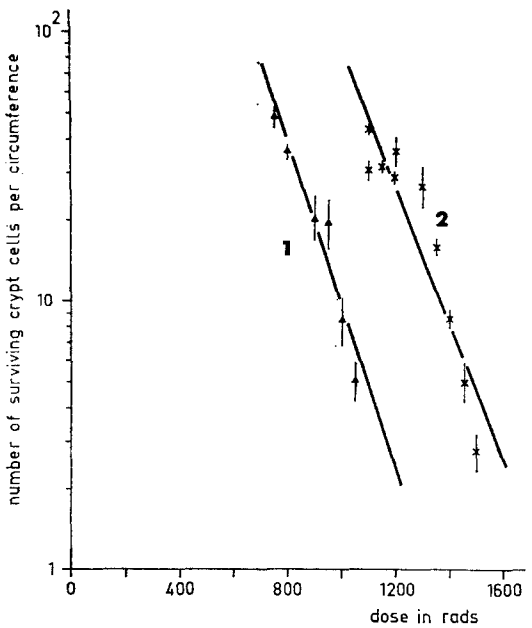


Fig. 3. *Survival of jejunal-crypt stem cells in mice after single dose irradiation with* 15 *MeV neutrons (curve* 1) *and* 300 *kV X-rays (curve* 2). *The standard deviations pertain to the variation within each separate experiment.*

This agreement is gratifying from the point of view of interpreting such very complex reactions in intact animals on the basis of responses of a single type of cells.

The RBE for the $LD_{50/30\ d}$ is in agreement with the value of 1·14 found by Sawada and Yoshinaga [10]. In accordance with this low RBE value, Davis *et al.* [11] found no consistent qualitative difference in lymphocyte and granulocyte counts after the administration of equal absorbed doses of 14 MeV neutrons and X-rays. Recently largely different values have been reported for the $LD_{50/30\ d}$ in mice exposed to 14 MeV neutrons. Strike [12] found RBE values of 1·6 and 1·5 for C57BL and C3H mice and Graul *et al.* [13] obtained an RBE of 1·9 for NMRI mice. With regard to the latter results it can be concluded, however, that death due to the intestinal syndrome interfered with death due to the bone marrow syndrome. Additional evidence for the low RBE value reported in this paper was obtained from collaborative experiments with Duncan *et al.* at Manchester on the CFU survival in mouse bone marrow after *in vivo* irradiation with D–T neutrons, 300 kV X-rays and ^{60}Co and ^{137}Cs gamma-rays [14, 15]. At a surviving fraction of 0·1% the Manchester group obtained an RBE of 1·25 for CFU survival. As discussed in detail elsewhere [15] the discrepancy with our results is partly explained by a 8% difference in neutron dosimetry and partly by differences in the neutron spectra used. The Manchester experiments were performed inside a steel collimator. It has been demonstrated that in their experimental conditions a proportion of 10–20% of the neutron dose is delivered by scattered neutrons [16]. The contribution of these low energy neutrons will tend to increase the RBE, since it has been shown in other studies [17] that the RBE increases with decreasing neutron energy.

The RBE of 1·4 for the $LD_{50/5\ d}$ derived from our data is very close to the RBE values of 1·5 to 1·6 obtained by Yamamoto [18] for the intestinal syndrome in mice. Withers *et al.* [19] studied the response of jejunal crypt cells in mice after single dose and split dose irradiation with 14 MeV neutrons. At a dose of about 900 rads of fast neutrons, at which mortality due to the intestinal syndrome will occur, an RBE of 1·4 can be calculated for a single dose irradiation. This value is in close agreement with the RBE values which we have found for the $LD_{50/5\ d}$ and the crypt cell survival. For the irradiation with two doses separated by a 24-hr interval an RBE of 1·5 can be calculated from their survival curves.

For the survival of cultured cells of human kidney origin it has already been shown that the RBE values increase with decreasing neutron energy [17]. A similar dependence has been found for the RBE for the bone marrow syndrome and the intestinal syndrome in dependence on the neutron energy as shown in Table 2. The RBE values for the two radiation syndromes show a distinct difference for the three different neutron energies. A few years ago we postulated that there are intrinsic differences in radiosensitivity between

Table 2. *RBE values for bone marrow syndrome and gastro-intestinal syndrome in mice after fast neutron irradiation*

	15 MeV neutrons [6]	7 MeV neutrons [20, 21]	Fission neutrons [22]
Bone marrow syndrome	1·1	1·5	1·9
Gastro-intestinal syndrome	1·4	2·3	3·0

bone marrow cells and intestinal cells, which are related to differences in the shapes of the survival curves for intestinal cells and bone marrow cells after neutron- and X-irradiation [23]. The recent experimental results on the dose survival characteristics of the stem cells of the bone marrow and the jejunal crypt cells support this hypothesis. In RBE studies of cyclotron produced neutrons for mammalian tissues a similar distinction has been made between the response of haemopoietic tissues and the response of a large number of other normal tissues [24].

A summary of the RBE values for effects on normal tissues and tumours has been given in Table 3. The tumour results will be discussed in detail elsewhere [1–3]. As far as the effects on mouse intestine and rat skin are concerned very limited data are available at present. The results of the fractionated irradiation of these normal tissues indicate an increase in RBE with decreasing dose per fraction. It has to be concluded, however, that more data have to be obtained for the responses of normal tissues after fractionated irradiation with 15 MeV neutrons and X-rays. At present no definitive evaluation can be given with respect to a possible therapeutic advantage of 15 MeV neutrons.

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Table 3. RBE values of 15 MeV neutrons relative to 300 kV X-rays for single and fractionated irradiations of animal tumours and normal tissues

Biological system	Irradiation conditions	RBE
Rhabdomyosarcoma in the rat (cloning technique*)	*in vitro*, single dose	1·6
	in vivo, single dose	2·9
	in vivo, 10 daily fractions	3·0
	in vivo, 15 daily fractions	2·9
Osteosarcoma in the mouse (end-point dilution assay*)	*in vitro*, single dose	1·6
	in vivo, single dose	1·8
	in vivo, 5 daily fractions	2·5
Haemopoietic tissue in the mouse (spleen colony assay*)	*in vitro*, single dose	1·10
	in vivo, single dose	1·03
	in vivo, 5 daily fractions	0·96
(Haemopoietic syndrome)	single dose	1·12
Gastro-intestinal tract in the mouse (microcolony technique)	*in vivo*, single dose	1·4
(G.I. syndrome)	single dose	1·42
Skin of the rat (20–60% reduction in surface of graft)	single dose	1·45
	2 daily fractions	1·65
	5 daily fractions	>2·0

*RBE values for cell survival are calculated at surviving fractions of 0·001.

REFERENCES

1. G. W. Barendsen and J. J. Broerse, Experimental radiotherapy of a rat rhabdomyosarcoma with 15 MeV neutrons and 300 kV X-rays. I. Effects of single exposures. *Europ. J. Cancer* **5**, 373 (1969).
2. G. W. Barendsen and J. J. Broerse, Experimental radiotherapy of a rat rhabdomyosarcoma with 15 MeV neutrons and 300 kV X-rays. II. Effects of fractionated treatments, applied five times a week for several weeks. *Europ. J. Cancer* **6**, 89 (1970).
3. L. M. van Putten, P. Lelieveld and J. J. Broerse, Response of a poorly reoxygenating mouse osteosarcoma to X-rays and fast neutrons. *Europ. J. Cancer* **7**, 153 (1971).

4. J. J. Broerse, Dosimetry for fast-neutron irradiations of cultured cells and intact animals. 2. Comparison between activation and ionization methods. *Int. J. Radiat. Biol.* **10,** 429 (1966).
5. J. J. Broerse and H. van Ammers, Dosimetry for fast-neutron irradiations of cultured cells and intact animals. 1. Characteristics of tissue-equivalent ionization chambers. *Int. J. Radiat. Biol.* **10,** 417 (1966).
6. J. J. Broerse, Dose-mortality studies for mice irradiated with X-rays, gamma-rays and 15 MeV neutrons. *Int. J. Radiat. Biol.* **15,** 115 (1969).
7. J. E. Till and E. A. McCulloch, Direct measurement of radiation sensitivity of normal mouse bone marrow cells. *Radiat. Res.* **14,** 213 (1961).
8. H. R. Withers and M. M. Elkind, Microcolony survival assay for cells of mouse intestinal mucosa exposed to radiation. *Int. J. Radiat. Biol.* **17,** 261 (1970).
9. S. K. Lahiri and L. M. van Putten, Modification of growth kinetics of colony-forming units *in vivo*. In *Radiation-Induced Cancer*, p. 109. IAEA, Vienna (1969).
10. S. Sawada and H. Yoshinaga, The relative biological effectiveness of X-ray, Co^{60} gamma-ray, and 14·1 MeV fast neutron for acute death in mice. *Nippon Acta Radiol.* **23,** 1080 (1963).
11. M. L. Davis, E. B. Darden and G. E. Cosgrove, Early hematologic effects of whole-body 14 MeV neutron irradiation in mice. *Acta Radiol.* **3,** 87 (1965).
12. T. A. Strike and R. H. Crutcher, Acute mortality of mice and rats exposed to 14 MeV neutrons. In *Armed Forces Radiobiology Research Institute Annual Research Report* ARR-3, p. 13 (1969).
13. E. H. Graul, W. Rüther and H. Krüger, Untersuchungen über die relative biologische Wirksamkeit von 14-MeV-Neutronen an Laboratoriumstieren und HeLa-Zellen. *Strahlentherapie* **138,** 699 (1969).
14. W. Duncan, D. Greene, A. Howard and J. B. Massey, The RBE of 14 MeV neutrons. Observations on colony-forming units in mouse bone marrow. *Int. J. Radiat. Biol.* **15,** 397 (1969).
15. J. J. Broerse, W. Duncan, A. C. Engels, C. W. Gilbert, D. Greene, J. H. Hendry, A. Howard, P. Lelieveld, J. B. Massey and L. M. van Putten, The survival of colony-forming units in mouse bone marrow after *in vivo* irradiation with D-T neutrons, X- and gamma-radiation. To be published in *Int. J. Radiat. Biol.*
16. D. Greene and R. L. Thomas, An experimental unit for fast neutron radiotherapy. *Brit. J. Radiol.* **41,** 455 (1968).
17. J. J. Broerse, G. W. Barendsen and G. R. van Kersen, Survival of cultured human cells after irradiation with fast neutrons of different energies in hypoxic and oxygenated conditions. *Int. J. Radiat. Biol.* **13,** 559 (1968).
18. O. Yamamoto, On the variation of LD-50 in time after 14·1 MeV fast neutrons- and 180 kVp X-rays-irradiation for young and adult mice. *Nippon Acta Radiol.* **26,** 1361 (1967).
19. H. R. Withers, J. T. Brennan and M. M. Elkind, The response of stem cells of intestinal mucosa to irradiation with 14 MeV neutrons. *Brit. J. Radiol.* **43,** 796 (1970).
20. S. Hornsey, Private communication (1970).
21. S. Hornsey, S. Vatistas, D. K. Bewley and C. J. Parnell, The effect of fractionation on four-day survival of mice after whole-body neutron irradiation. *Brit. J. Radiol.* **38,** 878 (1965).
22. J. A. G. Davids, Relative biological effectiveness of fission neutrons for production of the bone marrow syndrome in mice. *Int. J. Radiat. Biol.* **10,** 299 (1966).
23. J. J. Broerse, Effects of energy dissipation by monoenergetic neutrons in mammalian cells and tissues. Thesis, Amsterdam (1966).
24. S. B. Field, The relative biological effectiveness of fast neutrons for mammalian tissues. *Radiology* **93,** 915 (1969).

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Summary of Discussions in the Session on Fast Neutron Radiobiology of Tumours and Normal Tissues

H. S. REINHOLD

Radiobiological Institute TNO, 151 Lange Kleiweg, Rijswijk Z.H., The Netherlands

THE DISCUSSIONS in this session dealt with various aspects of tumour and normal tissue radiobiology, the OER and its possible change with depth in tissue and with the dependence of the RBE on dose and dose fractionation. In particular questions were raised about whether general conclusions can be drawn from the phenomena observed in a few animal tumours and normal tissues.

If all tumours showed an adequate degree of reoxygenation during a course of conventional radiotherapy with X- or γ-rays, then neutron radiotherapy would hardly be expected to provide an advantage. This was pointed out by Thomlinson in his paper and was also touched upon by van Putten in discussing the results of neutron radiotherapy for the poorly reoxygenating osteosarcoma. Elkind inquired whether it would be possible to predict in general the degree of reoxygenation of a tumour during a given treatment, taking into account that some tumours shrink rapidly while others increase in size for one or more days before starting to regress. Similar questions of how to obtain information about the rate of redistribution of oxygen and the optimal time intervals for treatment of a tumour, were also raised by Kaplan and van Putten. Thomlinson answered that, with respect to reoxygenation, there seems to be no direct correlation with tumour histology or with the volume regression curve of the tumour. Redistribution of oxygen depends probably in part upon a diminished demand by the radiation-killed cells. Thomlinson also pointed out that tumour regression is an active biological process, involving cell proliferation, phagocytosis and endothelial cell proliferation, which in itself is a radiosensitive process!

In the context of optimal fractionation schedules in relation to reoxygenation. Thomlinson considered the possibility that the therapeutic improvement, which was only observed with the trials of hyperbaric oxygen if less than ten fractions were given, might be interpreted by the assumption that by giving the dose in too few fractions without hyperbaric oxygen, one might exceed the dose per fraction which is optimally effective due to reoxygenation. The apparent improvement of the use of hyperbaric oxygen would thus have to be interpreted by the hyperbaric oxygen compensating for inadequate reoxygenation, in cases of fractionation schedules with only a few dose fractions! When planning for clinical trials with fast neutrons, such factors will have to be taken into account.

Kaplan raised the question of how an optimal strategy of a combined therapy with neutrons and X-rays might be devised and Thomlinson replied that, in order to kill as many hypoxic cells as possible, while keeping normal tissue damage low, one might start therapy with neutrons, and change to X-rays when the process of reoxygenation is well under way.

With regard to the use of neutrons for tumours such as the osteosarcoma investigated by van Putten, Fowler emphasized that, although this tumour showed no special *advantage* for the use of neutrons, there were certainly no *disadvantages*.

After Berry's paper discussing OER measure-

ments as a function of neutron energy and as a function of depth in a phantom, it was noted first that a decrease of the OER at 10 cm depth, although unexplained, would enhance a possible advantage of fast neutrons. Berry confirmed that all precautions against possible artifacts had been taken, with special regard to dose-rate effects, repair processes and the influence of temperature changes. Elkind felt that the observation that the D_0 for aerobic cells changes in a different way with depth in tissue than the D_0 for hypoxic cells, may have important implications with respect to repair processes. Without suggesting to offer a physical explanation for the discrepancies, Rossi indicated that the RBE as well as the OER may quite well change extremely rapidly with the energy density, and that the mean LET is not the relevant parameter. Particles of a given LET produce a range of energy depositions when traversing e.g. a cell, and thus the deposition of a given amount of energy in a microstructure depends upon a probability distribution.

Curtis discussed some of his calculations showing that the α-particle contribution may be very important in causing a low OER for fast neutrons. This conclusion was reached from calculations using track segment data to predict the relative contributions of protons, α-particles and heavy recoils to cell killing by 3 MeV and 15 MeV neutrons.

In the discussion about the various aspects of the RBE values for different objects as presented by Field and Broerse, Fowler noted that the tumour-RBE was in all instances higher than the RBE for the normal tissues. Ebert reported results by Gregory in Manchester, who derived RBE values for spleen plaque-forming cells, irradiated with 14 MeV neutrons, as measured by the Jerne technique for immunocompetent cells. The RBE for 7 S antibody forming cells was about 1·16, and independent of the surviving fraction of cells and thus also independent of the dose applied. The RBE for 19 S antibody forming cells on the other hand changed with dose and ranged from 1·6 at low doses to 1·2 at higher doses. All RBE values for 14 MeV neutrons, as determined by these systems, were below 2. Hall discussed results, obtained with the Vicia Faba system, showing that the RBE was strongly dependent on the dose per fraction. These results matched well the data presented by Field. Hall also investigated whether or not the OER changed with the dose-rate and reached the conclusion that the OER of 1·6 for acute exposure to 14 MeV neutrons is very similar to the value of 1·66 for chronic exposure to fission neutrons from ^{252}Cf at 16 rads/hr. With respect to Field's observation that the RBE for late effects is very similar to the one for early effects, Duncan raised the question of how to compare the extent of early and late reactions, especially in view of the fact that late reactions do not develop after low doses but at doses in excess of a given threshold they increase very rapidly with the dose. Field explained that, in his experiments, the intensity of the late reactions was scored on an identical scale as the one used for the early reactions. However, while the relationships mentioned are correct for the animal population as a whole, differences in severity between early and late reactions of individual animals were occasionally observed. This implies that those tissue components involved in the development of early reactions are not necessarily the same as those causing the late reactions. Morrison and Thomlinson suggested that late effects might be caused by damage to blood vessels and connective tissues. Thomlinson drew attention to the point that we must realize that, for the recovery of any normal tissue, a minimum number of clonogenic cells is required. If one reduces the number of viable cells in a given tissue unit beyond a certain threshold, the unit may never reappear. This is a factor which, for the study of normal tissue responses, should be taken into consideration. Finally Franke discussed some data indicating that RBE values for damage to brain caused by 14 MeV neutrons might differ for grey and white matter.

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Cellular Responses Determining the Effectiveness of Fast Neutrons Relative to X-Rays for Effects on Experimental Tumours

G. W. BARENDSEN
Radiobiological Institute TNO,
151 *Lange Kleiweg, Rijswijk Z.H., The Netherlands*

Abstract — *Relations between the relative biological effectiveness of fast neutrons and the dose or daily dose fractions are presented for cultured cells, tumours and normal tissues. These relations are dependent on a variety of factors, namely the neutron energy spectrum, the shapes of dose-survival curves, repair of sub-lethal damage, the presence of anoxic cells, reoxygenation during intervals between fractions and the dependence of radiosensitivity on the age of cells in their cycle. The relative influence of these factors is discussed.*

Comparison of the RBE values of fast neutrons derived from published data for experimental tumours and normal tissues indicates that overall gain factors, $RBE_{(tumour)}/RBE_{(normal\ tissue)}$, *range between* 1·1 *and* 1·3. *A possible advantage of fast neutrons will depend on the type of tumour and the type of dose-limiting normal tissue.*

INTRODUCTION

THE AIM of a radiotherapeutical treatment is to administer radiation according to a schedule which, by the proper choice of various parameters, namely the spatial distribution of the dose in the patient, the sizes of the dose fractions, the intervals between fractions and the total dose, will just be tolerated by normal tissues while causing maximum damage to the tumour. In comparison with orthovoltage X-rays, the introduction of high energy X-rays and fast electrons has allowed considerable improvement to be made in the dose distribution in the patient. With fast neutrons this distribution presumably cannot be improved further, and this radiation can only provide an advantage if the relative biological effectiveness (RBE) is larger for damage to the tumour than the RBE for damage to the dose-limiting normal tissues. Values of the RBE may depend on the parameters mentioned, i.e. the sizes of the dose fractions, the intervals between fractions and the total dose, as well as on the type of tumour and normal tissue irradiated and consequently a large amount of experimental and clinical data is required before possible advantages of fast neutrons can be adequately evaluated. In this paper the influence of a number of factors on the RBE will be discussed.

Results of radiobiological studies, obtained during the past decade, have shown that radiation-induced damage to normal tissues and tumours in animals is determined to a large extent by responses of those constituent cells which are capable of unlimited proliferation, i.e. the clonogenic cells. Furthermore, responses of individual cells after irradiation *in vivo* have been shown with a number of experimental systems to be similar to responses of the same type of cells grown and assayed *in vitro*, provided various environmental conditions, e.g. oxygen concentration, are made equal.

Investigations of cells cultured *in vitro* have demonstrated that a variety of factors can influence their radiosensitivity with respect to the capacity for proliferation, e.g. radiation quality, the distribution of dose in time, the presence of oxygen and other sensitizing compounds, the presence of protecting compounds, the proliferative state of the cells and their age in the cell cycle [1]. These factors, which in experiments *in vitro* have been studied in well controlled conditions, may cause large variations in the responses to fractionated irradiation of tumours and of normal tissues in patients, where cellular conditions are in general unknown and cannot be easily determined or controlled.

Experimental data obtained with respect to the radiosensitivity of cells in culture, in experimental tumours or in various normal tissues, have often been used to emphasize similarities between the characteristics of dose survival curves of different types of mammalian cells. It should not be forgotten however that differences by a factor of 2–3 in D_0 values, which range from 70 to 200 rads, and differences in extrapolation numbers by at least a factor of 10, have been observed. These differences in intrinsic radiosensitivity of various types of mammalian cells may be augmented by certain conditions in tumours or tissues due to some of the dose modifying factors mentioned and this can result in very large differences in the responses of these multicellular systems to a fractionated treatment [1].

In addition to factors influencing the radiosensitivity of individual cells, the responses of tumours and normal tissues after irradiation or during a fractionated treatment depend on the proliferative activity and changes in physiological conditions of those cells that have retained the capacity for unlimited proliferation. Irradiated cells will generally experience a phase of mitotic delay, after which proliferation is resumed. The rate of proliferation of parenchymal cells and the rate of removal of dead cells in irradiated tumours and in normal tissues may depend on various factors, which have not yet been sufficiently investigated, e.g. tumour architecture and the vascular system. Increased as well as decreased rates of cell production in comparison with pre-irradiation values have been observed in irradiated tumours, depending on the dose administered and the time interval after irradiation [2–7]. Furthermore changes in oxygenation status of cells have been demonstrated in tumours containing a fraction of severely hypoxic cells at the start of a treatment [6, 8–10]. Finally the volume response of a tumour to irradiations may depend on factors concerned with the architecture of the tumour, e.g. the amount and type of stroma, the blood vessels and the surrounding tissues.

As a consequence of all these factors which can cause variations in responses of tumours as well as of normal tissues, it is not inconsistent with radiobiological data that tumours in patients show large differences in their clinical radiosensitivity to fractionated treatments with X-rays or γ-rays. The main problem in experimental tumour radiobiology is to evaluate the relative importance of the various factors mentioned and to detect systematic differences between responses of various types of tumours and various types of normal tissues. Comparison of the effectiveness of fast neutrons relative to X-rays for inducing damage to different types of cells, tumours and normal tissues, can provide data for this evaluation, because some of these factors play a smaller part with neutron irradiations.

Dose-effect relations for X-rays and fast neutrons differ in two main aspects, namely a reduced shoulder of the survival curve for fast neutrons as compared with X-rays, with a consequent diminished contribution of repair of sub-lethal damage in fractionated treatments, and a relatively low oxygen enhancement ratio of fast neutrons, which does not depend greatly on neutron energy in the range between 5 and 30 MeV [11]. As a result of these differences, the relative biological effectiveness (RBE)* of a given dose of fast neutrons for effects on tumours and normal tissues depends on the dose, the fractionation schedule, the presence of anoxic cells and on changes in oxygenation status. Finally, differences between effects of fast neutrons and X-rays have been observed with respect to the dependence of radiosensitivity on cell age in the division cycle. The influence of this factor may also depend on the dose fractionation schedule, because the degree of induced synchrony depends on the dose, and the desynchronization before the next dose is given depends on the time interval.

All these factors, which can cause variations of the RBE of fast neutrons in dependence on the dose and dose fractionation schedule, have been listed in Table 1A. In part B of this table, three other factors have been listed which are important with respect to the final result of a

*The RBE of a dose of neutron radiation is defined as the ratio of a dose of X-rays and the dose of neutron radiation required for producing quantitatively equal effects.

Table 1. *Factors determining responses of tumours and normal tissues to single and fractionated irradiations*

Factors	Characteristics
A. *Factors which influence the RBE of fast neutrons*	
1. Neutron energy spectrum	RBE increases with decreasing neutron energy [11]
2. Size of dose or dose fraction	RBE increases with decreasing dose, dependent on shapes of survival curves of cells [1]
3. Intra-cellular repair of sub-lethal damage	RBE increases with decreasing dose fraction, if intervals between fractions are long enough to allow repair [1, 12, 13]
4. Hypoxia of proportion of cells	Because $(OER)_{neutrons} < (OER)_{X-rays}$ the RBE of neutrons is larger for damage to hypoxic cells [1, 4–6, 18]
5. Reoxygenation of hypoxic cells during intervals between fractions	Decreases the effect of hypoxic cells [5, 6, 8–10]
6. Dependence of radiosensitivity on cell age	This dependence is smaller for fast neutrons as compared with X-rays [14]
B. *Factors which do not influence the RBE of fast neutrons*	
1. Proliferation of cells in intervals between dose fractions	Rate can increase or decrease during and after fractionated treatment [2, 3, 7]
2. Non-lethal damage	Causes death in part of the progeny of surviving cells and slow rate of regrowth of tumours [15, 16]
3. Mitotic delay	Influence is presumably small in practical treatment schedules

radiotherapeutical treatment, but which do not cause further variations of the RBE of fast neutrons in dependence on the fractionation schedule employed. The first of these factors, proliferation of cells in intervals between dose fractions reduces the effect of a given treatment, i.e. a larger dose is required in order to kill all cells. The limited experimental data available indicate that changes in the rate of proliferation and of cell loss do not depend on the type of radiation employed if doses are compared which cause equal damage to the proliferative capacity of the clonogenic cells [1, 5]. Non-lethal damage has also been demonstrated to be caused to an equal degree by different radiations provided doses are compared which cause the same degree of cell reproductive death. Mitotic delay, as far as indicated by the limited data available, is caused with the same RBE as cell lethality. Thus if a treatment with fast neutrons and a treatment with X-rays produce equal effects with respect to cell killing, then cell proliferation, impaired cell production due to non-lethal damage or mitotic delay will not cause other differences between the effects of these treatments with respect e.g. to the probability of cure or the growth rate of recurrent tumours.

RBE VALUES OF FAST NEUTRONS FOR REPRODUCTIVE DEATH IN CULTURED CELLS

With *in vitro* systems, RBE values can be determined for cells in well controlled conditions, whereby variations due to, e.g. partial hypoxia or changes in age distributions of the cells can be eliminated. Survival curves for cultured asynchronous mammalian cells of human kidney origin (T-1 g cells) have been determined with fast neutrons of different energies by the clone technique [11]. From these curves RBE values relative to 250 kVp X-rays have been derived for various levels of the neutron dose. The results are presented in Fig. 1. These curves illustrate some general features of the dependence of the RBE on the neutron dose and energy. The logarithmic scales are used because some fundamental radiobiological considerations indicate that relatively simple shapes of the curves might be obtained [17].

The curves of Fig. 1 show that the largest RBE values are derived for the lowest neutron energy and conversely. The mean value of "10 MeV" assigned to the neutron spectrum obtained by bombarding Be with 20 MeV ^{3}He-ions, is quite arbitrary because the spec-

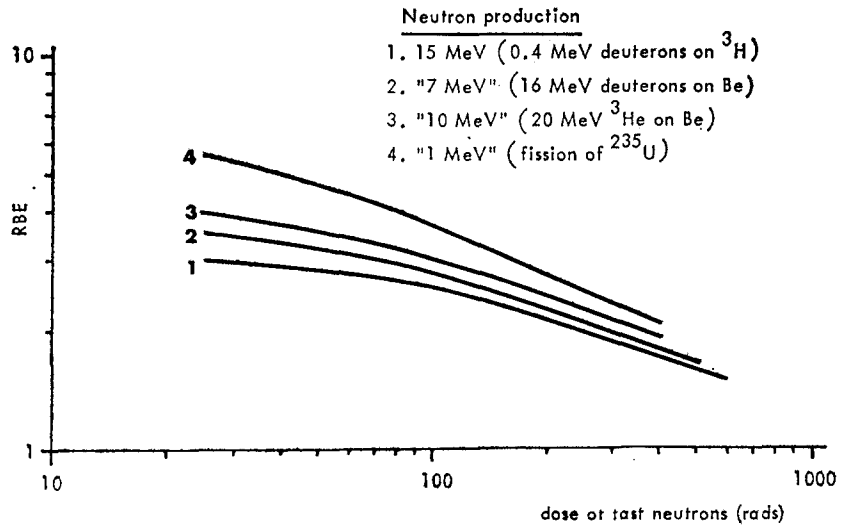


Fig. 1. Relative biological effectiveness as a function of the dose of fast neutrons relative to 250 kVp X-rays. Data derived from survival curves for cultured cells of human kidney origin [11]. *Curve 1: 15 MeV neutrons produced by the $^3H(d, n)^4He$ reaction. Curve 2: "7 MeV" neutrons produced by the $^9Be(d, n)^{10}B$ reaction. Curve 3: "10 MeV" neutrons produced by the $^9Be(^3He, n)^{11}C$ reaction. Curve 4: "1 MeV" neutrons produced by fission of ^{235}U.*

trum is complex and extends from very low values up to about 33 MeV. The mean value for the energy spectrum of neutrons produced by bombarding Be with 16 MeV deuterons, which extends from 0 to 18 MeV, is 7 MeV according to Bewley [18]. However, the fact that the RBE values of the "10 MeV" neutrons are larger than the values for "7 MeV" neutrons cannot be assigned a special significance with regard to the dependence of the RBE on neutron energy, because the contributions of the different parts of the energy spectrum cannot yet be adequately evaluated.

In addition to the dependence on neutron energy, the RBE is shown to increase with decreasing level of damage, i.e. with decreasing size of the doses compared. This is due to the fact that survival curves obtained with fast neutrons generally exhibit a smaller shoulder width as compared with survival curves obtained with X-rays or γ-rays [1]. In cases where fractionated irradiation is given with intervals which are sufficiently long to allow for complete repair of sub-lethal damage, the RBE value for a given dose in this Fig. 1 also applies to the dose per fraction. The problem of whether at very low doses or dose fractions a constant RBE value is approached has been discussed elsewhere [1, 17].

For the application of fast neutrons in radiotherapy, the region between 50 rads and 250 rads of fast neutrons is presumably the most important, because very small dose fractions would require a very large number of fractions to attain the total dose necessary for cure, while dose fractions in excess of 250 rads would be expected to be too large to be administered.

In radiotherapy, doses of radiation are generally applied in fractionated treatments in a number of weeks. The intervals of one or a few days can be expected to be sufficient for repair of sub-lethal damage to be completed and the extent of this repair is related to the shape of the shoulder region of the survival curves [13]. Consequently the RBE of a given *total dose* of fast neutrons increases with increasing number of fractions, i.e. with decreasing dose per fraction and to a first approximation the RBE is determined by the size of the dose fraction employed.

In addition to the smaller dependence of the effect of a given dose of neutrons, compared with X-rays, on dose fractionation, the oxygen enhancement ratio $(OER)_{neutrons}$ is generally smaller than $(OER)_{x\text{-}rays}$ by a factor 1·6–1·8 [18]. The maximum radiotherapeutic gain with respect to the "effective" dose to the tumour as compared to normal tissue, which can be obtained from this difference, would be attained for single acute doses, applied to tumours in which all cells were severely hypoxic, assuming that all cells in normal tissues were well oxygenated. This hypothetical oxygen effect gain factor $(OEG)_{max}$ is equal to $(OER)_{x\text{-}rays}/(OER)_{neutrons}$. In practice the gain factor will be smaller than $(OEG)_{max}$ because not all cells in tumours are severely hypoxic and because reoxygenation may occur during intervals between fractions, diminishing the influence of hypoxic cells.

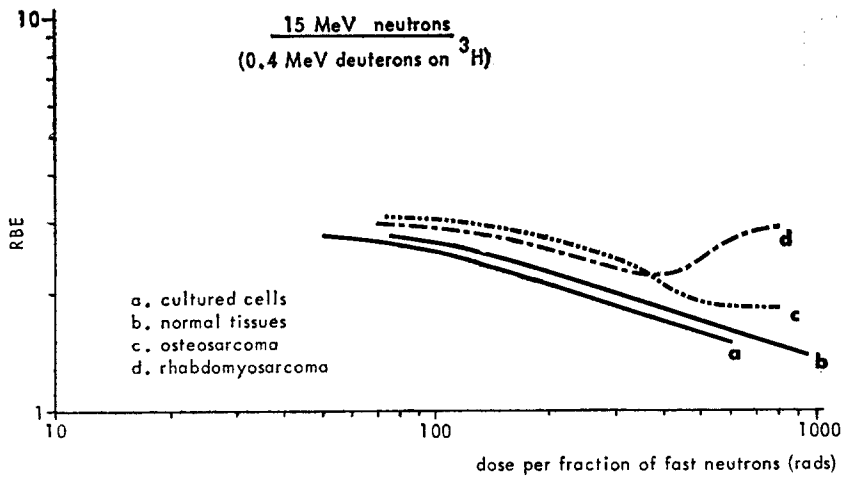


Fig. 2. Relations between the relative biological effectiveness of 15 MeV neutrons and the neutron dose or daily dose fraction, relative to 250 kVp X-rays. Curve "a" represents data obtained from survival curves for cultured cells of human kidney origin [11]. *Curve "b" represents data for normal epithelial tissues* [21]. *Curve "c" represents data for a mouse osteosarcoma. From van Putten* et al. [20]. *Curve "d" represents data for a rat rhabdomyosarcoma* [4, 5].

RBE VALUES MEASURED FOR RESPONSES OF EXPERIMENTAL TUMOURS AND NORMAL TISSUES

Fast neutrons will only provide an advantage relative to X-rays or γ-rays for the treatment of cancer if the RBE value for damage to the tumour is larger than the RBE for producing damage in those normal tissues which are dose-limiting for the treatment considered.

For two types of experimental tumours, a rhabdomyosarcoma transplantable in WAG/Rij rats and an osteosarcoma transplantable in (CBA/Rij × C57BL/Rij)F_1 hybrid mice, effects of fast neutrons with an energy of about 14–15 MeV produced by the ^{3}H(d, n)^{4}He reaction, have been studied with a sufficiently wide range of doses and dose fractions, to allow estimates of the RBE as a function of dose or daily dose fractions [4, 5, 19, 20]. In Fig. 2 these data are represented by curves "d" and "c" respectively. Curve "a" of this figure represents the dependence of the RBE of these neutrons on the dose for damage to cultured cells of human kidney origin, i.e. curve "a" is identical to curve 1 of Fig. 1. Curve "b" represents the dependence of the RBE on the dose or daily dose fraction for damage to skin and intestinal epithelium, derived from experiments with rats and mice [1, 21, 22]. This curve "b" can only be considered as an approximation, because the data are limited and not accurate enough to evaluate whether small differences exist between RBE values for the various systems investigated. Moreover, most of the data on which curve "b" is based have been measured for doses in excess of 500 rads and for low doses, curve "b" has been extrapolated parallel to curve "a" obtained from experiments on cell cultures.

A discussion of the relations between the curves of Fig. 2 can be given by referring to the factors summarized in Table 1. In the region between 70 and 350 rads of 15 MeV neutrons, all curves of Fig. 2 are within experimental errors parallel and the RBE increases with decreasing dose or magnitude of the daily dose fractions. This variation can be ascribed to the differences between shapes of the survival curves for neutrons and X-rays (factor A2) and to differences in the influence of repair of sub-lethal damage (factor A3) in the 24-hr intervals employed. The fact that the curves in Fig. 2 are within experimental errors parallel in the region between 70 and 350 rads suggests that the influence of these factors on the RBE is approximately equal for the cultured cells, the epithelial cells of skin and intestinal tract and the cells of the two tumours investigated.

It is important to note however that, for cells of the haemolymphopoietic system, X-ray survival curves generally exhibit a smaller shoulder as compared with epithelial cells. As a consequence the differences in the shapes of the survival curves for X-rays and fast neutrons are smaller and the RBE of fast neutrons for damage to these cells generally depends less on the dose or dose per fraction as compared with epithelial cells. Moreover, the values of the RBE of fast neutrons for damage to the haemolymphopoietic system are significantly lower than for epithelial cells [21, 23]. For instance, the RBE of 14 MeV

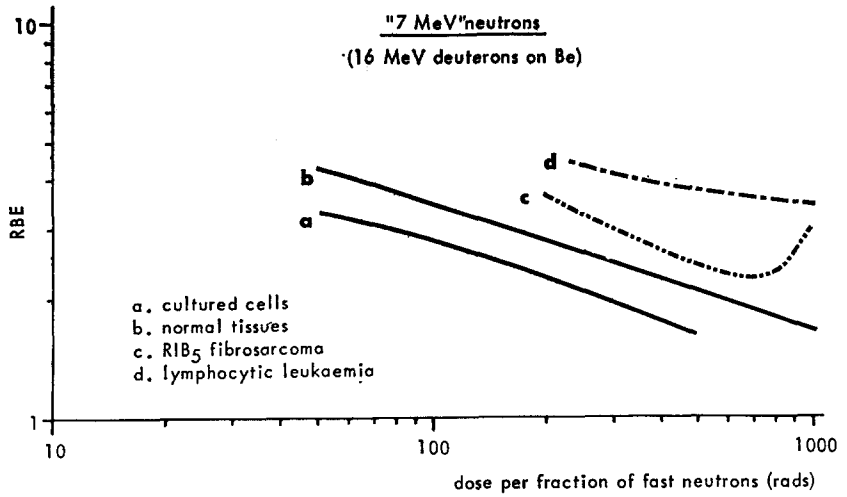


Fig. 3. Relations between the relative biological effectiveness of "7 MeV" neutrons and the neutron dose or daily dose fraction relative to 250 kVp X-rays. Curve "a" represents data obtained from survival curves for cultured cells of human kidney origin [11]. *Curve "b" represents data obtained for reactions of normal epithelial tissues of animals. From Field* [23]. *Curve "c" represents data for the RIB_5 rat fibrosarcoma. From Field* [23]. *Curve "d" pertains to a mouse lymphocytic leukaemia. From Berry* et al. [28].

neutrons for lethality of haemopoietic stem cells in mice ranges between 0·9 and 1·2 [21].

It can be concluded that, for cells in different types of tumours and for cells in different types of normal tissues, considerable differences can exist with respect to the width of the shoulder of survival curves. With respect to the influence of such differences on effects of dose fractionation in radiotherapy, two possibilities can be envisaged.

The first possibility is that the response of the dose-limiting tissue is characterized by a cell survival curve with a shoulder which has a greater width than the shoulder of the survival curve for the tumour cells. In such a case fractionation of a given total dose would have a greater sparing effect for the normal tissue as compared with the tumour. Consequently the use of a type of radiation which enhances this difference, would be indicated, i.e. X-rays or γ-radiation should give optimal treatment results.

A second possibility which can be envisaged is that the shoulder width of the survival curve of the tumour cells is larger than that of cells of the dose-limiting normal tissue. In this case, fractionation of a given total dose of X- or γ-rays in a large number of fractions would have a greater sparing effect on the tumour as compared with the dose-limiting normal tissue. A treatment with fractionated doses of X- or γ-rays would then result in a relatively small effect on the tumour, i.e. the tumour would be judged to be radioresistant. In this case the use of fast neutrons, which would eliminate a large part of the shoulder of the survival curve of tumour cells, might provide an advantage as compared with X- or γ-rays, although the shoulder of the survival curve for the cells in normal tissues would also be eliminated. At present methods are not yet available however to distinguish in clinical practice those tumours for which survival curves of the cells are characterized by large shoulders.

Curve "d" of Fig. 2 shows at doses in excess of 350 rads a distinct increase of the RBE with the dose. Experiments described in detail elsewhere have shown that this rhabdomyosarcoma contains a proportion of about 15% of severely hypoxic cells [4]. Furthermore $(OER)_{x\text{-rays}}/(OER)_{neutrons}$ was measured at approximately 1·7 [4]. At 800 rads of 15 MeV neutrons, the ratio of the RBE for damage to the rhabdomyosarcoma relative to the RBE for damage to normal epithelium is approximately 1·9. This indicates that for large doses or daily dose fractions the total gain factor can almost completely be explained by factor A4 of Table 1.

With fractionated doses of 300 rads of X-rays it has been demonstrated that reoxygenation, mentioned as factor A5 of Table 1, occurs during intervals of 24 hr and this presumably accounts for the fact that the influence of the presence of a proportion of anoxic cells on the RBE of 15 MeV neutrons is diminished for fractionated treatments [5]. It can be derived from Fig. 2 that with decreasing dose per fraction the overall gain factor decreases from 1·9 at 800 rads to 1·3 at 400 rads and approximately 1·2 at 100 rads of 15 MeV neutrons.

Curve c of Fig. 3 for damage to the reproduc-

tive capacity of cells in a mouse osteosarcoma has a shape which at neutron doses smaller than 400 rads is not significantly different from curve "d" and the increase in RBE with decreasing dose or daily dose fraction can be ascribed to the factors A2 and A3 mentioned in Table 1. For this osteosarcoma it has been demonstrated that reoxygenation of severely hypoxic cells occurs relatively slowly [19]. Consequently it might have been expected that for this tumour the RBE for fractionated treatments with daily doses of 100 rads or more would be considerably larger than for normal tissues. As discussed in detail elsewhere, this is not observed, because the oxygen-effect gain factor $OEG = (OER)_{x\text{-rays}}/(OER)_{neutrons}$ is equal to only approximately 1·2 [20]. This low value can also explain why the RBE does not increase at doses in excess of 400 rads.

With respect to the influence of the dependence of radiosensitivity on cell age, i.e. factor A6 in Table 1, on the RBE of fast neutrons, no quantitative experimental data have been published for tumours. For cultured cells irradiated with "fission spectrum" neutrons, the variation of the response throughout the cycle was less than for X-rays [14]. As a consequence the RBE showed variations by a factor of about 1·3 in dependence of cell age [14]. It is important to note that this is the maximum variation between well synchronized sub-populations of Chinese-hamster cells where differences in sensitivity, as a function of cell age, are larger than in many other cell types. Moreover with "fission spectrum" neutrons this difference in RBE is likely to be larger than for higher energy neutrons. In tumours treated with relatively small daily dose fractions, the degree of synchronization will presumably be too small to cause significant changes in the RBE of 15 MeV neutrons for different fractionation regimes. A special case in which such changes might occur is provided by low-dose rate treatments as applied in interstitial and intracavitary treatments of cancer. Data obtained with the rat rhabdomyosarcoma, irradiated at a dose rate of about 100 rads/hr of γ-rays and a dose rate of 25 rads/hr of 15 MeV neutrons, indicate that for treatments in excess of 12 hr large cells accumulate, probably in late S or in G_2. The RBE of 15 MeV neutrons for the induction of cell reproductive death in this tumour by low-dose rate irradiation was calculated at about 4. This value is larger than those obtained for fractionated treatments, but definite proof that this is due to induced synchrony is not yet obtained [24].

Finally it can be concluded from the RBE values derived for these experimental systems that for treatments with daily dose fractions of between 70 and 200 rads of 15 MeV neutrons, the overall gain factor does not exceed a value of 1·2. This might however still provide a significant advantage in clinical applications if the dose distributions in the patient obtained with 14 MeV neutrons are not considerably worse than those obtained with high energy X-rays.

With respect to the factors listed in Table 1B, it can be noted that for the rat rhabdomyosarcoma proliferation of cells in intervals between daily dose fractions of 50, 70 and 100 rads of 15 MeV neutrons and of 200 and 300 rads of X-rays applied during a three-week treatment, plays an important part in determining the responses of these tumours [5]. After a single dose of 2000 rads of X-rays, Hermens has demonstrated that an increased rate of production of cells can occur due to a shortening of the cell cycle [2]. However, for daily doses of 15 MeV neutrons and 300 kV X-rays respectively, which cause equal effects with respect to cell lethality, the influence of repopulation due to proliferation of cells was not significantly different, i.e. factor B1 of Table 1 does not cause changes in the RBE which are independent of the RBE for cell lethality [5]. Furthermore, after treatments which reduce the fraction of clonogenic cells by at least a factor 10^4, it has been shown for this experimental tumour that the rate of growth, after the time interval required to regrow to its pre-irradiation volume, is slower than for unirradiated tumours of the same volume. This can be ascribed to the non-lethal damage mentioned as factor B2 in Table 1 [1, 3–5, 25]. This slower growth of recurrent tumours is observed both after treatments with X-rays and 15 MeV neutrons, but it does not significantly influence the RBE [5]. Investigations of non-lethal damage detected as "small colony formation" for cells cultured *in vitro*, have also shown that the RBE for this type of damage is not significantly different from the RBE for cell lethality, but only depends on the level of damage induced [1, 15, 16].

With respect to mitotic delay, measurements for cultured mammalian cells treated with α-particles have indicated that the RBE for this effect is similar to the RBE for reproductive death. Consequently with respect to this factor, fast neutrons would not be expected to provide an advantage or disadvantage over X- or γ-rays. Moreover, the influence of partial synchrony due to mitotic delay is presumably

small for dose fractions of between 200 and 400 rads of X-rays or equivalent doses of neutrons applied with 24-hr intervals [26].

A discussion of the influence of various factors, as given for results of experiments with 15 MeV neutrons, can also be presented for data obtained with "7 MeV" neutrons from the cyclotron at Hammersmith. In Fig. 3, curves "c" and "d" represent RBE values published for a rat fibrosarcoma (RIB[5]) and a mouse lymphocytic leukaemia [23, 27, 28]. Curve "b" of Fig. 3 represents a relation of the RBE as a function of the dose or daily dose fraction for normal epithelium of skin and intestine in mice and rats. This curve represents only an average and no distinction has been made between data for skin and intestinal epithelium [29]. Curve "a" of Fig. 3, derived from data for cultured cells of human kidney origin, is included for comparison.

Curve "b" of Fig. 3 for normal epithelial tissues is within experimental errors parallel to curve "a" for cultured cells, but the values of curve "b" are approximately a factor of 1·2 larger than corresponding values of curve "a". Both curves show a distinct increase of the RBE with decreasing dose or daily dose fraction which can be ascribed to factors A2 and A3 of Table 1. Values of the RBE of "7 MeV" neutrons for damage to normal tissues are for equal doses larger than RBE values of 15 MeV neutrons, in agreement with the general characteristic listed as factor A1 of Table 1.

Curve "c" of Fig. 3 represents RBE values for growth delay of a solid fibrosarcoma (RIB[5]), known to contain a proportion of severely hypoxic cells. The shape of this curve is similar to the curve "c" of Fig. 2 for the rat rhabdomyosarcoma, i.e. at large doses in excess of 700 rads the RBE increases due to the influence of hypoxia, factor A4 of Table 1, while, at small daily dose fractions, reoxygenation influences the RBE significantly (factor A5 of Table 1). Unfortunately no data are available yet for doses lower than 200 rads. Comparison of curve "c" and "b" of Fig. 3 indicates that at 200 rads of "7 MeV" neutrons an overall gain factor of 1·3 is obtained while at 500 rads this factor is about 1·2. These values are smaller than the oxygen-enhancement gain factor of about 1·7, measured for this neutron beam with various systems [18].

Curve "d" of Fig. 3 represents RBE values for reproductive death of cells of a lymphocytic leukaemia growing in mice in conditions in which all cells are severely hypoxic [28]. The RBE values are a factor of about 1·7–1·9 larger than corresponding values of curve "b". This overall gain factor is almost entirely due to hypoxia of the cells, factor A4 of Table 1, because if these cells are oxygenated, the RBE values are decreased to values very close to curve "b" [28]. The increase in RBE with decreasing dose for these severely hypoxic cells indicates that the survival curve for X-rays is not an exponential but differs in shape from the neutron survival curve (factor A2 of Table 1).

CONCLUDING REMARKS

From the RBE values of fast neutrons derived from published data for tumours and normal tissues as a function of the dose and dose fractionation it can be concluded that overall gain factors ($RBE_{(tumour)}/RBE_{(normal\ tissue)}$) of between 1·1 and 1·3 might be obtained for fast neutrons relative to X-rays. The magnitude of this factor depends on a variety of parameters and also on the types of tumours and the types of tissues which are dose-limiting. Even if clinical applications of fast neutrons would eventually provide only a relatively small advantage over X-rays or γ-rays for only a limited group of tumours in man, the insights gained from the comparison of effects induced by the different types of radiations with respect to the influence of repair of sub-lethal damage and of anoxic cells relative to other factors e.g. repopulation in intervals between fractions might provide a basis for the design of optimal treatment schedules for fast neutrons as well as for X-rays and γ-rays.

REFERENCES

1. G. W. Barendsen, Responses of cultured cells, tumours and normal tissues to radiations of different linear energy transfer. In *Current Topics in Radiation Research* (Edited by M. Ebert and A. Howard), Vol. IV, p. 332. North-Holland, Amsterdam, (1968).
2. A. F. Hermens and G. W. Barendsen, Changes of cell proliferation characteristics in a rat rhabdomyosarcoma before and after X-irradiation. *Europ. J. Cancer* **5,** 173 (1969).
3. R. J. Berry, "Small clones" in irradiated tumour cells *in vivo*. *Brit. J. Radiol.* **40,** 285 (1967).

4. G. W. BARENDSEN and J. J. BROERSE, Experimental radiotherapy of a rat rhabdomyosarcoma with 15 MeV neutrons and 300 kV X-rays. I. Effects of single exposures. *Europ. J. Cancer* **5,** 373 (1969).
5. G. W. BARENDSEN and J. J. BROERSE, Experimental radiotherapy of a rat rhabdomyosarcoma with 15 MeV neutrons and 300 kV X-rays. II. Effects of fractionated treatments, applied five times a week for several weeks. *Europ. J. Cancer* **6,** 89 (1970).
6. R. H. THOMLINSON, Changes of oxygenation in tumours in relation to irradiation. In *Frontiers of Radiation Therapy and Oncology* (Edited by J. M. VAETH), Vol. 3, p. 109. Karger, Basel (1968).
7. E. FRINDEL, F. VASSORT and M. TUBIANA, Effects of irradiation on the cell cycle of an experimental ascites tumour of the mouse. *Int. J. Radiat. Biol.* **17,** 329 (1970).
8. L. M. VAN PUTTEN and R. F. KALLMAN, Oxygenation status of a transplantable tumor during a fractionated radiation therapy. *J. nat. Cancer Inst.* **40,** 441 (1968).
9. R. F. KALLMAN, Repopulation and reoxygenation as factors contributing to the effectiveness of fractionated radiotherapy. In *Frontiers of Radiation Therapy and Oncology* (Edited by J. M. VAETH), Vol. 3, p. 96. Karger, Basel (1968).
10. A. E. HOWES, An estimation of changes in the proportions and absolute numbers of hypoxic cells after irradiation of transplanted C3H mouse mammary tumours. *Brit. J. Radiol.* **42,** 441 (1969).
11. J. J. BROERSE, G. W. BARENDSEN and G. R. VAN KERSEN, Survival of cultured human cells after irradiation with fast neutrons of different energies in hypoxic and oxygenated conditions. *Int. J. Radiat. Biol.* **13,** 559 (1967).
12. M. M. ELKIND, Cellular aspects of tumor therapy. *Radiology* **74,** 529 (1960).
13. G. W. BARENDSEN, Possibilities for the application of fast neutrons in radiotherapy: recovery and oxygen enhancement of radiation induced damage in relation to linear energy transfer. *Europ. J. Cancer* **2,** 333 (1966).
14. W. K. SINCLAIR, Radiation survival in synchronous and asynchronous Chinese hamster cells *in vitro*. In *Biophysical Aspects of Radiation Quality,* 2nd Panel Report, p. 39. IAEA, Vienna (1968).
15. A. WESTRA and G. W. BARENDSEN, Proliferation chracteristics of cultured mammalian cells after irradiation with sparsely and densely ionizing radiations. *Int. J. Radiat. Biol.* **11,** 477 (1966).
16. A. H. W. NIAS, Clone size analysis: a parameter in the study of cell population kinetics. *Cell Tissue Kinet.* **1,** 153 (1968).
17. H. H. ROSSI, The effects of small doses of ionizing radiation. *Phys. in Med. Biol.* **15,** 255 (1970).
18. D. K. BEWLEY, Fast neutron beams for therapy. In *Current Topics in Radiation Research* (Edited by M. EBERT and A. HOWARD), Vol. VI, p. 249. North-Holland, Amsterdam (1970).
19. L. M. VAN PUTTEN, Tumour reoxygenation during fractionated radiotherapy: studies with a transplantable mouse osteosarcoma. *Europ. J. Cancer* **4,** 173 (1968).
20. L. M. VAN PUTTEN, P. LELIEVELD and J. J. BROERSE, Response of a poorly reoxygenating mouse osteosarcoma to X-rays and fast neutrons. *Europ. J. Cancer,* this issue.
21. J. J. BROERSE, G. W. BARENDSEN, G. FRERIKS and L. M. VAN PUTTEN, RBE values of 15 MeV neutrons for effects on normal tissues. *Europ. J. Cancer* **7,** 171 (1971).
22. H. R. WITHERS, The response of stem cells of intestinal mucosa to irradiation with 14 MeV neutrons. *Brit. J. Radiol.* **43,** 796 (1970).
23. S. B. FIELD, The relative biological effectiveness of fast neutrons for mammalian tissues. *Radiology* **93,** 915 (1969).
24. H. B. KAL, Impairment of cell reproductive integrity in an experimental rhabdomyosarcoma by fast neutron and gamma-irradiation at low dose rates. *Proc. IVth Int. Congress of Radiation Research,* Evian (1970). In press.
25. W. K. SINCLAIR, X-ray-induced heritable damage (small colony formation) in cultured mammalian cells. *Radiat. Res.* **21,** 584 (1964).
26. J. M. YOUNG and J. F. FOWLER, The effect of X-ray induced synchrony on two-dose cell survival experiments. *Cell Tissue Kinet.* **2,** 95 (1969).

27. S. B. Field, T. Jones and R. H. Thomlinson, The relative effects of fast neutrons and X-rays on tumour and normal tissue in the rat. II. Fractionation: recovery and reoxygenation. *Brit. J. Radiol.* **41,** 597 (1968).
28. R. J. Berry, D. K. Bewley and C. J. Parnell, Reproductive capacity of mammalian tumour cells irradiated *in vivo* with cyclotron-produced fast neutrons. *Brit. J. Radiol.* **38,** 613 (1965).
29. S. B. Field and S. Hornsey, RBE values for cyclotron neutrons for effects on normal tissues and tumours as a function of dose and dose fractionation. *Europ. J. Cancer* **7,** 161 (1971).

Europ. J. Cancer Vol. 7, pp. 191–194. Pergamon Press 1971. Printed in Great Britain

Therapeutic Margins in Clinical Radiotherapy with Conventional Types of Radiations and their Implications for Neutron Therapy

MORTON M. KLIGERMAN

Department of Radiology, Yale University School of Medicine, New Haven, Connecticut 06510, *U.S.A.*

Abstract — *For various groups of tumors, clinical characteristics are discussed which are important with respect to responses to fractionated treatments with supervoltage X-rays and with respect to problems to be expected with fast neutron treatments. Physical characteristics of supervoltage X-ray beams, with small penumbra, skin sparing and large depth dose, provde important advantages which are unlikely to be equalled by fast neutron beams. As a consequence the usefulness of fast neutrons might be limited in cases where either a critical structure is near the tumor bearing volume or where large areas of skin receive doses equal to the tumor dose. Different groups of tumors are distinguished which currently are highly curable or are not suitable for neutron treatment for other reasons, whereas others are discussed which seem to be candidates for neutron therapy, although methods are not available to predict their responses.*

Approximately half of all patients seen in a general hospital with cancer will be benefited by current radiotherapeutic management at some time during the course of their disease. However, the concern to-day is with those patients for whom curative radiotherapy is proposed. These constitute about 50% of all patients who present themselves to a radiotherapy department. The first condition necessary to place a patient in the possibly curative group is that the tumor must be localized. However, certain sensitive tumors such as lymphomas and seminoma can be treated with expectation of cure even though relatively large volumes of the body are involved. Secondly, irrespective of what biological factors may be involved, one must be able to deliver a dose of radiation to that localized tumor which will completely eliminate the tumor and yet permit the normal tissues to recover to a clinically acceptable functional and cosmetic state.

In selecting patients for treatment with conventional radiotherapy, it would be helpful if it were known in advance that certain groups of tumors have such a characteristic arrangement of capillary supply that all of the cells could be expected to be well oxygenated. If it could be known in advance which tumors would undergo efficient reoxygenation and the time sequence after the start of a particular treatment plan that this would occur, conventional radiotherapy would be enhanced. Or if means could be found to differentially sensitize tumors to irradiation as compared with the normal tissues either by improved methods to oxygenate the tumor or by the appropriate use of radiosensitizing or radioprotective agents, conventional therapy would be enhanced. If it were known in which tumour patients immune

factors could be utilized, and how to utilize them, conventional radiotherapy would be enhanced. However, none of this information is available to us when a patient first arrives for treatment. Therefore, all tumors arising in a particular anatomic site within a certain extent or stage are treated similarly, assuming that the individual tumor will behave like the majority in that group, i.e. respond favorably. The same tumor group testing which established these treatment schemes will have to be carried out for any new treatment method. It should be pointed out that the tumor-bearing volume, that volume of tissue which must be treated to include the gross disease and that amount of surrounding normal appearing tissue beyond which it is reasonable to assume no tumor cells have extended, is usually fairly large, that is, over 8 cm in at least one diameter, and frequently from 10 to 18 cm. Furthermore, consideration must be given to treating the lymph node drainage of this tumor by radiotherapy either concomitantly or sequentially. In some instances, especially in head and neck tumors, this is accomplished surgically. In most situations, therefore, the volume of tissue to be treated is relatively large. It is the total volume of the patient treated, and the particular region of the body under treatment, that determines the maximum daily body tolerance. When the volume is large and the region of the body relatively sensitive (abdomen), treatment must be protracted. This could bring certain patients into undesirably long fractionation schemes because of an adverse neutron RBE in the oxygenated normal tissues (the intestine).

It should also be pointed out that though protracted fractionation schemes with conventional therapy generators have theoretical as well as clinically observed advantages, protraction must be limited by a daily dose of irradiation which is large enough to prevent a net increase in the number of cells in the tumors. Indeed, tumors are observed to grow under treatment in protracted therapy schemes and this growth is interrupted by increasing the daily dose. Clinically, there are tumors which will regrow with still larger daily doses and these indeed seem to be candidates for neutron therapy. The problem is that it is difficult to know in advance which tumors will behave in this fashion except for certain rare tumors such as grade III carcinoma of the thyroid, and some soft tissue sarcomas.

Inspection of the isodose curve for neutrons reveals it to be relatively poor compared to modern X-ray supervoltage equipment. (^{60}Co units should not be considered as modern.) The depth–dose for 14 MeV neutrons is poor, and the penumbra is unsatisfactorily large. This former characteristic would require an increase in the number of portals to treat a tumor at the depth, especially since the skin sparing effect of supervoltage X-rays is reduced with neutrons. The lack of skin sparing would also limit the usefulness of the neutron beam in treating eccentric lesions which approach or involve the skin. Cartilage in adults is non-proliferating, anoxic and, therefore, a candidate for greater damage as compared to supervoltage X-rays.

The large penumbra would limit the usefulness of the neutron beam if there is a critical structure, such as the eye or spinal cord, near the tumour-bearing volume. Abutment of portals would be difficult and potentially dangerous especially to the spinal cord. With available supervoltage X-ray generators one can expect to miss critical structures by appropriate treatment planning, patient immobilization, and beam direction.

A number of tumor groups currently are highly curable within their early stages (see Table 1). The sensitive tumors — lymphomas,

Table 1. *High cure rate in early stages*

Skin
Lip
Alveolar ridge
Buccal mucosa
Floor of mouth
Soft palate
Tongue
Larynx
Uterus
Seminoma
Lymphomas

seminomas, neuroblastomas, and Wilm's tumor — probably would not benefit significantly from neutron therapy as compared with conventional generators since they already appear to have little ability to repair sublethal damage. There are other tumors which could be added to this group but their incidence is too small for practical consideration.

It was pointed out above that the tumor must be localized to make cure possible. Table 2 presents those lesions which characteristically metastasize early and widely beyond regional nodes. Again, uncommon tumors such as melanoma, which also metastasize early, have been omitted. However, between Tables 1 and 2 are included a large proportion of patients

Table 2. *Metastasize early*

Breast
Lung
Stomach
Ovary
Rectosigmoid
Prostate
Embryonal cell CA of testis
Leukemia and lymphomas
Common tumors of children

who present themselves for radiotherapy treatment.

This at least leaves for consideration of neutron therapy the advanced lesions of those listed in Table 1. These same tumors in which conventional treatment yields a high cure rate do not respond well when advanced because of their size if localized; because the tolerance of the normal tissue in which they occur does not tolerate a dose high enough to control the localized tumor (brain tumors are a good example); or, because the metastatic pattern, though localized, can be massive as in the cervical lymph node metastasis in advanced head and neck lesions. Under treatment these advanced lesions require large tumor volumes, and the treatment plans bring either large portions of the skin or intestine to doses that approach or equal the tumor dose. It would seem that neutron therapy with its known interference with the large recovery processes that skin and intestine are capable of when treated with fractionated conventional therapy equipment could be at a disadvantage. Table 3 lists the tumors in which skin or intestine would be so affected.

Table 3. *Skin or intestine in tumor volume*

Brain
Head and neck, advanced
1. Bulky lesions
2. Neck node metastasis
Anterior mediastinal lesions
Stomach
Pancreas
Intestine, large and small
Kidney
Ovarian, advanced
Uterus, advanced
Bladder, advanced

Treatment of brain tumors should be attractive for the radiotherapist because they do not metastasize. However, in general the common highly malignant gliomas require that a large volume be treated before a cone down can be effected. The latter is also reasonably large. Currently one is limited by the tolerance of the normal brain (vasculature) though the skin, whose dose approaches the tumor dose, is able to tolerate the treatment. The high dose to the skin and the decrease in the ability to recover from neutron irradiation could limit this type of treatment. In advanced head and neck lesions, bulky tumors which cross the mid-line also bring large volumes of mucosa and skin to dose levels which approach or equal the tumor dose. Large metastatic nodes in the neck are necessarily treated in a manner which results in skin dose equalling tumor dose. In the abdomen, in treating large lesions, the intestine is either contiguous with or even intimately related to the tumor bearing volume. In all instances treatment with high energy photons is dependent on the high degree of recovery in the mucosa and skin.

The physical characteristics of supervoltage X-ray beams with their minimal penumbra, skin sparing, and large depth dose make them ideal for sophisticated treatment planning using beam direction and wedge filters. It is possible to treat a maxillary sinus with the upper margin of the portal just under the globe of the eye with the well defined beam of modern conventional supervoltage equipment. One can raise the dose to masses relatively near the sensitive spinal cord to high levels by direction of beams of high energy photons with this small penumbra. Wedge filters for eccentric lesions especially in the head and neck permit sparing normal mucous membrane by lateralizing the deposition of ionizing radiation. This not only has made it possible to maintain nutrition in patients because they can swallow down the "untreated" contralateral side, but this technique has markedly reduced the incidence of aspiration pneumonia, which is a serious problem in the older age group into which the majority of patients with such lesions fall. Aspiration results from difficulty in swallowing. Furthermore, healing of the mucosa takes place more rapidly, presumably by migration of untreated cells into the treated area, and because better nutrition permits healing. In a series of larynx patients treated at Yale-New Haven Hospital, the relationship between total volume of tissue treated and the daily tolerance of the specific structure, the laryngeal mucosa, has been demonstrated [1]. Tolerance is inversely proportional to the volume of neck treated, even though the size of the larynx itself is approximately the same in all patients of the same sex.

It should also be remembered that the use of radium implantation (or equivalently-used isotopes), where applicable, gives excellent results because a high dose of ionizing radiation is limited to the tumor bearing volume, and apparently because of the low dose rate. This is also a conventional radiotherapy method. Supervoltage units have unfortunately, and in this author's opinion erroneously, replaced radium in many institutions to the point where this valuable technique has been lost.

It would seem that groups of tumors which are well vascularized and, therefore, presumably well oxygenated would be good candidates for supervoltage photon beams. Renal cell carcinomas and thyroid carcinomas are so vascular that one of the differential physical diagnostic signs is the presence of a bruit heard on auscultation due to the rush of the large volume of blood through the tumor. Embryonal cell sarcomas, angiosarcomas and most tumors of endocrine organs are highly vascularized. However, this vascularization does not necessarily mean that in all parts of the tumor there are well oxygenated cells. Previous observations by the author [2], through Algire chambers in an animal system, mammary adenocarcinoma in C3H mice, demonstrated sluggish, stagnant or variable blood flow through capillary sinusoids in the tumor. Clinically, Dr. Robert Hutter, Professor of Pathology at Yale University, points out that these groups of human tumors are not consistently vascular, and secondly that though much of the tumor can be highly vascular, the remainder is similar to other tumors in that microscopically many of the cells are far from capillaries. There is currently no clinical way to ascertain this microscopic situation so that if neutron beams are appropriate therapy in clinical tumors, this so-called vascular rich group must also be tested.

Finally, alteration in current fractionation schedules might take advantage of reoxygenation more effectively in conventional therapy possibly by utilizing a few large doses early in a split course treatment. At the other extreme, many radiotherapy centers have shortened their fractionation schemes departing from the possibly optimum low dose rate provided by more protracted fractionation. This departure has been mainly due to economic reasons. However, if a return to the 8 to 12-week treatment schedule has merit, it should be pointed out that with the relatively low cost and high output of modern supervoltage X-ray generators, that sufficient equipment could probably be added to existing centers for a cost that would be similar to that of installing a single clinical neutron generator.

REFERENCES

1. M. Tepper, J. A. Kirchner and M. M. Kligerman, Glottic carcinoma, with examination of failures by serial sections, *Radiology* In press.
2. M. M. Kligerman and D. K. Henel, Some aspects of the microcirculation of a transplantable experimental tumor, *Radiology* **76,** 810 (1961).

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Criteria for Selection of Types of Tumours for Radiotherapy with Unconventional Radiation

HENRY S. KAPLAN

Department of Radiology, Stanford University Medical Center, Stanford, California, U.S.A.

Abstract — *Consideration has been given to a number of criteria for the selection of appropriate types of tumors for randomized clinical trials to test the efficacy of fast neutron radiotherapy. On the basis of these criteria, a broad spectrum of human neoplasms has been assessed as unsuitable, doubtful, or suitable for selection. It appears that the numbers of tumors considered suitable, and the limited numbers of cases of such tumors available, may seriously hamper and restrict the establishment of fast neutron clinical trials.*

CLINICAL trials employing neutrons and other unconventional radiations may be expected to be of three different types: (a) preliminary studies aimed at the clinical assessment of the RBE and tissue tolerance data required for definitive clinical trials; (b) clinical trials in which the entire course of radiotherapy for some tumors will be given with fast neutrons; and (c) clinical trials in which combinations of neutrons and photon therapy are employed, in which the neutrons will be used either to deliver the first dose, the last dose, or some other combination.

In studies of type (a), such as have been under investigation at Hammersmith Hospital in London during recent years, it is desirable to use very far-advanced cases, with little or no hope of cure. Patients with disseminated metastases, either in the lungs or in subcutaneous sites, are entirely suitable for such investigations, as are selected patients with more localized but nonetheless far-advanced neoplasms. In short, the principal criteria for the selection of such cases are that the tumors be accessible, either by direct vision and palpation or by radiographic methods, for serial measurements of their response and that the patients have little or no chance for cure by conventional techniques.

However, when one turns to consideration of definitive, randomized clinical trials, particularly those in which neutrons are to be used for an entire course of radiotherapy, the criteria for the selection of appropriate types of tumors become appreciably more stringent and restrictive. I would list the most important criteria as follows: (1) histopathologic sections of the tumor must reveal a high frequency of foci of necrosis, adjacent to which zones of tissue anoxia or hypoxia can reasonably be expected to exist; (2) there must be a relatively high rate of local failure to cure the tumor with conventional radiations or surgery; (3) the tumor must exhibit a low incidence of distant blood-borne metastasis; (4) the tumor must be sufficiently abundant to provide practical numbers of cases for a clinical trial; (5) it is preferable, though not absolutely essential, that the tumor be accessible, either directly or radiographically, for serial observation and measurement; (6) it is preferable that the tumor be rapidly lethal when it has not been successfully treated so that survival rates can be obtained without undue delay; (7) it is

Table 1. Unfavorable types of tumors

I. *High frequency of blood-borne metastases*	
Ca of breast (most cases)	Ca of kidney
Ca of bronchus	Malignant melanoma
Ca of esophagus	Wilms' tumor
Ca of stomach	Neuroblastoma
Ca of colon and rectum	Osteosarcoma
Ca of ovary (most adenocarcinomas)	Diffuse histiocytic lymphoma (reticulum cell sarcoma)
II. *Too rare to provide practical numbers of cases*	
Thyroid cancer	
Cylindroma of the upper air passages	
Others	
III. *Too highly curable with conventional techniques*	
Ca of skin	Ca of cervix, Stages I and IIA
Ca of lip	Seminoma of testis
Ca of larynx	Hodgkin's disease
Ca of oral cavity, T_1, N_0	Nodular lymphomas
IV. *Benign or of low-grade malignancy*	
Pituitary tumors	
Astrocytomas, Grades I and II	
Craniopharyngiomas	
Meningiomas	

preferable that the tumor be located in regions in which no serious dosimetry problems due to tissue inhomogeneity may be anticipated; (8) it is preferable that the tumor not be so large as to exceed currently available beam sizes; (9) conversely, it would be desirable that the tumor not be so small or so close to vital organs as to exceed the capabilities of currently available collimation techniques; (10) it would be desirable that the tumor not be located in sites in which the physical limitations of neutron dosimetry and treatment planning, relative to those of photon beams, might be likely to cancel out the potential advantages of the high RBE and low oxygen dependence of neutrons.

With respect to histological type, it seems generally more likely that the carcinomas, both by virtue of their far greater frequency and their greater propensity for necrosis, will be more suitable for such clinical trials than the sarcomas. Within the carcinoma category, the squamous cell tumors have been more extensively studied and appear to reveal foci of necrosis and presumed hypoxia or anoxia in a very high percentage of cases. Less information on this point is available for the adenocarcinomas or transitional cell carcinomas.

Based on these considerations, I have reviewed a broad spectrum of neoplasms of different sites and types and have classified them as unfavorable, doubtful, or favorable for the purposes of a randomized clinical trial designed to test the efficacy of neutron radiotherapy (Tables 1–3). This compilation is not intended to be exhaustive, but it does cover

Table 2. Tumors of doubtful suitability

Soft tissue sarcomas
Ca of breast, locally inoperable, without distant metastases
Ca of prostate, locally inoperable, without distant metastases
Ca of oral cavity, T_{3-4}, N_{2-3}, M_0
Ca of oro-hypopharynx, T_{3-4}, N_{2-3}, M_0
Ca of urinary bladder, transitional cell
Ca of testis, embryonal

Table 3. Tumors probably favorable for clinical trials

Squamous cell Ca of oral cavity, T_{2-3}, N_{0-1}, M_0
Squamous cell Ca of oro-hypopharynx, T_{2-3}, N_{0-1}, M_0
Squamous cell Ca of cervix, Stages IIB and III
Squamous cell Ca of urinary bladder
Gliomas of brain (astrocytomas, Grades III and IV)

the vast majority of neoplasms of various sites. It is clear that the number of sites and types of neoplasms available for a practical clinical trial are indeed severely limited. Since the numbers of fast neutron installations in various parts of the world will initially be very small and since it seems undesirable to advocate the rapid proliferation of such expensive installations until the superiority of neutron radiotherapy has been clearly established in one or more clinical trials, the device of the cooperative clinical trial, in which numbers of cases from various institutions are pooled to hasten the attainment of statistically significant numbers, will unfortunately not be feasible. Accordingly, radiotherapists in institutions proposing to install fast neutron generators and to explore their efficacy in clinical trials should be fully aware that they are undertaking a long, difficult, and tedious program of clinical investigation which may well require as much as ten years to complete and which is by no means certain, even after so much time and effort, to reveal a statistically significant superiority for neutron therapy. This is indeed a daunting prospect.

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Possible Clinical Models for Quantitative Comparison of Effects of Different Radiations on Tumours

K. BREUR and H. A. VAN PEPERZEEL

Department of Radiotherapy, Netherlands Cancer Institute,
Department of Radiotherapy, University Hospital, Wilhelmina Gasthuis,
Amsterdam, The Netherlands

Abstract — *It cannot be expected that the use of fast neutrons instead of conventional radiation, will cause large improvements in cure rate of human tumours.*

Therefore controlled clinical trials of different types of tumours, requiring many years of observation will be necessary to assess possible gains.

In addition to values from experiments with animal tumours, there is a need for clinical models which can provide quantitative data on the direct effects and on the RBE of fast neutrons in human tumours.

The possibilities and properties of some of these models are discussed. Pulmonary metastases presumably offer the best possibilities for a comparative study of effects of photons and fast neutrons.

INTRODUCTION

IN STARTING clinical applications of a new therapeutic agent, based on results obtained in laboratory experiments, a number of problems are encountered with respect to the selection of patients and the evaluation of the results. One of the most important difficulties is that, because of uncertainties about the efficacy of any new treatment method for a neoplastic disease, clinicians initially select patients that have a bad prognosis with the conventional methods of treatment. If for such a group of cancer patients the proportion of cures, e.g. assessed as survival rates is taken as an end-point for evaluation, the probability to prove a beneficial effect is small due to the many factors influencing the final result. To reduce the risk to the patient a selection of cases has to be made and the clinical experiments have to be planned in such a way that reliable conclusions can be drawn after a limited period of time.

The demonstration of an advantage of treatments with fast neutrons as compared with conventional radiations would not present problems if the gain factor were very large, but this can hardly be expected on the basis of presently available experimental data. With regard to the relative biological effectiveness (RBE) of fast neutrons, it has not been shown that due to intrinsic differences in radiosensitivity substantial differences exist between the RBE values for tumour tissues and for normal tissues. The possible exception of haemopoietic tissues is not of great importance for clinical applications, because effects on this system seldom present limitations in regional radiotherapy. With respect to the second main factor which might provide an advantage of fast neutrons over conventional radiations, the low oxygen enhancement ratio (OER), it should be noted that little is known about the fraction of anoxic cells in human tumours. Although certainly necrosis is observed in a

number of tumours, clinical experience does not show a clear correlation between radiosensitivity or curability of tumours and the amount of necrosis. For instance necrosis is often observed in the very radiosensitive Wilms' tumours and in lymphogranulomas while conversely very little necrosis is observed in radioresistant fibrosarcomas.

It is worthwhile to note also that the masses of keratinized material in squamous cell carcinomas cannot be considered to be necrotic, but must be seen as end-products of differentiation which cannot be eliminated from the tumorous tissue for instance by exfoliation.

Furthermore it is important to state that the results of irradiations of patients in hyperbaric oxygen have not provided great improvements in cure rates, indicating that, presumably due to reoxygenation, anoxic cells might not be a major factor in the failure of treatments of many types of tumours with commonly employed fractionated irradiations.

In addition to these fundamental radiobiological aspects, it is important to note that even the best fast neutron beams available at present have some disadvantages compared to high energy photon beams. Firstly, the dose distribution of fast neutron beams in tissues is inferior to that of photon beams presently available. This makes conditions for the treatment of deep-seated tumours by neutron beams unfavourable. Of secondary importance is probably the limited flexibility of the machines and the less satisfactory beam collimation, which can be overcome in most instances by using more complex and elaborate treatment planning. Finally treatments with fast neutrons will be more expensive but if an advantage can be demonstrated this should not deter clinical applications.

From the discussion of all these uncertainties and difficulties it can be concluded that clinical applications of fast neutrons must be started with the realistic view that carefully planned controlled clinical trials, which are carried out on a sufficiently large number of patients during many years, will be required to demonstrate an improvement in cure rate. A large number of patients, selected by applying a number of rigid criteria, can eliminate many factors which, apart from the type of radiation used, might influence the results. It will be clear however that before starting with such elaborate trials it is of utmost importance to have available in addition to data from experiments with animals, a number of quantitative data on the RBE of fast neutrons for human tumours and normal tissues.

CLINICAL MODELS

For a direct quantitative evaluation of effects of fast neutrons in comparison with photons in human tumours it would be ideal to compare two tumours which:

1. have about equal sizes;
2. consist of identical types of tumour cells;
3. have closely similar growth characteristics, cell kinetics and immunological properties;
4. grow in the same type of normal tissue;
5. have similar vascular supply, and
6. can be measured accurately without influencing growth conditions.

Such a model would be complete if a quantitative estimate could be made of effects on normal tissues in the irradiated volume.

A review of the practical possibilities indicates that for such optimal models it is necessary to consider multiple lesions of the same tumour in one patient. The systems of choice are then confined to different locations of a systemic tumorous disease or to multiple metastases of a known primary tumour.

Malignant systemic diseases are not very suitable for a comparative study, because most of the malignant lymphomas are very radiosensitive and local cures can be achieved in a large percentage with moderate doses. It might be possible however to compare responses of symmetrically localized lesions, for instance on both sides of the neck, after photon and fast neutron radiation. This would provide also the opportunity to study the effects on normal skin. Measurements of changes in volume will be difficult, so the main criterium for evaluation would be differences in local recurrence rate, observed within a certain number of years.

For direct measurements metastases of less radiosensitive solid tumours seem to offer better possibilities. Skeletal metastases are not suitable because it is impossible to measure their volume accurately and changes in the tumour volume are not demonstrable on the X-ray pictures within many weeks.

Skin metastases offer better possibilities.

Adenocarcinomas of the breast and malignant melanomas often give rise to multiple cutaneous and subcutaneous metastases, which usually show a very low radiosensitivity. Most of them are of small size, making accurate measurements of changes in volume rather difficult. In addition they tend to become confluent with others when left untreated. Moreover time for observation will be limited with breast carcinoma because other types of treatment, e.g. with hormones or chemotherapeutic

agents, will often interfere with the evaluation. If radiation treatment is indicated, a type of radiation with a limited penetration in tissue is preferred. Low energy neutrons could be applied in these cases for instance in comparison with fast electron beams. The reactions of the surrounding normal skin could also be compared for both types of radiation.

Pulmonary metastases fulfil most of the conditions required for a quantitative study of the effects of different types of radiations on human tumours. Blood born metastases in the lungs occur from a variety of human tumours, differing in place of origin and histological type. They all grow under comparable conditions in a tissue that offers little mechanical resistance and ample blood-supply. On radiographs of the chest the solid metastasis gives a shadow with a distinct contrast against the surrounding air-containing lung tissue. The selection of cases with pulmonary metastases, which are sharply defined and spherical on the films, has been shown to provide the opportunity to determine fairly accurately the volume of the tumour by measuring the diameter. Changes in volume with time can be closely followed in this way without influencing the growth process. A number of authors [1–8] already have published data obtained with this model for studying growth characteristics. It has been shown that in the great majority of cases these metastases grow with a constant growth rate during the period they can be observed radiographically. With only few exceptions, the lung metastases in one patient have the same growth rate. There is a great variety in growth rate, however, between metastases of different histological type and also within one group with the same histological classification. Volume doubling times range from about 10 to 300 days and more. Only exceptionally human tumours are observed to grow faster than corresponding with a doubling time of 10 days.

These patients have a short survival time, while on the other hand, patients with slowly growing metastases, can be examined during many years. The diagnosis of pulmonary metastases practically always means a very bad prognosis. In many cases these metastases can continue to grow during an appreciable time interval without causing symptoms which will require palliative treatment. In cases treated palliatively with a course of fractionated radiotherapy it has been demonstrated [9] that:

1. these metastases start to shrink concentrically a short time after the start of the treatment;
2. there is an exponential decrease in volume during the series of fractionated therapy, afterwards followed by a resumption of growth with approximately the original growth rate;
3. the percentage of volume reduction caused by equal doses of radiation differs greatly between different tumours;
4. there probably exists a correlation between growth rate and radiosensitivity; slowly growing tumours show less effect of a given treatment than fast growing ones, and
5. the radiosensitivity of tumours with different growth rates, as determined from the volume reduction, can differ by factors of at least 8.

These results might be interpreted by the assumption that growth rate is one of the main factors influencing the radiation response of tumours. Furthermore it can be deduced that the marked differences in radiosensitivity between different types of tumours, observed in clinical radiotherapy, cannot be completely explained by the differences due to the presence of anoxic cells only. The results also suggest that one could make serious errors if data obtained by cell, or tumour systems in the laboratory, growing with doubling times of less than one day or at most of a few days, are applied directly to human tumours as a guide for radiotherapy. The differences observed might be explained by assuming that in experimental tumours the majority of cells are proliferating and that the slow growth of many human tumours results from a small proportion of proliferating cells.

It remains hard to explain variety in radiosensitivity only by differences in proliferative fractions between different types of tumours.

Recently data have also been obtained from a study on the effects of a single dose of radiation on different tumours, using the lung metastases model [8]. In 10 patients doses ranging from 300 rads to 700 rads were administered in a single session. On chest radiographs made at very short intervals, it was shown that there is a decrease in volume in the first days after the single dose irradiation. After 4–7 days an increase in the volume is observed, initially with a far greater growth rate which gradually diminished until after a few weeks the tumour continues to grow with the original growth rate.

In addition to human tumours pulmonary metastases in dogs have also been studied. Most of these grew faster than the metastases of human tumours. The effect of a single dose

of irradiation in two dogs followed the same pattern as that of the metastases in man.

By injecting a suspension of tumour cells, derived from a murine mammary carcinoma, in the tail vein of mice, artificial lung metastases could be induced and measured on radiographs. From the 15th day on until death, the growth was exponential with a doubling time of 24 hr. A single dose of 210 rads resulted in a substantial decrease of the volume followed after about 45 hr by a period of fast regrowth which again gradually decreased to the original growth rate.

For all tumours it could be demonstrated that the effect of a single dose is smaller if the original growth rate of the tumour is greater. An attempt was made by combining the data obtained with tumours of comparable growth rate to draw "tumour survival curves". The results suggest that with the great variety of human tumours with regard to the growth rates, the "survival curves" can differ extensively not only in the steepness of the curve (D_0), but also in the width of the shoulder part. Of course these "tumour survival curves" cannot be regarded as equivalent to cell survival curves obtained in laboratory experiments.

Nevertheless the data suggest the possibility that survival curves of human tumour cells *in situ* may be different from those frequently encountered in radiobiological literature.

Especially a greater width of the shoulder part could be of importance for a differential effect of fast neutrons compared with photons. The model of the artificial lung metastases in mice provided an opportunity to study cell kinetics before and after a single dose of radiation. It could be shown that in the first days after a single dose, the cycle time of the proliferative cells was shortened in all phases. At the same time the proliferating fraction increased to almost 100%. Comparable results are obtained by Hermens and Barendsen in cell kinetic studies with rhabdomyosarcomas in rats [10]. The observations of an initial fast regrowth following a dose of radiation could be of importance with regard to the effect of different fractionation schemes. The results obtained with the lung metastasis model show that quantitative measurements of effects on human tumours are essential for progress in clinical radiotherapy especially with respect to the problem to what extent experimental data from the laboratory can be applied in the clinical situation.

In addition the data indicate that this model can serve very well to study differences in effects of photons and fast neutron radiation. It is quite well possible to irradiate metastases in one lung with X-rays or γ-rays and those in the other lung with fast neutrons using a comparable dose. In this way differences in the RBE of fast neutrons for different types of tumours could be established. By applying split dose techniques or courses of fractionated therapy, the effects of differences in the shoulder part of "survival curves" could be analyzed for a variety of tumours.

A number of disadvantages of the lung metastasis model for investigating effects of fast neutron radiation should also be mentioned. There is the possibility that the proportion of hypoxic tumour cells in these metastases is relatively small compared with that in tumours growing in dense tissues, because of the ample blood supply by the lung capillaries. This could make the influence of the low OER less clearly demonstrable. Furthermore the dose to the metastases is commonly applied by using anterior and posterior opposing fields on the thorax. The diameter of the thorax usually ranges between 20 and 30 cm, and consequently with 14 MeV neutrons the dose will be lower in the centre than in the periphery.

In addition the inhomogeneity caused by the air-containing lungs will require accurate dose measurements in phantoms.

The model employing lung metastases will not provide possibilities to study effects on normal tissue. At best skin reactions and the occurrence of lung fibrosis could be compared after series of fractionated therapy to very high total doses.

Finally it is important to note that in all models mentioned one will have to deal with problems of an ethical nature as will always arise with experiments on human beings. These, however, are the same as with controlled clinical trials.

REFERENCES

1. K. Breur, Growth rate and radiosensitivity of human tumours. I. Growth rate of human tumours. *Europ. J. Cancer* **2,** 157 (1966).
2. P. Chahinian and L. Israel, Survival gain and volume gain: mathematical tool in evaluating treatments. *Europ. J. Cancer* **5,** 625 (1969).
3. P. Chahinian, Le temps de doublement des cancers broncho-pulmonaires. *Thesis.* Paris (1969).

4. V. P. COLLINS, R. K. LOEFFLER and H. TIVEY, Observations on growth rates of human tumours. *Amer. J. Roentgenol.* **11,** 988 (1956).
5. V. P. COLLINS, Time of occurrence of pulmonary metastasis from carcinoma of colon and rectum. *Cancer* **15,** 387 (1962).
6. E. GERSTENBERG, Die Tumorverdopplungszeit, ihre röntgenologische Bestimmung und ihre Bedeutung für die Röntgendiagnostik. *Röfo* **101,** 39 (1964).
7. L. R. HOLSTI, M. W. BRENNER, P. HOLSTI and Y. PERTHALA, Clinical studies on the growth rate of human tumours. *Brief Report of a Symposium, on Control of Cellular Growth in the Adult Organism,* Helsinki, October 4–6 (1965).
8. H. A. VAN PEPERZEEL, Patterns of tumor growth after irradiation. *Thesis,* University Amsterdam (1971).
9. K. BREUR, Growth rate and radiosensitivity of human tumours II. Radiosensitivity of human tumours. *Europ. J. Cancer* **2,** 173 (1966).
10. A. F. HERMENS and G. W. BARENDSEN, Cellular proliferation patterns in an experimental rhabdomyosarcoma in the rat. *Europ. J. Cancer,* **3,** 361 (1967).

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The Influence of Cell Proliferation in Tumours and Normal Tissues During Fractionated Radiotherapy

J. DUTREIX, M. TUBIANA, A. WAMBERSIE and E. MALAISE
Institut Gustave Roussy, Villejuif, France

Abstract — *Proliferation rates of human tumours and normal tissues can be derived from clinical observations. They are in agreement with experimental data obtained on animals. The proliferation rates do not remain constant after an irradiation or during the course of fractionated irradiation. Thus the usual regular fractionation does not seem optimally adapted to the kinetics of the cell populations. However, there is no evidence for any superiority of few and large fractions.*

INTRODUCTION

CELL PROLIFERATION in normal and tumour tissues exhibits different characteristics dependent on the type of tumour or tissue. Thus for the discussion of cell proliferation with a view to its influence in radiotherapy the problem must be related to a definite type of tumour and a definite type of normal tissue. We shall only consider:

(a) as far as the tumour is concerned, the most usual type of growth, known as gompertzian growth which becomes progressively slower when the tumour size increases;

(b) as far as normal tissues are concerned, the covering epitheliums, which are most often the dose-limiting tissues and are the tissues for which a good amount of clinical and experimental data is available.

TUMOUR CELL PROLIFERATION AFTER IRRADIATION

The assumption of a constant rate of cell proliferation after an irradiation or during the course of a fractionated irradiation seems to be inadequate for the solid tumours. It usually leads to an under-estimation of the proliferation ability of the tumour cells.

Some animal experiments [1–3] have demonstrated that the growth rate after a single dose is faster than it was before irradiation and that it decreases progressively during the regrowth of the tumour. Clinical observations on human tumours [4–7] show the same kind of variation. The growth rate increases as the number of surviving cells decreases; it is not however related only to the cell survival or the tumour size [8]: from experiments by Barendsen and Broerse [2] one can compute that the time required for the tumour to regrow to the same size as at the moment of irradiation is 17 days after a dose of 2000 rads and 46 days after 4000 rads. Thus, the growth rate at any particular size seems to be smaller when the dose has been larger. The mechanism responsible for the increases of the growth rate after irradiation has not yet been demonstrated. It may either be due to the depopulation which increases the availability of nutrients or to a stimulation by the killed cells [9].

Malaise [10] has shown for a NCTC mice fibrosarcoma that the tumour growth is faster when 10^7 heavily irradiated cells (15,000 rads) are added to the inoculated cells: the initial doubling time is reduced from 2·3 days to 1·2 days (*in vitro* cell cycle: 0·75 day). Furthermore, the 50% tumour transplantation number (TD 50) is reduced from 10^3 to 10^2. If the risk of recurrence were directly related to the

TD 50, the cure of a tumour would require the reduction of cell survival by a factor one-tenth lower when killed cells are mixed with surviving cells. This effect could have a particular importance after a large dose when the viable surviving cells are mixed with a very great number of killed cells. It is not demonstrated whether this stimulation of the viable cells by the killed cells occurs in all types of tumours. It has also been observed by Malaise [10] on the solid forms of L1210 mouse leukaemia (subcutaneous implant) but an opposite effect, i.e. inhibition, has been obtained on the ascitic form of this tumour.

The general shape of the regrowth curve after a single irradiation suggests that during the course of a fractionated irradiation the cell production rate will progressively increase as the cell survival decreases. The consequence of this phenomenon is that too small fractions and too large intervals between the fractions may lead after a few fractions to a steady state in which continuing the irradiation will not cause any further decrease of the cell population. This problem has been studied by Barendsen and Broerse [11] on the rat rhabdomyosarcoma:

while daily doses of 400 rads and 300 rads are able to eradicate the tumour when given for 4 and 6 weeks respectively, a daily dose of 200 rad fails to cure any tumour even for an 8-week treatment.

the number of clonogenic cells as a function of the dose keeps decreasing for daily fractions of 300 rads while for fractions of 200 rads there is a tendency to level off after 10 fractions.

Human data do not allow any precise assessment of the critical limit at which such a steady state situation may occur: there is clinical evidence that daily fractions of 200 rads can sterilize epidermoid carcinomas that is to say that 24-hr repopulation is not sufficient to repair the depletion produced by a dose of 200 rads. However, this is not a general rule and, for instance, Friedman and Pearlman [12] quote a case of undifferentiated squamous cell carcinoma, in which a permanent histological maturation was observed despite an average daily dose of 285 rads up to a total tumour dose of 8000 rads. We can consider that this risk of stumbling on a steady state situation is the main problem the radiotherapist is confronted with.

Barendsen and Broerse's data [11] suggest that the cell production increases rapidly during the first sittings. This would imply that almost all the fractions of the usual fractionated irradiation have to deal with the maximum rate of tumour cell proliferation. Thus the maximum rate of proliferation is the most important parameter for the radiotherapist.

Some observations on patients with squamous cell carcinoma irradiated by a concentrated irradiation (2×850 rads) show that the first clinical sign or recurrence appears at about 5 weeks. On the assumption that this delay corresponds to 17 doubling times a rough approximation for the average doubling time is 2 days. However, there is no clinical way prior to the treatment for the assessment of the potential proliferation capacity. The growth rate of the tumour before the irradiation does not seem to give any reliable indication.

During the first sittings, the cell proliferation rate may be significantly smaller than afterwards. This first phase of the irradiation during which the tumour growth rate increases progressively may correspond to a significant depletion (10^{-2} to 10^{-3}) of the cell population. Thus the optimal time dose pattern based on the comparison of proliferation kinetics of the tumour and of the normal tissues should be considered separately for the first sittings and the rest of the treatment. Fowler [13] has discussed some other factors which lead to the same separation of these two phases of the treatment.

NORMAL TISSUES

Dose limiting tissues are different according to the site and the extent of the irradiated volume.

We shall only consider the skin and the mucosa of the initial parts of the digestive and respiratory tracts since

they most often determine the immediate tolerance to the irradiation;

these stratified epitheliums have common features, and

the clinical observations provide some data on the kinetic response of these tissues to irradiation.

We shall recall briefly the histologic and kinetic features which are necessary for the analysis of the clinical reactions of these tissues. We shall then consider the reactions observed after a concentrated irradiation, a conventionally fractionated irradiation and a concentrated irradiation followed after a 3-week rest by a fractionated irradiation. The variation of the reactions gives some information on the cell proliferation capacity after large doses and during the course of a fractionated irradiation.

Table 1. *Mouse Skin —from Iversen and Bjerknes* [14]

Basal cells: 60% Differentiating cells: 40%	} of the total cell mass
Mean generation time of the basal cells: about 5 days	
Mean turn-over time of the differentiating cells: about 3 days	
Mean turn-over time of the horny layer: about 1–3 days	
Mean mitotic count at 10 a.m.: $0{\cdot}6\pm0{\cdot}03\%$ of all cells	
Mean number of DNA-synthesizing cells at 10 a.m.: $2{\cdot}94\pm0{\cdot}15\%$ of all cells	
Mean mitotic duration at 10 a.m.: about 1 hr	
Mean DNA synthesizing time at 10 a.m.: about 5 hr	
Mean rate of cell proliferation (mitotic rate): 0·6% of all cells per hr, or: 0·9% of all basal cells per hr	
Mean rate of DNA synthesis: 0·6% of all nuclei per hr	
Duration of premitotic "resting" phase, G_2: about 1–3 hr	

(a) *Kinetics of cell proliferation under steady state conditions*

The deepest layer of these stratified epitheliums is the basal layer where all the mitoses take place under normal circumstances. The basal layer and a few layers of cells above it, build up in the terminology of the histologists the germinative region (stratum germinativum). In the outer region of the epithelium, cells do not divide any more but experience a process of differentiation. The number of cells in the germinative region and in the region of differentiation is approximately the same. The general characteristics given by Iversen and Bjerknes [14] for mouse skin are indicated in Table 1.

A detailed review of the mechanisms of response of the epithelium to stress has been given by Lamerton and Steel [15].

If a limited number of divisions is allowed to the basal cells the numerical problem can be solved by a kinetic model (which is now well established for erythopoietic tissues) with three kinds of cells [16]:

"young cells" with a short history of generations which may be considered as actual stem cells,

proliferating cells committed to differentiation, some of which are normally dormant in the layers above the basal layer, but may have some further mitotic activity if needed,

and

differentiating cells.

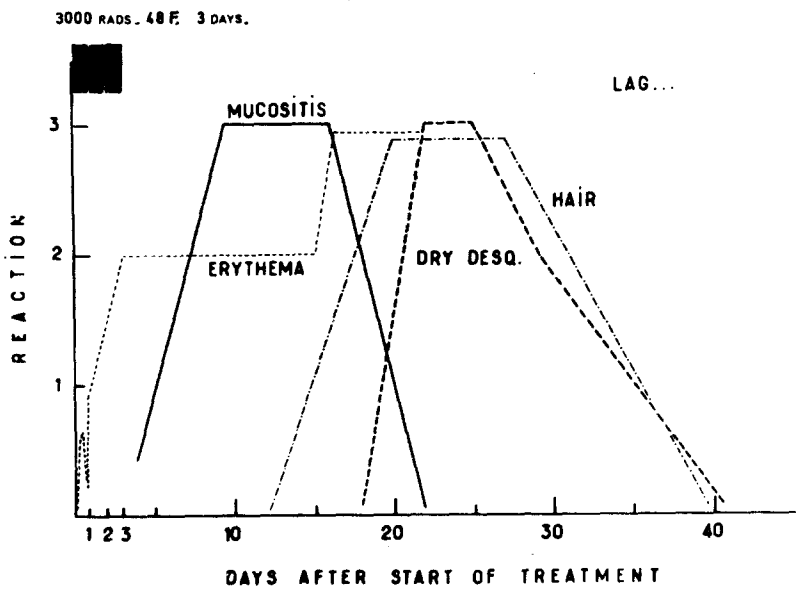


Fig. 1. *Variations of the reactions of skin, mucosa, hair as a function of time after a concentrated irradiation* (3000 *rads*/48 *fractions*/3 *days*). *Epithelioma of the tongue—lateral field:* 8×8 *cm*^{2}, 220 *kV X-rays. A similar variation is observed after* 1700 *rads*/2 *fractions*/3 *days.*

Table 2. Quantitative data concerning re-population rate after irradiation for skin

Reference	Material	Method	Delivered doses (rads)	No. of fractions of the compared irradiations	Overall-time (days)	"Recovered dose" (rads/day)	Doubling time
Skin							
Withers [18]	mouse albino WH/HT	*in situ, in vivo* cell cloning technique	695+655	2	1–6	100	0·9 day
Emery *et al.* [19]	mouse albino SAS/TO	*in situ, in vivo* cell cloning technique	900+variable (control: 600 and 1200+variable)	2	1–7 1–14 (1–21)	48 42 (29)	2·2 days 3·1 days —
Fowler *et al.* [20]	pig English middle white	gross skin reactions	total doses 3400–4400 (equal fractions)	5	5–28	mean value 25	(3·7 days)*
Comas [21]	mouse C3H	gross skin reactions	total doses 2300–6000 (equal fractions)	2	1– 7	70	(1·3 days)*
Denekamp *et al.* [22]	mouse albino SAS/TO	gross skin reactions	1000+variable	2	1–14 1–21	30 29	(3·1 days)
Dutreix & Shaich (unpublished)	mouse C3H	gross skin reactions	total doses 4500–5580 (equal fractions)	15	17–35	32	(2·9 days)
Cohen [23]	human skin	clinical observation (erythema, tolerance)	1250–5775	up to 25	up to 33	28–34	(3·0 days)*
Dutreix	human skin human mucosa	clinical observation (duration of repair processes)	1700–3000	concentrated irradiations		—	estimated 1·5 day 1·0 day
Intestine (for comparison)							
Withers & Elkind [24]	mouse CDF_1	*in situ, in vivo* cell cloning technique	$660+1415/_{1825}$	5	interval for proliferation assessment 2·5–4 days after first dose (see text)	—	8 hr
Wambersie (unpublished)	mouse BALB/c	LD 50 intestinal death	total doses 2000–3500 (equal fractions)	5	4– 8	125	14 hr (see text)

*The doubling time T_d "corresponding" to a "recovered dose" D_r/t (in rad/day) is estimated by the formula $T_d = \frac{D_o \cdot \text{Log. } 2}{D_r/t}$, with $D_o = 135$ rads for skin epithelial cells.

(b) *Response of skin and mucosa after a concentrated irradiation*

Clinical observation shows some variation in the reactions of a given tissue at a definite site from one patient to another. However, there is a great similarity for most of the patients particularly in the chronology of the clinical reactions.

Figure 1 shows the variation of epithelitis and mucositis after a concentrated irradiation (3000 rads/48 fractions/3 days). The reaction reaches its maximum at 10 days for the mucosa and 20 days for the skin. Assuming that the differentiating cells are insensitive to the irradiation, these delays may be considered equal to the transit time of the cells across the differentiation compartment.

The repair is clinically complete at 25 days for the mucosa and 40 days for the skin. Assuming that at that time the normal equilibrium is recovered for all compartments, a reasonable approximation is that the proliferating compartment had recovered its normal population 15–20 days after the irradiation. A similar chronology is observed after 2 fractions of 850 rads at 48-hr intervals and does also agree with that observed by Fowler [17] on the skin of mice.

Assuming that the cell survival which results from the irradiation is approximately 10^{-5}, the average doubling time would be 20–30 hr. These figures may be compared to more accurate measurements made on mouse skin (Table 2). The assumption of a constant proliferation rate is oversimplified as shown by Emery *et al.* [19] and as will become apparent in the next paragraph.

(c) *Fractionated irradiation*

The time-dose pattern of fractions of 330 rads, 3 times a week, for 9 weeks (20 MV X-rays) was applied to 10 patients with extensive epithelioma of the tonsil. The average variation of the mucositis (Fig. 2) suggests a cyclic variation with a minimum around 40 days after the start of the treatment and 2 maximums at days 15 and 55. This cyclic variation is more evident on the individual curves but the fluctuations in the time for the minimum to occur smoothed the amplitude of the trough on the average curve. This kind of cyclic variation has been observed by Friedman and Pearlman [12] — who proposed for it the term of "periodicity" — for daily doses of 107 rads over 81 days: the maximums lay at 26 and 70–80 days and the minimum at 50–60 days.

Assuming that the transit time across the differentiation compartment causes a lag of 10 days (as seen in the previous paragraph) between the depletion of the proliferation compartment and the clinical consequence of this depletion, one can build a tentative dynamic model illustrated in Fig. 3:

in the initial phase a deep depletion of the population is achieved in a couple of days; the homeostatic regulation brings the popula-

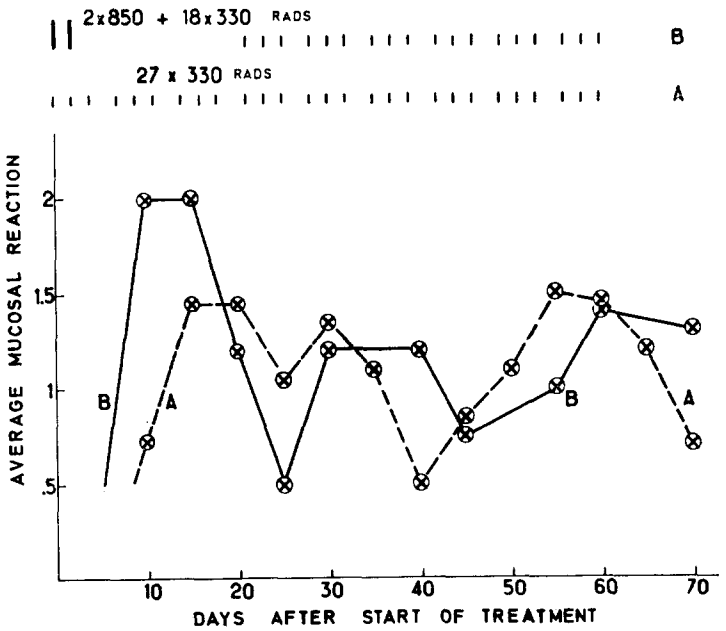


Fig. 2. Variation of the mucosal reaction as a function of time after start of the treatment. Curve A: fractionated irradiation: 330 *rads/fraction* — 3 *times a week* — 9 *weeks. Curve B: split course:* 2 × 850 *rads at a* 48*-hr interval:* 3 *weeks rest;* 330 *rads/fraction* — 3 *times a week* — 6 *weeks. Epithelioma of the tonsil — lateral fields —* 22 *MV X-rays. Each curve corresponds to the average for* 10 *patients.*

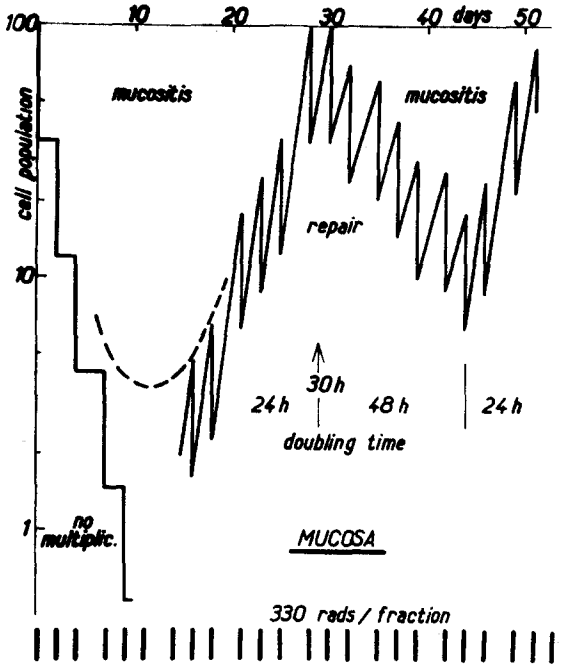


Fig. 3. Tentative model fitting the cyclic variation of the mucosal reaction observed during a fractionated irradiation, Fig. 2, curve A. Each fraction of 330 rads is assumed to cause a cell survival of 35%. Moderate variations of the doubling time, from 24 to 48 hr may account for the variations of the clinical reactions.

tion back to its normal size at the 30th day. This variation corresponds to the classical model given by Lajtha and Oliver [25];

the process of stimulation collapses during the following 15 days and then starts again.

Many mechanisms may be responsible for this cyclic variation:

a loose feedback system in which the stimulation is triggered when the population is in "low position" and stopped when the population is in "high position";

a two-step regulation system for instance acting firstly on the proliferating cells which exhaust their ability to divide around the 30th day and secondly on the stem cells;

the participation around the 30th day of an additional cause of tissue reaction, for instance, vascularization troubles.

These mechanisms are merely hypothetical; however, as the mucositis varies somewhat in the course of a regularly fractionated irradiation this suggests that the tissue repair is not controlled by a single mechanism.

(d) *Concentrated irradiation followed by a fractionated irradiation after a rest interval*

The capacity of repair retained by the tissues after recovering from a first large irradiation has been investigated on patients irradiated for epithelioma of the tonsil by a particular split-course:

2 fractions of 850 rads at 48-hr interval followed after 21 days of rest by a 6-week fractionated irradiation of 330 rads, 3 times a week (Fig. 2B).

The average (10 patients) variation of the mucositis shows that the reaction produced by the first series (2×850 rads) (cf. paragraph (b)) is in the process of disappearing at the start of the second series. The second series causes a wave of mucositis which reaches its maximum 10 days after start and tends to vanish 15 days later. A second wave of reaction follows, keeps increasing up to the end of the irradiation and diminishes thereafter. During the following weeks there is no noticeable difference between the effects of the split-course and the conventionally fractionated irradiation. The first wave following the start of the second series causes a reaction which seems to be less important and shorter than the first reaction caused by the conventionally fractionated irradiation (cf. paragraph (c)).

Thus it seems that 3 weeks after the concentrated irradiation there remains some potential stimulation of the repair system. This duration is longer than observed by Van den Brenk [26] on the skin of the rat: the reaction for 2×1800 rads, 3×1200 rads or 6×600 rads does not depend on the overall time when it exceeds 14 days.

(e) *Other tissues and later injuries*

Many other tissues may be injured by the irradiation depending on the site of the irradiated volume (lung, kidney, liver, spinal cord) or its extent (haemopoietic tissues). Kinetic data are scarce for most of these tissues; however, they indicate important differences with epithelial tissues. On the other hand late reactions must be considered since they are responsible for very severe complications. The irradiated epithelium shows histologic signs of premature aging. This may be attributed to the exhaustion of the proliferative capacity of the surviving cells or to progressive vascular disorders. There is some striking parallelism between the overall dose-time curves for skin tolerance and late CNS tolerance [27]. This suggests that the critical structures may be the same for both tissues and perhaps for all tissues. This lead one to consider, as actual critical structures, the structures which are common to all tissues, namely the capillaries and the connective tissues.

Vascular disorders may have at least two origins:

fibrosis of the connective tissues, hindering the metabolic exchanges between the blood and the cells, and

injuries to the capillaries.

The responsibility of capillary damage for the late necrosis is not obvious at histological examination where the capillaries can appear as numerous and broader than in normal tissues. But the parameter of interest is the blood-flow rate which depends also on other factors. The reaction of the capillaries to irradiation has been studied experimentally [28, 29]. However, the data are so far not sufficient to give a full understanding of the kinetics of the vascular cells and of their response to different time–dose patterns.

DISCUSSION

It is trivial to say that there is only a possibility of (local) cure when the net result of a fraction at the moment of the following fraction is more important on the tumour population than on normal tissues. Many factors are responsible for this result and the optimal time–dose pattern cannot be assessed by considering only the cell proliferation.

However, it seems from the examples which have been considered that a regular fractionation is not the optimal pattern on account of the variation of cell proliferation in tumour as well as on normal tissues. Keeping the tumour in a negative cell balance requires that the size of the fractions exceeds a minimum which depends on the interval between fractions and increases as a function of the time at the start of the treatment. It seems that this minimum may be relatively low during the first sittings when the growth rate is small. But on the other hand, if the repopulation is triggered faster for normal tissues, large doses could be more efficient.

It should be reasonable to take account of the tumour size and its growth rate before the irradiation: starting with relatively small fractions would be advisable for large and slow growing tumours, in order to achieve an important reduction of the tumour mass at the cheapest cost for normal tissues. When both tissues are at their maximum capacity of repopulation, the safer way is to press the normal tissues to their extreme limit of tolerance. The usual weekly dose distribution, for instance 5×200 rads or 3×300 rads, corresponds approximately to the end-point tolerance of normal tissues for 8–9-week treatment. However, there may be some periods of time when the treatment could be somewhat accelerated or should be given more slowly. Some split-course treatments may empirically achieve a synchrony of this kind; however, on account of the delay between the actual condition of the cell population and its clinical consequence, non-uniform time distribution cannot yet be based on a rational guide-line.

As far as repopulation is concerned there is no convincing evidence to support the superiority of few and large fractions over conority of few and large fractions over conventional fractionation. It would be interesting to try increasing the size of the fractions; however, any change should be moderate, as long as other factors are not proved to advocate a drastic departure from the conventional fractionation.

REFERENCES

1. E. Malaise and M. Tubiana, Croissance des cellules d'un fibrosarcome expérimental irradié chez la souris C3H. *C.R. Acad. Sci. (Paris)* **263,** 292 (1966).
2. G. W. Barendsen and J. J. Broerse, Experimental radiotherapy of a rat rhabdomyosarcoma with 15 MeV neutrons and 300 kV X-rays. I. Effects of single exposure. *Europ. J. Cancer* **5,** 373 (1969).

3. A. F. Hermens and G. W. Barendsen, Changes in cell proliferation characteristics in a rat rhabdomyosarcoma before and after X-irradiation. *Europ. J. Cancer* **5,** 173 (1969).
4. P. Rambert, E. Malaise, A. Laugier, M. Schlienger and M. Tubiana, Données sur la vitesse de croissance de tumeurs humaines. *Bull. du Cancer* **55,** 323 (1968).
5. H. A. van Peperzeel, Patterns of tumour growth after irradiation. Thesis Amsterdam. Van Denderen, Groningen (1970).
6. E. Frindel, E. Malaise and M. Tubiana, Cell proliferation kinetics in five human solid tumors. *Cancer* **22,** 611 (1968).
7. M. Tubiana, E. Frindel and E. Malaise, *In vivo* cell kinetics of human cancers. In *Normal and Malignant Cell Growth.* (Edited by R. J. M. Fry, M. L. Griem and W. H. Kirsten), p. 202. Springer Verlag Berlin (1969).
8. M. Tubiana, E. Frindel and E. Malaise, The application of radiobiologic knowledge and cellular kinetics to radiation therapy. *Amer. J. Roentgenol.* **102,** 822 (1968).
9. L. Revesz, Effect of tumor cells killed by X-rays upon the growth of admixed viable cells. *Nature (Lond.)* **178,** 1391 (1956).
10. E. Malaise, Influence des cellules stérilisées sur la croissance des cellules isologues *in vivo* et *in vitro. Proc. IVth Int. Congress of Radiation Research,* (1970), in press.
11. G. W. Barendsen and J. J. Broerse, Experimental radiotherapy of a rat rhabdomyosarcoma with 15 MeV neutrons and 300 kV X-rays. II. Effects of fractionated treatments applied five times a week for several weeks. *Europ. J. Cancer* **6,** 89 (1970).
12. M. Friedman and A. W. Pearlman, Time–dose studies on human epithelium and tumors. *Proc. Symp. Dose Rate in Mammalian Radiation Biology* — 19 Oak Ridge, US Atomic Energy Commission, Conf. 680410 (1968).
13. J. F. Fowler, Biological foundations of radiotherapy. Proc. XIth Int. Congr. Radiology, Rome, 1965. *Excerpta Medica* **105,** 731 (1966).
14. D. H. Iversen and R. Bjerknes, Kinetics of epidermal reactions to carcinogens. A cybernetic model analysis. *Acta Radiol. Microbiol. Scand.*, suppl. 165 (1963).
15. L. F. Lamerton and G. G. Steel, Cell population kinetics in normal and malignant tissues. In *Progress in Biophysics and Molecular Biology.* (Edited by J. A. V. Butler and D. Noble), Pergamon Press, Oxford (1968).
16. L. F. Lamerton, Cell population kinetics in relation to hemostasis. In *Ciba Foundation Symp. on Homeostatic Regulators.* (Edited by E. W. Wolstenholme and J. Knight), Churchill, London (1969).
17. J. F. Fowler, Effects of fractionated irradiation on the skin of pigs and other animals and on tumors in rats. *Proc. Symp. Dose Rate in Mammalian Radiation Biology* — 18 Oak Ridge, US Atomic Energy Commission, Conf. 680410 (1968).
18. H. R. Withers, Recovery and repopulation *in vivo* by mouse skin epithelial cells during fractionated irradiation. *Radiat. Res.* **32,** 227 (1967).
19. E. W. Emery, J. Denekamp and M. M. Ball, Survival of mouse skin epithelial cells following single and divided doses of X-rays. *Radiat. Res.* **41,** 450 (1970).
20. J. F. Fowler, R. L. Morgan, J. A. Silvester, D. K. Bewley and B. A. Turner, Experiments with fractionated X-ray treatment of the skin of pigs. I. Fractionation up to 28 days. *Brit. J. Radiol.* **36,** 188 (1963).
21. F. V. Comas, *Proc. Symp. Dose Rate in Mammalian Radiation Biology,* p. 18.1, Oak Ridge. US Atomic Energy Commission, Conf. 680410 (1968).
22. J. Denekamp, M. M. Ball and J. F. Fowler, Recovery and repopulation in mouse skin as a function of time after X-irradiation. *Radiat. Res.* **37,** 361 (1969).
23. L. Cohen, Theoretical "iso-survival" formulae for fractionated radiation therapy. *Brit. J. Radiol.* **41,** 522 (1968).
24. H. R. Withers and M. M. Elkind, Radiosensitivity and fractionation response of crypt cells of mouse jejunum. *Radiat. Res.* **38,** 598 (1969).
25. L. G. Lajtha and R. Oliver, Cell population kinetics following different regimes of irradiation. *Brit. J. Radiol.* **35,** 131 (1962).
26. H. A. S. van den Brenk, Relation of dose fractionation of X-rays and its spacing in time to skin damage. *Amer. J. Roentgenol.* **97,** 1023 (1966).
27. M. Lindgren, On tolerance of brain tissue and sensitivity of brain tumours to irradiation.— *Acta Radiol.* Suppl.1 70 (1958).

28. P. RUBIN and G. CASARETT, Microcirculation of tumors. Part II. The supervascularized state of irradiated regressing tumors. *Clin. Radiol.* **17,** 346 (1966).
29. H. S. REINHOLD, The response of tumor blood supply to fractionated irradiation. *Proc. Symp. Time and Dose Relationships in Radiation Biology as Applied to Radiotherapy*, p. 210. Carmel, Calif. (1969).

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Summary of Discussions in the Session on Experimental and Clinical Radiotherapy

A. F. HERMENS

Radiobiological Institute TNO, 151 *Lange Kleiweg, Rijswijk Z.H., The Netherlands*

THE DISCUSSIONS of the papers presented in this session pertained to fundamental factors which are known to determine the responses of tumours and normal tissue tolerance to ionizing radiations, and to practical factors relevant for the selection of types of tumours as candidates for clinical trials.

1. *Questions about fundamental aspects of neutron applications in tumour therapy*

Referring to Table 1 of Barendsen's paper, Fowler asked a further explanation of why repopulation, non-lethal damage, and mitotic delay have no effect on the RBE of neutrons. Barendsen emphasized that it is important to distinguish between factors which are associated with large differences, and those which are associated with small differences with respect to the responses observed after X-ray and neutron irradiation. Equally large effects or at most small differences in responses to equivalent doses of X-rays and neutrons have been observed for the rate of re-population of tumours by clonogenic tumour cells, as well as for non-lethal damage of cells cultured *in vitro*. In addition Barendsen noted that differences of the shapes of dose–effect curves might be of interest with respect to tumour responses, if various types of tumour cells which had intrinsic differences in radiosensitivity were present in different structures of a single tumour.

Field commented on the overall gain factor of 1·2, mentioned by Barendsen (compare discussion of session on: *Present status and future developments of fast neutron radiotherapy*). With a gain factor in this range, Field argued that e.g. compared to a standard dose of 6000 rads in 5 weeks, one would expect to be able to cause additional damage equivalent to an extra dose of 1200 rads to a tumour, without increasing the damage to the normal tissues. Barendsen agreed that such a gain may be considerable, but a gain factor of 1·6 or 1·7, which has often been mentioned in the literature, has never been observed, except for large single doses, or for completely anoxic tumours.

van Putten concluded that with so many variable factors, it will be nearly impossible to interpret clinical results of tumour treatments on the basis of mathematical models derived only from dose response curves for single doses and assumptions about repair of sub-lethal damage and re-population. The results of Barendsen have shown that a variation in re-population rate during a course of fractionated tumour therapy can be an important factor determining the final fraction of clonogenic cells. Barendsen agreed that the manifold factors make the construction of useful models more difficult.

2. *Questions about causes of unsuccessful curative therapy*

Many results of intended curative tumour treatment, even for groups of tumours considered highly curable, showed cure rates which are considerably less than 100% (Kligerman, Kaplan, Breur and Rossi). It is therefore important to analyze which factors determine the results of a treatment in order to obtain

insight about whether hypoxia is a relevant factor associated with many of the failures (Fowler).

Failures are a composite of several untoward events, which Kaplan classified as follows:

(1) the occurrence of distant metastases;

(2) the occurrence of a second primary tumour, especially in the group of patients with head and neck cancer;

(3) cervical lymph node involvement, which cannot be eradicated surgically, although the primary lesion has been controlled.

(4) persistence or recurrence of the primary lesion during or after treatment. The cause for this type of failure may be either technical, e.g. errors in dosimetry and beam alignment or due to radiobiological factors, among which hypoxia.

From the point of view of clinical therapy, failure to cure tumours locally is the consequence of the limited tolerance of normal tissues, which prevents the application of doses of radiation large enough to eradicate the tumour (Kligerman). Possibly the ratio of damage to the tumour relative to normal tissue could be improved by increasing the overall treatment period, by using shrinking fields, or supplementary implantation of radioactive isotopes (Kligerman).

3a. *Selection of tumours for neutron therapy trials*

Morrison noted that the lists of "favourable" tumours suggested by Kaplan and Kligerman, limits the application of fast neutrons to at most 10% of all cancers. External therapy of cervix carcinoma and cancer of the bladder requires high depth doses, which might present problems for neutron therapy, because of their poor penetration.

If one of the criteria used for the selection of tumours favourable for neutron therapy was their possibility of being hypoxic, Elkind suggested that such tumours would also be of interest for studies of modified fractionation regimes and reoxygenation, using low LET radiations.

3b. *Application of neutrons in advanced stages of cancer*

Brennan mentioned the possible application of fast neutrons for palliative treatments of advanced cancer of the head and neck, such as are included in preliminary studies at Hammersmith. Kaplan noted that these cases have been selected mainly on the basis of accessibility for measuring tumour size and for close observation of the effect of the treatment e.g. on tumour shrinkage and skin reactions.

Withers added to this that, if a clinical trial were not to show a benefit from neutron therapy over X-ray therapy with respect to improvement of cure rate, then it is not reasonable to expect much better palliative effect by using neutrons.

Another category of tumours which might be treated with neutrons are those to be irradiated pre-operatively (Rodriguez-Antunez, van der Werf-Messing).

According to Kaplan one should distinguish two types of pre-operative irradiation:

(1) irradiation with a *high* dose to eradicate all of the tumour if possible, with surgery used only to remove "microscopic" residue;

(2) irradiation with a *low* dose, merely to shrink the tumour to a size which improves chances for technically successful surgery.

The application of neutrons in the first category is similar to the situation of a clinical trial, but because the surgery complicates the evaluation, Kaplan felt that the use of neutrons should be postponed till they have been shown to be advantageous for other types of tumours.

In the second group there is no reason to use neutrons because of the low dose applied.

van der Werf-Messing suggested that it might be of interest to compare the effects of neutrons on the primary tumour and on its lung metastasis, e.g. for osteosarcomas, because sometimes it is observed that the rates of volume reduction induced by treatment with X-rays, are different for primary tumours compared with their metastases.

4. *Co-operation between centres for clinical application of neutrons*

van Bekkum suggested that in order to obtain uniformity in selecting patients and in methods and parameters for clinical evaluation of the results, it might be of great advantage to promote co-operation between centres, e.g. through the European Organization for Research on Treatment of Cancer. Breur agreed that the conduction of clinical trials requires an experienced group of workers, who are also interested in radiobiological problems and have technical facilities at a high level available.

5. *Quantitative evaluation of effects observed on tumours and normal tissues*

Fowler explained that changes of tumour volume are determined by the fraction of proliferating cells, the mean cell cycle time, and the fraction of cells eliminated from the tumour per unit time. Changes of tumour volume induced by radiation are associated with complex alterations of the contributions of

these factors to tumour volume. Fowler therefore emphasized that curves of log volume versus dose, as presented in the paper by Breur and van Peperzeel, cannot be interpreted as being cell survival curves.

A problem which was repeatedly mentioned, concerned the relationship between curability and the rate of shrinkage of the tumour volume during and after treatment.

Berry recalled the work published by Suit [1] which showed that over a wide range of human tumours, the correlation of volume shrinkage and cure of the tumours was very poor. Breur stated that assessment of volume changes before, during and after treatments with single and fractionated doses, will provide useful information about the clinical response of the tumour. Furthermore it was observed by Van Peperzeel and Breur that differences in radiosensitivity of clinical tumours as derived from the volume response to irradiation, more or less parallel differences of local curability of these tumours, in contrast to Suit's observations.

Berry noted that radiation induced changes of cell proliferation characteristics, such as observed in a mouse mammary carcinoma studied by van Peperzeel, and in a rhabdomyosarcoma reported by Hermens and Barendsen, after different doses and with different time intervals do not occur in every tumour type after irradiation. Brown and Berry [2] have shown that in tumours induced in hamster cheek pouch there is a slight prolongation of the cell cycle time at 72 hr after a single dose of 1000 rads of X-rays. Berry pointed out that for radiotherapists it is important to have information about changes of the rates of cell proliferation of both tumour tissues and normal dose-limiting tissues.

Djordjevic asked whether cells which have lost their capacity for unlimited proliferation show alterations of their cell kinetics before dying. Barendsen explained that as far as the rhabdomyosarcoma is concerned both surviving cells and cells destined to die after irradiation can proliferate with reduced cell cycle times. The altered kinetics of surviving cells can explain the fast rate of re-population by clonogenic cells observed after X-ray treatment. Because re-population occurs at the same rate in tumours treated with an equivalent dose of neutrons, Barendsen concluded that similar changes of proliferation properties occur in both neutron and X-irradiated tumours. In addition to this, as shown by Westra and Barendsen [3], the mean doubling time of the progeny of cells which sustained non-lethal damage after irradiation with X-rays or α-particles, was shown to be prolonged due to many unsuccessful cell divisions. Such prolongation was similar after X- or α-irradiation with doses which gave equal cell survival. Obviously the proportion of these cells will decrease very rapidly if surviving cells without such non-lethal damage re-populate the tumour.

With respect to the possibility of steady state proliferation kinetics during daily fractionation therapy of clinical tumours, it is important to know whether cells in human tumours can proliferate with cell cycle times of less than 24 hr (Elkind). Dutreix referred to studies by Frindel *et al.* [4] of five different types of human tumours, in which cell cycle times were observed ranging from 1 to 4 days. According to Dutreix it is reasonable to assume that for some tumours the depletion of cells caused by relatively small daily radiation doses may be completely compensated for during the time interval between two subsequent fractions. Breur reported his observations on re-epithelization of human skin after moist desquamation during a course of fractionated radiotherapy. It was shown that the average doubling time of foci of skin cells is equal to 24 hr and thus regeneration could compensate for a daily cell loss of about 50%.

Kligerman reported observations made during fractionated radiotherapy of carcinoma of the rectum. In spite of doses of 200 rads/day, given 5 times a week, microscopic analyses of biopsy material of the mucosa never showed lesions at day 20 which were worse than observed 10 days after starting the treatment. No ulceration of normal mucosa was ever found. However, of 24 cases of rectal carcinoma treated with a total dose of 4500 rads in 30 days, the majority of the tumour cells was destroyed, and in 5 cases no tumour cells could be detected. Presumably the normal rectal mucosa was able to maintain a steady state during treatment. Furthermore Kligerman mentioned clinical observations, showing that prolonged daily fractionation, over a period of 8–12 weeks, can eradicate locally inoperable cancers of the breast with little or no reactions of the overlying skin. According to Kaplan these observations suggest that when the daily dose is small enough, the proliferation response evoked in surviving clonogenic tumour cells, may be less than that in the normal cells. Dutreix agreed with these suggestions explaining that the rate of proliferation of the normal tissue cells must be higher than that of the tumour cells in order to obtain a cure.

REFERENCES

1. H. Suit, R. Lindberg and G. H. Fletcher, Prognostic significance of extent of tumor regression at completion of radiation therapy. *Radiology* **84,** 1100 (1965).
2. J. M. Brown and R. J. Berry, Effects of x-irradiation on cell proliferation in normal epithelium and in tumours of the hamster cheek pouch. *Proc. Symp. Effects of Radiation on Cellular Proliferation and Differentiation*, p. 475, Monaco. IAEA, Vienna (1968).
3. A. Westra and G. W. Barendsen, Proliferation characteristics of cultured mammalian cells after irradiation with sparsely and densely ionizing radiations. *Int. J. Radiat. Biol.* **11,** 477 (1966).
4. E. Frindel, E. Malaise and M. Tubiana, Cell proliferation kinetics, in five human solid tumors. *Cancer* **22,** 611 (1968).

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Evaluation of Past Experience with Fast Neutron Teletherapy and its Implications for Future Applications*

J. T. BRENNAN† and T. L. PHILLIPS‡

†*Dept. of Radiology, University of Pennsylvania, Philadelphia, Penna., U.S.A.*
‡*Dept. of Radiology, University of California, San Francisco, Calif., U.S.A.*

Abstract — *A review has been given of past experience with fast neutron teletherapy and the development of fast neutron therapy sources. The first clinical applications of fast neutrons, initiated by Stone and Larkin at Berkeley, from* 1939–1944, *have been re-examined. The re-evaluation confirmed the interpretation arrived at earlier by investigators at Hammersmith Hospital that the patients had been over-exposed. Details of four cases of the Stone-Larkin series are presented to illustrate the narrow reserve tolerance present in radiotherapy and to indicate the necessity of proceeding with extreme care in determining neutron treatment schemes.*

The second part of the review deals with the production of adequate beams for fast neutron teletherapy. In view of the limited penetration of 14 *MeV neutrons, large source to skin distances have to be employed, which require a total yield of* 4×10^{12} *neutrons/sec produced by the D–T reaction. The characteristics of various types of D–T neutron generators are discussed in view of source strength and target life-time problems. These two primary problems might be solved by using a mixed deuterium-tritium beam and a bare copper drive-in target. The developments of this machine are described. Although the required neutron yield has not been demonstrated up to the present, the operation experience on this type of machine seems promising.*

INTRODUCTION

WITH RESPECT to past experience in fast neutron teletherapy two important areas will be discussed in this review, namely clinical trials on patients and physical problems in obtaining adequate beams of fast neutrons. Excluded from this discussion are the interesting but early efforts started recently, employing ^{252}Cf for fast neutron brachytherapy.

Up to the present fast neutron teletherapy has been applied in three clinical series: (1) The Stone-Larkin series, Berkeley, California, 1939–1944; (2) The Hammersmith series, London, England, 1965–1970; (3) the effort at the Christie-Holt Institute, Manchester, England, 1970 (just initiated). In the first part of this paper the experience in the Stone-Larkin series will be extensively discussed. The second part deals with the experience on production of fast neutrons for teletherapy.

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EXPERIENCE WITH FAST NEUTRON THERAPY IN THE STONE-LARKIN SERIES

In 1939 the 60-in cyclotron designed by E. O. Lawrence became available for biomedical use. In the ensuing 5 yr, 226 patients were treated using this machine. The beam was fixed in a horizontal plane. The dose rate varied from 6 to 10 rads/min and three field sizes were used (7×7 cm, 10×10 cm and 15×15 cm). The SSD (source to skin distance) was 100 cm and the end of the collimator was covered by a filter (0·5 mm Cu plus 1·0 mm

Al). Beam localization was achieved by placing the skin of the treatment port in contact with the aluminium side of the filter.

Patient treatment at Berkeley was terminated in 1944, primarily because of logistic pressures that resulted from the local World War II effort. Follow up on the patients continued in so far as circumstances permitted. On 9 June 1947, at Atlantic City, N.J., Stone delivered the now famous twelfth Janeway lecture entitled "Neutron Therapy and Specific Ionization" [1].

1. *The Janeway lecture of* 1947

The principal message in this presentation was that fast neutrons can eradicate cancer, but that the associated late effects on normal tissues are unacceptable and more severe than one would expect when compared with early effects on skin in the same patients.

The title of the subject lecture contains the phrase "specific ionization", which is a measure of the LET. The phrase appears in the title because it was Stone's intention to explain, as far as possible, the responses observed in his patients in terms of what was known at that time about the influence of LET. This approach assumed that the influence of time-dose relationships had already been taken into account properly but this was not the case. In the era in which the patients were treated, it was widely believed that "the number of fractions makes no difference. Only total dose and duration of treatment count." This idea appeared in 1944 as the Strandquist curves, in which fraction size and number of fractions are neglected. This was essentially the philosophy that Stone adopted in selecting fractionation schemes for his patients. Ultimately, in writing the Janeway lecture, he found it impossible to explain the patient data in terms of LET differences. To his credit he identified the troublesome area, for among his nine conclusions is the following: "6. Biological work showed that for some effects of radiations the distribution of ion pairs 'in time' played a part similar to that of the distribution in space. The rate of administration was not sufficiently weighted in the treatment of patients". This is a clear recognition of the fact that time-dose relationships had not been properly evaluated.

During the 1950s the importance of the oxygen effect was realized and mammalian cell survival curves having a "shoulder" were demonstrated. This led to a renewed interest in the use of high LET radiations for therapy purposes. In particular the Hammersmith group developed a theory that explained Stone's results [2]. This theory took into account fraction size and number of fractions as well as the other parameters that Stone had not allowed for. The theory indicated that the California patients were overexposed because the RBE of a neutron dose fraction increases as the fraction size decreases, a fact unknown to Stone in 1939.

2. *Re-evaluation of the Stone-Larkin series*

Beginning in 1966 the Radiology Department of the University of Pennsylvania undertook a study of the advisability and feasibility of initiating new trials of fast neutron teletherapy in the U.S.A. As part of this study, and in collaboration with Dr. Sheline and Dr. Phillips of the University of California, a detailed re-examination of the Stone-Larkin patient records was undertaken [3]. Sheline had the primary responsibility for the review, which depended on the fact that Stone's records were of good quality and had been carefully preserved along with a number of clinical, coloured photographs of the patients.

A further fortunate circumstance was the fact that Dr. Field of Hammersmith was on a sabbatical year at Stanford University nearby. Field had just completed his work on RBE versus fraction size for the Hammersmith neutron beam [4]. Stone's Berkeley neutron spectrum was similar to the Hammersmith spectrum. Using the curve for RBE versus fraction size it became possible to apply the Ellis formula [5] to each of the neutron dose fractions on Stone's patients. It was noted immediately that the most distressing results in the series were the consequence of very high NSD's (nominal single doses) in the range from 2000 to 3200 rets*. The upper limit is now regarded in most centres as being about 1800 rets for moderate to large fields. The Hammersmith interpretation of Stone's results was thus confirmed.

Another group of severe late effects was accounted for on the basis of previous X-ray or gamma-ray therapy. Previous therapy was carefully noted in the records, but was given little weight in the matter of deciding upon neutron dosage. Also the fact that the exit dose was neglected probably accounted in part for some instances of overexposure to skin.

When these factors were considered it became quite clear that there was only one group of patients whose late responses appeared to support Stone's conclusions. This was a group of 23 cases of malignancy of the prostate

*It has been recommended that the NSD should be expressed in rets (rads equivalent therapeutic) [5].

which were treated through one anterior field and two angled buttock ports. The NSDs for this group were in the range from 1650 to 2100 rets and the late effects on skin (ulceration and induration) were much more severe than one would expect from the NSDs. Several ideas have been suggested that might explain this phenomenon but the chief lesson to be learned is that one must take special care in treating the lower abdomen with fast neutrons. Four to 6 ports, or rotation therapy would seem to be indicated. Also one must be aware of the possibility that the relative sensitivity of gut to fast neutrons may be high and dependent on neutron energy [6, 7].

2.1. *Four illustrative cases from the Stone-Larkin series*

Details are presented below on four of Stone's cases. The first two illustrate the disastrous results of very high NSDs. The second two cases illustrate the occurrrence of distressing late effects in prostate cases in spite of relatively reasonable NSDs.

Photographs are available of these patients illustrating the reactions. All patients were treated on the 60-in cyclotron in Berkeley. The original doses in "*n*" units were converted to rads and also to a 6-week dose (SWD, 30 fractions of gamma-rays in 6 weeks) and to an NSD equivalent for gamma-rays. In all of our calculations the exit dose was taken into account.

Case 1 (*T.W.*). A 58-year old man was treated in March 1941 for a moderate sized carcinoma of the vocal cord and represents the first long term cure of a patient by neutron therapy. Opposing 7×7 cm fields were used and each received 1920 rads in 19 fractions over 48 days. The SWD was 8600 rads and the NSD 2500 rets. The reaction at the completion of treatment was 4+. At 5 years after treatment marked telangiectasis was present. At 8 years an ulcer developed but healed. At 15 years a severe ulcer developed and a graft was attempted, but failed. The patient died 18 years after neutron treatment of a carcinoma of the stomach.

Case 2 (*E.M.*). The patient was a 44-year old woman treated in November 1940 for a recurrent fibrosarcoma of the left shoulder. Using a single 10×10 cm field 2570 rads in 15 fractions over 34 days were given. The SWD was 11,000 rads and the NSD 3200 rets. The 4+ early reactions did not heal and a deep ulcer developed between July 1941 and August 1942. This 4+ late reaction never healed and a shoulder girdle amputation, removing all of the irradiated tissues, was done at 26 months. No tumour was present in the specimen and the patient remains alive to the present day (age 74).

Case 3 (*W.K.*). A 61-year old man with carcinoma of the prostate was treated in October 1941 with 3 fields, each 15×10 cm, two posterior and one anterior. The posterior fields received 1200 rads in 11 fractions over 29 days for a SWD of 6500 rads and an NSD of 1900 rets. The anterior field dose was 1320 rads in 11 fractions over 29 days, the SWD was 6900 rads and the NSD 2000 rets. The anterior early reaction was 4+ and the posterior 3+. An ulcer at the base of the penis persisted until September 1943, but healed in February 1944. By September 1946 severe telangiectasis was present posteriorly and in January 1947 there was fibrosis of the penis and an ulcer on the buttocks. The patient also developed rectal fibrosis and a vesico-colic fistula requiring a colostomy. He died of complications 9 years after neutron treatment.

Case 4 (*J.W.*). A 62-year old man was given treatment for carcinoma of the prostate in April 1942. The three 15×10 cm fields were used to give 960 rads in 4 fractions over 29 days posteriorly and 1060 rads in the same time anteriorly. The SWD was 5340 rads anterior and 6000 rads posterior. The NSD's were 1560 and 1700 rets. The early reactions were grade 2+ and the late 3+. These late reactions seem severe for the doses given.

These four cases illustrate the narrow reserve tolerance present in radiotherapy and warn the therapist to be extremely careful in determining neutron dose schemes. Certainly doses equivalent to 1800 rets NSD or 6300 rads SWD should not be exceeded except in very special circumstances. The tolerance would appear to be relatively low for neutrons in the pubic and buttock areas, perhaps lower than for gamma-rays in terms of the equivalent doses noted above.

2.2. *The sensitivity of tissues in lower abdominal fields to fast neutrons*

The most probable explanation for the severe late effects seen in the lower abdominal fields is the relatively thick layer of subcutaneous fat which exists in these regions. With fast neutrons having the energies used at Berkeley and Hammersmith, absorbed dose to fat is about 20% higher than absorbed dose to muscle. Blood vessels which lie in such a thick layer of fat must be especially vulnerable

and an excess of 20% in dose to these vessels could account for the severe fibrosis seen. It should be noted further that neutron beams of different energy may produce an absorbed dose in fat which differs from that produced in the Stone-Larkin patients. Consequently each new beam must be studied individually.

FAST NEUTRON SOURCES FOR TELETHERAPY

1. *Cyclotrons*

The first cyclotron was a table top model, built by E. O. Lawrence in 1930. Larger models followed in rapid succession. The first neutron source of any kind, having a source strength sufficient to be considered for radiotherapy, was the 37-in model which became available for medical purposes in 1937. Although the depth dose with this machine was inadequate for any but near surface lesions, valuable data was obtained by Stone *et al.*, on normal skin responses in 23 patients [8].

In 1939 the 60-in cyclotron became available for biomedical use. This machine, like the present Hammersmith cyclotron, could accelerate deuterons to a maximum energy of 16 MeV. This maximum was chosen because at energies greater than 16 MeV there is a relativistic increase in the mass of the deuterons which causes them to lag behind and arrive at the accelerating gap too late. The solution of this problem requires a much more complicated and expensive cyclotron. Thus it is not a coincidence that the Berkeley 60-in and the present Hammersmith cyclotron have the same maximum deuteron energy and therefore the same neutron spectrum. This circumstance did much to facilitate our study since it permitted direct comparison of Stone's early results with recent Hammersmith results [3].

The relativistic mass-increase problem referred to above was first overcome in the 1940s by the invention of the synchrocyclotron. Although these machines achieved very high energies, the beam current and hence the neutron output of the early synchrocyclotrons was too low for therapy purposes. It was not until the 1960s that another new kind of cyclotron appeared. This was the Azimuthally Varying Frequency, or AVF machine. The name arises from the fact that the strength of the magnetic field between the magnet poles varies not only as a function of radius, but also as a function of azimuthal angle. With this arrangement it is possible to have deuteron energies well above 16 MeV along with adequate neutron output. Six machines of this type are now operating in the U.S.A. and of these the one best suited to radiotherapy purposes is the TAMVEC (Texas A and M Variable Energy Cyclotron) at College Station, Texas. Measurements made on this machine in 1969 indicate that for a cyclotron, the minimum deuteron energy required to produce a therapy beam having satisfactory depth dose characteristics is about 30 MeV [9].

2. *D–T neutron generators*

Deuterium (D) and tritium (T) are neutron-rich isotopes that readily liberate a neutron when they collide. In the case of a D–T collision the maximum yield of neutrons occurs at a deuteron energy of only 100–140 keV. Also the Q value is high (17 MeV) and the neutrons emitted are nearly monoenergetic at 14 MeV. Even so, these neutrons are not as penetrating as ^{60}Co gamma-rays. A collimated beam of 14 MeV neutrons, at an SSD of 125 cm, has depth–dose properties similar to those of a standard ^{60}Co machine (SSD = 80 cm). A major difficulty arises from the fact that there is no D–T neutron generator available which has the source strength (4×10^{12} neutrons/sec) sufficient for use at an SSD of 125 cm. The highest performance machine now available has a source strength of only 1×10^{12} neutrons/sec [10].

In practice the D–T neutron generators may employ a beam of accelerated deuterons or a "mixed beam" of deuterons and tritons. If deuterons only are used the target surface must be impregnated with tritium. With a mixed beam there is no hydrogen in the target at the outset, but the metal target is loaded with entrapped D and T as the beam starts up. These trapped nuclei provide the targets for D and T nuclei which arrive later. This is called a "drive-in" target system.

2.1. *Generators with gas targets*

The metal targets discussed above are inefficient in that only a very small fraction of the accelerated ions produce neutrons. Most of the ions simply heat up the metal target. Obviously an all tritium target (consisting of tritium gas) would be more efficient in this respect. The difficulty here is in compressing the tritium into a small enough volume to make a desirable source. This is now being investigated at the University of Wisconsin by Boone *et al.*, using a modern differential pumping system to contain the tritium. At Hammersmith, Vonburg *et al.*, are constructing a steel cylinder target which will hold deuterium gas at 30 atmospheres of pressure. This is to be exposed in the cyclotron to a beam of 16 MeV deuterons which

can penetrate the wall of the gas containing cylinder.

2.2. *Generators with a mixed beam and sealed tube*

The Services Electronics Research Laboratory (S.E.R.L.) at Baldock, Herts., has developed a mixed beam machine which employs a drive-in target, and in which both ion source and target are sealed within an evacuated glass tube. The useful life-time of one tube is about 200 hr after which the complete modulus is replaced. At present one of these machines produces a maximum of 5×10^{11} neutrons/sec. A machine with a yield of 2×10^{12} neutrons/sec is programmed for 1971.

2.3. *Generators with a mixed beam and bare copper target*

Targets used heretofore consist of a copper backing upon which a thin layer of titanium or erbium is electroplated. These two metals trap hydrogen well, thereby creating a high yield target. However, during operation the titanium or erbium tends to flake off the copper ("sputtering"). This degrades the neutron output. It had been observed that bare Cu, with no titanium at all, still has some neutron yield (20%) when used as a drive-in target. The lower yield from Cu could be overcome by using a higher ion beam current. The merit of this idea is that Cu conducts heat well and a Cu target can be cooled in such a way as to provide an indefinite target life-time.

3. *The source strength and target life-time problems in 14 MeV neutron generators*

As of 2 years ago the best available D–T generator had an acceptable target life-time of about 200 hr but the neutron output was a factor of 4 less than that required for a therapy machine [9]. It was necessary, therefore, to advance the state of the art of target design in order to permit the construction of a therapy machine.

It was clear at that time that source strength and target life-time were primary problems which must be solved first. All other machine design problems were, and are less difficult. Accordingly we set about to mount a direct attack, concentrating on the two primary problems.

3.1. *The AGC approach*

The Pennsylvania feasibility study group was required to employ the open bidding system whenever possible. The first formal step was the issuance, by the University of Pennsylvania, of a Request for Proposal or "RFP" for assembling and testing a new target system. At the time the RFP was written there was still a possibility that a small (30-in) cyclotron could be the best answer if some new, and as yet unexploited, nuclear reaction could be uncovered. Accordingly, the RFP was written to be compatible with either a cyclotron or a D–T machine (or possibly some other solution).

At the time the RFP was issued, it seemed likely that the winning bid would be on a rotating target D–T machine of the type developed by Booth at L.R.L. However, by going through the standard bidding and advertising procedures we uncovered an unsuspected and relevant effort underway at the Aerojet General Corporation (AGC) plant at San Ramon, Calif. At that location, AGC had for some time been working on an Air Force project which called for a D–T neutron generator having a source strength of 1×10^{13} neutrons/sec. The AGC approach was attractive because the target was a piece of bare copper which might have an indefinite target life-time (as in the case of a tungsten X-ray target).

Two other well-conceived bids were based on the rotating, tritium impregnated target. Analysis of the theoretical justifications contained in these bids led to increasing scepticism regarding the rotating target. There appeared to be a possibility that the problem could be solved only by giving the rotating annular target a very large diameter, perhaps 24 in. This would create serious beam collimation problems and the target itself would cost thousands of dollars. On the other hand, further analysis of the rationale behind the AGC, bare Cu target idea, made it seem more attractive. For these reasons the contract was awarded to AGC.

3.2. *The AGC results*

In accordance with our Request for Proposal the AGC approach was set up as an experiment for testing targets. This was not an attempt to build a prototype therapy machine. It was, rather, a physics experiment intended to determine whether or not the problems of neutron output and target life-time could be solved.

In developing a new D–T neutron generator, it is customary to begin by accelerating ^1H (protons) into whatever target is to be used. This is convenient because no secondary, hazardous radiations are created and there is no significant residual radioactivity problem. The next step is to proceed to the use of the D–D reaction. This produces some neutrons and some residual radioactivity, but the cross-

section for the D–D reaction is only 1% of the cross-section for the D–T reaction and the radiation hazard problems are proportionately less. The third and final step is to employ the D–T reaction. This maximizes the neutron and residual activity hazards and, in addition introduces the radiation hazard from the tritium itself.

The principal results obtained by AGC can be summarized as follows: by January 1970, they finished assembling our accelerator and began accelerating protons. By 16 February 1970, they had proceeded into the second, or D–D phase.

The operating conditions used with the D–D reaction on a bare Cu target are given in Table 1. Under these conditions the yield was 9×10^{8} neutrons/sec implying a yield of 9×10^{10} neutrons/sec if a D–T mixed beam were used.

Table 1. The operating conditions used with the D–D reaction on a bare Cu target to obtain a yield of 9×10^{8} *neutrons/sec*

Current	20 mA
Energy	120 keV
Target diameter	0·7 cm
Target temperature	230°C

Continuing with the D–D reaction, and using the operating conditions of Table 1, liquid nitrogen was substituted for cold water. This produced a marked increase in the neutron output but the reason for this increase is not fully understood at this time. For example, deuterated ice may have been formed on the front surface of the target. Discounting temperature effects, it now appears that 4×10^{12} neutrons/sec can be obtained under the conditions shown in Table 2 if a slanting target is used. Moreover, the bare Cu target showed no evidence of radiation damage at high power densities and, with the closed D–T replenishment system used, it may be possible to run indefinitely with one target and one charge of D–T gas. Thus it appears that two primary problems of sufficient neutron flux density and target lifetime have probably been solved.

Table 2. The operating conditions calculated to yield 4×10^{12} *neutrons/sec with the D–T reaction*

Current	80 mA
Energy	175 keV
Effective target diameter	~2·0 cm
Target temperature	250°C

An abrupt decision was made by AGC to end experiments on the drive-in target as of 27 February 1970. For this reason the work stopped at the end of the D–D stage and their final report contains no D–T data.

During the period March to August 1970 analysis of the Aerojet results continued and, on 20 August 1970, the apparatus and personnel involved in the effort were acquired by the Cyclotron Corporation of Berkeley, Calif. The project was transferred to Berkeley and experimental work was resumed. Final results are anticipated during 1971.

3.3. *Theoretical models for a drive-in Cu target*

During the course of the work at AGC, periodic on-site observations were made by the staff of the University of Pennsylvania and their consultants. When the AGC final report became available in 1970, it was possible to perform a careful analysis of the experimental data. This analysis revealed a textbook error in the application of hydrogen diffusion coefficients to the model proposed by AGC. As a result of this analysis a new model [11, 12] was proposed for the storage of the large amount of hydrogen in the target needed to explain the neutron yield. A detailed explanation of the mechanism of storage is needed and will require further study.

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REFERENCES

1. R. S. Stone, Neutron therapy and specific ionization. *Amer. J. Roentgenol.* **59,** 771 (1948).
2. J. F. Fowler, R. L. Morgan, C. A. P. Wood, D. K. Bewley, R. H. Thomlinson, S. Hornsey, G. Silini, T. Alper, B. A. Turner and J. A. Silvester, Pre-therapeutic experiments with the fast neutron beam from the Medical Research Council cyclotron. I-VIII. *Brit. J. Radiol.* **36,** 77 (1963).
3. G. E. Sheline, T. L. Phillips, S. B. Field, J. T. Brennan and A. Raventos, Effects of fast neutrons on human skin. *Amer. J. Roentgenol.* **111,** 31 (1971).
4. S. B. Field, The relative biological effectiveness of fast neutrons for mammalian tissues. *Radiology* **93,** 915 (1969).

5. F. Ellis, The relationship of biological effects to dose-time fractionation factors in radiotherapy. In *Current Topics in Radiation Research* (Edited by M. Ebert and A. Howard), Vol. IV, p. 357. North-Holland, Amsterdam (1968).
6. S. Hornsey and G. Silini, Recovery of tumor cells cultured *in vivo* after X-ray and neutron irradiations. *Radiat. Res.* **16,** 712 (1962).
7. H. R. Withers, J. T. Brennan and M. M. Elkind, The response of stem cells of intestinal mucosa to irradiation with 14 MeV neutrons. *Brit. J. Radiol.* **43,** 796 (1970).
8. R. S. Stone, J. H. Lawrence and P. C. Aebersold, A preliminary report on the use of fast neutrons in the treatment of malignant disease. *Radiology* **35,** 322 (1940).
9. J. T. Brennan, Fast neutrons for radiation therapy. *Radiol. Clin. N. Amer.* **7,** 365 (1969).
10. R. Booth, Rotating neutron target system. *IEEE Transactions on Nuclear Science*, 938 (1967).
11. D. F. Herring, C. D. Pruett, R. M. Potenza, J. Dobson and D. M. J. Compton, Review of the program for development of a prototype fast-neutron therapy system for the University of Pennsylvania. *Enviro-Med. Report* No. ESD-211 (1970).
12. D. M. J. Compton and D. F. Herring, The use of copper as a drive-in target for neutron radiation therapy sources. *Enviro-Med. Report* No. ESD-213 (1970).

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Clinical Experience with Fast Neutrons from the Medical Research Council's Cyclotron at Hammersmith Hospital*

MARY CATTERALL

Cyclotron Unit, Hammersmith Hospital, London W.12, England

Abstract — *Forty patients suffering from advanced malignant tumours of the breast, head, neck and skin have been treated with 7 MeV fast neutrons from the M.R.C.'s cyclotron at Hammersmith Hospital. This machine has an output of 40 rads/min and treatment times did not exceed 4 min. Ten of the patients had multiple or very large tumours and were treated also with low LET radiations.*

The responses of the skin in the treated areas to the two types of radiation have been observed and the reactions quantitated in order to obtain the RBE of fast neutrons for effects on skin. From the data available this appears to be approximately 2·9. A skin sparing effect has been demonstrated. The tumours have been measured as objectively as possible and rates of regression have been recorded. These have varied among different histological types and also in the same patient when multiple nodules have been treated with both types of radiation. All the tumours treated with neutrons showed a favourable response which was not qualitatively different from that seen with low LET radiation.

THE present clinical investigation into the effects of fast neutrons on advanced tumours and the normal tissues included in the treated area was started in November 1969. It was a preliminary investigation before a controlled clinical trial is undertaken, and the aims were as follows:

1. To investigate the RBE for effects on normal and malignant tissues.
2. To select a practical therapeutic regimen.
3. To investigate the effects of field size, bolus and skin sparing.
4. To develop methods of measuring more precisely the effects of all types of radiation on normal and malignant tissue.

*Please note: This is a summary of the paper presented at the meeting on "Fundamental and Practical Aspects of the Application of Fast Neutrons in Clinical Radiotherapy", at Rijswijk, the full text of which is to be published in the *British Journal of Radiology*.

METHODS

Dosimetry

The depth doses for the fast neutron beam were intermediate between those of 250 kV X-rays and gamma-rays from ^{60}Co. The output was about 40 rads/min and the time for each treatment was less than 4 min. Collimation was achieved by borated wood diaphragms and a range of field sizes from 27 to 440 cm^2 was available. Wedged filters made of paraffin wax were employed when necessary.

Aluminium activation

Pure aluminium (^{27}Al) was activated by fast neutrons and became ^{27}Mg. This isotope decayed with a half life of 9·52 min emitting gamma rays of 0·84 and 1·01 MeV which could be counted with an error of less than 0·5% on a 1 g sample.

Small pellets of aluminium were placed

within the treatment area on the patient's skin beneath bolus, wax moulds and in cavities such as the mouth, before treatment. They were removed immediately afterwards and counted within 15 min. One pellet, placed at the site of the incident beam, received a known dose and the others were related to this.

Measurement of dose to the eyes

Special neutron film badges were marked with the patient's names and were attached to the skin near the patient's eyes during each treatment. They were then sent to the Radiation Protection Centre for measurement of the dose received during that course of treatment. The distance of the site irradiated from the eyes was measured and also the approximate angle of the beam to the lens.

Observations on the skin

(a) *Clinical observations.* The radiotherapists recorded the reactions of the skin to the treatment at least 3 times weekly during treatment and usually daily for the first 2 or 3 weeks after treatment. Thereafter observations were recorded twice weekly for a further 7 or 8 weeks and subsequently fortnightly up to 3 months after the end of treatment and at longer intervals thereafter.

(b) *Skin scoring.* The system of giving a numerical score to the skin damage produced by varying doses of neutrons, which has been used previously by workers in this Hospital, was modified and applied to patients' skin reactions in this study.

The scoring was done twice weekly starting on day 15 of the treatment and continuing for 70 days by 4 non-clinical scientists.

The statistician computed the mean of the scores and the standard error for each scoring day. At the end of the 70 days, all the scores given to a patient were integrated to yield a single score representing reactions which appeared 5 days before to 5 days after the peak reaction. The scores were plotted as effect or reaction against time after treatment.

The presence and degree of skin sparing with the neutron beam was investigated by placing 1 mm tissue equivalent plastic over half the field irradiated. This resulted in proton build-up so that the maximum effect of ionization was brought to the skin surface and the reactions so produced were compared with those where there was full skin sparing. A total of 96 fields was scored in 35 patients. Of these 67 were treated with neutrons and 29 with X-rays. Of the 67 neutron fields 28 had tissue equivalent plastic applied to part of them.

Observations on tumour response

Changes in size, induration, ulceration, mobility, pain and tenderness were annotated by the radiotherapist. Two scientific officers trained in the measurement of animal tumours measured the tumours twice weekly before, during and after treatment. These scientists were not clinicians and where they measured 2 or more tumours in the same patient, they did not know what treatment had been given. Measurements were made by callipers in 2 or 3 dimensions and averaged, then these results were expressed as fractions of the size on the first day of treatment and plotted against time.

General condition

After a full physical examination and the necessary special investigations, peripheral blood counts, serum electrolytes and weight measurements were made at weekly intervals.

The patients

Five of the 40 patients treated died before any assessment of the effects of treatment could be made. Thirty-five patients were divided into three groups.

Group I. Eight patients suffered from Stage T_4 breast cancer, 7 were also N_3 and 4 were also M_1 or M_2. Each had at least 2 areas irradiated, for example, the breast by fast neutrons and the glandular areas by orthovoltage X-rays. The skin changes produced by the 2 types of radiation were observed and provided information on the comparability of the effects produced by the neutrons and the X-rays.

Group II. Ten patients formed this group and these all suffered from very large tumours of more than 400 cm^2 or multiple skin nodules. These tumours were divided into areas varying from 48–80 cm^2, some of which were treated with fast neutrons and some with low LET radiation. The purpose was to compare the tumour response and ascertain that neutron therapy was not inferior to low LET radiation. The normal skin adjacent to the tumours which was included in the treated area was also observed and the reactions compared. So long as the skin reactions were the same, any difference in tumour response was due to the type of radiation.

Group III consisted of 17 patients suffering from advanced but superficial tumours which could be easily seen and palpated, 6 with metastatic glands in the neck, 4 with mouth tumours and 3 with advanced epidermal tumours. Four other patients in this group had adenocarcinomas. The purpose of treating

this group was to measure tumour response and observe skin and mucous membrane reactions.

All treatments, by whatever radiation, were given three times weekly and the standard overall time was 4 weeks or 26 days. The doses of both neutrons and X-rays were varied within a small range in order to give a maximum tolerance dose which produced the same skin reactions with both types of radiation. To an area of 90 cm^2, giving 12 fractions in 4 weeks, the range of total dose for neutrons was from 1200 to 1760 rads and for orthovoltage X-rays from 3000 to 4800 rads. The present regimen to an area of 90 cm^2 is 1440 rads neutrons in 12 fractions over 26 days. For areas other than this, a factor, based on X-ray data, was applied to the dose given.

CONCLUSIONS

1. 1440 rads fast neutrons in 12 fractions over 26 days, to a 90 cm^2 area was a practical therapeutic regimen.
2. It was clinically approximately equivalent to 4100 rads orthovoltage X-rays in the same fractionation and overall time. By the skin scoring system, the RBE was about 2·9 but more data are required before this value can be ascertained statistically.
3. This dose caused an exudative skin reaction which healed. The peak occurred 3–30 days after the end of the treatment.
4. When tissue equivalent plastic covered part of the irradiated field, a greater skin reaction was observed under the plastic. Where the fields were open, there was a clinically significant skin sparing effect.
5. The early tumour response was comparable with that of the X-ray dose although the rates of regression of tumours varied in some cases, with the type of radiation. Fast neutrons were shown to be effective against a variety of tumours, but the follow up has been only a few months.
6. Aluminium activation was a useful means of measuring dose.
7. The addition of carbon to wax produced an attenuation coefficient similar to human tissue.
8. The lining of wax moulds by 0·1 mm lead absorbed the protons and restored the skin sparing effect.
9. A fixed horizontal beam was not a significant disadvantage because the high output enabled treatment to be given in about 4 min and allowed flexibility of treatment positions.

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Considerations in Planning a Clinical Trial with Fast Neutrons

H. R. WITHERS, P. R. ALMOND and B. W. BROWN
The University of Texas M. D. Anderson Hospital and Tumor Institute at Houston Texas Medical Center, Houston, Texas 77025, *U.S.A.*

Abstract — *The problems of instituting a clinical trial are discussed and some qualities of a cyclotron at Texas A. & M. University are outlined. It is clearly impossible to test all possible dose fractionation regimes before beginning a clinical trial. Therefore, selection of a treatment regime must be based on previous clinical and radiobiological experience, without certainty that the regime chosen is the best. However, the main problem in a clinical trial lies in the difficulty of proving with statistical satisfaction the superiority of one form of treatment.*

INTRODUCTION

To BEGIN a clinical trial of fast neutrons for radiotherapy, it is an advantage to have a good neutron source, an adequate number of patients with suitable tumors and an understanding of the radiobiology of the neutrons to be used. The radiobiology will be discussed thoroughly elsewhere in these proceedings. We shall consider mainly a neutron source available in Texas and some representative problems that emerge when the feasibility of a clinical trial is analyzed.

THE CYCLOTRON AS FAST NEUTRON SOURCE

Texas Agricultural and Mechanical University, 90 miles from The University of Texas M. D. Anderson Hospital and Tumor Institute at Houston, has a variable energy, sector-focused 88-in cyclotron. This machine is capable of accelerating protons from 6 to 60 MeV, deuterons from 12 to 65 MeV, and alpha-particles from 25 to 130 MeV with extracted beam currents of several microamperes. For irradiation of patients, neutrons would be produced by bombarding a beryllium target with deuterons.

The characteristics of this machine that favor its use in a clinical trial are:

1. *Output*

The output (measured at 125 cm target to surface distance) using a five microampere beam can range from 13 to 60 rads/min, depending on the thickness of the target chosen and on whether 30 MeV or 50 MeV deuterons are used.

2. *Half-value thickness*

For a 6 cm square field, the half-value layer in tissue-equivalent solutions for the "30 MeV" beam is 9 cm and for the "50 MeV" beam, 11 cm. For a 16 cm diameter field using 50 MeV deuterons on a thin (0·32 cm) target, the half-value layer in tissue is 14·5 cm and for a thick (1·21 cm) target, 16·5 cm. Furthermore, the angular distribution of the beam is peaked highly in the forward direction, which may reduce the problems of collimation of a therapy beam.

3. *Dosage build-up*

Using a thin target and 50 MeV deuterons, the surface neutron dose is 76% of the maxi-

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mum. The maximum occurs at about 1 cm, indicating that the average neutron energy is about 30 MeV.

4. *Linear Energy Transfer (LET)*

The LET ranges from about 1·55 to about 580 keV/μ. It is expected that with increasing depth a higher proportion of the dose will be found in higher LET intervals. This may have some biological significance in that the normal tissues traversed by the beam may be partially "spared".

These characteristics all suggest that the Texas A. & M. cyclotron would be very well suited to clinical use. Furthermore, it should be possible to deliver a beam of neutrons horizontally, vertically, or at intermediate angles.

THE PROBLEM OF ACHIEVING A SIGNIFICANT RESULT

The crucial question that arises in planning a clinical trial is, "Are there enough patients with suitable tumors to ensure a conclusive result within a reasonable time?" A reasonable time is about 5 yr. Prolonging the time may foster the spread of neutron generators before any real clinical advantage or disadvantage of neutron therapy is established.

Selection of patients

Selecting potential patients for a trial is restricted by the uncertainty regarding the tolerance of the intestine to neutron irradiation, particularly fractionated irradiation. The response of the intestine to fractionated radiotherapy is governed by the large capacity for repair of sublethal damage exhibited by the stem cells of the intestinal mucosa after X- or γ-irradiation. If this protective repair capacity were reduced disproportionately by neutron irradiation, the clinical use of neutrons would have to be limited to extra-abdominal tumors, a restriction which could reduce the usefulness of neutrons, regardless of any benefit shown by a clinical trial. The response of mouse intestine to neutrons from the Hammersmith cyclotron seems to be different from that following exposure to 14 MeV monoenergetic neutrons, there being more recovery in the intestine exposed to 14 MeV neutrons [1]. The response of mouse intestine to Texas A. & M. cyclotron neutrons would, of course, be investigated with the hope that the mucosal cells would show repair of sublethal damage proportionate with that in other tissues, i.e. that the relative biological effectiveness (RBE) for intestinal injury would not be greater than that of other tissues, particularly at low doses.

At present, however, tumors of the head and neck were considered to be the only ones suited to a randomized trial for the following reasons:

1. There would be no intestine in the path of the irradiation beam.
2. A reasonable number of patients with the chosen types of tumor would be available for study.
3. It is possible to assess the results of local treatment because of a relatively low probability of early death of the patient from metastatic disease.

Therefore, we analyzed the number of patients presenting with tumors of the head and neck and the results of treatment at Anderson Hospital and decided that tumors of four sites could be used: the retromolar trigone, tonsillar fossa, base of tongue, and pharyngeal wall. Tumors of other sites were excluded for a variety of reasons. In the nasopharynx, the most common tumor is the lympho-epithelioma. It was excluded because the local control rate with conventional radiotherapy was too high to expect improvement by neutron therapy. Squamous carcinoma of the nasopharynx was excluded because there were too few patients and the technical aspects of avoiding overdosage to the spinal cord might have been too great. Most of the other tumors around the head were excluded because there were too few patients presented per year. Although cerebral glioblastomas may represent an additional study group because of the ineffectiveness of conventional therapy, our discussion here is limited to squamous carcinomas of the four sites mentioned.

In the four tumors chosen, a high proportion of T1 and T2 lesions are controlled locally by conventional treatment; therefore it would be difficult to demonstrate an improvement. Furthermore, the risk of worsening results (in terms of both tumor control or normal tissue damage) precluded inclusion of patients with these tumors. Therefore only Stage T3 and T4 lesions have been considered. The larger tumors are a reasonable choice since it is usually believed that they are characteristically less oxygenated and thus would stand to gain most from neutron therapy.

Although it would be necessary to pool the results of treatment of the four tumor-bearing sites, this is not too serious a disadvantage if, in specifying treatment, each of the four sites is randomized separately and according to the stage of the disease. In planning the treatment of these patients, only the choice of external beam therapy would be randomized. Thus, the patients treatment might include local inter-

stitial implantation of radium and/or surgical dissection of the lymph nodes of the neck in addition to the external beam therapy. In other words, the patients' treatment would be individualized, striving to obtain the best possible result with no randomization other than the choice of γ-rays or neutrons for the external beam therapy.

Number of patients and duration of trial

In a clinical trial, the results of treatment for head and neck cancer can be assessed fairly well two years after therapy, since nearly 90% of local recurrences will have become evident by that time. In fact, it would be unjustifiable in a trial of neutron therapy to delay assessment longer than 2 yr.

Analysis of the results of conventional treatment for Stage-T3 disease in the four sites selected shows that between 1963 and 1966 2-year local control of the disease was achieved by radiotherapy alone in about 75% of cases. (Surgical salvage of locally recurrent disease and deaths from metastases are not considered when assessing the tumor response to irradiation.) For example, if a tumor recurs locally within 2 yr of radiotherapy and surgical intervention then salvages the patient, the result is noted as a recurrence. If the treated area is clear of disease and the patient dies of metastases, that is scored as local control. A similar analysis of Stage-T4 lesions shows that about 40% of the tumors were controlled locally for two years or more using sparsely ionizing radiations.

How much better must neutrons be to be worthwhile and how difficult is it to prove a difference between the results of conventional and neutron irradiation? It may be calculated [2] that to have a three-to-one chance of showing that a 20-percentage point improvement in the results of treatment of T3 tumors, that is, to show that an improvement from 75% to 95% local control was significant with a probability of 0·9 (not 0·95 as is usually sought), would require that about 60 patients be treated, 30 conventionally and 30 with neutrons. Stated in another way, if the true effect of neutrons is to improve local control from 75 to 95%, then in four randomized clinical trials, each treating 60 patients, there would be one trial (on the average) that would fail to show that degree of improvement at the 0·1 significance level. If one wanted a higher probability of obtaining the true result, more patients would have to be treated. Obviously, if the improvement in local control were less than 20-percentage points, it would require that more patients be treated to have the same chance of achieving an equally significant result — a 15-percentage point improvement would require about twice as many patients. Likewise, if one were to demand that the probability of significance be 0·95 rather than 0·9, about 30% more patients would be required.

If one were to achieve a 20-percentage point improvement in the local control of T3 tumors in a series of 60 patients, the trial would last about 5 yr at the present rate of patient recruitment at Anderson Hospital. This would be followed by another 2 yr of follow-up, and as discussed later, would be preceded by a pilot study lasting at least 2 yr. Therefore, an optimistic assessment of the time required to conclude the trial would be 9 yr after the first patient was irradiated, if the results had to satisfy the usual statistical criteria of significance. However, the choice of 0·9 or 0·95 as the desirable probability of significance is an arbitrary one and, from a practical point of view, may be unrealistic for some clinical trials.

A 20-percentage point improvement in local control from 75% to 95% is somewhat optimistic. A 15-percentage point improvement would be worthwhile. Yet in planning a clinical trial one is faced with the prospect of having to treat 140 patients to have a three-in-four chance of showing a 15-percentage point improvement to be significant with a probability of 0·9. One would have to plan on treating 180 patients to achieve the 95% probability of significance commonly sought in animal experimental work. At the present rate of patient recruitment and assuming complete and continued cooperation between surgeons, radiotherapists, and other interested physicians, this would require 10 to 15 years of randomized treatment.

Some would say that a 10 percentage point improvement from 75% to 85% local control would be sufficient justification for the use of neutron therapy. Unfortunately, it would be virtually impossible to establish that such an improvement was significant; one may be left with having to accept only a 50–50 probability that a 10% improvement was not just fortuitous. Clearly, some compromise is necessary in deciding how significant a result must be before accepting that the form of treatment tested is worthwhile.

In the case of T4 tumours, the 2-year local control rate with radiotherapy at Anderson Hospital is about 40%. To have a four-to-one chance of showing that a 40-percentage point improvement was significant ($P=0·95$) would require that 36 patients be randomized evenly

between cobalt γ-ray and neutron therapy. A 30-percentage point improvement would require about 70 patients so randomized. Anything less than a 30-percentage point improveprovement would be difficult to establish as being highly significant. The recruitment rate of suitable patients at Anderson Hospital has been about seven per year. Therefore, randomized treatments would probably need to be planned for at least 5 yr. The actual duration of the trial would be determined by the degree of improvement or worsening of results obtained and the probability of significance required.

It might be possible to obtain results earlier than this for two reasons: (1) When we actually have the data we may be able to make a more powerful analysis using parametric assumptions, which take into account the time to recurrence, as well as the fact of recurrence or nonrecurrence at a fixed time. (2) If the distribution of times to recurrence is analyzed, then data can be used from patients lost to follow-up or dying of intercurrent disease or metastasis in the absence of recurrence within 2 yr.

Stopping the trial

Having begun the trial with the knowledge that a significant result would be difficult to achieve, the next problem, and a potentially more difficult one, is when to stop. This decision would not be difficult if a decided advantage or disadvantage from neutron therapy were apparent. However, what if there appeared to be a 10% better rate of local control in the neutron group after five years of randomized treatment? Should the trial be continued, should all patients thereafter be treated with neutrons, or should this 10% improvement, which has about an even chance of being fortuitous, be considered not worth the extra trouble, expense, and protection hazard associated with neutron therapy? There is no simple answer to what is a worthwhile improvement, since ethical and subjective factors must be counterbalanced by a broader consideration of priorities. In fact, the organizations funding medical research may make the decision.

RADIOBIOLOGICAL PROBLEMS ASSOCIATED WITH A TRIAL OF NEUTRONS

It is not proposed to consider in depth the clinical radiobiological problems associated with fast neutrons. The main concern clinically is in selecting the best dose-fractionation regime. It is obviously impossible to test all the possible combinations of dose-per-fraction, fractionation interval, and overall treatment time. Therefore, some guesses must be made, based on known radiobiological characteristics of fast neutrons and on the experience of radiotherapists with conventional radiation.

The overall treatment time should probably be the same for neutron therapy as for the controls, because two radiobiological phenomena related to the overall treatment time, reoxygenation and repopulation, will probably influence equally the responses of tumors and normal tissues to neutrons and γ-rays.

The number of fractions will probably be less than 5 per week because cyclotron time is valuable and there is no conclusive proof that 5 fractions per week has any advantage over 3. Conversely, the smaller the dose per fraction, (i.e. the larger the number of dose fractions) the more likely it is that reoxygenation will "sensitize" a sufficient proportion of the tumor cells to ensure that hypoxic cells do not influence the response. The use of large doses per fraction in which most of the oxic cells are eliminated by the initial part of the dose leaving only hypoxic survivors during the latter part of each exposure, although less of a disadvantage to neutron than to γ-ray therapy, is nevertheless still a disadvantage to neutrons because the oxygen enhancement ratio is greater than one. At present there is no firm radiobiological basis for choosing 2, 3, 4, or 5 fractions per week, but, since clinicians generally favor a larger number of fractions and since this preference is based upon decades of observation, it may be better to use three rather than two fractions per week.

The RBE (neutrons/X-rays) at low doses is large and changes rapidly and may change differently in different tissues. However, this is probably because of diversity of the responses of tissues to X-rays, at least as much as to diverse neutron responses. There is no reason to assume that the responses to small fractional doses of neutrons will be any more varied than are the responses to small doses of X-rays.

The total dose given as 2 or 3 fractions per week over 6 or 7 weeks would have to be established by trial and error in palliative treatment of patients during a pretrial pilot study. It should be the dose that produces the same degree of normal tissue injury as is obtained in conventional therapy. This pretrial pilot study should last at least two years in order that the late responses to neutrons can be assessed. It would also be necessary to establish that post-irradiation surgery was equally feasible in X-ray and neutron-irradiated tissues.

As this pilot study progressed, patients of the type that were to be included in the subsequent randomized trial would be treated. The work already in progress at Hammersmith should make other pilot studies easier.

Although one aim of the pilot study would be to determine the maximum tolerable dose of Texas A. & M. cyclotron neutrons that could be given as a certain fractionated regime, another purpose would be to develop the necessary technical skills for treating patients. The importance of the pilot study lies in the fact that it is necessary to develop, in as short a time as possible, neutron treatment techniques and schedules that are as sophisticated as those used in conventional radiotherapy.

Other radiobiological problems need not be discussed here, except to mention that in treatment of head and neck cancer in human beings it may be necessary to be concerned with activation of gold and mercury and other heavy metals embedded in patients' teeth.

REFERENCES

1. H. R. Withers, J. T. Brennan and M. M. Elkind, The response of stem cells of intestinal mucosa to irradiation with 14 MeV neutrons. *Brit. J. Radiol* **43,** 796 (1970).
2. W. G. Cochran and G. M. Cox, In *Experimental Designs*, p. 24. John Wiley & Sons, New York (1957).

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Possibilities for the Application of Fast Neutrons in Manchester

W. DUNCAN

Christie Hospital and Holt Radium Institute, Manchester, England

Abstract — *The present experimental* 14 *MeV neutron generator facility at the Christie Hospital, Manchester, is described. A fully engineered neutron therapy machine, 'Hiletron', is soon to be installed and the main features of its design are reported. Experiments have been started to determine the RBE for effects on skin of selected patients and this will be followed by a study of the effects of neutrons on other normal tissues. Formal clinical trials should be started in* 1971 *and the choice and stage of tumours to be studied are detailed.*

BECAUSE the possibilities for the application of fast neutrons are primarily determined by the fast neutron facilities available, firstly a brief description will be given of the present installation at Manchester and the fully engineered therapy machine which we are to receive at the end of 1970.

The present experimental facility has been described in detail by Greene and Thomas [1]. The sealed tube neutron generator [2] in its insulated container is provided on loan by S.E.R.L.* The generator is shielded in a stack of steel, polythene and lead from which a fixed horizontal beam is collimated by a series of interchangeable applicators. When patients are being treated the apparatus is concealed behind a curtain wall from which the applicator protudes for about 5 cm. The neutron dose rate on the skin is at present 3 rads/min and treatment times have to be very long. This arrangement thus allows us to begin clinical investigations on skin reactions. Although this set-up limits the possible clinical applications it is expected that we will be able to complete our skin studies with this experimental unit.

At the end of 1970 the Elliott Automation neutron therapy machine, known as the Hiletron, is to be installed at the Christie Hospital and Holt Radium Institute in Manchester. The pear-shaped head is constructed by combinations of steel, polythene and lead and reduces the radiation around the treatment head to 1% of the dose rate in the main beam. The beam will be collimated by a series of interchangeable applicators handled by a mechanical trolley. The limits of each field may be illuminated and light sources indicate the correct source to skin distance which we have chosen to be 80 cm. Field sizes from 5×5 cm to a 30 cm circle are available. The treatment head is mounted iso-centrically, mechanized for rotation therapy and can be angled to 110° from vertical on each side. The couch has the usual freedom of movement and the vertical movement is motorized. Special features in design have been used to minimize the induced radio-activity in the couch assembly. The S.E.R.L. tube producing 14 MeV neutrons by the $^{3}H(d,n)^{4}He$ reaction should given an output of about 10^{12} neutrons/sec and a dose rate of 6 rads/min. There is good reason to expect that outputs of 3×10^{12} neutrons/sec will be attained [3]. The power supply is mounted on the same annulus counterbalancing the treatment head. The 250 kV cable pierces a dividing partition which

*Services Electronics Research Laboratories, Baldock, Herts.

separates the treatment area from the service area.

The machine is to be operated by radiographers and is controlled by a simple on-off button. The required dose will be preset and a time switch on the control desk will also cut off the radiation. Double dosimetry is provided. We expect that early in 1971 we will be able to start clinical trials with patients who have locally advanced tumours which may possibly respond better to fast neutrons than to X-rays [4, 5].

In the meantime our aim is to determine normal tissue tolerance, firstly for skin and then for the mucosa of mouth and pharynx. The dose of fast neutrons must be found which produces the same severity of reaction as radiotherapists are accustomed to accept with X- or γ-rays. This seems a simple enough requirement which is considered of primary importance since, in certain trials using hyperbaric oxygen, more severe reactions were experienced as a result of treatment in oxygen than in air. In order to avoid this, normal tissue tolerance has to be determined initially as a distinct and separate aim without consideration of the tumour response.

For assessment of skin reactions a four field technique is used to treat the pelvis in patients with advanced cancer of the bladder or uterine cervix. Using telecobalt radiation two anterior and posterior fields are treated together with the left lateral field which has build up of dose on the skin. The right lateral field is treated with fast neutrons with build-up through polythene. The exit doses complicate the situation but for the neutron beam this amounts to about 3% and for the γ-ray beam effectively to about 10%. The technique does, however, allow the comparison of reactions of symmetrical sites treated simultaneously in the same patient. The site is attractive too as it is normally protected from sunlight, is relatively insensitive to the discomfort of reactions and, if poor cosmetic results follow high doses, this will not be an embarrassment. There is usually a good layer of subcutaneous fat in this region, so that subdermal atrophy may be observed. The technique implies that only 20% of the tumour dose is delivered by the neutrons and the remainder by conventional irradiation. This is advantageous although no assessment of tumour response is being made. The reactions are to be scored by independent radiotherapists using an arbitrary scale as suggested by the Hammersmith M.R.C. cyclotron group.

When the skin studies are completed, a minor modification of the collimator will allow treatment of the head and neck region to find RBE values for reactions in the mouth, pharynx and larynx. Again the RBE will be measured in relation to telecobalt γ-rays which would seem to be the best reference radiation in this investigation. The definition of beam quality is easy and telecobalt therapy units are generally available throughout the world. The radiation quality of ^{60}Co γ-rays is in the region of low LET, where large variations in γ-ray energy have little influence on RBE. Absorption of this γ-radiation in body tissues is nearly uniform and so removes the complications of variability of absorbed doses in different tissues.

Finally the skin sparing effect due to build-up dose in the superficial 4 mm of tissue below the skin surface can be compared with the skin sparing properties of 14 MeV fast neutrons with build-up in proton dose occurring at 2 mm depth [1].

The clinical evaluation of fast neutrons must be designed to answer three questions:

1. Do fast neutrons increase the therapeutic ratio — i.e. are more tumours cured for the same morbidity as accepted now with X- or γ-ray treatments?
2. Do patients with large incurable tumours obtain better palliation, both qualitatively and quantitatively, with fast neutrons as compared with X- or γ-rays?
3. May certain tumours, at present regarded as unresponsive to radiotherapy, be more successfully treated with fast neutrons?

The first question is obviously the most important and requires assessment by carefully planned clinical trials. We wish to select for inclusion in this trial tumours in which we think the response to irradiation is limited by a proportion of hypoxic cells [4], and we would include in this group most squamous cell carcinomas. We are fortunate in Manchester in having sufficient numbers of new registrations each year to undertake trials using randomly selected groups of patients with the more common types of tumour. Table 1 gives a list of tumours considered suitable for such a trial, with 5-year survival figures presently obtained with X- or γ-ray therapy and the number of patients suitable for inclusion in the trial each year. The locally advanced cancers of the head and neck region are the most attractive group for assessment as they are accessible, making evaluation of local control of the tumour simple and the recognition of high dose effects easy. In addition, it is possible to obtain meaningful results in terms of 2- or 3-year survival figures within a relatively short

Table 1. *Tumours suitable for clinical trials of fast neutron radiotherapy*

Tumour site Squamous cell carcinoma	Stage (All M_0)	5-yr survival (%)	Annual number
Larynx and hypo-pharynx	T_{3-4} N_{0-3}	30	50
Mouth	T_{3-4} N_{0-3}	25	35
Oro-pharynx	T_{2-3} N_{0-3}	30	30
Bladder	T_3	25	40
Cervix	IV	10	50
Cerebral gliomata	—	20	30

Table 2. *Tumours which may be more responsive to neutrons than to X- or γ-rays*

Metastatic melanoma
Soft tissue sarcomata
Teratoma testis
Post-cricoid carcinoma

time. The patients with advanced cancer of the bladder and uterine cervix will be assessed simply on survival data.

Patients with cerebral tumours have been included and again survival rates can be the only measure of the value of the treatment. Many of these tumours do show histological features of necrosis which may be due to hypoxia and survival rates at present are very poor indeed. It is accepted that survival figures are a relatively poor and perhaps confusing indication of the real merit or possible dangers of a treatment and this is particularly true in the treatment of cerebral tumours — where reduced survival may be due to overdosage and radionecrosis of nervous tissue rather than to poorer control of the tumour. It is hoped to treat about 100 patients in the first year after accepting the Hiletron and perhaps half as many again in the second year. It is also intended to look at the response of a miscellaneous group of tumours listed in Table 2, to determine if these so-called radioresistant tumours respond to neutrons than to X- or γ-rays.

A critical factor in the design of clinical trials is the unsolved problem of optimum fractionation. The importance of producing identical levels of reactions with neutrons and X- or γ-rays has been stressed, but this does not imply that the fractionation schedule has to be the same. It seems unlikely that the best fractionation schedule for fast neutrons will be the same as for low LET radiations.

Neutron therapy has to be compared with what appears at present to be the best treatment programme for X- or γ-rays. Centres should not change their orthodox treatment programme for X- or γ-rays as it seems unwise to change a well-established scheme of fractionation to another which might seem more suitable for fast neutrons. It may be advisable to give fewer and larger fractions of neutrons than X-rays [6, 7], and it may be that in the final analysis one has to compare the effects of only 2 or 3 fractions of neutrons with perhaps 25 daily fractions of X- or γ-rays. The number of variables should be as few as possible and the techniques of treatment should be matched as closely as possible so that the size and shape of the high dose volume and the distribution of dose are comparable in the two treatment groups.

We are now in a position where for the first time fundamental research [8] has provided good reason for developing a new technique in radiotherapy and it seems timely to evaluate the possible advantages of fast neutron therapy by clinical trials.

REFERENCES

1. D. Greene and R. L. Thomas, An experimental unit for fast neutron radiotherapy. *Brit. J. Radiol.* **41,** 455 (1968).
2. M. F. Paris, D. W. Downton and C. W. Glanford, An experimental fast neutron radiotherapy system. *J.R.N.S.S.* **23,** 232 (1968).
3. M. Hillier, P. D. Lomer, D. S. Stark and J. D. L. H. Wood, Performance of targets in sealed-off neutron tubes. *Proc.* 3rd *Conf. Accelerator Targets designed for the Production of Neutrons*, p. 125. Euratom, Brussels, EUR 3895, d-f-e (1967).
4. T. Alper and J. L. Moore, The interdependence of oxygen enhancement ratios for 250 kVp X-rays and fast neutrons. *Brit. J. Radiol.* **40,** 843 (1967).
5. H. S. Kaplan, Criteria for selection of types of tumors for radiotherapy with unconventional radiation. *Europ. J. Cancer* **7,** 195 (1971).
6. S. B. Field, T. Jones and R. H. Thomlinson, The relative effects of fast neutrons and X-rays on tumour and normal tissue in the rat. Part II. Fractionation: recovery and reoxygenation. *Brit. J. Radiol.* **31,** 597 (1968).

7. J. F. Fowler, Fast neutron therapy — Physical and biological considerations. In *Modern Trends in Radiotherapy* (Edited by T. J. Deeley and C. A. P. Wood), p. 145. Butterworths, London (1967).
8. L. H. Gray, A. D. Conger, M. Ebert, S. Hornsey and O. C. A. Scott, The concentration of oxygen dissolved in tissues at the time of irradiation as a factor in radiotherapy. *Brit. J. Radiol.* **26,** 638 (1953).

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Summary of Discussions in the Session on "Present Status and Future Developments of Fast Neutron Radiotherapy"

H. S. REINHOLD

Radiobiological Institute TNO, 151 *Lange Kleiweg, Rijswijk Z.H., The Netherlands*

IN THE discussion, following this session, comments were made about the absorption of neutrons by various tissues, the assessment of the dose given in clinical neutron radiotherapy, problems concerned with the collimation of the fast neutron beam and questions of how to perform clinical trials.

ABSORPTION OF NEUTRONS

After Brennan's paper concerning Stone's results, Field suggested that the severe late reactions of the buttocks after the prostate treatment might be accounted for by the high neutron absorption in fatty tissues, which may be as much as 20% higher than in other tissues. Brennan thought that this might indeed be the case, but also pointed out that the question which really needs an answer is: "How thick must a fat layer be to cause trouble?" Brennan suggested that in a layer of only a few mm thick it is possible that the blood vessels become obliterated, but are replaced by new vessels growing in from both sides. Similar ideas were developed by Kaplan later, when the discussion dealt with the radiosensitivity of the brain. Kaplan then pointed out that the brain contains a high concentration of sphingomyelins and other compound lipids and that therefore the absorption of neutrons might be greater in the central nervous system (C.N.S.).

It is therefore possible that with fast neutrons damage to structures with a high hydrogen content will differ from the type of damage caused by the conventional X-rays.

ASSESSMENT OF DOSE

It has always been a difficult matter how to evaluate the actual dosages, given by Stone. Rossi once determined the *n*-values for a number of Victoreen chambers and found them quite variable. They ranged from 1·7 to 2·3 rads/*n*-unit. Rossi suggested to re-calibrate the chambers if they were still around. Field then commented that at Hammersmith a conversion factor of 2·4 was derived between the *n*-values as measured by Aebersold and the rads as measured at Hammersmith. In both cases this was done *via* a biological dosimeter, namely the threshold erythema reaction.

Dr. Catterall also used in the Hammersmith patients the iso-skin reaction and concluded that 1440 rads of neutrons was clinically equivalent to 4100 rads of X-rays. This was in reply to a question of Herring of how she was able to compare the effect of X-rays with that of neutrons, while the tumour responses (i.e. the regression of chestwall nodules) were not the same.

The question of how to derive RBE values from clinical applications was also elaborated by Fowler after Duncan's presentation. For the assessment of the RBE for effects on skin, for neutrons, relative to ^{60}Co γ-rays it is important not only to employ full build-up in both cases,

but also to keep the dose rate the same. This becomes especially important when changes are made later from a small to a big machine involving a change of the dose rate from e.g. 3 rads/min to 6 rads/min. At present, according to Hall, it is not possible to foresee what implications this will have for the RBE.

BEAM COLLIMATION

One of the difficulties in performing clinical trials with fast neutrons, as compared with the present mega-voltage X-ray apparatus, is that for the latter extremely sophisticated beam collimating devices are in operation. This, together with the small focal area on the anode, ensures that tissues outside the treatment field receive a negligible dose of radiation. This is certainly not the case with neutrons, and so Herring asked Withers how they intend to cope with this problem. Withers and Almond explained that no modifications in the control ^{60}Co γ-ray treatment will be made and that the 125 cm target to skin distance in their cyclotron design will allow e.g. blocking the corners of the fields. With regard to the total body dose a patient will receive from neutron beam therapy, Rodriguez-Antunez asked Kaplan what he expected on the basis of his experience with the mantle technique. Kaplan then pointed out that the situations are hardly comparable; with the mega-voltage X-rays used for the mantle technique the patient receives something like a half percent total body dose, or about 30–35 rads. In the neutron radiotherapy, as discussed, the treatment field is much smaller and the total body component much higher. Treatments like the mantle fields for lymphomas will therefore also be unfeasible.

CLINICAL TRIALS

With regard to the tumours which may be selected for clinical trials, Kuipers was not very happy that Duncan had included in his choice the group of stage IV carcinoma of the cervix. Kuipers argued that (a) most patients will have para-aortic node involvement, and also distant metastases; (b) many of the patients will have bilateral parametrial involvement, so that, treating one side with neutrons and the other side conventionally, one will encounter serious difficulties in evaluating the effects of any treatment, because local control on one side would be obscured by a failure on the opposing side; (c) the low survival rate (10%) is unfavourable for statistical analysis. Duncan replied that this initial application was primarily aimed at evaluating skin responses. In addition he noted that some of the extensive pelvic tumours can be cured with conventional radiation and that he expects that as few as 50 cases should give sufficient information for statistical analysis.

With regard to Stone's treatment of carcinoma of the prostate as reported by Brennan, Kaplan made clear that if this disease is treated with rotational techniques, using 6–8 MeV X-rays [1], the results are excellent. A long-term disease free survival of 55–60% can be obtained, with a local complication rate of only about 5%. Thus, any modern clinical trial utilizing neutrons must be prepared to compete with this kind of result.

In designing clinical trials it will be very difficult to subject the control patients to exactly similar treatment as the test group. The problems regarding beam collimation and total body dose have already been mentioned, but in addition Greene wondered whether or not one should shuttle control patients over the same distance for their treatment outside the hospital (in Texas 90 miles) as one does the test group! This approach may seem somewhat immoderate, but it stresses the point that one should take extreme precautions to assure that the *only* difference between the control and the treatment groups is the type of radiation used.

Kaplan then raised the important question of what will be the rules for stopping a trial and what are the estimated significance levels for the (Manchester) clinical trials? Duncan felt one should obtain data by extrapolating the dose response curves. If, for a given therapeutic gain factor of e.g. 1·2, one expects an improvement of 15%, then the trial should be designed to pick up this figure. Kaplan thought this approach was a dangerous one; it has been shown by statisticians that a spurious outcome can result if a trial is continued until the desired effect is encountered. One should therefore decide in advance when to stop the trial. He also warned that we tend to demand a higher level of significance of trials in general when we do not wish to believe their results rather than for trials where a bias is in our favour. Anyhow, one has to know how many patients it will take for some final chance of revealing e.g. a 10% difference and be prepared to close a trial at that point. However, as was emphasized by Duncan, there is at present insufficient information available about the magnitude of therapeutic improvement that one may expect. Morrison then suggested the possibility of using the already available Manchester data of conventional therapy for the control part of the trial, assuming that the figures should not change appreciably from

year to year. Morrison, on the other hand, fully realized that statisticians would like control groups to be treated at the same time. Duncan indeed preferred to perform a trial with matching groups, and discussed with Withers the numbers of patients required. For an improvement of 70%–80%, 290 patients are needed on each side, a very large number for any hospital. Obviously, only co-operative interdepartmental trials can provide such a number of patients.

GAIN FACTOR

The gain factor for neutron radiobiology has been defined as the ratio of the OER values for X-rays relative to the one for neutrons [2]. Radiotherapists however, as Field explained, relate "gain" with clinical improvement; i.e. a higher proportion of patients being cured because of an increased probability of the local sterilization of tumours. In order to avoid confusion therefore the expression "oxygen effect gain factor" (OEG) was introduced by Barendsen [3].

The *therapeutic* gain factor must then be regarded as the ratio of the RBE for local tumour control relative to the RBE for normal tissue tolerance, both for a given treatment schedule. It was pointed out by Withers that, when plotted against dose, both the tumour cure probability as well as the probability of exceeding the normal tissue tolerance rise quickly. If, by using neutrons, the two could be separated further, then better cure rates will be obtained for any rate of complications.

Radiotherapists have an amazing confidence in the radiobiologist. Van Putten therefore warned the therapists never really to believe the radiobiologists. Radiobiologists may be able to explain certain clinical phenomena, but they are not in a position to predict what will happen in a certain clinical situation. The empiricism in radiotherapy, based on observation and evaluation of results is still the only way of improving treatment regimens.

REFERENCES

1. M. A. Bagshaw, H. S. Kaplan and R. H. Sagerman, Linear accelerator supervoltage radiotherapy. *Radiology* **85,** 121 (1965).
2. T. Alper, Comparison between the oxygen enhancement ratios for neutrons and X rays, as observed with *Escherichia coli* B. *Brit. J. Radiol.* **36,** 97 (1963).
3. G. W. Barendsen, Cellular responses determining the effectiveness of fast neutrons relative to X-rays for effects on experimental tumours. *Europ. J. Cancer* **7,** 451 (1971).

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Summary of General Discussion on Technical Aspects of Fast Neutron Therapy Facilities

J. T. BRENNAN
Department of Radiology, University of Pennsylvania, Phila., Penna., U.S.A.

1. *The principle of comparability*

COMPARISON of the results of modern 6–8 MV supervoltage X-ray therapy with results from the period in which only orthovoltage X-rays-(200–300 kV) were available shows that better machine performance produces better results. It is generally agreed, moreover, that this improvement is not due to any fundamental difference between the biological effects of 6–8 MV X-rays and of orthovoltage X-rays, but rather to the mechanical and geometrical advantages, e.g. with respect to depth dose provided by the greater flexibility of super voltage equipment. This suggests strongly that a clinical trial comparing fast neutrons and 6–8 MV X-rays, in which the properties of the neutron beam, with respect to depth dose, beam sharpness etc., are not better than those of 250 kVp X-rays, is an unfair test of neutrons. If, under these conditions, neutrons prove to be superior to high energy X-rays, then the issue is settled. However, in case the opposite result is obtained, the trial will be inconclusive.

In a similar way it is essential that all of the techniques used on the fast neutron part of a trial are equivalent in overall effectiveness to the best techniques employed with high energy X-rays. This principle of comparability applies not only to depth dose but also to beam localization, patient stabilization, dosimetry systems, data-processing systems and treatment planning systems. At present, compromises on neutron techniques shouid be made reluctantly if at all.

2. *Maximum treatment times*

For all patient positions, except standing, the consensus of opinion was that a treatment time of 10 minutes is permissible. It is recognized that a few patients will be so debilitated that they can only be treated in a horizontal body position. However, such patients are not numerous and in most cases they have a life expectancy so short as to make it advisable to exclude them from a trial if this can be done by a legitimate procedure. The maximum treatment time to be considered for any body position is thirty minutes, but opinion was divided on this point. Some experienced therapists felt that any treatment time that exceeds 10 minutes will introduce psychological and physiological stresses that could invalidate a controlled trial. A majority considered a 20-minute maximum as acceptable.

3. *Maximum dose per fraction*

If neutrons are used only for the first and last increments of dose, single fractions as high as 300 rads may be permissible. In case all fractions are given with neutrons, the maximum permissible dose per fraction is difficult to estimate. Estimating *optimum* fraction size is, of course, a major unsolved problem in all of radiotherapy and will be discussed elsewhere.

A dose as high as 200 rads of fast neutrons per fraction would, judging from current Hammersmith experience, reduce the total number of fractions to about six. Also, based on van den Brenk's experience with large fractions in hyperbaric radiotherapy, it seems likely that considerable experience with fractions of less than 200 rads will have to be gained before this value can be exceeded. This

could change if, for any reason, total treatment times in excess of the four to seven week range were used.

4. *Depth dose*

With a field size of 5×5 cm and an SSD of 80 cm, the 50% depth dose for a ^{60}Co γ-ray beam is attained at 10 cm. SSDs larger than 80 cm are not used with ^{60}Co sources due to limitations of the specific activity of the cobalt, imposed by the heat dissipation problems encountered at higher specific activities. The consensus was that a depth dose inferior to that of a ^{60}Co γ-ray beam would be an unfair handicap in the fast neutron part of a clinical trial. The first applications by the group in Manchester might proceed with a 50% depth dose at only 8 cm [1]. Duncan mentioned that the number of patients treated in their radiotherapy department, 4000 new malignancies per year, will permit selection of cases in which a 50% depth dose at 8 cm may be used. The majority opinion, however, was against accepting anything less than the 10 cm value for the depth at which the dose is reduced to 50% and some experienced therapists held that even 10 cm is inadequate in view of the depth dose distributions achieved with 6–8 MeV photon therapy.

5. *Beam collimation—beam sharpness*

Data presented by Greene and Major [2] with respect to 14 MeV neutrons show that for an SSD of 75 cm and a 1% leakage through the shield portion of the treatment head, the hazard to tissues outside the treatment volume is a serious problem. Since with this shield a gap of 25 cm of air is present between the patient and the collimator surface, it is presumably possible to interpose more shielding and reduce this component of the whole body dose to an acceptable level. Secondary radiations originating in tissues within the 'useful beam' present a more difficult problem. This hazard varies, of course, with field size and, in addition, the ratio of the neutron dose to gamma dose changes rapidly with distance from the central axis of the beam. This effect is important because of the large RBEs associated with small increments of neutron dose. It is essential that this point be evaluated for each neutron beam prior to any general use on patients. LET spectrum measurements are helpful in this respect and, when the dose rates are high enough, biological determination of RBE is indicated.

Beam sharpness, beam flatness and depth dose, all improve with increasing SSD and, 14 MeV neutrons become competitive with ^{60}Co on these points only if the SSD is 125 cm or greater. In the case of a cyclotron employing the D-Be reaction and an SSD of 125 cm the depth dose equals that of ^{60}Co only if the deuteron energy is 30 MeV or greater. Recent measurements indicate that the use of a heavy water target may reduce this requirement to 25 MeV or less.

6. *Dosimetry and treatment planning requirements in a clinical trial of fast neutron teletherapy*

Neutron dosimetry is inherently more complicated than photon dosimetry and, with respect to man-hour and financial requirements, perhaps ten times more expensive. In the case of photons, only one kind of secondary particle is produced in tissue. In the case of neutrons absorbed in tissue, secondary alpha- beta-, gamma- and neutron radiations contribute to the dose. Special difficulties are caused by the fact that neutron spectra and neutron dose to gamma dose ratios change with depth in tissue in such a manner that both must be measured routinely.

With fast neutrons, absorbed dose in fat is 20% higher than absorbed dose in muscle and neutron dose to bone is significantly less than the dose absorbed in muscle. In addition, special care must be taken in irradiating tissues, such as cartilage, which may be naturally hypoxic.

These difficulties vastly increase the number and complexity of the dosimetry measurements required in fast neutron therapy.

6.1. *Required measurements and instrumentation*

Certain dose parameters are sufficiently important to require routine consideration in each individual patient treatment plan. The most useful single instrument is the proportional counter which can measure neutron dose separately, gamma dose separately and total dose. But, in addition, tissue equivalent ionization chambers must be included to determine the total dose, whereas thermoluminescent dosimeters or paired ionization chambers have to be employed for the determination of the gamma dose separately. Since measurement of these parameters must be made at numerous positions within and outside the treatment volume, the quantity of data generated requires automatic data processing.

6.2. *Auxiliary dosimetry methods*

Although not required in the routine treatment planning for each individual patient, some capability for measuring neutron spectra is necessary. Threshold detectors, ^{3}He spec-

trometers and emulsion methods are available. At least one of these systems should be used.

Since a Rossi proportional counter is required for other purposes it is natural to use it also for the measurement of LET spectra. Such measurements can provide an insight with regard to: (a) the RBE which should be assigned to radiation doses outside the treatment volume, (b) the quality factor to be used for evaluating hazards to operating personnel and others in the radiation area, and (c) the interpretation of radiobiological experiments.

Both neutron and LET spectrum measurements require automatic data processing and LET spectra are best determined if an on-line analysis can be employed.

6.3. *Treatment planning*

The photon part of a clinical trial must employ the best possible current techniques. This necessarily includes a computerized treatment planning system, which can take into account tissue inhomogeneities, wedge filters, bolus, etc. In principle, the same techniques can be applied to the neutron and gamma components of fast neutron dose. But, regardless of the particular methods used, the treatment-planning system employed in the neutron part of the trial must be comparable in overall effectiveness to the best methods now in use in photon therapy.

6.4. *Financial aspects*

Assuming that an organization already has in operation an advanced treatment-planning system for photons and if charges for computer time are excluded, some estimates of the financial requirements may be made. During the initial, or "setting-up" phase, which should last about ten years, capital expenditures for equipment will range from $40,000 to $80,000 and about 4 man-years will be required. Thereafter the availability of one man, employed full-time in patient dosimetry, may be sufficient.

Treatment planning costs are difficult to estimate because they depend upon what kind of photon treatment planning methods are available.

6.5. *Time schedule of applications of fast neutrons*

The dosimetry and treatment-planning systems to be used should be well along in their development before any patients are irradiated. Of particular importance is the necessity of cross-calibration with other clinics using fast neutrons.

7. *Skin-sparing*

Recent tests on patients at Hammersmith [3] have demonstrated conclusively that fast neutron beams have a definite skin-sparing effect. Physical measurements on neutron beams with different energies have shown that secondary particle equilibrium is attained at depths varying from 2 mm to 8 mm, depending on neutron energy. It is highly probable that all of the fast neutron beams under consideration for clinical applications have significant skin-sparing properties. However, each new beam should be studied independently, because neutrons having energies in excess of 10 MeV produce very high LET alpha-particles and recoil nuclei in tissue. Equilibrium for these particles is reached in skin at depths of 0·1 mm or less. Consequently, for these components of neutron dose there can be no skin-sparing.

8. *Use of a fixed horizontal beam*

In the case of neutrons produced with a cyclotron it is difficult to obtain anything but a fixed horizontal beam. Such a beam is well suited to cases that can be treated in the sitting position. However, some problems arise in treating the pelvis and lower abdomen. Two cases have been treated recently at Hammersmith with the patient in the kneeling position and good results are reported. The standing position is considered as unacceptable by most therapists. Radiotherapists differ considerably in their choice of patient body position during treatment and an experienced therapist who has used one system for many years may be reluctant or unwilling to use a neutron beam that requires a different positioning system.

9. *Radiological safety requirements for a fast neutron teletherapy facility*

The physical properties that make neutron dosimetry more complicated than photon dosimetry, have a similar influence on the assessment of neutron radiation hazards. Investigators who use ^{252}Cf have found that commercial "neutron dosimetry" film badge services may provide much too low values for neutron dose. In addition, the commonly used TLDs are so energy dependent that they must be backed up by T.E. ion chamber measurements. Only one satisfactory T.E. ion chamber, designed as an "on-body" personnel dosimeter, is available in the U.S.A. The instrument problems in fast neutron hazard control are formidable.

Further difficulties may arise from the activation of the patient and patient excreta

and of materials in the treatment room. Solutions to these problems require skills that are not part of a conventional photon therapy capability. Finally, and most importantly, neutron hazard control is not a simple problem of measurement only, because of the very high, but as yet not well determined RBEs, which might be associated with small increments of neutron dose in relation to late effects. While this consideration does not preclude the use of neutrons in clinics and elsewhere, it does mean that with respect to the late biological effects we will be operating without a large safety factor that is part of photon hazard control systems. Because of this, a national and, if possible, an international register of those occupationally exposed to neutrons in therapy clinics should be maintained on a permanent basis.

In the case of D–T neutron generators tritium radiation hazards can pose a prohibitively difficult problem. Sealed tube D–T generators have only a minimal problem in this respect but some of the generators with evacuated acceleration tubes now under design, require as much as 3000 c of tritium. Regulations differ, of course, in different countries. If the governing agency rules that the maximum credible accident is the instantaneous release of all of the tritium in the system, and if the installation is in a populated area, one may have to install a high capacity air handling system which will be activated automatically when tritium escapes. In addition release in the air through a tall smoke stack (~100 feet) may be required. Such air movement systems are possible but expensive.

In some countries (the U.S.A. for example) possession of 3000 c of tritium requires a special licence which would be granted only after the responsible body has satisfied itself, by an on-site inspection, that the radiation hazard control practices will be sound. Furthermore since tritium radiation hazards are additive with other radiation hazards (neutrons, gamma-rays) the local capability for dealing with all radiation hazards is reviewed and evaluated prior to granting a licence.

REFERENCES

1. W. Duncan, Possibilities for the application of fast neutrons in Manchester. *Europ. J. Cancer* **7,** 237 (1971).
2. D. Greene and D. Major, Collimation of 14 MeV neutron beams. *Europ. J. Cancer* **7,** 121 (1971).
3. M. Catterall, Clinical experience with fast neutrons from the Medical Research Council's cyclotron at Hammersmith Hospital. *Europ. J. Cancer* **7,** 227 (1971).

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Summary of General Discussion on Radiobiological Aspects of Fast Neutrons in Radiotherapy

M. M. ELKIND

Biology Department, Brookhaven National Laboratory, Upton, New York 11973
U.S.A.

As THE discussions of the physical and clinical aspects of the potentialities of fast neutrons in radiotherapy illustrate, it is hard to confine radiobiological questions to biology alone. Similarly, as what follows will illustrate, various questions initiallly introduced in a biological context soon spill over into physical or clinical considerations. From this, one conclusion is already clear: the subject matter of *neutrons in radiotherapy involves a triad inextricably mixed.*

In spite of the difficulties in attempting to compartmentalize, for present purposes the various radiobiological discussions of the more formal parts of the Rijswijk meeting may be grouped under four headings. These are: (1) RBEs* and single-dose effects; (2) possible variations of RBEs and OERs with depth in a phantom; (3) properties of experimental tumors relative to fractionation, and (4) considerations of normal, cell renewal systems and their response to fractionation.

*Abbreviations used in this paper:
RBE = relative biological effectiveness;
OER = oxygen enhancement ratio;
LET = linear energy transfer (in general, LET refers to the total rate of energy loss);
D_0 = the dose required to produce an $1/e$ effect in the exponential portion of a dose-effect curve;
D_q = the quasi-threshold dose (the dose at which an extrapolation of the exponential portion of a dose-effect curve intersects the abscissa);
n = the extrapolation number (the ordinate relative to 1·0 intersected by an extrapolation to zero dose of the exponential portion of a dose-effect curve).

RBEs vs. single doses

A property of high LET radiation of principal importance in therapy is the reduction in the sensitizing effect that oxygen has. Relative to survival curves, in brief this is made evident by the fact that while the D_0 of oxic cells decreases with the increasing LET, on a relative basis the D_0 of hypoxic cells decreases more rapidly. In the limit at maximally effective LETs (e.g. 100–200 keV/μ), the OER = 1 because the hypoxic cell survival curve becomes steep enough to be essentially coincident wth the oxic curve.

Also relative to survival curves, a covariant of OERs which decrease with increasing LET is shoulder widths which decrease. A full discussion of interrelationships between OER, LET, RBE and D_q/D_0 (= in n, where D_q/D_0 is the shoulder width normalized to the D_0 value at a given LET) lies beyond present purposes. But the relevance of a decreasing *capacity* for sublethal damage with LET — i.e. decreasing D_q/D_0 — to a decreasing ability to *repair* sublethal damage makes essential a discussion of the variation of survival curve shape with LET.

There exists in the literature several fairly complete sets of data on the dependence of the shape of survival curves on LET. However, in the main these were obtained using "track segments" of charged particle beams. The situation is appreciably less complete relative to neutron irradiations even if beam specifications are limited to modal energies and

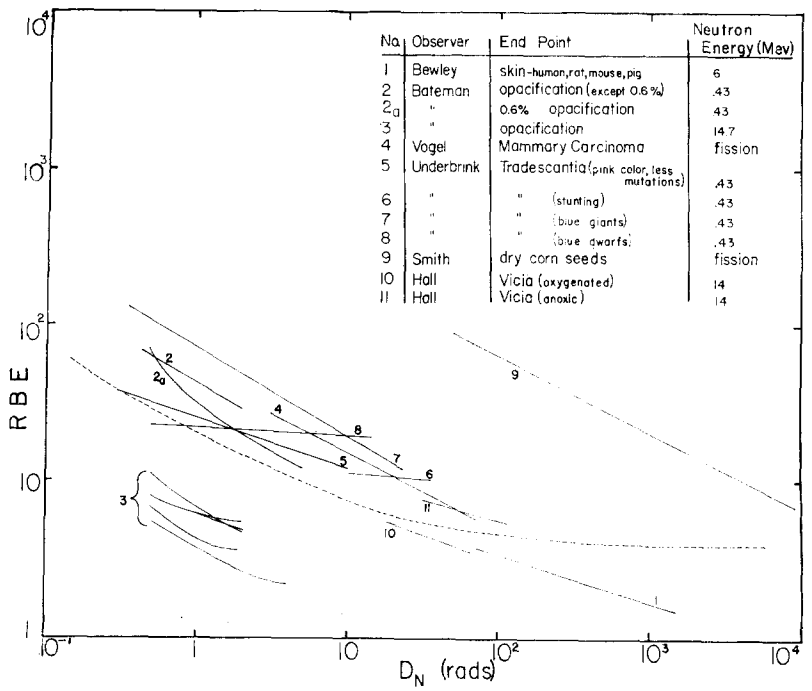


Fig. 1. Variation of RBE with neutron dose, D_n, for a number of neutron beams and endpoints. (From data presented by Rossi.)

average LETs. Some results obtained with neutron beams broad in energy spectrum will be discussed presently. At this point, I turn instead to an approach to questions of the shape of dose-effect curves which while indirect, potentially is quite powerful.

Rossi, with the collaboration of Kellerer, has been engaged in an analysis of the effects produced by different neutron beams relative to a number of different endpoints. Figure 1 shows a summary of the data he presented which, while dealing with effects in plant as well as animal systems, has already at this stage the interest of being broad relative to kinds of end points. Rossi and Kellerer have analyzed these data in the framework of microdosimetry (statistical considerations of energy deposition in microtargets) in an effort to see if constraints on target size and organization can be derived from plots of RBE *vs.* neutron dose, D_N (or X-ray dose, D_x). Without pursuing details, theory implies that curves which approach a slope of $-\frac{1}{2}$ at low doses (-1 for RBE *vs.* D_x)—as does the dashed curve result from end points effected by one or two neutron events, or two low LET events. At high doses, a constant RBE should be approached as both X-ray and neutron dose-effect curves tend toward exponential.

An important feature of these RBE plots as a function of dose is that they are based on the concept of an "equivalent biological effect" as proposed by the ICRU. This means that RBE is simply a ratio of an X-ray to a neutron dose when an equivalent effect is scored with a particular endpoint. Presumably, this avoids the need to have equivalence at all stages of the development of a lesion and consequently many difficulties from the point of view of measurement. While this notion is not always justified, it may be in sufficiently many instances to be used with advantage.

Thus, *biological dose equivalence* in the Rossi/Kellerer model may be used as a way of inferring the degree of "single-hit" character in an effect produced by neutron irradiation. In connection with therapy, the particular position of a given curve relative to the co-ordinates shown may or may not be of use. Still the degree to which the slope of a curve in the low dose region suggests single-hit inactivation and how this depends on the neutron beam used is quite relevant because of implications which follow. A single-hit dose-effect process is one not involving damage accumulation (i.e. $D_q/D_0 = 0$), and therefore, is one relative to which damage repair between dose fractions would not be expected. Furthermore, while the degree of relative variation of OER and D_q/D_{0q} with LET may not be the same for a particular endpoint, let alone different endpoints, we know that in connection with cell survival at least, OERs$\simeq 1$ at LETs which yield $D_q/D_{0s} \simeq 0$. Hence, a Rossi/Kellerer plot may also afford an insight in a given situation relative to the ability of oxygen to sensitize.

It seems clear, therefore, that more single-dose data analyzed in the way the end points in Fig. 1 have been thus far would be worth obtaining. We might wonder, to begin with, how tight the connection will be between neutron energy, low-dose region curve shape, OER, and 2-dose repair. We might wonder further, whether a given neutron source will continue to yield similar curves for endpoints conceivably even more diverse than those in Fig. 1. And finally, we might wonder if the low-dose slope of a curve ultimately can be used to predict, for a given neutron spectrum, values for OER, and D_q/D_0.

The foregoing points to a need for more single-dose data. More such data particularly at low doses is needed for another reason, however. As shown in Fig. 1, extrapolation to low doses of many of the curves suggests that RBEs increase without limit. In view of the fact that neutron beam collimation may not be as easily or effectively managed as for X- and γ-rays (e.g. see Greene's paper) it is important to know to what degree incidental irradiations of a patient with neutrons might be expected to produce significant effects.

RBE (and OER) dependence on depth

As already noted, reduced OERs are associated with high LETs. Broadly speaking, this propery of charged particles is shared by neutron beams. For the treatment of deep-seated lesions, however, fast neutrons have the critical advantage of penetrability. Even so, with depth the intensity of a fast neutron beam decreases appreciably. Because of this and the possibility that spectral changes may also be involved, the question has been considered: do the biological properties of a given beam change with depth? Information relative to this point was introduced by several people. Unfortunately, the data are conflicting and hence, from the outset it is apparent that more information is needed. In what follows, the principal observations are described starting with those of Evans *et al.* [1].

The group at the Sloan-Kettering Institute in New York City [1], following up earlier dosimetry measurements of the beam produced by a small cyclotron [2], used neutrons of modal energy 6 MeV, $^{9}Be(^{3}He,n)^{11}C$, for measurements of the survival of HeLa S3 cells with depth. Some of their data are reproduced in

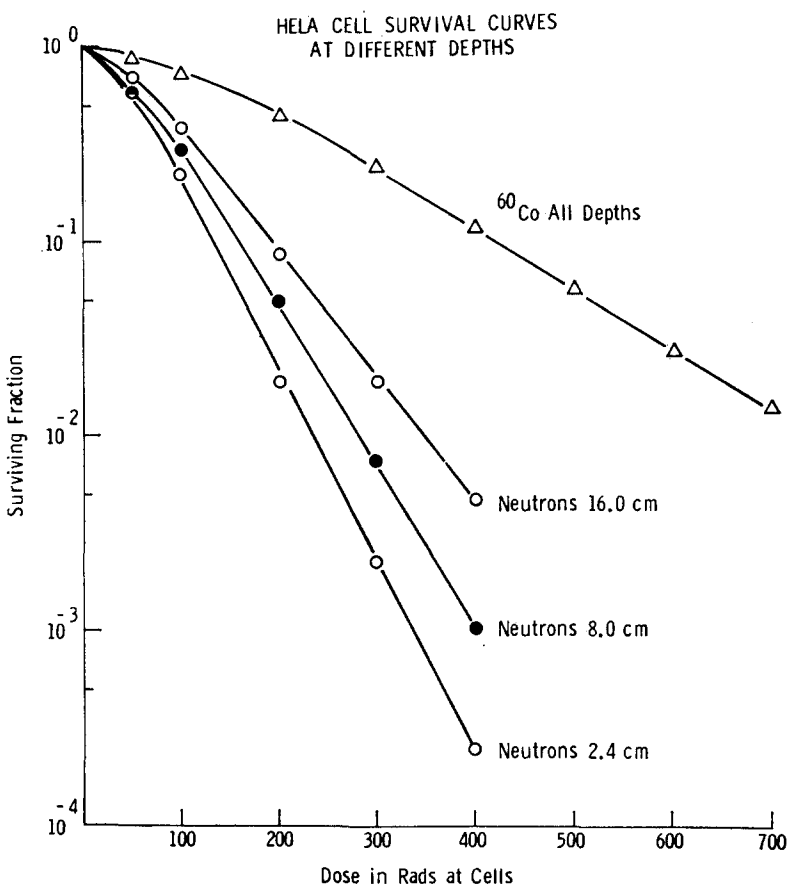


Fig. 2. Survival curves for HeLa S3 cells irradiated in tissue equivalent material after an overnight growth period. Neutrons were produced by $^{9}Be(^{3}He,n)^{11}C$ reaction and had a modal energy of 6 MeV. Dose rates: at 2·4 cm, 15 rads/min; at 8 cm, 8 rads/min, and at 16 cm, 4 rads/min. (From Evans et al. [1]).

Table 1. *RBE of neutron beam compared to* ^{60}Co *at different depths*

Depth in cm	RBE values at different surviving fractions				D_0, rads.
	0·80	0·50	0·37	0·20	
2·4	3·8	3·4	3·2	3·1	40
8·0	3·2	2·9	2·7	3·1	~49
16·0	2·4	2·3	2·2	2·6	65

Summary of results with HeLa S3 cells. (From Evans *et al.* [1]).

Fig. 2 and the variation of RBE with depth in a tissue equivalent phantom is shown in Table 1. These data were obtained with cells permitted to attach and grow for an overnight period before irradiation. When the resulting multiplicity was accounted for (see chapter 2 of Ref. 3), the neutron survival curves at the three depths shown became exponential (i.e. $D_q/D_0=0$) maintaining the same final slopes as in Fig. 2.

To insure the reduced dose rate with depth did not influence their data, the Sloan-Kettering group measured survival at 2·4 cm using the three dose rates corresponding to those used in Fig. 2. Dose rate variation was accomplished by varying the ^3He ion current of the cyclotron. No effect of dose rate over the range involved was noted. Finally, Evans and associates reported that although at 2·4 cm 11% of the total dose was due to γ-rays and at 8 cm this increased to 15%, at 16 cm the γ-ray contamination was down to 6%. Hence, an explanation of these data based simply on an increasing contribution to cell killing from low LET radiation is not readily apparent. (Although the data in Fig. 2 were for an uncollimated beam, Evans *et al.*, state that a 10× 10 cm field gave similar results.)

In qualitative agreement with the depth-dependent D^2 changes in Table 1 are Berry's data summarized in Table 2 [4]. These results were obtained with *P*-388 leukemia cells assayed *in vivo* and, in addition to the different neutron beams involved, OERs are also listed. Aside from the inconsistency of the D_0 changes for 14 MeV neutrons (Lawrence Radiation Laboratory *vs.* Aldermaston), the OERs show a trend to lower values with depth although, statistically, most of the OER values for a given beam probably do not significantly differ [4].

Field, referring to data obtained by McNally and Bewley with the neutrons generated by the Hammersmith cyclotron, noted that results with Ehrlich ascites cells do not support the trends observed by Evans *et al.* and by Berry. McNally and Bewley found no change in D_0 values and also no change in OER when cells irradiated at 8·7 cm in a phantom were compared to those irradiated at the surface. Brennan pointed out that while the neutron beam was the same as one of those used by Berry (Table 2), the manner of exposing the cells was different in the experiments of McNally and Bewley. To eliminate any influence from this source, Berry is planning to repeat his measurements with *P*-388 cells and the same physical set-up of the experiments with Ehrlich ascites cells. However, in support for depth-independencies of D_0 and OER, Hall noted that

Table 2. *Summary of results with P-388 leukemia cells (From Berry* [4])

Neutron source	D_0, rads.		OER	
	air	10 cm (tis. eq. phantom)	air	10 cm (tis. eq. phantom)
Fission (TRIGA, reactor)	91	96	1·1	1·2
Cyclotron (Hammersmith, 7 MeV)	77	99	1·6	1·3
Cyclotron (Texas A. & M.)	129	139	1·5	1·1
14 MeV (Lawrence Rad. Lab.)	87	115	1·8	1·3
14 MeV (Aldermaston)	76	73	1·8	1·5

with shoots of *Vicia faba* and 14 MeV neutrons (uncollimated) no effect of depth was observed.

Before leaving Table 2, an implication should be noted. To have decreasing OERs with increasing D_0s (oxic) implies that with depth the effective LET increased beyond the maximally effective value since only under such conditions would "wasted energy" events contribute to less effective killing independent of state of oxygenation. In view of the large shift in D_0s in Fig. 2 and Table 1, an extension of the results with HeLa cells to hypoxic irradiations would seem desirable.

The possibility that RBE and OER may depend on depth is of considerable importance. Of primary relevance, to begin with, is the complication introduced into treatment planning. In addition to a significant depth-dependence of dose, we would now have to account for a possible change in effectiveness as well. And if there is a large reduction of RBE with depth, we would have to be alerted to the possibility of unacceptable levels of damage to overlying normal tissue and to shifts in biological parameters with field size and collimation. Of perhaps secondary relevance in the present context but important nevertheless is the reasons for the RBE and/or OER changes. These imply changes in beam quality and possibly concomitant changes in curve shape and hence repair capacity. This latter factor is relevant not only because of fractionated dosage schedules but also because dose rates may be low enough to involve repair during irradiation. Further comments relative to these questions come later.

Properties of experimental tumors

In addition to various kinds of studies with cellular and acellular systems of possible relevance to therapy, the response of communities of cells or tumors is particularly important. Here, for experimental work, animal tumors are used and generally tumors which originated in or have been adapted to a particular strain of rat or inbred strain of mouse. While the number of tumor systems studied still remain few, some of these have received detailed examination. Among these are: a rat rhabdomyosarcoma used principally by investigators at Rijswijk by Reinhold *et al.*; (e.g. see Ref. 5, 6); a mouse mammary carcinoma used by Suit and collaborators (e.g. see [7]); and a rat fibrosarcoma used by Thomlinson and associates (e.g. see [8]). The work of Barendsen and Broerse, in the context of neutron therapy, is particularly interesting since not only have they developed their system to permit survival measurements of single cells (*in vitro*) as well as tumors, but they have also made comparative measurements with X-rays and neutrons.

In addition to studies with the aforementioned systems, some of which are reviewed in these proceedings, several more general properties of tumors were discussed. Denekamp drew attention to the question of tumor cell kinetics following single irradiations and during fractionation. Some observers have reported (e.g. Hermens and Barendsen, van Peperzeel) that following irradiation tumors grow more rapidly. Although in a given instance this could result from a reduction in cycle time, the point was developed that changes in the growth fraction, probably associated with a change in oxygenation, is probably responsible for the apparent increases in tumor growth. After an acute exposure, mainly oxic cells are lethally affected. Depending on the specific cell type involved plus, quite likely, conditions specific to tumor vasculature and tumor/host interactions, these oxic cells will continue to metabolize and to increase in size initially during a division delay period before undergoing one to a few divisions. When division ceases, some cells form giants while most of the others start to lyse. With cell breakdown, oxygen and others nutrilites become available for those cells initially hypoxic. This plus a relief in the movement of cells away from capillaries and toward zones of necrosis permits these hypoxic cells to start cycling or at least to cycle more rapidly. The net effect following irradiation is tumor shrinkage followed by regrowth which for a period might be at a rate more rapid than that just prior to irradiation. Insofar as initially hypoxic cells are concerned, their cycle time very likely may be decreased when they become oxic. Relative to the cycle time of cells initially oxic, however, there may not be a reduction in cycle time. In kinetic terms, a more appropriate explanation of the resulting enhanced, but usually temporary, radiation stimulation of tumor growth (volume doubling time) is that the growth fraction has increased and concomitantly there may be a transient decrease in the rate of cell death as well.

The alterations in tumor growth outlined above, point to a possibility that in some instances at least, reoxygenation may be sufficiently effective and/or rapid to have an important bearing on the course of fractionation even when conventional low LET sources are used. An example of this comes from the work of Howes [9] who showed that the total dose needed to sterilize mouse mammary

tumors reached a minimum when 2–3 days separated the two fractions used. An analysis of the principle involved in utilizing reoxygenation to improve low LET fractionation therapy is also available [10].

A second general tumor property to which attention was drawn by van Bekkum concerns their immunological relationships with their host. Accumulating evidence suggests that even in some human, presumably spontaneous, tumors a degree of antigenicity may exist. The likelihood of immunological involvements is large in animal tumors except where inbred strains and at least early isotransplants are used. The point to which van Bekkum drew attention is that if the antigenic status of the tumor is not known, then it may be at best confusing to study tumor kinetics grossly or on a cellular basis following irradiation. A strongly antigenic situation could involve, for example, interactions between host and tumor which depend upon tumor size and as a consequence could dominate the course of events in certain periods following irradiation.

In addition to cell kinetic and immunological questions, a last point relative to tumor properties needs to be borne in mind. Most animal tumor systems are rapidly growing — a a property which might reflect the relatively rapid rates of development and aging of rodents. Whatever the cause may be, however, cell cycle times are often found to be less than a day and tumor doubling times usually about one or a few days. While there are a few reports of tumors in humans involving comparable growth rates, and while it is true that the number of measurements of human tumors is not large, still the vast majority of the data show that human tumors grow considerably more slowly than tumors in rodents. Cell cycle times are frequently two or more days and tumor doubling times may be weeks or months.

In reference to fractionation studies with animal tumor systems as models for clinical strategies, one must question the adequacy of such studies at least when daily fractionation is used. When the fractionation interval (FI) is small compared to the cell cycle time (T), as is likely for many human tumors treated daily, we can expect a number of cell-based parameters (e.g. parameters related to repair, aging, division delay, and damage expression) to have an important bearing on the outcome of a fractionation sequence. But in the opposite situation, when FI is appreciably greater than T, some of these cell-based responses will have relatively less influence on the effectiveness of each fraction (e.g. because of the larger roles played by repopulation, and cell degeneration as it can affect the oxygen status of a tumor). Presently available, experimental, rodent tumor systems irradiated daily generally fall into this second class and hence might not be useful.

Cell renewal systems

As with any other new modality, neutron therapy has to be justified on the basis that with it an improved differential treatment can be managed relative to a more conventional modality. "Differential" in the context of radiotherapy means effectiveness in tumor tissue relative to normal tissue. Hence, to assess the likelihood of improved treatment it is necessary to understand and evaluate neutron effects in normal cells and tissues as well as in tumors.

Normal tissues involve a variety of elements and structures for many of which adequate quantitative assays are not yet available. In connection with cell renewal systems, however, assays for stem cell survival have been developed in several instances; namely for skin, bone marrow, and intestinal crypt cells. The responses of stem cells of the latter two systems are sufficiently different to serve well to illustrate the range of possibilities that might be encountered.

From the work of McCulloch and Till [11] and others who used the spleen colony assay of marrow stem cell viability that they devised, we know that the low LET, single-dose survival curve of these cells, has only a small shoulder, $D_q/D_0 = 0{\cdot}4$–$0{\cdot}9$. Consistent with this, only a small positive 2-dose effect is observed [12]. In view of this small capacity for sublethal damage, if irradiation with a high LET beam enhances cell killing, we would expect this to result mainly in a D_0 reduction and indeed at least with neutron beams of modal energies less than 14–15 MeV, this is what is observed (see chapter 5 of Ref. 3, or Ref. 13 for a review). This situation is sketched in Fig. 3A; curve *a* for X-rays and curve *b* for neutrons.

In contrast to marrow, we know, principally from the work of Withers and his associates [14], that intestinal stem cells have a considerable ability to repair sublethal damage when irradiated with X-rays. Accordingly, we may infer that the single-dose survival curve of such cells has a broad shoulder as suggested by curve *c* in Fig. 3B. (From 24-hr fractionation results, it can be estimated that $D_q/D_0 \simeq 3$.) As would be inferred from track-segment irradiation experiments, with increasing LET a reduction in D_q/D_0 should result as a consequence of a reduction both in extrapolation number as well as in D_0. Ordinarily, the curve for X-rays

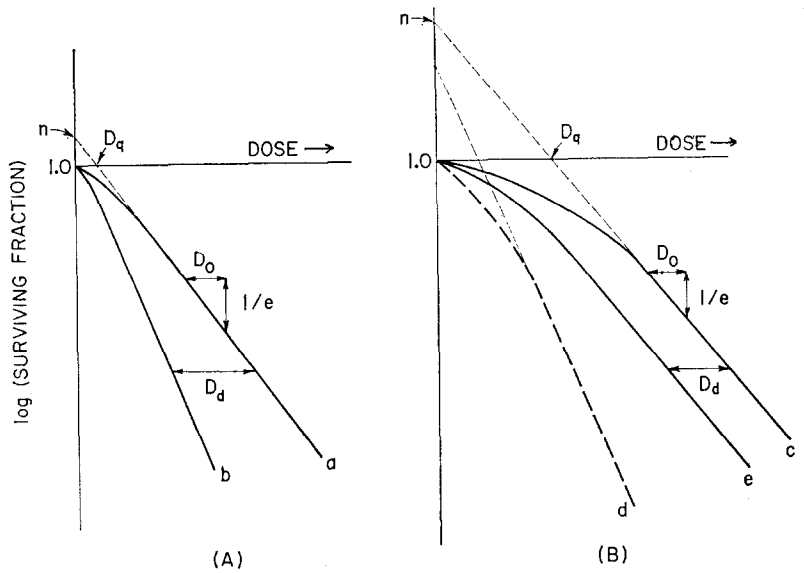


Fig. 3. Panel A — Sketches of survival curves of marrow stem cells exposed to X-rays (curve a) or 14 MeV neutrons (curve b).
Panel B — Sketches of survival curves for intestinal crypt stem cells. Curve c after X-rays, high dose rate curve d after 14 MeV neutrons high dose rate; and curve e after 14 MeV neutrons, low dose rate.

would be expected to be shifted to curve *d*. What was observed by Withers *et al.*, using 14 MeV neutrons [15] is essentially curve *e*; that is, a curve having reduced D_q/D_0 mainly as a consequence of a decrease in extrapolation number with little concomitant change in D_0 (about a 15% reduction).

To analyze curve *e*, we must recall that the LET distribution that results from 14 MeV neutrons has significant contributions from values comparable to those resulting from X- or γ-ray absorption as well as from maximally effective and more than maximally effective LETs. Further, while we would expect LETs in the maximally effective range and larger to give rise to exponential survival ($D_q/D_0=0$), we would not expect "wasted energy" LETs to make a large enough contribution to result in an increase in D_0 from that of curve *d* to curve *e* (e.g. see Ref. [13]). Consequently, another reason must be found for the almost equal final slopes of curves *c* and *e*.

A likely explanation of the situation in Fig. 3B is one that accounts for repair during irradiation when dose rates are low and other conditions are favorable (e.g. temperature, nutritional supply, etc.). Withers *et al.* [15] showed that in addition to almost equal final slopes, gut stem cells could repair sublethal damage between two doses albeit to only about half the extent compared to X-rays (in a 24-hr period). This observation by itself introduces the possibility that sublethal damage was repaired during irradiation since while the X-ray dose rate was about 140 rads/min, the 14 MeV neutron dose rate was about one-tenth as large.

Thus the possibility must be considered that the almost equal final slopes of curves *c* and *e* result from: (1) a very large capacity for sublethal damage and hence, a large repair capacity (low LET radiation); (2) a distribution of LETs as a result of 14 MeV neutron absorption which include appreciable contributions from low LET recoil protons; and (3) a low dose rate for the neutron irradiation. In contrast to the situation in Fig. 3B, that in Fig. 3A, would be expected even if conditions (2) and (3) obtained providing that the cells in question have a small capacity for sublethal damage as appears to be the case for marrow stem cells.

For present purposes, the analysis presented illustrates several points. *First*, it identifies conditions under which qualitatively different responses would be obtained depending on the normal tissue incidentally being irradiated in a given case. If the *dose deficit*, D_d, is defined as the difference between the X-ray and neutron doses to reach a given level of survival (or damage), as shown in Fig. 3A this would be expected to increase without limit for marrow cells. In contrast when gut cells are involved D_d would at first increase but then remain fairly constant as a result of the low dose rate and sublethal damage repair. Thus when comparing neutron irradiation with X-irradiation the allowable exposure to normal tissue depends on the tissue and the degree of damage. For gut cells, the

dose D_q by which the X-ray dose should be reduced to yield the same effect with neutrons approaches a fixed value whereas with marrow cells, the amount by which the neutron dose must be decreased relative to the X-ray dose increases with increasing levels of damage.

Second, the analysis points to the likelihood that repair effects in general (e.g. relative to fractionation) while they may be reduced in extent they may not be negligible with 14 MeV neutrons. The results of Withers *et al.* [15], which underlie this discussion, were with a neutron dose rate which might be higher by a factor of 2–3 than those to be encountered in practice barring significant advances in neutron generation technology. Consequently, even though dose fractions will probably be appreciably smaller in therapy than in the study in question, this will be offset to some degree by the lower dose rate.

And *third*, although illustrations of characteristically different normal cell responses, the examples sketched in Fig. 3 should also alert us to the gamut of tumor cell responses which may be encountered in practice. Tumor cells which respond like marrow cells will probably not be majorly influenced by repair during irradiation nor between fractions. The reverse should be expected with cells whose low LET response is more like gut cells.

SUMMARY AND CONCLUSIONS

The Rijswijk meeting has served to identify a number of broad issues which should be pursued. These in part denote our incomplete knowledge of basic radiobiological phenomena in general and, therefore, efforts to help solidify the base from which clinical strategies can be planned and/or improved will be of considerable use in other connections.

To begin with, we need more single-dose data relative to a variety of endpoints and with different neutron beams and preferably monoenergetic neutrons. Relative to survival alone, in addition to RBE, OER, and fractionation studies, effects of dose rate under conditions of optimal and reduced metabolism should be examined in view of the low dose rates to be available in the near future.

For neutron studies with tumors, the requirements for more suitable material are not unique. In the area of chemotherapy, it has been recognized for some time that tumors which more closely simulate human tumors are needed. In some respects the problem in radiotherapy is simpler since the ability to direct the beam ensures that at least the tumor will be treated. Still, simulation of the growth of human tumors *vis-à-vis* daily or near daily fractionation must be recognized as an important criterion in the choice of suitable experimental tumor systems.

Finally, it is worth identifying an area where potentially biological and physical measurements may aid and abet each other. As noted, the important question of RBE and possibly OER changes with depth in an absorber remains unresolved. Here, an appropriately chosen cell culture system, under good experimental control, together with careful dosimetry and hopefully LET measurements as well could be used to advantage. With time and experience, it is possible that biological measurements alone could serve to map out quality and dose rate changes in absorbers for different beams and with different collimators. Thus, mammalian cells might serve as a "biological dosimeter" measuring the net effect of the complex interactions involved in neutron absorption not easily accomplished for practical purposes in other ways.

Neutrons in radiotherapy have thus far had an uncertain history. Hopefully, the amours of the eternal triangle of radiotherapy, *physics*, *biology*, and *medicine*, can be effectively managed in order that the potentialities of this new treatment mode may be realistically assessed.

REFERENCES

1. R. G. Evans, A. Pinkerton, J. M. Djordjevic and J. S. Laughlin, Changes in biological effectiveness of a fast neutron beam with depth in tissue-equivalent material. *Radiat. Res.* **45,** 235 (1971).
2. A. Pinkerton, J. P. Mamacos and J. S. Laughlin, Dosimetry of fast neutron beams from a small medical cyclotron. *Radiology* **96,** 131 (1970).
3. M. M. Elkind and G. F. Whitmore, *The Radiobiology of Cultured Mammalian Cells.* Gordon & Breach, New York (1967).
4. R. J. Berry, The role of factors modifying neutron damage in irradiation of mammalian cells *in vitro* and *in vivo*. *Proc. Symp. Neutrons in Radiobiology*, p. 483. Oak Ridge, Tennessee (1969).
5. H. S. Reinhold, Quantitative evaluation of the radiosensitivity of cells of a transplantable rhabdomyosarcoma in the rat. *Europ. J. Cancer* **2,** 33 (1966).

6. G. W. Barendsen and J. J. Broerse, Experimental radiotherapy of a rat rhabdomyosarcoma with 15 MeV neutrons and 300 kV X-rays. I. Effects of single exposures. *Europ. J. Cancer* **5,** 373 (1969).
7. H. D. Suit and M. Maeda, Hyperbaric oxygen and radiobiology of a C3H mouse mammary carcinoma. *J. nat. Cancer Inst.* **93,** 639 (1967).
8. R. H. Thomlinson and E. A. Craddock, The gross response of an experimental tumour to single doses of X-rays. *Brit. J. Cancer* **21,** 108 (1967).
9. A. E. Howes, An estimation of changes in the proportions and absolute numbers of hypoxic cells after irradiation of transplanted C3H mouse mammary tumours. *Brit. J. Radiol.* **42,** 441 (1969).
10. M. M. Elkind, Reoxygenation and its potential role in radiotherapy. *Proc. Conf. Time and Dose Relationships in Radiation Biology as Applied to Radiotherapy*, p. 318. Carmel, Cal., BNL Publ. nr. 50203 (C–57), (1970).
11. E. A. McCulloch and J. E. Till, The sensitivity of cells from normal mouse bone marrow to gamma-radiation *in vitro* and *in vivo*. *Radiat. Res.* **16,** 822 (1962).
12. J. E. Till and E. A. McCulloch, Early repair processes in marrow cells irradiated and proliferating *in vivo*. *Radiat. Res.* **18,** 96 (1963).
13. M. M. Elkind, Damage and repair processes relative to neutron (and charged particle) irradiation. In *Current Topics in Radiation Research Quarterly* (Edited by M. Ebert and A. Howard), Vol. VII, p. 1. North-Holland, Amsterdam, (1970).
14. H. R. Withers and M. M. Elkind, Radiosensitivity and fractionation response of crypt cells of mouse jejunum. *Radiat. Res.* **38,** 598 (1969).
15. H. R. Withers, J. T. Brennan and M. M. Elkind, The response of stem cells of intestinal mucosa to irradiation with 14 MeV neutrons. *Brit. J. Radiol.* **43,** 796 (1970).

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Summary of General Discussion on Radiotherapeutic Aspects of the Clinical Application of Fast Neutrons

J. F. FOWLER
Department of Medical Physics, Hammersmith Hospital, Ducane Road, London, W.12, England

THE AUTHOR feels strongly that his summary cannot adequately convey the complexities of the subject. Readers are therefore reminded to study the papers presented in earlier sessions.

1. *Aims of clinical applications*

It was agreed that there are two stages of clinical assessment with new methods of treatment such as neutron beams. Definitive clinical trials will eventually be essential to evaluate whether improved local and regional control can be achieved with fast neutrons as compared with the best supervoltage X-ray methods. Before this stage is reached, however, preliminary data for the determination of RBE values must be obtained from treatments of types of tumours and normal tissues which allow the quantitative assessment of responses. Such studies can be made, for example, with metastatic skin nodules in breast cancer, inoperable breast cancer, or pulmonary metastases.

2. *Dose-limiting normal tissues*

It is obvious that the responses of tissues which might be dose-limiting should be examined at the earliest possible stage in the clinical use of any fast neutron beam. Special attention should therefore be given to responses of skin, pharyngeal mucosa, epithelium of intestinal tract, the rectum and the lens of the eye. Dr. Catterall's paper reported observations of skin reactions in patients treated with the "8 MeV" neutron beam from the MRC cyclotron, Hammersmith Hospital, London. In addition, long-term reactions must also be studied. For cells of the haemolymphopoietic system the RBE of fast neutrons is expected to be lower than for most other tissues but at very low doses per fraction, as received at points outside the treated area, the RBE values might instead be considerably higher because the effectiveness of the X-rays becomes lower at such small doses. Blood counts should therefore be carried out regularly on all patients, and chromosomal abnormalities should be looked for. Occasional marrow puncture tests would enable aplastic anaemia or leukaemia to be detected. Lenses of the eye should be examined regularly both in patients and staff exposed to neutrons. A long-term register of all such patients and staff should be maintained for the duration of their lives.

Whenever a new type of neutron generator becomes available, its clinical use must be preceded by physical and radiobiological tests to ensure that the physical distribution and spectral components of the beam are as expected. Then clinical tests should be carried out to establish values of RBE for the actual beam. Different RBE values might be found for different tissues and RBE is certainly dose-dependent (i.e. dependent on dose per fraction). Results of investigations with other types of neutron beams can only provide enough guidance for the first pilot irradiations.

The possibility of increased radiation injury in partly hypoxic tissues was discussed in relation to cartilage and spinal cord. The increased injury to these tissues observed when high

pressure oxygen (HPO) therapy was used did not provide definite evidence about whether the problem might also be expected to arise with neutron beams but a cautious approach was indicated. The high incidence of myelitis in HPO treatments found by Van den Brenk might have been due either to the few and large fractions used, or to the HPO, or to both [1].

3. *Types of tumours to be considered for clinical trials*

Possible sites and types of tumours for definitive clinical tests were discussed in detail by Kligerman and Kaplan (see papers in this symposium). In addition, a distinction might be made between:

(a) tumours which regress after conventional treatment, but recur in a large fraction of the cases, and
(b) tumours which respond little (as judged from a decrease in volume) after treatments up to the tolerance of normal tissues.

The fact that early stages of some types of tumours have a high probability of eradication with X- or gamma-rays might be interpreted by assuming that a large capacity for repair of sub-lethal damage or the presence of hypoxic cells are not major causes of failure for these types of tumours and overall gain factors for neutron therapy might then be low. If these factors (a large amount of repair or the presence of hypoxic cells) have a greater influence in the advanced stages of tumour growth, the overall gain factors might then be somewhat higher.

Tumours belonging to group (b) are at first sight not attractive for testing any new treatment modality, but a comparison of neutron and X-ray results might provide important insights into the causes of the lack of response to X-rays. If this lack of response is due to the presence of hypoxic cells and to the inability of the tumour to reoxygenate these cells, possibly because it fails to shrink, then neutrons might indeed give an improved result. Dr. Denekamp showed that animal sarcomas, unlike carcinomas, did not usually shrink until several days after an X-ray dose of 1000–2000 rads. She pointed out that in several of these sarcomas reoxygenation had been shown to be less in extent than in the only mouse carcinoma investigated, which shrinks within one day after X-irradiation. It was agreed that soft tissue sarcomas might be of interest for a neutron trial with the reservation that conventional treatments were giving good results for small soft tissue sarcomas. The low numbers of these tumours occurring would make inter-centre collaboration essential.

The treatment of nodes in the neck using radiation with HPO had given better results than without HPO; but the meaning of this improvement was questioned, because it was possible that the best available conventional technique had not been used in obtaining those results.

4. *Quantitative considerations in clinical trials*

One consideration in the selection of tumours to be used for neutron trials is that the probability of 2-yr survival of the patient should be greater than about 20%, because otherwise evaluation of late normal tissue reactions would be impossible. Most tumours considered suitable for a trial with neutrons in the session on experimental and clinical radiotherapy, satisfied this criterion. An exception was the glioblastoma, which, it was felt, should still be included on the grounds that other cases would in any case provide information about long-term effects on normal tissues.

It was noted that proposals outlined by Withers included a number of tumour types where present local control rates at 2 yr were between 70 and 75%. Since these tumours were in other respects suitable for such a trial, it was not felt that this local control rate was so high as to exclude their consideration for this purpose.

Duncan and Breur pointed out that larger tumours might show a more marked improvement over the results using conventional techniques than small tumours. Duncan mentioned that his colleague radiotherapists at Manchester felt that an improvement in cure rate of as little as 7% would be worth achieving. Of course, such a small increase would be difficult to demonstrate.

More definitive data is desirable on the rate at which the proportion of patients cured increased with dose. Withers quoted Shukovsky [2] who found a steep response in supraglottic tumours stage 2 and 3. In this series the probability of local control of supraglottic squamous cell carcinoma had increased from 20% to 80% when the nominal single dose was increased from 1750 to 2100 rets: an increase of only about 20% in effective dose. The response in the case of larger tumours, stage 3b and 4 of the cervix carcinomas, did not show this steep rise. Withers and Fowler showed a fairly steep increase of tumour cure rate with dose in C_3H mice with spontaneous mammary carcinoma, where an increase of 10% in dose caused the cure rate to rise from 20% to about 50%.

Table 1. *Total numbers of patients in two groups required for a given chance of demonstrating a specified improvement of local control rates*

Prospective chances of demonstrating an improvement	Improvement in local control		
	40–50%	40–80%	75–95%
Boag [3] 1 chance in 2 of significance at 95% level of confidence	2×180	2×14	2×20
Withers [4] 4 chances in 5 of significance at 90% level of confidence	2×180*	2×20	2×30
Haybittle [5] 9 chances in 10 of significance at 95% level of confidence	2×475†	2×35	2×50

*This figure refers to an improvement in local control from 60–70% instead of from 40–50%.
†Improvement from 30–40%.

An increase of 25% in dose caused an increase in cure rate from 20% to 70%. These increases would correspond, of course, to a therapeutic gain factor of 1·10 and 1·25 respectively, if the normal tissue responses were not increased. The slope of this curve was equally steep if 5 fractions instead of a single dose were given to these mouse tumours.

The number of patients required for prospective clinical trials depends upon two statistical criteria, as well as upon the cure rate by the conventional technique and the extent of the improvement which the new technique might bring. One of the statistical criteria is well known: the level of significance or "*P* value" accepted for the difference found, usually $P \leqslant 0{\cdot}05$ but sometimes $P \leqslant 0{\cdot}10$. The other criterion arises from the fact that the trial is only one sample of patients and not the universal group. This was expressed by the chance of 1 in 2 [3] or 4 in 5 [4] or 9 in 10 [5] of obtaining the difference expected (at the level of significance agreed) in one clinical trial, as shown in Table 1.

These numbers, together with the prospect of a 6-yr intake totalling only 40 patients of one type in a medium-large radiotherapy centre, indicate that co-operation between centres will be required. The same problem, of course, arises in clinical trials of other modalities.

Stratification of pairing should be carried out *before* randomization, so that numbers in comparative groups should be kept as equal as possible. It was emphasized that *all* the relevant factors should be included in the stratification at the outset: tumour site, TNM classification, and histology, were suggested as essential. It has been shown [6] that even large numbers of categories do not result in severe losses of information in pairing systems: about two-thirds of all the patients contribute to a comparison which is more valid than in an unpaired series.

5. *Modes of application of fast neutrons to tumours in patients*

Thomlinson had pointed out that neutrons might be most effective if used for the *first* and/or for the *last* treatment of a series of fractionated doses. Barendsen suggested that X-ray dose fractions might be used for the *first half* of a split-course treatment; then if after the rest period the tumour had responded inadequately, neutrons would be used for the *second half*. These suggestions were interesting, but were judged not appropriate at present when patient numbers were barely adequate to test an all-neutron against an all-X-ray method. The clinical trial of neutrons-only should come first. Such splitting might eventually be appropriate if economic or time factors caused a shortage of treatment time, for example where negative pi mesons were produced at facilities devoted mainly to nuclear physics rather than to therapy.

Finally the mode of neutron fractionation was discussed. It was agreed that fast neutrons should be tested *first* against the *best conventional* therapy: linear accelerators, betatrons, and possibly (for head and neck treatments) cobalt-60, used in daily fractions over 4 weeks or longer. It was pointed out that 3 fractions per week was probably not different from 5 fractions per week. Neutron fractionation

should be chosen to give the best results that neutrons could give, but the ideal fractionation was not known. Three, two, or one fraction per week might be investigated. Should neutrons prove better, then the effect of any non-standard fractionation regime used could later be investigated using the X-rays. The overall time should probably be the same as for the X-ray course. Cell proliferation factors were not expected to be different after fast neutrons or X-rays; this might suggest not too great a departure from conventional fractionation regimes, i.e. 3 or 2 per week rather than one per week, although the latter should not be excluded.

REFERENCES

1. H. A. S. VAN DEN BRENK, W. RICHTER and R. H. HURLEY, Radiosensitivity of the human oxygenated cervical spinal cord based on analysis of 357 cases receiving 4 MeV X-rays in hyperbaric oxygen. *Brit. J. Radiol.* **41,** 205 (1968).
2. L. J. SHUKOVSKY, Dose, time, volume relationships in squamous cell carcinoma of the supraglottic larynx. *Amer. J. Roentgenol.* **108,** 27 (1970).
3. J. W. BOAG, Statistical problems which arise in cancer therapy. *Clin. Radiol.* **11,** 150 (1960).
4. H. R. WITHERS, P. R. ALMOND and B. W. BROWN, Considerations in planning a clinical trial with fast neutrons. *Europ. J. Cancer* **7,** 231 (1971).
5. J. HAYBITTLE, Quoted in "Preliminary Report of the Working Party of the British Institute of Radiology on the Effects of Dose Fractionation in Radiotherapy". *Brit. J. Radiol.* **36,** 382 (1963).
6. T. J. DEELEY, Paper read at Fac. Radiol. Annual Meeting at Hammersmith Hospital, 20th June 1970. See also: A clinical trial to compare two different tumour dose levels in the treatment of advanced carcinoma of the bronchus. *Clin. Radiol.* **17,** 299 (1966).

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Concluding Remarks on the Application of Fast Neutrons in Clinical Radiotherapy

G. W. BARENDSEN and J. J. BROERSE
Radiobiological Institute TNO, 151 Lange Kleiweg, Rijswijk Z.H., The Netherlands

THE AIM initially stated in the invitations to participants of the meeting, was to review available knowledge of technical, radiobiological and radiotherapeutic aspects of the application of fast neutrons in radiotherapy and to arrive at some measure of agreement about a number of statements and recommendations concerning various problems which can arise in the clinical use of fast neutrons. The discussions during the meeting demonstrated however, that with respect to many aspects the available information is as yet insufficient to allow general conclusions to be drawn or recommendations to be made and obviously further research and development of techniques are urgently required. The main recommendation which was generally made implied the necessity of close co-operation and exchange of information between the various centres involved in or planning the application of fast neutrons in radiotherapy. The most important subjects on which more information must be obtained and co-operation is required can be summarized as follows:

1. For the dosimetry of fast neutrons and of mixed beams of fast neutrons and gamma-rays, standardization of methods and intercomparison on an international scale of measurements of absorbed dose and radiation quality and variations of these parameters with depth in tissue are required. These measurements are not only necessary for tumour therapy but also with respect to radiation protection of personnel. In addition, for assessment of skin sparing properties of fast neutron beams, changes of radiation quality in the superficial few millimetres of skin must be evaluated, because build-up of secondary particle equilibrium is only due to protons, which may have an RBE different from that of heavy recoil nuclei.

2. Further development of fast neutron sources is required to obtain neutron beams which have good penetration and are sufficiently intense to deliver dose rates in excess of 10 rads/min, at SSD values of at least 100 cm, necessary to allow adequate collimation and shielding.

3. In the area of neutron radiobiology, it must be emphasized that RBE values for effects on different normal tissues as well as for effects on different types of tumours are not only dependent on the dose per fraction, the time interval between the fractions and the dose rate, but also on the neutron energy spectrum and the amount of gamma-radiation present. Consequently, for each individual beam extensive radiobiological studies should be carried out and effective therapeutic gain factors should be evaluated for comparison with data from other centres.

4. From the extensive discussions of types of tumours considered suitable for clinical applications of fast neutrons, it became abundantly clear, that because of the limitations in the numbers of patients with suitable tumours, close co-operation and exchange of information between all centres involved is an absolute requirement if possible advantages of fast neutrons in radiotherapy are to be evaluated adequately within a period of 5–10 yr. This

co-operation obviously requires that techniques, dosimetry and evaluation of responses of tumours and normal tissues are standardized.

We hope that the uninhibited and fruitful exchange of data and opinions between radiotherapists, radiobiologists and physicists at this symposium will be followed up soon by similar meetings necessitated by the accumulation of new data.

Acknowledgements—We want to express our sincere thanks to Miss M. W. Th. Wagener for her expert assistance in connection with the planning of this meeting and editorial processing of the proceedings, and to Mr. J. S. Groen for efficient recording of the extensive discussions.

Europ. J. Cancer Vol. 7, pp. 265–267. Pergamon Press 1971. Printed in Great Britain

List of Participants at the Meeting on Fundamental and Practical Aspects of the Application of Fast Neutrons in Clinical Radiotherapy

held at the

Radiobiological Institute TNO

151, *Lange Kleiweg, Rijswijk Z.H., The Netherlands*

June 21–24, 1970

P. R. ALMOND, Dept. of Physics, The University of Texas, M.D. Anderson Hospital and Tumor Institute, 6723 Bertner Ave., Houston, Texas 77025, U.S.A.

J. G. VAN ANDEL, Rotterdamsch Radiotherapeutisch Instituut, 297, Groene Hilledijk Rotterdam, The Netherlands.

G. W. BARENDSEN, Radiobiological Institute TNO, 151 Lange Kleiweg, Rijswijk Z.H., The Netherlands.

J. BATAINI, Service de Radiothérapie A, Fondation Curie, 26 rue d'Ulm, Paris (Ve), France.

D. W. VAN BEKKUM, Radiobiological Institute TNO, 151 Lange Kleiweg, Rijswijk Z.H., The Netherlands.

J. A. BELLI, Harvard Medical School, 50 Binney Street, Boston, Massachusetts 02115, U.S.A.

R. J. BERRY, Radiobiology Laboratory, Churchill Hospital, Headington, Oxford, England.

D. K. BEWLEY, Cyclotron Unit, Hammersmith Hospital, Ducane Road, London W12, England.

V. P. BOND, Brookhaven National Laboratory, Upton, L.I., N.Y. 11973, U.S.A.

M. BOONE, Radiotherapy Dept., University of Wisconsin, Madison, Wisconsin 53706, U.S.A.

J. BOOZ, Biology Division, Euratom CCR, 21020 Ispra/VA, Italy.

J. T. BRENNAN, Dept. of Radiology, Hospital of University of Pennsylvania, 3400 Spruce Street, Philadelphia, Penn. 19104, U.S.A.

K. BREUR, Radiotherapy Dept., Wilhelmina Gasthuis, Amsterdam, The Netherlands.

J. J. BROERSE, Radiobiological Institute TNO, 151 Lange Kleiweg, Rijswijk Z.H., The Netherlands.

MARY CATTERALL, Cyclotron Unit, Hammersmith Hospital, Ducane Road, London W12, England.

M. R. CLELAND, Radiation Dynamics, 1800 Shames Drive, Westbury, L.I., N.Y. 11590, U.S.A.

S. B. CURTIS, Radiobiological Institute TNO, 151 Lange Kleiweg, Rijswijk Z.H., The Netherlands.

JULIE DENEKAMP, Hammersmith Hospital, Ducane Road, London W12, England.

B. DJORDJEVIC, Division of Physical Biology, Sloan-Kettering Institute for Cancer Research, New York, N.Y. 10021, U.S.A.

D. W. DOWNTON, Services Electronics Research Laboratory, Baldock, Herts, England.

W. DUNCAN, Christie Hospital and Holt Radium Institute, Withington, Manchester 20, England.

J. Dutreix, Institut Gustave-Roussy, 16 bis avenue P. Vaillant-Couturier, 94-Villejuif, France.

A. van Dijk, N.V. Philips' Gloeilampenfabriek, Medical Systems Division, Bldg. TQ 111-3, Eindhoven, The Netherlands.

M. Ebert, Paterson Laboratories, Christie Hospital and Holt Radium Institute, Manchester M20, 9BX, England.

M. M. Elkind, Dept. of Biology, Brookhaven National Laboratory, Upton, L.I., N.Y. 11973, U.S.A.

S. B. Field, Hammersmith Hospital, Ducane Road, London W12, England.

A. C. Ford, Radiobiological Institute TNO, 151 Lange Kleiweg, Rijswijk Z.H., The Netherlands.

J. F. Fowler, Dept. of Medical Physics, Hammersmith Hospital, Ducane Road, London W12, England.

H. Franke, Radiologische Universitätsklinik und Strahleninstitut, 52 Martinistrasse, 2 Hamburg 20, Germany.

J. van de Geijn, Ziekenhuis van den H. Joannes de Deo, Westeinde, The Hague, The Netherlands.

D. Greene, Physics Dept., Christie Hospital and Holt Radium Institute, Withington, Manchester 20, England.

G. Griggs, Vanderbilt Hospital, Vanderbilt University, Nashville, Tennessee 37203, U.S.A.

E. J. Hall, Dept. of Radiology, Radiological Research Laboratories, 630 West 168th Street, New York, N.Y. 10032, U.S.A.

K. E. Halnan, Institute of Radiotherapeutics, Western Infirmary, Glasgow W.1, Scotland.

A. F. Hermens, Radiobiological Institute TNO, 151 Lange Kleiweg, Rijswijk Z.H., The Netherlands.

D. Herring, Enviro-Med Inc., P.O. Box 2324, La Jolla, Cal. 92037, U.S.A.

S. den Hoed-Sytsema, Rotterdamsch Radiotherapeutisch Instituut, 297 Groene Hilledijk, Rotterdam, The Netherlands.

B. Hogeweg, Radiobiological Institute TNO, 151 Lange Kleiweg, Rijswijk Z.H., The Netherlands.

C. F. Hollander, Experimental Gerontology Unit TNO, 151 Lange Kleiweg, Rijswijk. Z.H., The Netherlands.

K. L. Jackson, Division of Radiation Biology, School of Medicine and University Hospital, University of Washington, Seattle, Washington 98105, U.S.A.

H. B. Kal, Radiobiological Institute TNO, 151 Lange Kleiweg, Rijswijk Z.H., The Netherlands.

H. S. Kaplan, Dept. of Radiology, Stanford University School of Medicine, Stanford, Cal. 94305, U.S.A.

C. A. Kelsey, Medical Physics Dept., University Hospitals, Madison, Wisconsin 53706, U.S.A.

J. C. Klein, Radiobiological Institute TNO, 151 Lange Kleiweg, Rijswijk Z.H., The Netherlands.

M. M. Kligerman, Dept. of Radiology, Yale Medical School, 333 Cedar Street, New Haven, Connecticut 06510, U.S.A.

G. Kok, Ziekenhuis van den H. Joannes de Deo, Westeinde, The Hague, The Netherlands.

Tj. Kuipers, Rotterdamsch, Radiotherapeutisch Instituut, 297 Groene Hilledijk, Rotterdam, The Netherlands.

H. A. Künkel, Institut für Biophysik und Strahlenbiologie, 2 Hamburg 20, Martinistrasse 52, Germany.

S. K. Lahiri, Radiobiological Institute TNO, 151 Lange Kleiweg, Rijswijk Z.H., The Netherlands.

R. C. Lawson, Institute of Radiotherapeutics, Western Infirmary, Glasgow W.1, Scotland.

D. A. Lundberg, Neutron Division, Elliott Brothers (London) Ltd., Elstree Way, Borehamwood, Hertfordshire, England.

R. Morrison, Hammersmith Hospital, Ducane Road, London W12, England.

J. M. Nelson, Radiobiological Institute TNO, 151 Lange Kleiweg, Rijswijk Z.H., The Netherlands.

B. P. Offermann, AEG-Telefunken, 29 Industriestrasse, 2 Wedel, Germany.

R. G. Parker, Division of Therapeutic Radiology, School of Medicine and University Hospital, University of Washington, Seattle, Washington 98105, U.S.A.

H. A. van Peperzeel, Radiotherapy Dept., Wilhelmina Gasthuis, Amsterdam, The Netherlands.

T. L. Phillips, Section of Therapeutic Radiology, University of California, San Francisco Medical Center, San Francisco, Cal. 94122, U.S.A.

J. A. van de Poel, Rotterdamsch Radiotherapeutisch Instituut, 297 Groene Hilledijk, Rotterdam, The Netherlands.

W. Porschen, Institut für Medizin, KFA Jülich, Postfach 365, 517 Jülich 1, Germany.

L. M. VAN PUTTEN, Radiobiological Institute TNO, 151 Lange Kleiweg, Rijswijk Z.H., The Netherlands.

O. REIFENSCHWEILER, Philips Natuurkundig Laboratorium, Willem Alexanderlaan, Geldrop, The Netherlands.

H. S. REINHOLD, Radiobiological Institute TNO, 151 Lange Kleiweg, Rijswijk Z.H., The Netherlands.

A. RODRIGUEZ-ANTUNEZ, Dept. of Radiation Therapy and Nuclear Medicine, Cleveland Clinic, Cleveland, Ohio 44106, U.S.A.

H. H. ROSSI, Dept. of Radiology, Radiological Research Laboratories, 630 West 168th Street, New York, N.Y. 10032, U.S.A.

R. SCHITTENHELM, 8520 Erlangen, Von-Bezzel-Strasse 6, Germany.

A. SOMERWIL, Rotterdamsch Radiotherapeutisch Instituut, 297 Groene Hilledijk, Rotterdam, The Netherlands.

F. STÄHLER, Institut für Biophysik und Strahlenbiologie, 52 Martinistrasse, 2 Hamburg 20, Germany.

S. K. THE, Rotterdamsch Radiotherapeutisch Instituut, 297 Groene Hilledijk, Rotterdam, The Netherlands.

P. THOMAS, Radiotherapy Dept., Academisch Ziekenhuis, Leiden, The Netherlands.

R. H. THOMLINSON, Exp. Radiopathology Research Unit, Hammersmith Hospital, Ducane Road, London W12, England.

E. VERNIN, Tunzini-Sames, Division Electrostatique, Electronique et Nucléaire, 21 rue Jean Macé, 38 Grenoble, France.

B. VAN DER WERF-MESSING, Rotterdamsch Radiotherapeutisch Instituut, 297 Groene Hilledijk, Rotterdam, The Netherlands.

H. R. WITHERS, M.D. Anderson Hospital and Tumor Institute, 6723 Bertner Avenue, Houston, Texas 77025, U.S.A.

P. WOOTTON, Division of Medical Radiation Physics, School of Medicine and University Hospital, University of Washington, Seattle, Washington 98105, U.S.A.

H. O. WYCKOFF, Armed Forces Radiobiology Research Institute, National Naval Medical Center, Bethesda, Maryland 20014, U.S.A.

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Announcement

European Organization for Research on Treatment of Cancer (E.O.R.T.C.)

COURSE IN CLINICAL PHARMACOLOGY

with special reference to cancer

at

THE INSTITUT JULES BORDET, BRUSSELS

May 10–13, 1971

(1) Animal screening for anti-tumour agents;
(2) Animal toxicity as a tool for prediction in man;
(3) Biostatistics and design;
(4) Human pharmacology.

With the participation of: Cooper (Leeds), Garattini (Milan), Heuson (Brussels), Kenis (Brussels), Mathé (Paris), Munk (Heidelberg), van Putten (Rijswijk), Sachsenmaier (Innsbruck), Schwartz (Paris), Stryckmans (Brussels), Staquet (Brussels), Tagnon (Brussels), Tubiana (Paris).

Simultaneous translation — English/French, French/English.

For further information and enrolment, please contact:

Dr. M. Staquet,
E.O.R.T.C.,
Institut Jules Bordet,
1 rue Héger-Bordet,
1000 BRUSSELS.
Tel.: 38.57.90

Fee: 25 U.S. dollars.

Europ. J. Cancer Vol. 7, p. 270. Pergamon Press 1971. Printed in Great Britain

Announcements

The Netherlands Cancer Institute has organized an international symposium on occasion of the retirement of Prof. Dr. O. Mühlbock. The title, place and date are:

RNA Viruses and Host Genome in Oncogenesis

Amsterdam 12–14 *May* 1971

The main topics to be dealt with are the genetics of avian and murine leukaemia viruses, the mouse-mammary tumour virus and biochemical aspects.

Registration fee is $30 — (U.S.A.-dollars). Further details and application forms can be obtained from the secretariat, Dr. L. M. Boot, Netherlands Cancer Institute, Sarphatistraat 108, Amsterdam.

Because of the shortage of hotel accommodation in Amsterdam a timely reservation is indicated.

The Second Congress of the European Association of Radiology will be held in Amsterdam, the Netherlands, from June 14th to 18th, 1971.

The Secretary's office is located at 16 Lange Voorhout, The Hague, Netherlands.

Europ. J. Cancer Vol. 7, p. 271. Pergamon Press 1971. Printed in Great Britain

Erratum

MAGDA M. STILMANT, GEORGES M. VAMECQ, WILLY F. PIESSENS and ROBERT R. BADJOU, Evaluation of extent of metastatic liver disease: A proposed discriminant. *Europ. J. Cancer* **7**, 92 (1971).

In the first column, 8 lines from the bottom, BSP should have read bilirubin.

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Improved Microcirculation in Irradiated Tumours*

H. S. REINHOLD†
Radiology Department, Yale University Medical School, New Haven, Conn. 06510, *U.S.A.*

Abstract — *The response of the tumour micro-circulation of a mouse mammary tumour to fractionated irradiation was investiagted by the quantitative analysis of cinematographically recorded micro-angiograms made of tumours, growing in a sheet-like fashion with a thickness of about* 50 μ. *The results indicate that, during a course of fractionated radiotherapy with daily fractions of* 576 *rads, there is an improvement of the tumour microcirculation, which is most marked at the 7th treatment day when the tumours have just regressed beyond their initial size. The technique of growing sheet-like tumours in a modification of the Algire chamber system (called the "sandwich" system), does not only allow the direct observation, recording or measurement of the various aspects of the tumour microcirculation, but also permits direct estimation of the local oxygenation (redox) state of the tumour tissue by using micro-fluorometric methods.*

INTRODUCTION

THE VASCULAR architecture of tumours has been studied intensively since the turn of the century, mainly because of histo-pathological and radiodiagnostic interest. The importance of the abnormalities of the blood vessel patterns for the radiosensitivity of tumours was brought out by the classical work of Thomlinson and Gray in 1955 [1]. Employing the Krogh–Ehrlang diffusion equations and using the available data with regard to the diffusion of oxygen in tissues, the oxygen consumption of tumours and the concentration of oxygen in capillaries, Thomlinson and Gray evaluated the maximum distance from the capillaries at which oxygen would still be available for the tumour cells. They concluded that there will be a limit to the range of the diffusion of oxygen into tumour cords if the radius of the cord exceeds a value of about 150 μ. If tumour cells would subsist beyond this range, they must do so under hypoxic conditions. Thomlinson and Gray's theoretical model agreed very well with their histological observations in which necrosis was found invariable when the intercapillary distance exceeded the critical value. Recently Tannock, in his careful studies about the rates of cell proliferation at different locations in tumour cords, made similar calculations and also reached the conclusion that, among the various metabolites and catabolites, oxygen will be the one with the shortest diffusion length [2].

In a surprising number of experimental solid tumours examined so far, a radioresistant fraction of cells has been found, and this is generally attributed to hypoxia of those cells [3]. For experimental tumours this fraction has been determined to vary between 1% to 100%, most experimental solid tumours seem to contain between 10% and 20% hypoxic cells. Due to a lack of suitable techniques no data about human tumours are available yet.

The determination of the proportion of

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†Present address: Radiobiological Institute TNO, Lange Kleiweg 151, Rijswijk, Z.H., The Netherlands.

hypoxic cells in experimental tumours is generally performed by irradiating the tumours under different conditions of oxygenation and assaying for differences in the number of surviving cells. To this end the Hewitt-type of transplantation assay can be used [4, 5]. The analysis of tumour regrowth curves or cure rate data have been shown also to yield valuable information [6, 7]. These methods have extensively been used during the past decade to determine cell survival curves of experimental tumours. From these cell survival curves the size of a radioresistant compartment of hypoxic cells can be derived. In addition it has been shown for a number of tumours that preceding radiation treatments tend to decrease the proportion of hypoxic cells [8–11].

Apparently the existence in tumours of viable, but hypoxic malignant cells is determined by physical parameters. It may therefore seem inconsistent that the determination of the proportion of hypoxic cells, or actually the regional distribution of oxygen in tumours, is still being carried out by means of bio-assays. It must be realized, however, that with regard to tumour cure, cell survival is an extremely relevant parameter and that the techniques employed in physiology do not yet meet our requirements.

The aim of the present communication is to report a technique which allows the quantitative study of the micro-circulation of tumours *in vivo*. The results obtained with this method indicate a real improvement of the tumour micro-circulation during fractionated radiotherapy.

In short, a modification of the Algire chamber system was developed in which it was possible to grow tumours in a sheet-like fashion. The micro-circulation in those tumours was evaluated by analyzing cinematographic recordings of the inflow of an intravenously injected fluorescent dye in the tumour vessels.

MATERIAL AND METHODS

Male C_3H/HeJ mice (Jackson Lab, Bar Harbor, Maine) were fitted with the Algire

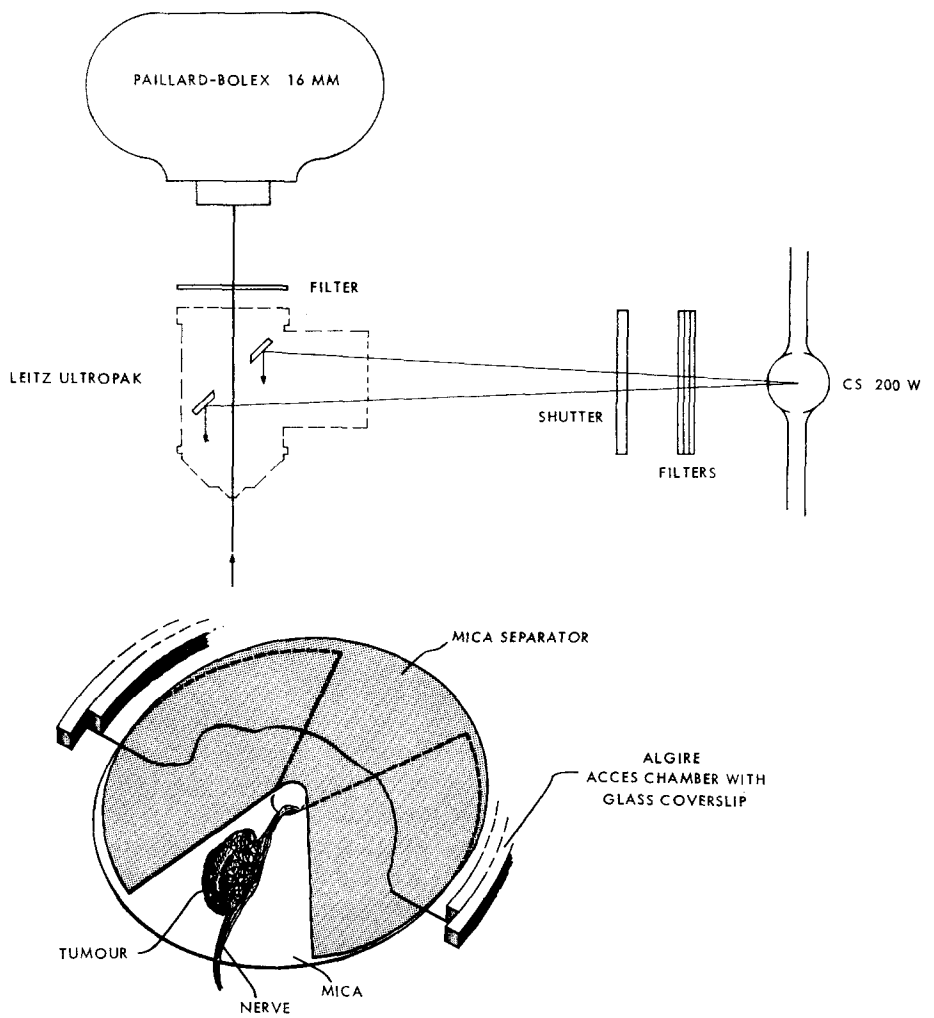


Fig. 1. *Schematic presentation of the "sandwich" tumour system.*

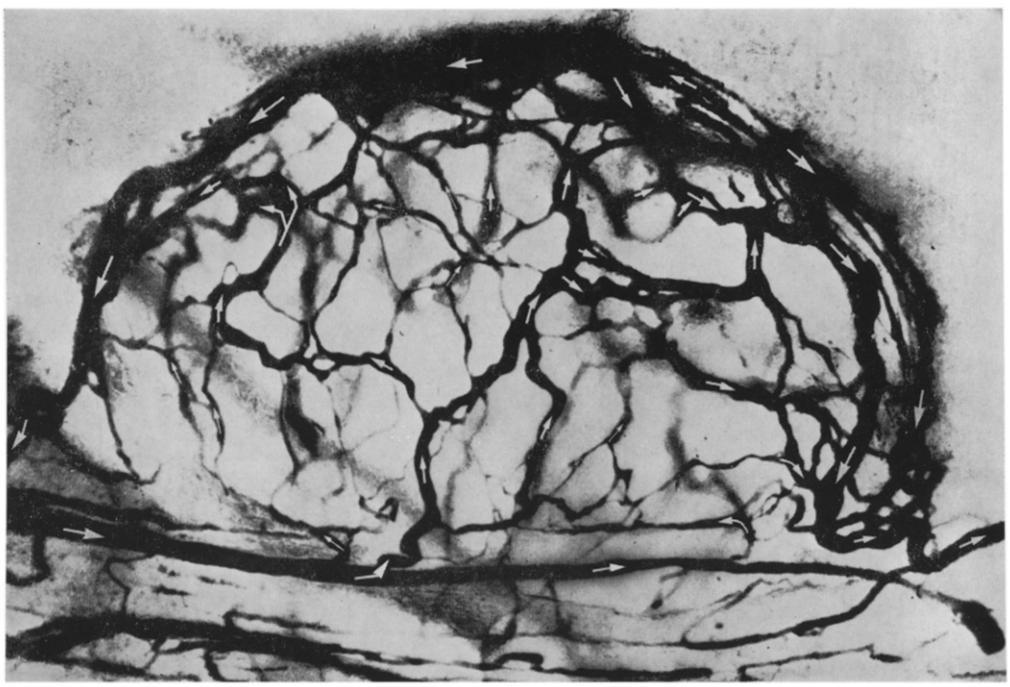


Fig. 2. Blood flow in a "sandwich" tumour. Mouse mammary tumour C_3HBA. *Picture derived from photographs and observations* ($\times 50$).

(to face p. 274)

access chamber [12, 13]. Next day the chamber was opened, and one of the vascularized skin-nerves was dissected free from the underlying skin for about 5 mm. Then a U-shaped piece of mica with a thickness of about 0·1 mm was interposed between the nerve and the underlying skin and a similar shaped piece of mica of about 0·04 mm thick was put on top of it, but in a reverse position (see Fig. 1). In this way the nerve, and thus its blood-vessels, ran isolated from the surrounding tissues for a distance of about 5 mm and was protected from being squeezed by a mica separator. Before closing the chamber with the coverslip, a tiny piece of Mammary Carcinoma C_3HBA measuring about 0·1 mm^3 was placed right next to the isolated nerve.

This tumour inoculum grew in such a "sandwich" chamber in about ten days to a size of about 5 mm^2; its thickness being limited to about 50 μ. The size of the tumours was expressed in terms of tumour area rather than tumour volume. This, however, will have hardly any consequence for the interpretation of the results. The "sandwich" tumours were sufficiently translucent to allow the observation of the circulation in virtually all capillaries. Employing the 546 nm wave-length of a high-pressure mercury arc and high speed Polaroid No. 57 film, photomicrographs of the tumour vasculature could be obtained with excellent contrast.

Micro-angiography was performed by giving the nebutal-anesthetized animals a single intravenous injection of 0·15 ml of 10% Pyranin* in 0·9% NaCl, and recording the flow of this fluorescent dye through the tumour vessels on Kodak No. 2479 film. The camera set-up was similar to the one used by Kligerman [14]. A Leitz ultropak 3·8×objective was used for incident ultraviolet light (mainly 366 nm) illumination. The film speed was 11 frames per second. The important advantage of the *in vivo* micro-angiography is that it can be repeated after the dye has cleared from the tumour-area. This usually takes about one day. Therefore most of the micro-angiograms were repeated every other day. In this way considerably more information per animal could be obtained.

The irradiations were carried out with a 250 kV Westinghouse machine; 15 Ma, HVL 0·8 mm Cu, dose rate 157 rads/min. The field size was limited to 8 mm in diameter; allowing the irradiation of only the tumour-area and avoiding the irradiation of the rim of the Algire access chamber.

Twenty-two preparations entered the experiments mentioned in this paper, furnishing the material for 82 micro-angiograms.

OBSERVATIONS AND RESULTS

The first indication of specific vascular supply for these "sandwich" tumours was observed at about the third day after implantation. This blood supply was a venous one; this is in accordance with Kligerman's finding [14]. One or two days later arterial blood supply was established and from that moment on the tumours grew with a doubling time of about two days.

The vascular pattern changed gradually but continuously during this period of growth, and although every individual tumour could readily be distinguished by its vascular pattern, it proved to be extremely difficult to follow in detail the development of the capillary system. This has also been the experience of others [13], and is due to the fact that the entire vasculature of a growing tumour is subject to continuous change. While at some sites new vessels emerge, others are simultaneously being stretched, displaced, squeezed or even occluded. This prevents the use of blood-vessels as landmarks and, because of the translucency of the few cell layers of tumour tissue, no other landmarks are available.

The vascular system of these small "sandwich" tumours showed evidence of the abnormalities for which tumour blood-vessels are notorious [13, 15]. Figure 2 represents a combination of an *in vivo* photograph and drawings of the vasculature of a C_3HBA carcinoma. Striking features are the apparently random way the blood-vessels, and blood-flow, criss-crosses through the tumour. There is a rapid flow in some of the central vessels, but there frequently shunt right into the venous sinusoids. These sinusoids are mainly located at the tumour periphery and show a sluggish circulation. The flow rate of the blood in the sinusoids could only be estimated by direct observation and was thought to be of the order of 100 μ per second, sometimes slower, but sometimes faster; especially where shunts empty in the sinusoids.

A peculiar observation was that in tumours with volumes over 1 mm^3 there was occasionally a kind of dual current in some of the larger sinusoids. The marginal blood flow in those sinusoids was extremely sluggish, while the midstream current was of higher velocity.

Another observation was that frequently very tiny tumour capillaries existed in which only

*Pyranin: fluorescent dye, Messrs. Bayer, Germany.

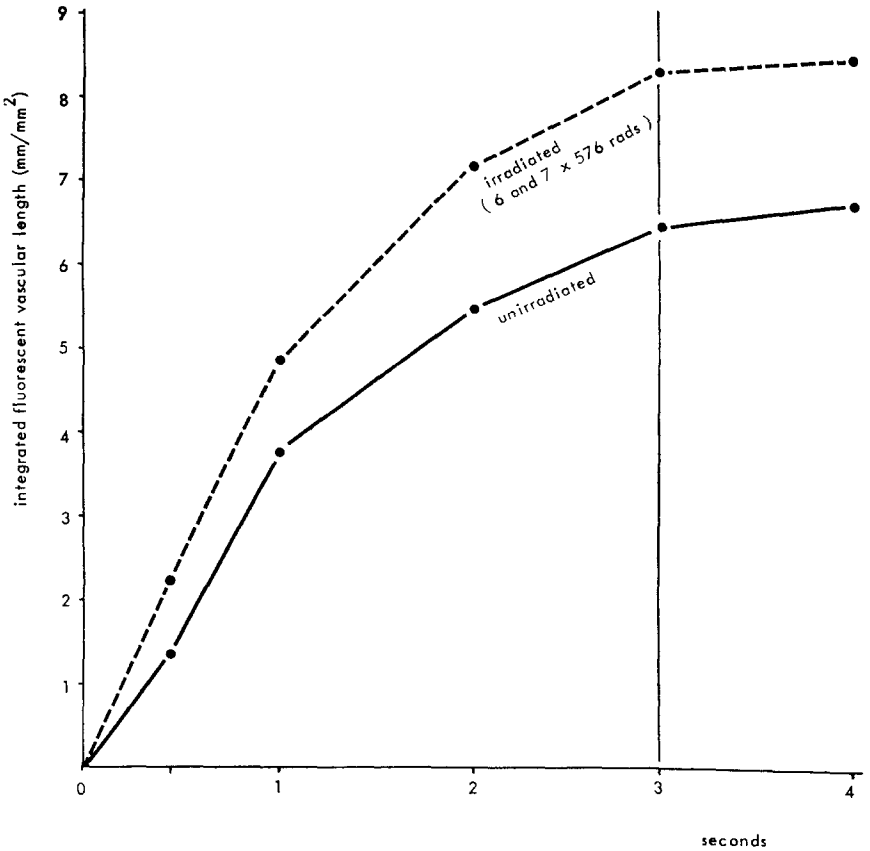


Fig. 3. The rate of inflow of intravenously injected fluorescent dye in the capillary system of the C_3HBA "sandwich" tumours.
Solid line: pooled data from 23 unirradiated tumours (day 0 and 1). Broken line: pooled date from 13 irradiated tumours (day 5 and 6).
Time 0 is the moment the dye enters the tumour vascular system.

now and then an erythrocyte passed through. Such capillaries could of course be easily overlooked by direct observation, but the same applies for those "arterial" capillaries in which the flow rate is very high. This is probably due to a different orientation of the erythrocytes in the blood stream at higher velocities.

The cinematographic fluorescent micro-angiograph facilitated the analysis of some properties of the intravascular blood flow. In order to derive measurable values the 16 mm movie film was projected frame by frame on a sheet of paper, and tracings of the progress of the dye in the blood-vessels were made by hand at $\frac{1}{2}$, 1, 2, 3 and 4 seconds after the first appearance of the dye in the supplying artery. The length of those tracings—and thus of the progress of the dye—was measured by the use of a simple curvimeter; the tumour surface was determined with the aid of a planimeter. The dye is dissolved in the plasma, and because there may be differences in velocity between erythrocytes and the plasma [16] it seems better to speak about the flow rate of the blood *plasma* rather than about the velocity of the blood as such.

It was found that the velocity of the blood plasma decreased while traversing the tumour. From the average of at least 150 measurements it was estimated that the mean flow rate of the blood plasma during the first second of its passage was of the order of 650 μ per sec (150–1500) and during the next few seconds about 300 μ per sec (100–500). Differences in flow rates of a factor of 5 to 10 were observed, even in adjacent capillaries.

It is of interest to compare the micro-circulation of different tumours, or the same tumour at different time intervals. This requires that some parameters, like the tumour size, are normalized. In addition, a value expressing the rate of flow through the blood-vessels has to be found, but also a value indicating the "density" of the capillary network. As has been discussed before: while the dye is being transported by the blood plasma through the tumour vessels, more and more capillaries emerge fluorescent. This results in an increase in the measured total length of the blood-vessel tracings, or the "integrated fluorescent vascular length". Taking also the tumour size into account, the value of the

"integrated fluorescent vascular length per mm²" is derived. This value can be regarded as a suitable indicator for the average vascular density. As can be seen from Fig. 3, the "integrated fluorescent vascular length" increases with time. The very first one or two seconds after the dye entered the tumour there is a rapid increase which tends to level off after about 4 sec, indicating that the vast majority of the vessels have become fluorescent. After 4 sec, most tumours show such a degree of extravasation of the dye, which obscures the picture, that further analysis is unrewarding. The speed of this extravascular diffusion, or extravascular streaming, differs from place to place. It was estimated to range from $<1\mu$ to 25μ per sec.

The effect of irradiation is also apparent from Fig. 3. In this figure two curves are shown; one representing the mean values of 23 unirradiated tumours (day 0 and −1), the other representing the mean values of 13 tumours (day 5 and 6) which have been irradiated for 6 or 7 days with daily doses of 576 rads. The curves show a clear difference ($P<0{\cdot}05$ at 2, 3 and 4 sec); indicating an improvement of the microcirculation of the latter group of tumours. From this graph it was

Table 1. *The response of the blood supply of the C_3HBA "sandwich" tumours to fractionated irradiation*

Day	Dose of X-rays (rads)	"Micro-circulation"*	*n*	Percentage†
−3		7·65	3	119
−2		7·06	9	109
−1		6·36	12	99
0	576	6·48	11	101
1	576	7·61	3	118
2	576	7·71	9	120
3	576	7·94	5	124
4	576	6·88	7	107
5	576	7·83	4	122
6	576	9·19	9	143
7	576	7·29	3	113
8				
9		7·6	4	118
2 and 3	unirradiated controls	4·4	4	69

*The value of the "micro-circulation" is derived from the "integrated fluorescent vascular length" (mm/mm²), at 3 sec after the first appearance of the dye in the tumour (Fig. 3).

†The 100% value refers to the mean of the pooled unirradiated tumours at day 0 and—1.

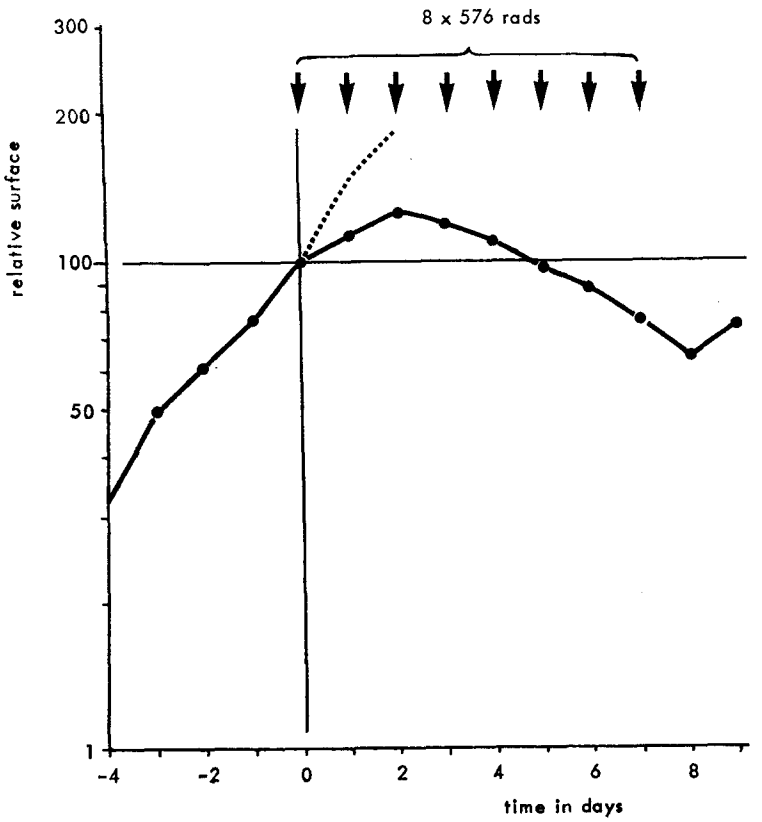


Fig. 4. [*Graph showing relative tumour size against time. Solid line: irradiated tumours. Dotted line: unirradiated controls.*

also evident that the "integrated fluorescent vascular length" value at 3 sec would yield a reasonable reliable figure for comparing the microcirculatory values of different tumours. In this way a single, absolute value per analysed filmstrip was obtained, in which not only a measure of the vascular density is incorporated, but also a dynamic factor, indicating what length of blood-vessels has been perfused in that 3-sec period.

Table 1 shows the values derived from the analysis of 82 filmstrips. It is apparent from this Table that the microcirculation in the tumours tends to increase during the irradiation treatment; the highest values are reached at about the seventh day. This is even more apparent when comparing this with the trend of the unirradiated tumours whose microcirculation tends to decline while the tumours increase in size (see Fig. 4).

DISCUSSION

The results obtained from the micro-angiographic analysis of the micro-circulation of the "sandwich" tumours indicate an improvement in micro-circulation during a course of radiotherapy with daily fractions of 576 rads. The use of the term "micro-*circulation*" seems justified in this system because a factor representing the flow rate is incorporated in the analysis of the micro-circulation. The majority of the tumours showed an improved microcirculation, outweighing the slight decrease which occurred in a few tumours. Thus the mean micro-circulation improves and the question arises whether or not the findings would be applicable to larger tumours. One could argue that a large tumour is composed of numerous small units like the ones used in these investigations. This represents a somewhat limited view however, because our tumours were strictly limited in size (less 5 mm diameter) and never reached the size in which necrosis develops. The system will require further development for this purpose.

An important question is to what extent the present findings can be helpful in explaining the mechanisms underlying the tumour "reoxygenation" during radiotherapy. Recently it has become evident that some tumours, like murine mammary tumours (Suit *et al.* [17, 18], Cheshire and Lindop [19], Dusault [11]), murine carcinomata and sarcomata (van Putten and Kallman [8], Suit [20]) and sarcomata of the rat (Thomlinson [10], Barendsen and Broerse [21]) show an improvement of the availability of oxygen for the surviving tumour cells as a result of irradiation. This phenomenon is called "reoxygenation" and some workers found that the time required for this process to take place can be very short; taking in some tumours only a few hours (Howes [22], Kallman [23]). Reoxygenation after irradiation seems however not to be a property of all experimental tumours, because at least one exception has been reported (van Putten [24]). To what extent reoxygenation of surviving malignant cells occurs in human tumours remains an open question, especially because the first hypoxic, living malignant cell in human tumours has still to be demonstrated. Howes and Field however, deduced that there may be an indication for an early reoxygenation in Friedman and Pearlman's data about chestwall recurrences [25]. Also polarographic measurements of oxygen tensions in accessible human tumours seems to indicate a facilitation of oxygen diffusion to the electrode through the irradiation treatment. Bergsjø and Evans confirmed this with their colposcopic system [26], Cater in breast tumours [27], Babib *et al.*, in a variety of tumours and metastases [28], and Evans and Naylor in carcinomata of the breast and its metastases and also in metastases of a tumour of the pyriform fossa [29]. The latter authors, using their sophisticated instrumentation, found a barely significant indication of differences in oxygen tensions before and after irradiation, but also pointed to the important question of the difficulty of obtaining representative (point) samples in an inhomogeneous tumour mass.

A radiation induced decrease of the proportion of hypoxic cells in tumours may be caused by a diminished tissue respiration as well as by an improvement of its oxygen supply.

A fall in oxygen consumption in the Sarcoma RIB_5 was reported by Thomlinson [10]. The improved oxygenation was attributed to extensive cell-death rather than to an improved circulation.

The present results seem to indicate that the improved oxygenation which has been reported for other mouse mammary tumours [11, 17–19, 22] may be related to an improved blood supply. The apparently improved flow rate (Fig. 3) as well as the increased "vascular density" [30, 31] are most marked at the 7th treatment day, when the tumour has regressed beyond its original size (Fig. 4). This seems also to be consistent with the idea that at least part of the effect is in some way related to cell decay and elimination [10, 32].

The technique presently described opens the possibility to investigate other tumours, especially the non-reoxygenating ones [24], with

regard to the response of their micro-circulation to irradiation. But, in addition, many more investigations about the tumour physiology, especially those related with the regional distribution of oxygen, become feasible when employing the "sandwich" tumour system.

The method has aready shown its usefulness in measuring the local response of the NADH fluorescence to variations in the oxygen content of the animal's blood. The intensity of the NADH autofluorescence is an indication for the local intra-cellular redox state and is measured by means of a fluorescence-microscopic set-up in conjunction with a photomultiplier [33]. Preliminary results indicate large local differences in the "sandwich" tumours as to the fluorescence response (Gosalvez *et al.* [34]). The distribution of the NADH autofluorescence-response was related to the vascular pattern, indicating heterogeneity in the distribution of oxygen through the tumour tissue. This non-destructive method may very well provide us in the future with an alternative method to bio-assays for the examination of tumours with respect to oxygenation and reoxygenation. If, in additon, the combined "sandwich" tumour/NADH fluorescence method were to be associated with the recently improved polarographic techniques (Albanese [35]) and optical oximetry (Reichert [36]), then the scrutinous examination of the factors controlling the topographical distribution of oxygen in tumour tissue would finally become within the reach of the investigator.

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REFERENCES

1. R. H. Thomlinson and L. H. Gray, The histological structure of some human lung cancers and the possible implications for radiotherapy. *Brit. J. Cancer* **9,** 539 (1955).
2. I. F. Tannock, The relation between cell proliferation and the vascular system in a transplanted mouse mammary tumour. *Brit. J. Cancer* **22,** 258 (1968).
3. H. B. Hewitt, D. P. S. Chan and E. R. Blake, Survival curves for clonogenic cells of a murine keratinizing squamous carcinoma irradiated *in vivo* or under hypoxic conditions. *Int. J. Radiat. Biol.* **12,** 535 (1967).
4. H. B. Hewitt, Studies of the dissemination and quantitative transplantation of a lymphocytic leukaemia of CBA mice. *Brit. J. Cancer* **12,** 378 (1958).
5. W. E. Powers and L. J. Tolmach, Demonstration of an anoxic component in a mouse tumor-cell population by *in vivo* assay of survival following irradiation. *Radiology* **83,** 328 (1964).
6. R. H. Thomlinson and E. A. Craddock, The gross response of an experimental tumour to single doses of X-rays. *Brit J. Cancer* **21,** 108 (1967).
7. H. D. Suit and M. Maeda, Hyperbaric oxygen and radio biology of a C3H mouse mammary carcinomas. *J. nat. Cancer Inst.* **39,** 639 (1967).
8. L. M. van Putten and R. F. Kallman, Oxygenation status of a transplantable tumor during fractionated radiation therapy. *J. nat. Cancer Inst.* **40,** 441 (1968).
9. H. D. Suit, Hyperbaric oxygen in radiotherapy of four mouse tumors. In: *Proc. Int. Conf. on Radiation Biology and Cancer, Kyoto, Japan,* p. 39 (1966).
10. R. H. Thomlinson, Changes of oxygenation in tumours in relation to irradiation. *Front. Radiation Ther. Onc.* **3,** 109 (1968).
11. L. A. Du Sault, Reoxygenation of tumours during fractionated radiotherapy. *Radiology* **92,** 626 (1969).
12. G. H. Algire, An adaptation of the transparent-chamber technique to the mouse. *J. nat. Cancer Inst.* **4,** 1 (1943).
13. R. Merwin, G. H. Algire and H. S. Kaplan, Transparent-chamber observations of the response of a transplantable mouse mammary tumor to local roentgen irradiation. *J. nat. Cancer Inst.* **11,** 593 (1950).
14. M. M. Kligerman and D. K. Henel, Some aspects of the microcirculation of a transplantable experimental tumor. *Radiology* **76,** 810 (1961).
15. C. M. Goodall, A. G. Sanders and P. Shubik, Studies of vascular patterns in living tumours with a transparent chamber inserted in hamster cheek pouch. *J. nat. Cancer Inst.* **35,** 497 (1965).
16. M. Kampp, O. Lumdgren and N. J. Nilsson, Extravascular shunting of oxygen in the small intestine of the cat. *Acta physiol. scand.* **72,** 396 (1968).

17. H. D. Suit and J. V. Schiavone, Effect of a single dose of radiation on proportion of hypoxic cells in a C3H mouse mammary carcinoma. *Radiology* **90,** 325 (1968).
18. H. Suit, R. Lindberg, C. Suchato and A. Ozenne, Radiation dose fractionation and high pressure oxygen in radiotherapy of the DBA mouse mammary carcinoma. *Amer. J. Roentgenol.* **99,** 895 (1967).
19. P. J. Cheshire and P. J. Lindop, The influence of intracellular recovery and hypoxic cells on the radiation response of mammary tumours and skin in C3H mice. *Brit. J. Radiol.* **42,** 215 (1969).
20. H. D. Suit, C. Suchato, Hyperbaric oxygen and radiotherapy of a fibrosarcoma and of a squamous-cell carcinoma of C3H mice. *Radiology* **89,** 713 (1967).
21. G. W. Barendsen and J. J. Broerse, Experimental radiotherapy of a rat rhabdomyosarcoma with 15 MeV neutrons and 300 kV X-rays—I. Effects of single exposures. *Europ. J. Cancer* **5,** 373 (1969).
22. A. E. Howes, An estimation of changes in the proportions and absolute numbers of hypoxic cells after irradiation of transplanted C3H mouse mammary tumours. *Brit. J. Radiol.* **42,** 441 (1969).
23. R. F. Kalmann, In the discussion of an article of R. H. Thomlinson: Reoxygenation as a function of tumor size and histopathological type. In *Conf. on Time and Dose Relationships in Radiation Biology as Applied to Radiotherapy*, p. 248, held at Carmel, Ca., September 1969.
24. L. M. van Putten, Tumour reoxygenation during fractionated radiotherapy; studies with a transplantable mouse oestosarcoma. *Europ. J. Cancer* **4,** 173 (1968).
25. A. E. Howes and S. B. Field, The oxygen effect: a new look at "Friedman and Pearlman". *Brit. J. Radiol.* **41,** 554 (1968).
26. P. Bergsjø and J. C. Evans, Oxygen tension of cervical carcinoma during the early phase of external irradiation—I. Measurements with a Clark micro electrode. *Scand. J. clin. Lab. Invest.* **22** (suppl. 106) 159 (1968).
27. D. B. Cater, Oxygen tension in neoplastic tissues. *Tumori* **50,** 435 (1964).
28. A. O. Badib and J. H. Webster, Changes in tumor oxygen tension during radiation therapy. *Acta Radiol.* **8,** 247 (1969).
29. N. T. S. Evans and P. F. D. Naylor, The effect of oxygen breathing and radiotherapy upon the tissue oxygen tension of some human tumours. *Brit. J. Radiol.* **36,** 418 (1963).
30 P. Rubin and G. Casarett, Microcirculation of tumors—II. The supervascularized state of radiated regressing tumors. *Clin. Radiol.* **17,** 346 (1966).
31. H. S. Reinhold, The post-irradiation behaviour of transplantable solid tumours in relation to the regional oxygenation. In *Proc. 11th int. Congr. Radiology, Rome* p. 1482 (1965).
32. A. F. Hermens and G. W. Barendsen, Changes of cell proliferation characteristics in a rat rhabdomyosarcoma before and after X-irradiation. *Europ. J. Cancer* **5,** 173 (1969).
33. B. Chance and V. Legallais, A spectrofluorometer for the recording of intracellular oxidation–reduction states. *IEEE Trans. Bio-Med. Elect.* **10,** 40 (1963).
34. M. Gosalvez, R. G. Thurman and H. Reinhold, Local intracellular redox state of tumors *in vivo*. In *Fed. Europ. Biochem. Soc. Abstr. Commun.* Madrid, Spanish Biochemical Society, No. 748, 1969.
35. R. A. Albanese, C. H. Chang and W. B. Seaman, Determination of the absolute oxygen tension in tissues by a new microelectrode technique. In: *Third Int. Conf. on Hyperbaric Medicine, N.A.S., Washington*, p. 294, 1966.
36. W. J. Reichert, The theory and construction of oximeters. In *A Symposium on Oxygen Measurements in Blood and Tissues and their Significance* (Edited by J. P. Payne and D. W. Hill), p. 81. Churchill, London (1966).

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Studies on the Anti-Tumour Activity of Aliphatic Aldehydes—III.

Formation of Thiazoidine-4-Carboxylic Acid in Tissues*

L. LORETI, M. E. FERIOLI, G. C. GAZZOLA and G. G. GUIDOTTI

Centro di Studio per la Patologia Cellulare del C.N.R., Istituto di Patologia Generale, Università di Milano, Via Mangiagalli, 31, 20133 *Milan, Italy*

Abstract — *The formation of a thiazolidine-4-carboxylic acid in normal and neoplastic tissues as a result of the condensation of endogenous cysteine with an added aliphatic aldehyde (C^{14}-labelled α,β-dihydroxybutyraldehyde) has been followed by electrophoretic-chromatographic methods on paper and silica-gel thin-layer, radioactive scanning and autoradiographic techniques. The identification of thiazolidine carboxylic acids in tissues treated with aliphatic aldehydes indicated that the thiazolidine condensation at the expense of endogenous cysteine occurred freely under physiological conditions and reinforced previous evidence suggesting that a lowered availability of cysteine in tissue (and the consequent imbalance of the protein-synthesizing amino acid pool) could explain the inhibitory effect of aliphatic aldehydes on protein biosynthesis.*

INTRODUCTION

THE ANTI-TUMOUR activity of aliphatic aldehydes has been ascribed to their inhibitory effect on protein synthesis [1, 2]. Attempts to elucidate the mechanism of this inhibition suggested that aliphatic aldehydes might condense with cysteine to form thiazolidine carboxylic acids [3], thus depriving the tissues of this thio-amino acid. In turn, the resulting imbalance of the protein-synthesizing amino acid pool and the lowered availability of sulphydryl compounds would impair the synthetic process.

Under physiological conditions of temperature and pH the condensation of aliphatic aldehydes with L-cysteine occurs very rapidly and the equilibrium of the reaction favors the formation of the thiazolidine ring [3]. However, as far as the authors are aware, no direct proof has been given hitherto for the occurrence of this reaction at the expense of intracellular cysteine in tissues treated with aliphatic aldehydes.

It is the purpose of the present paper to define methods and conditions under which thiazolidine carboxylic acids can be detected in tissues. The formation of 2-(1',2'-dihydroxypropyl)-thiazolidine-4-carboxylic acid in normal and neoplastic tissues upon addition of α,β-dihydroxybutyraldehyde is reported below.

MATERIAL AND METHODS

In vitro *experiments*

Wistar albino rats of either sex, weighing 160–180 g were used in these experiments. They were maintained on a diet of commercial rat pellets supplied *ad libitum* and ordinary tap

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water. Livers, removed from fed animals, were quickly weighed and homogenized in 4–8 vol of ice-cold Krebs-Ringer phosphate [4] equilibrated with N_2. Yoshida ascites hepatoma cells (AH-130), harvested from tumour-adapted rats, were washed exhaustively to remove the ascitic fluid and resuspended in Krebs-Ringer phosphate as described previously [5]. Hepatoma cells were used either as suspension or after being homogenized in a Virtis waring blendor at 2°C under N_2 atmosphere (homogenization has been followed by microscopic examination). Samples of cell suspension were collected for tissue dry weight determinations.

Incubations were carried out in a Dubnoff metabolic shaker oscillating at a rate of 50 cycle/min at 37°C. Five ml of tissue homogenate or cell suspension (0·5–1 g of tissue wet wt.) were placed in the main compartment of a Thunberg vessel equipped with a two-way stopcock. The sideharm contained 0·5 ml of 4 mM DL-*erythro*, *threo*-α,β-dihydroxybutyraldehyde-U-C^{14} (s.a. 1·05 mc/mmole). After adequate evacuation to remove residual oxygen and equilibration with N_2, the content of the side harm was tipped into the main compartment. Incubation lasted 30 min with homogenates and 45 min with cell suspensions. Anaerobic conditions were selected to prevent the utilization of thiazolidine-4-carboxylic acid possibly formed during the incubation [6]. At the end of the incubation period, 20 ml of ice-cold ethanol were quickly added to stop the reaction and to precipitate proteins.

In vivo *experiments*

The animals used were female Swiss mice weighing 25–30 g. After a two-hour fast, mice were injected intravenously (dorsal vein of the tail) with 0·5 ml of 0·15 M DL-*erythro*, *threo*-α,β-dihydroxybutyraldehyde-U-C^{14} (s.a. 1·05 mc/mmole) and sacrificed by decapitation 5 min and 20 min after aldehyde inoculation. The liver was quickly removed, placed on the base of an aluminium cone immersed in liquid N_2. The frozen material in the form of small fragments was weighed and transferred into a Potter tube containing 15 vol. of 80% ethanol pre-cooled to 0°C and homogenized. In some experiments mice were injected intraperitoneally with L-cysteine (1 mg/g of body weight) 60 min before the aldehyde inoculation [7].

Analytical procedures

All the hydro–alcoholic suspensions were centrifuged at 12,000 **g** for 30 min in the cold. The supernatants were transferred into flasks equipped with Vigreux columns connected to a vacuum pump and evaporated to dryness under mild heating. The sediment was dissolved in 0·8 ml of water and the resulting cloudy fluid was centrifuged at 20,000 **g** for 30 min in the cold. The clear supernatant was frozen and maintained at −30°C until analyses could be carried out.

Electrophoresis was performed either on Schleicher & Schüll filter paper (type No. 2317) or on silica-gel-coated polyethylene terephthalate Eastman chromatogram sheets (type 6061) in a mixture of acetic acid–formic acid–water (10: 15: 75), pH 1·8, with 10 v/cm for 7 hr at −10°C. Chromatographic analyses, either independent or coupled to electrophoresis as descending (paper) or ascending (silica gel) right-angle development were performed at 2°C using the following solvent systems: a. water-saturated *n*-butanol-acetic acid (4: 1) for 8 hr (silica gel) and 12–18 hr (paper); b. *tert.*-butanol–methanol–water (4: 5: 1) for 8 hr (silica gel) and 12 hr (paper); c. water-saturated *n*-butanol–ethanol (5: 1) for 12–16 hr (paper); d. *tert.*-butanol–methylethylketone–water (3: 1: 1) for 7 hr (silica gel). Thiazolidine-4-carboxylic acid was located with ninhydrin; aldehydes were detected with aniline phthalate; labelled compounds were traced by autoradiography and autofluorography or by radioactivity measurements made with a radiochromatogram scanner (model 7201, Packard Instrument Co. Inc.).

Chemicals

DL-*erythro*, *threo*-α,β-dihydroxybutyraldehyde and 2-(1',2'-dihydroxypropyl)-thiazolidine-4-carboxylic acid were obtained from Laboratori Farmitalia, Milan, Italy. DL-*erythro*, *threo*-α,β-dihydroxybutyraldehyde-U-C^{14} has been synthesized from $Na_2C^{14}O_3$ (EURATOM Contract No. 046-64-1 RISI) and 2-(1',2'-dihydroxypropyl)-thiazolidine-4-carboxylic acid labelled with C^{14} was obtained by condensation of DL-*erythro*, *threo*-α,β-dihydroxybutyraldehyde-U-C^{14} with unlabelled L-cysteine. Proanalysis grade acetic acid, formic acid, methanol, ethanol, *n*-butanol, *tert*-butanol and methylethylketone were purchased from E. Merck A.-G., Darmstadt, Germany.

RESULTS

Evidence for the formation of a thiazolidine-4-carboxylic acid in tissues as a result of the condensation of endogenous cysteine with added aliphatic aldehyde has been obtained using one-dimensional chromatographic and two-dimensional electrophoretic–chromato-

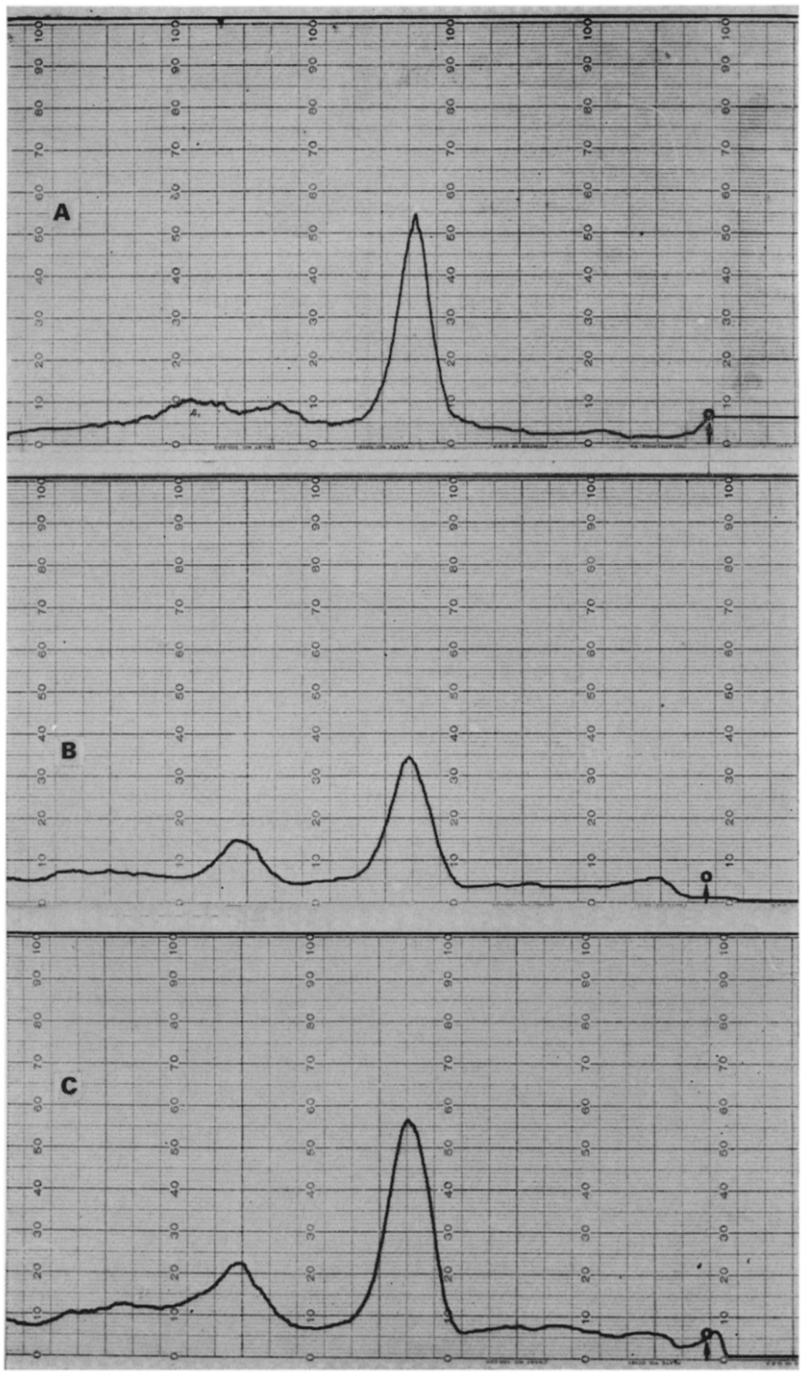


Fig. 1. *Formation of thiazolidine carboxylic acid by homogenates of Yoshida ascites hepatoma cells incubated with* α,β*-dihydroxybutyraldehyde-U-*C^{14}. *Radioscans of paper chromatograms developed in water-saturated* n*-butanol-acetic acid* (4: 1): (A) C^{14}*-labelled* 2-(1′,2′*-dihydroxypropyl*)*-thiazolidine-*4*-carboxylic acid standard* (1·5 μg, *s.a.* 5 μc/*mg*)*; the same compound* (*unlabelled*, 15 μg) *is located by ninhydrin on the underlying chromatogram;* (B) *supernatant of homogenate. The highest peak of radioactivity exhibits the same migration characteristics as the thiazolidine carbyxolic acid standards. Fast-moving peaks are over the migration area of* α,β*-dihydroxybutyraldehyde;* (C) *supernatant of homogenate plus* C^{14}*-labelled* 2-(1′,2′*-dihydroxypropyl*)*-thiazolidine-*4*-carboxylic acid standard* (1 μg, *s.a.* 5 μc/*mg*). *Note the increase of the peak of radioactivity associated with the zone of migration of thiazolidine carboxylic acid.*

(to face p. 282)

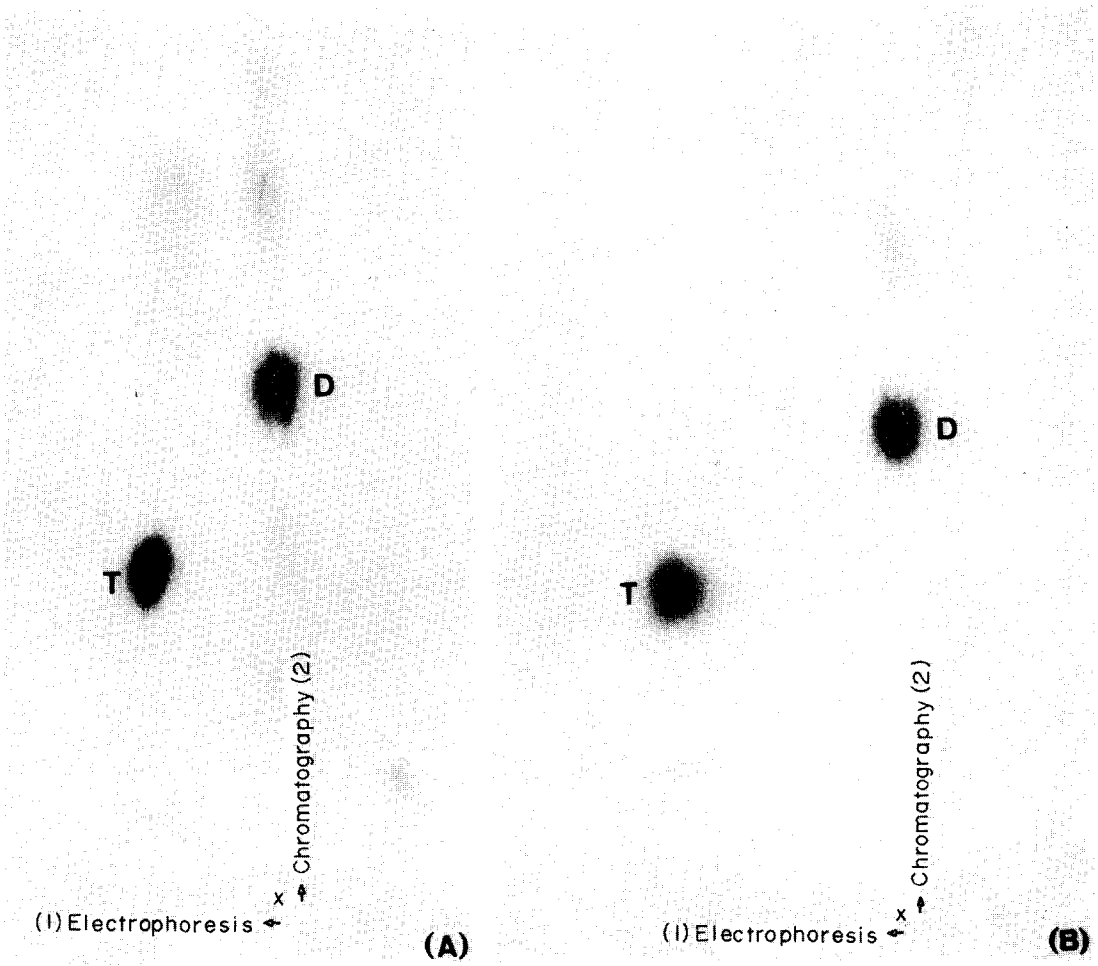


Fig. 2. (A) *Autoradiogram of two-dimensional electrophoretic–chromatographic separation on filter paper of supernatant of homogenate from Yoshida ascites hepatoma cells incubated with* α,β-*dihydroxybutyraldehyde-U-*C^{14}*;* (B) *Fluorogram of two-dimensional electrophoretic chromatographic separation on filter paper of extracts of liver from cysteine-preloaded mice injected with* α,β-*dihydroxybutyraldehyde* 5 *min before sacrifice. Electrophoretic separation carried out in acetic acid–formic acid–water* (10: 15: 75), *pH* 1·8; *chromatographic development, as right-angle descending migration, performed in water-saturated* n-*butanol-acetic acid* (4: 1). *Following autoradiography* (*Kodirex, Kodak Ltd.; exposure* 8 *days*) *or autofluorography* (*Regulix, Kodak Ltd.; exposure* 7 *days after treatment of the paper with the Omnispray scintillator; New England Nuclear*) *the chromatograms were stained with ninhydrin. T indicates the radioactive spots which coincide with the spots of added* 2-(1′.2′-*dihydroxypropyl*)-*thiazolidine*-4-*carboxylic acid* (*unlabelled standard*) *as located by the ninhydrin reagent. D indicates the radioactive spots corresponding to the zones of location of the major component of C-*^{14}*labelled* α,β-*dihydroxybutyraldehyde used in the present study along the chromatographic migration.*

graphic systems on paper and silica-gel thin-layer.

The radiochromatographic pattern of supernatants of homogenates from Yoshida ascites hepatoma cells incubated with C^{14}-labelled α,β-dihydroxybutyraldehyde showed a peak of radioactivity associated with a zone corresponding to that traced after independent migration or comigration of samples of authentic 2-(1',2'-dihydroxypropyl)-thiazolidine-4-carboxylic acid (Fig. 1). Essentially similar results were recorded when surviving cell suspensions of ascites hepatoma were incubated with the labelled aldehyde.

A single peak of radioactivity, corresponding to the area of location of pure 2-(1',2'-dihydroxypropyl)-thiazolidine-4-carboxylic acid, has been evidenced in supernatants of homogenates and extracts of cell suspensions from ascites hepatoma incubated with α,β-dihydroxybutyraldehyde when analysed by two-dimensional electrophoretic-chromatographic systems.

The radiochromatographic patterns of supernatants of rat liver homogenates incubated with α,β-dihydroxybutyraldehyde-C^{14} and of extracts of liver from C^{14}-labelled aldehyde-injected mice (untreated or preloaded with L-cysteine) exhibited a peak of radioactivity associated with a zone corresponding to the area of migration of authentic 2-(1',2'-dihydroxypropyl)-thiazolidine-4-carboxylic acid. This peak was higher in liver extracts from cysteine-preloaded than from untreated mice and decreased with time after aldehyde inoculation. A remarkable finding in liver was the presence of peaks of radioactivity associated with slow-moving compounds which were absent in aldehyde-treated neoplastic tissue.

The results obtained by the radiochromatogram scanning technique have been confirmed by autoradiography and autofluorography which allowed unambiguous identifications of radioactive spots on the autoradiogram with the corresponding spots located by specific reagents on the chromatogram. Figure 2 shows the appearance of radioactive spots coincident with the location of 2-(1',2'-dihydroxypropyl)-thiazolidine-4-carboxylic acid in supernatants of homogenates from ascites hepatoma cells incubated with α,β-dihydroxybutyraldeydeh-U-C^{14} (*A*) and in extracts of liver from C^{14}-labelled aldehyde-injected mice (*B*).

DISCUSSION

The reactivity of aliphatic aldehydes toward sulphydryl groups and, in particular, their condensation with cysteine to form thiazolidine carboxylic acids have been known for a long time [8, 9]. More recently the formation of thiazolidine-4-carboxylic acids at the expense of intracellular cysteine and the consequent imbalance of the protein-synthesizing amino acid pool has been suggested as an explanation for the inhibition of protein synthesis that occurs in tissues treated with aliphatic aldehydes [3, 5].

The identification of a thiazolidine-4-carboxylic acid in tissues after incubation *in vitro* or injection *in vivo* with C^{14}-labelled α,β-dihydroxybutyraldehyde, as reported in this paper, indicates that the condensation of exogenous aldehydes with endogenous cysteine occurs freely under physiological conditions.

We recognize that the various experiments described above do not allow to extrapolate much beyond this conclusion. Indeed, the occurrence of 2-(1',2'-dihydroxypropyl)-thiazolidine-4-carboxylic acid in supernatants of aldehyde-treated liver homogenates and in extracts of liver from aldehyde-injected animals suggests, but does not prove, that the intracellular cysteine is involved in the thiazolidine condensation, since the participation to the reaction of the amino acid present extracellularly (mainly in plasma) cannot be excluded. Perhaps, a more adequate support for the involvement of intracellular cysteine in the thiazolidine synthesis comes from experiments with ascites tumour cells. Used as cell suspension or as cell homogenate, this neoplastic tissue has been completely freed from ascitic fluid before being incubated *in vitro*. Yet, the synthesis of the thiazolidine ring has been shown to take place upon addition of aldehyde. Even in these experiments, however, our procedure does not discriminate between intracellular synthesis of thiazolidine carboxylic acid and extracellular synthesis of this compound at the expense of intracellular cysteine eventually released during the incubation.

In spite of these uncertainties, the identification of a thiazolidine carboxylic acid in tissues treated with aliphatic aldehydes supports the conclusion that the thiazolidine condensation must be regarded as a primary step leading to a decrease of the concentration of endogenous cysteine upon aldehyde treatment and reinforces previous evidence [3] that one of the mechanisms by which aliphatic aldehydes inhibit protein synthesis is by lowering the intracellular availability of cysteine. Results showing that the content of free cysteine in tissues, as estimated by the method of Gaitonde [10], decreases after aldehyde inoculation [11] lead to the same conclusion.

An additional finding in our study deserves consideration. The radiochromatographic patterns from C^{14}-aldehyde-treated liver tissue show the presence of peaks of radioactivity associated with slow-moving substances which do not appear in ascites hepatoma. So far it has been impossible to determine the nature of these compounds. The facts that these peaks of radioactivity are larger when the concentration of cysteine in liver tissue is expected to be low (untreated mice) whereas they are smaller at high cysteine concentrations in the tissue (cysteine-preloaded mice) and that they tend to increase as the peak of thiazolidine-4-carboxylic acid decreases with time, might indicate that the affinity of the aldehyde for cysteine is greater than that for the unidentified compounds and suggest that the slow-moving peaks of radioactivity found in liver tissue represent simple addition compounds of the aldehyde [8].

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REFERENCES

1. E. Ciaranfi, L. Loreti, A. Borghetti and G. G. Guidotti, Studies on the anti-tumour activity of aliphatic aldehydes — II. Effects on survival of Yoshida ascites hepatoma-bearing rats. *Europ. J. Cancer* **1,** 147 (1965).
2. G. G. Guidotti, A. F. Borghetti, M. E. Ferioli, B. Giglioni, L. Loreti, S. Ottolenghi and E. Ciaranfi, Studi sulla sintesi proteica nei tessuti neoplastici. Inibizione da aldeidi alifatiche. *Atti Soc. ital. Patol.* **11,** 139 (1969).
3. G. G. Guidotti, L. Loreti and E. Ciaranfi, Studies on the anti-tumour activity of aliphatic aldehydes — I. The mechanism of inhibition of amino acid incorporation into protein of Yoshida ascites hepatoma cells. *Europ. J. Cancer* **1,** 23 (1965).
4. W. W. Umbreit, R. H. Burris and J. F. Stauffer, *Manometric Techniques*, 3rd Edition. Burgess, Minneapolis (1959).
5. G. G. Guidotti, A. Fonnesu and E. Ciaranfi, Inhibition of amino acid incorporation into protein of Yoshida ascites hepatoma cells by glyceraldehyde. *Cancer Res.* **24,** 900 (1964).
6. L. Loreti, A. F. Borghetti, M. E. Ferioli and G. G. Guidotti, Il metabolismo degli acidi tiazolidin-4-carbossilici nei tessuti normali e neoplastici. *Atti Soc. ital. Patol.* **10,** 851 (1967).
7. C. R. Ball, Estimation and identification of thiols in rat spleen after cysteine or glutathione treatment: relevance to protection against nitrogen mustards. *Biochem. Pharmacol.* **15,** 809 (1966).
8. M. P. Schubert, Compounds of thiol acids with aldehydes. *J. biol. Chem.* **114,** 341 (1936).
9. S. Ratner and H. T. Clarke, The action of formaldehyde upon cysteine. *J. Amer. chem. Soc.* **59,** 200 (1937).
10. M. K. Gaitonde, A spectrophotometric method for the direct determination of cysteine in the presence of other naturally occurring amino acids. *Biochem. J.* **104,** 627 (1967).
11. M. E. Ferioli and G. C. Gazzola (unpublished results).

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Unfavourable Effects of Long-Term Adjuvant Chemotherapy with Endoxan in Radically Operated Bronchogenic Carcinoma

K. W. BRUNNER,* TH. MARTHALER† and W. MÜLLER‡
Swiss Chemotherapy Group§
and Departments of Surgery of the University Clinics of Switzerland¶

ADJUVANT, so-called prophylactic chemotherapy, is being more and more widely used in various carcinomas after radical operation with the intention or hope of preventing, or at least postponing recurrent disease resulting from minimal residual tumour and thus prolonging survival. Its effectiveness has, so far, only been proven in very few neoplasias such as Wilm's tumour [1, 2], choriocarcinoma [3] and possibly malignant testicular tumours [4].

A number of well controlled clinical studies have demonstrated that short-term prophylactic chemotherapy, given for a limited number of days before and after radical surgery of bronchogenic carcinoma has no effect on survival and, at certain dose levels, may increase the risk of post-operative death [1, 6–14].

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*Evaluation and manuscript.
†Biostatistisches Zentrum der medizinischen Fakultät der Universität Zürich.
‡Representant of the participating departments of surgery.
§Onkologische Station, Inselspital Bern and Center for evaluation: K. W. Brunner, R. W. Sonntag, H. J. Ryssel; Hämatologisch-Onkologische Station, Kantonsspital Zürich: G. Martz, Chr. Sauter; Centre d'Oncohématologie, Hôpital Cantonal, Genève: P. Maurice, P. Alberto, L. Wagenknecht; Onkologisch-hämatologische Station, Medizinische Universitäts-Poliklinik Basel: H. J. Senn, W. Rhomberg.
¶Chirurgische Universitätskliniken von Bern (B. H. Nachbur), Zürich (K. Schriber), Basel (W. Müller), Genf (R. P. Mégevand) und Lausanne (J. Pettavel).

Given the decreased viability of freshly released cancer cells entering the blood stream during or after operation and considering the long doubling time of solid tumours with their considerable pool of resting, non-dividing cells, these results are not surprising. Furthermore, simple chemotherapy, with one drug, is only marginally effective in bronchogenic carcinoma, with a tumour cell kill of less than one log, that is less than 90% in the most sensitive cases. There are no experimental data suggesting that tumour-cell kill is greater when there is only minimal residual disease.

Long-term prophylactic chemotherapy therefore seems to be more logical and promising. Few studies in this direction have so far been performed. In 1967 Karrer in a non-randomized study claimed beneficial effects of long-term adjuvant chemotherapy with Endoxan, given intermittently over a period of 3 years [15, 16]. He found that survival was only prolonged in patients who at least once during the long-term chemotherapy had a fall of leucocytes below 3000. The control group, to which survival of the treated group was compared, was not randomized and the criteria for selection of patients for treatment and no-treatment are not stated.

MATERIAL AND METHODS

In March 1967 the Swiss Chemotherapy Group, in co-operation with the Departments of Surgery of the University Clinics in Switzer-

land, initiated a strictly randomized and controlled study of long-term chemoprophylaxis with Endoxan in radically operated patients with bronchogenic carcinoma. All radically operated patients were randomized into a group treated with Endoxan and a group with no further treatment. The patients in both groups were seen at regular intervals and evaluated. Treatment with Endoxan was started between 1 and 2 weeks after radical surgery. It consisted of weekly injections of 12 mg/kg of Endoxan for a total dose of 100 mg/kg per course. Six intermittent courses were to be given over a period of 2 years, one course every 4 months. When local recurrence or metastases occurred, further treatment was given according to the judgment of the investigator, but all patients were to be followed until death. Patients in both groups had thorough clinical, radiological and laboratory controls once monthly. The number of recurrences, duration of the recurrence-free period and the time from recurrence to death were determined in both groups. The acceptance of new patients into the study was stopped in April 1969, when the prefixed sample size of 100 patients per group was almost reached and a constant unfavourable trend for the group treated with Endoxan was observed. In May 1970 a preliminary evaluation of all aspects of the study was performed. This provided a definite trend for the effects of post-operative Endoxan treatment.

Table 1 shows all procedures for the total number of patients evaluated by the member institutions up to the final randomization. Of a total of 504 patients, 156 patients were excluded before operation according to protocol: (1) because they were clinically judged inoperable (118 patients), (2) only palliative resection was planned (11 patients), (3) no strict follow-up seemed possible (foreigners etc.) (27 patients).

To the remaining 348 patients operated with the intent of a radical resection, a closed envelope was assigned, containing the randomization card with the group (Endoxan or control), to which the patient was allocated when radically resectable. The numbered

Table 1. *Randomization procedure*

		No. of cases	E	K
1.	Total No. of patients seen by the member institutions:	504		
2.	Patients excluded from randomization before operation:	156		
	Reasons for exclusion before operation in 156 patients:			
	—clinically judged inoperable:	118 pat.		
	—only palliative resection planned:	11 pat.		
	—no follow-up possible (foreigners, etc.):	27 pat.		
		156 pat.		
3.	Total No. of patients with intended radical surgery:	348		
4.	Patients excluded after operation according to protocol:	159	79	80
	Reasons for exclusion after operation:			
	—not radically resectable:	87 pat.	40	47
	—diagnosis other than bronchogenic carcinoma	37 pat.	22	15
	—post-operative deaths during first month:	28 pat.	12	16
	—lost to follow-up:	7 pat.	5	2
		159 pat.		
5.	Total No. of evaluable randomized patients with radically resected bronchogenic carcinoma:	189	95	94

% patients operated: 69%.
% radical operations: 48% (resectability rate).
% post-operative deaths: 12·5% (radically resected cases only).

Table 2. Endoxan prophylaxis; recurrences and deaths for equal total periods of observation

	Total patients	Died from other causes	Evaluable cases	Without recurrence (No.) (%)	With recurrence (No.) (%)	Died from carc. (No.) (%)	Living with recurrence
Endoxan	95	4	91	48=52·7	43=47·3	35=38·4	8
Controls	94	6	88	65=73·9	23=26·1	20=22·7	3
Total	189	10	179	113=63·1	66=36·9	52=29·0	11

envelopes were drawn in sequence according to the dates of operation and were opened after the operation. All envelopes and randomization cards together with detailed information on operative findings and histology were sent to the evaluation centre. This procedure was chosen in order to have detailed information on every patient considered for radical resection and to prevent exclusion from the study for undocumented reasons. The reasons for exclusion after the operation were stated in the protocol in advance. They were the following: (1) no radical or questionable radical resection possible, (2) a disease other than the clinically diagnosed lung cancer found at the operation, (3) post-operative death during the first month after operation. The reasons for exclusion after the operation had to be documented in detail for each patient. This was the case for 159 patients, including 7 patients lost to follow up after the operation. For 79 of the excluded cases, the randomization card in the envelope would have prescribed treatment with Endoxan, 80 patients would have been allocated to the control group. This proves, in addition to the correct documentation of every case excluded, a correct randomization.

RESULTS

This leaves 189 radically operated patients evaluable for the study, 95 patients in the treated and 94 patients in the untreated group.

Table 2 gives the total number of recurrences and deaths for equal lengths of observation in both groups up to a total period of 3 years. Four patients in the treated and 6 patients in the control group died of causes other than cancer and are not evaluable for the study. Of the remaining 91 patients in the Endoxan group 43 or 47·3% had local recurrence or metastases during the observation period and 35 or 38·5% have died from recurrent cancer. In the untreated control group, 23 of 88 patients or 26·1% demonstrated recurrent disease and 20 or 22·7% have died from cancer. The differences are statistically significant.*

In Fig. 1 the cumulative recurrence rate during the period of 3 years is demonstrated for both groups according to the life-table method. Post-operative deaths and patients dying from causes other than cancer are excluded. This, together with the strict criteria for radical operation in the selection of patients, accounts for the relative low recurrence rate in this series. Recurrence rate only was chosen for the evaluation of the study, because it was felt that this is a more valuable criterion for the effect of adjuvant chemotherapy than life-span, which may be influenced by additional therapy following recurrence. As can be seen, all recurrences so far have occurred during the first 24 months of observation in both groups. The difference of both curves is statistically significant at nearly all points ($P<0·05$). After 15 months the curves tend to become parallel and run strictly parallel after 24 months. This coincides with the end point of treatment with Endoxan and provides further evidence, that the higher rate of recurrences in the treated group is directly related to the Endoxan treatment. Contrary to the incidence of recurrences, the mean and median time to recurrent disease and death, calculated from the date of operation, is not significantly different in both groups. Endoxan therefore does not postpone recurrent disease or death (Table 3).

All tests for the randomization procedure (age distribution, distribution of histological types, distribution of operative procedure) demonstrate that randomization was effective and the difference of the treated and untreated

*For difference in recurrence rate: $x^2=4·49$, $0·05> Px^2 >0·025$; for difference in death rate: $x^2=7·69$, $0·01> Px^2 >0·005$ (x^2 using Yates' correction for continuity).

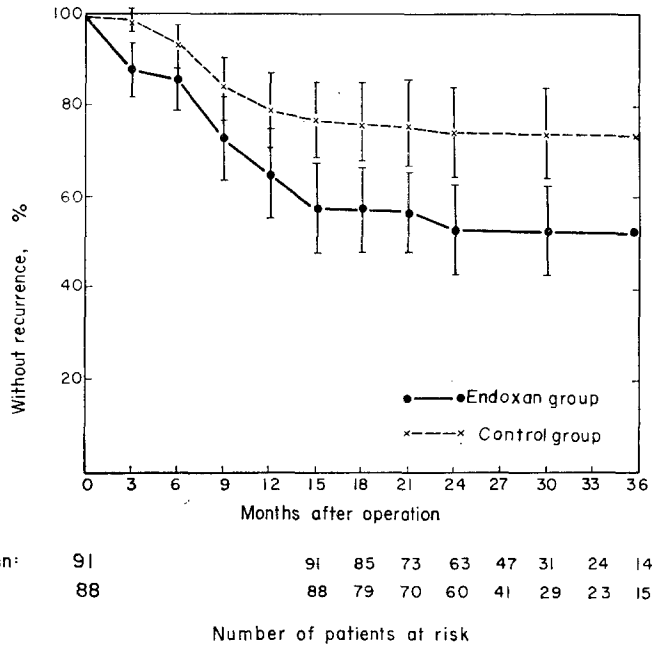


Fig. 1. *Endoxan prophylaxis; cumulative incidence of recurrences (life table methods).*

group is correlated to the Endoxan treatment alone.

Table 4 shows the distribution of histological types in both groups which is very similar. At the same time number and percentages of recurrences for each histological type is given. As can be seen, the total recurrence rate in both groups together is much higher for oat-cell and anaplastic carcinoma than for epidermoid and adenocarcinoma. Total recurrence rate in differentiated and undifferentiated epidermoid carcinoma is very similar, but in the Endoxan treated group the recurrence rate for undifferentiated epidermoid carcinoma is nearly double that in the control group. The difference in the treated and untreated group is even higher in patients with adeno-carcinoma, where more than 50% relapsed in the treated group, vs. only 13% in the control group.*

In Table 5 the distribution of operative procedures in both groups is presented. Of all patients, 3·9% had segmental resection, 44·1% had a lobectomy, 11·2% a bilobectomy and 40·8% a pneumonectomy. There are more pneumonectomies in the Endoxan group than in the control group. But the nearly identical recurrence rate in patients with lobectomy and pneumonectomy in this group suggests that the modality of operative procedure did not influence the rate of recurrences. This conclu-

*The subgroups according to histology or mode of operation are too small for a separate statistical evaluation and none of the differences are therefore statistically significant. (F.i. the difference in undifferentiated squamous cell carcinoma has a P value of 0·08.)

Table 3. *Endoxan prophylaxis; time from operation to recurrence, from recurrence to death and total survival*

	Time from operation to recurrence (months)		Time from recurrence to death (months)	Average total survival (months)	
	Mean	Median	Mean	Mean	Median
Endoxan	8·9	7·95	3·6	12·5	9·0
Controls	9·6	8·25	4·6	14·1	9·1

Table 4. Endoxan prophylaxis; histology

	Total patients	Differentiated squamous-cell carcinoma			Undifferentiated squamous-cell carcinoma			Adenocarcinoma			Oat-cell+anaplastic carcinoma		
		Total	Rec.	%	Total	Rec.	%	Total	Rec.	%	Total	Rec.	%
Endoxan	91	43	16	37	27	14	52	11	6	54	10	7	70
Controls	88	44	11	25	30	8	27	8	1	13	6	3	50
Total	179	87	27	31	57	22	38	19	7	37	16	10	62

For the difference in rate of recurrences in undifferentiated squamous-cell carcinoma: $P=0\cdot08$.
For the difference in rate of recurrences in adenocarcinomas: $P\pm0\cdot12$.

Table 5. Endoxan prophylaxis; modality of operation and rate of recurrences (Rec.)

	Total patients	Segmental resect.			Lobectomy			Bilobectomy			Pneumonectomy		
		Total	Rec.	%	Total	Rec.	%	Total	Rec.	%	Total	Rec.	%
Endoxan	91	4	2	50	36	17	47	6	2	33	45	22	49
Controls	88	3	0	0	43	9	21	14	4	28	28	10	36
Total	179	7	2	28	79	26	33	20	6	30	73	32	44

Table 6. Endoxan prophylaxis; age distribution

	All patients median age	Without recurrence median age	With recurrence median age
Endoxan	61·9	63·3	60·7
Controls	62·4	62·8	61·3

Distribution according to age groups

Age group (yr)	Endoxan No. of patients	Control No. of patients
<40	1	0
40–49	8	9
50–59	28	25
60–69	44	43
>70	10	11

sion can also been drawn from different operative series published in the literature. The difference in the rate of recurrences in treated and untreated patients therefore cannot be attributed to some imbalance in the distribution of operative procedures in these two groups.

Table 6 gives the age distribution in both groups for all patients and for patients with and without recurrences. The small differences do not explain recurrence rates. The same is true when the total number of patients is broken up into four age groups, namely 40–49 yr, 50–59 yr, 60–69 yr and over 70 yr. In no age category does the difference in number of patients in the treated and untreated group suggest other factors than chance, with P not below 0·8 in any group.

Table 7 summarizes the side effects of Endoxan treatment in patients with and without recurrences. It can be seen, that both groups had less than the prescribed total dose of Endoxan, mainly because of gastro-intestinal toxicity. In the group with recurrences, the average reduction in dosage per patient was 1·22 g, in the recurrence free group 3·88 g. Patients with recurrences therefore had, on the average, more Endoxan than patients without recurrence. Six patients in the group with recurrences had to be switched from weekly intravenous injections to daily oral doses of Endoxan, vs. 19 patients in the recurrence-free group. The average of lowest white counts observed during treatment is not significantly different in the group with and without recurrences. The same is true for lowest platelet counts.

Table 8 gives some more details about haematological toxicity of Endoxan treatment

Table 7. Endoxan prophylaxis; toxicity of Endoxan treatment in patients with and without recurrence

	Dose reduction per patient		Change to oral doses	Weight change 3 months after	Nadir of WBC and platelets	
	No. of patients	End. (g)	No. of pat.	operation	WBC × 10^3	Thromb. × 10^3
Recurrences	27/43	−1·22	6/43	−2·86 kg	3·83	172·8
Without recurrence	31/48	−3·88	19/48	+1·00 kg	3·54	154·3

Table 8. Endoxan prophylaxis; haematological toxicity

	WBC × 10^3				Platelets × 10^3		
	>5·0	3·5–5·0	2·0–3·5	<2·0	>150·0	100–150	50–100
	%	%	%	%	%	%	%
Without recurrences	13	38	45	4	43	43	13
Recurrences	28	32	34	6	57	24	19
Total	20	35	39	5	50	34	16

in patients with and without recurrent disease. There are no significant differences in both groups. Forty per cent of all patients with recurrences had at some time a white count below 3500 cm^3, vs. 49% in the group without recurrences. Fifty-three per cent and 56% respectively had a platelet fall below 100,000.

Table 9 summarizes some data of weight changes in patients with and without recurrences in the Endoxan treated group. Patients with recurrence had an average weight loss of 2·8 kg 3 months after the operation and 2·2 kg at the time of recurrence, compared to the weight before operation. These patients recovered poorly from the weight loss occurring in the post-operative period. On the contrary, patients without recurrence demonstrated an average weight increase of 1·0 kg 3 months after the operation, and 3·9 kg at the time of the last consultation, despite Endoxan treatment. Sudden weight loss has been observed to be a very sensitive indicator of recurrent disease before the disease became clinically manifest.

SUMMARY AND CONCLUSIONS

In a strictly controlled study with complete and careful follow-up of 179 radically operated patients, it is demonstrated that the rate of recurrence and death from cancer during the first 2 years of observation after surgery is significantly higher in a group of 91 evaluable patients who received adjuvant long-term intermittant treatment with Endoxan for 2 years, as compared with an untreated control group of 88 patients. The time of recurrence and death after operation is similar in both groups. A number of other observations, mentioned previously in this study, support the evidence that the higher recurrence and death rate is, in fact, directly related to the treatment with Endoxan.

No factors other than Endoxan treatment have been found to explain the constant and statistically significant difference between the treated and untreated group, despite a careful search for such possible influences. The randomization procedure was strict and correctly followed, according to protocol and included documentation of every case considered as radically operable. The allocation of a randomization card in a closed envelope before the operation, which was opened after the operation, resulted in no imbalances in the groups, with a nearly equal number of those patients in both groups excluded after operation and of those remaining in the study. This form of randomization effectively excluded any bias in favour of one or the other group as is demonstrated by the balanced distribution of patients concerning age, histology and modality of operation.

The results may be called preliminary. But two thirds of the patients have been observed for a minimal period of 24 months after operation. A constant and significant trend against Endoxan treatment prevails. It is well known from other survival studies in bronchogenic carcinoma that the overwhelming majority of recurrences, local or metastatic, occur during the first 2 years after operation. Furthermore, the median time for recurrence was 8–9 months in both groups and did not change with increasing time of observation. These considerations make it highly improbable that the results found in this study are due to chance and may be reversed or considerably changed with increasing length of observation.

It therefore seems safe to conclude that the constant and statistically significant differences in the number of recurrences for nearly all points of observation, as analysed by the life table method, can be attributed directly to the unfavourable effects of post-operative treatment with Endoxan.

These results have important theoretical and practical implications. From a theoretical

Table 9. *Endoxan prophylaxis; weight changes in Endoxan-treated patients*

	Three months after operation					At time of recurrence or last observation				
	Weight increase		Weight loss		Mean net change	Weight increase		Weight loss		Mean net change
	Patients %	(kg)	Patients %	(kg)	(kg)	Patients %	(kg)	Patients %	(kg)	(kg)
Recurrences	24	+3·8	76	−5·0	−2·8	44	+4·5	56	−7·6	−2·2
Without recurrences	58	+4·2	42	−3·4	+1·0	76	+6·7	24	−4·8	+3·9

stand point it is extremely important to know, that an agent such as Endoxan, which has limited therapeutic effect in advanced metastatic bronchogenic carcinoma, not only is unable to erradicate tumour cells in minimal residual disease, but actually seems to favour the growth and propagation of the tumour. The reason for this adverse effect is a matter for speculation. As the patients were strictly randomized, it has to be assumed that, in both the treated and untreated groups, an equal number of patients had viable tumour cells remaining after "radical" operation, which sooner or later may produce recurrent disease. If the difference in the number of recurrences, which so far has been observed, persists during future observation, as the curves in Fig. 1 suggest, then this seems to be good evidence for the assumption that in the untreated group, an unknown number of patients were able to eliminate residual tumour tissue. In the treated group this was not the case, possibly due to impairment of defence mechanisms by Endoxan. The unfavourable effects of Endoxan therefore may be due to its relative inability to kill residual tumour cells and its relative potency in suppressing specific or unspecific host defence mechanisms or "immunological control", which may play a role after surgery with minimal residual disease.

For practical purposes the results of the study caution against any uncontrolled postoperative so-called prophylactic chemotherapy. This is especially true for tumours where suppression of a possible antitumour immune response, which may prevail in a state of minimal residual tumour, outweighs the obtainable tumour cell kill and thus may increase, rather than diminish the chance of recurrent disease. The same may not be true for tumours highly sensitive to cytotoxic agents. But this has to be established by carefully controlled studies.

REFERENCES

1. S. Farber, G. D'Angio, A. Evans and A. Mitus, Clinical studies in actinomycin D with special reference to Wilms' tumor in children. *Ann. N.Y. Acad. Sci.* **89,** 421 (1960).
2. D. J. Fernbach and D. T. Martyn, Dactinomycin and improved survival in Wilms' tumor. *J. Amer. med. Ass.* **195,** 1005 (1966).
3. J. F. Holland, M. M. Hreshchyshyn and O. Glidewell, Controlled clinical trials of Methotrexate in Treatment and Prophylaxis of Trophoblastic Neoplasia. Abstract 747, Tenth International Cancer Congress, Houston (1970).
4. F. J. Ansfield, B. C. Korbitz, H. L. Davis and G. Ramirez, Triple drug therapy in testicular tumors. *Cancer* **24,** 442 (1969).
5. A. R. Curreri, Nitrogen mustard as an adjuvant to pulmonary resection in the treatment of the carcinoma of the lung. *Cancer Chemother. Rep.* **16,** 123 (1962).
6. E. G. Dolton, Combined surgery and chemotherapy for carcinoma of the bronchus. Letter to the Editor. *Lancet* **i,** 40 (1970).
7. G. A. Higgins, E. W. Humphrey, F. A. Hughes and R. J. Kuhn, Cytoxan as an adjuvant to surgery for lung cancer. *J. Surg. Oncology* **1,** 221 (1969).
8. F. A. Hughes and G. Higgins, Veterans administration surgical adjuvant lung cancer chemotherapy study: present status. *J. thorac. cardiovasc. Surg.* **44,** 295 (1962).
9. M. B. Shimkin and G. E. Moore, Adjuvant use of chemotherapy in the surgical treatment of cancer. *J. Amer. med. Ass.* **167,** 1710 (1958).
10. N. H. Slack, Bronchogenic carcinoma: Nitrogen mustard as a surgical adjuvant and factors influencing survival. *Cancer* **25,** 987 (1970).
11. Veterans Administration Surgical Adjuvant Chemotherapy Group, Status of adjuvant cancer chemotherapy. *Arch. Surg.* **82,** 156 (1961).
12. Veterans Administration Surgical Adjuvant Cancer Chemotherapy Group, Evaluation of chemotherapy agents as adjuvans to surgery in 22 Veterans Administration Hospitals: Experimental design. *Cancer Chemother. Rep.* **20,** 81 (1962).
13. Veterans Administration Surgical Adjuvant Cancer Chemotherapy Group, Adjuvant use of HN 2 and ThioTEPA: progress report. *Cancer Chemother. Rep.* **44,** 27 (1965).
14. H. V. Wingfield, Adjuvant chemotherapy in surgery. Letter to the Editor. *Lancet* 470 (1970).

15. W. Denk and K. Karrer, Combined surgery and chemotherapy in the treatment of malignant tumors. *Cancer* **14,** 1197 (1961).
16. K. Karrer, Kombinierte chirurgische und zytostatische Therapie des Bronchuskarzinoms. *Münch. med. Wschr.* **109,** 1320 (1967).

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Epidemiologic Features of Breast Cancer in Slovenia, 1965–1967

BOŽENA RAVNIHAR, BRIAN MACMAHON and JURIJ LINDTNER
Institute of Oncology, Medical Faculty, University of Ljubljana, Yugoslavia, and the Department of Epidemiology, Harvard School of Public Health, Boston, Massachusetts, U.S.A.

Abstract — *As part of an international collaborative investigation, the epidemiologic characteristics of breast cancer in Slovenia have been studied. An attempt was made to interview all cases of the disease newly diagnosed during a period of* 39 *months. For comparison purposes, hospital patients with other diseases were interviewed. A total of* 806 *eligible breast cancer cases were ascertained and* 772 *were interviewed. Two thousand three hundred and thirty-two control patients were selected and* 2308 *interviewed.*

The incidence of ascertained cases was quite similar to that obtained from the data of the Cancer Registry of Slovenia, the rates being approximately twice as high as those in Japan and other Asian populations, but substantially lower than those in North America and Northern Europe. In regard to both the overall level of incidence and the characteristic shape of the age–incidence curve, the data from Slovenia were similar to those from Athens, Greece — the other Southern European center in the international study.

There were no significant differences between cases and controls in socio-economic status, number of fetal deaths and frequency or duration of lactation. Even though some patients reported unusually prolonged lactation experience these were equally frequent among the cases as among the controls.

Women first pregnant under the age of 25 *had approximately* 70% *of the breast cancer risk of those whose first pregnancy was delayed until age* 30 *or older. While this trend is in the same direction as that seen in some other centers, the protective effect of early pregnancy seems substantially less in Slovenia than in the other areas studied.*

There was a significant association between early menarche and increased breast cancer risk. Median ages at menarche were quite late both in the cases and in the controls, similar in fact to those reported in the counterpart study in Japan. There was also an indication that late menopause was associated with increased breast cancer risk.

The breast cancer patients were taller and heavier than the controls.

As PART of an international collaborative investigation, an epidemiological study of breast cancer has been carried out in Slovenia. The primary purpose of the international study was to test the hypothesis that differences between populations in duration of breast feeding account, in part, for the observed international differences in breast cancer rates. Slovenia was one of three centers included in the international study as representative of areas where breast cancer rates are intermediate between the high rates characteristic of Northern Europe and North America and the low rates seen in certain Asian and African populations. A summary of the plan of the international study and of the findings with respect to breast feeding has been published [1].

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An additional purpose of the international study was to compare the reproductive and medical histories of breast cancer patients and of control groups in the areas included in the study to identify other factors associated with increased or decreased breast cancer risk. Although the methodology of the study was basically the same in all study centers, there are specific details of the procedures and certain aspects of the findings in each center which require individual description. In addition, the data on breast feeding have been analyzed in greater detail than in the summary report of the international study as a whole. This paper deals with special aspects of the study in Slovenia.

MATERIAL AND METHODS

In the international study it was planned to identify and interview all new cases of breast cancer appearing in selected defined populations. Slovenia, one of the six republics of the Socialist Federal Republic of Yugoslavia, is well-defined by geography and by nationality of population. The existence of a population-based cancer registry and the fact that more than 80% of breast cancer patients in the Republic receive diagnosis and treatment in the Institute of Oncology, Ljubljana, make the area particularly suitable for a population-based study. The study period in Slovenia was from 1 October 1964 to 31 December 1967*.

Breast cancer patients

Eligible breast cancer cases were female residents of Slovenia who, during the study period, were admitted to a hospital for a stay during which a diagnosis of breast cancer was made. The Institute of Oncology, Ljubljana, was the main source of study cases, and there was no difficulty contacting patients in this institution since the study staff were located there. Cases diagnosed and treated in other hospitals in Slovenia were brought to our attention by designated members of the staffs of the individual hospitals. In such instances the interviewer visited the hospitals, interviewed patients on the wards and reviewed medical records. Other than the Institute of Oncology, there were five sources of patients; these were the regional hospitals of the following cities and their regions: Brezice, Koper, Maribor, Novo mesto and Ptuj. No attempt was made to interview patients in their homes, and patients who had been discharged from hospital, prior to becoming known to the study staff were not interviewed unless they were re-admitted to hospital during the study period.

A total of 806 eligible cases was ascertained during the study period, and 772 (96%) of these were interviewed. Twenty-nine patients were too ill, too senile, or too deaf to be interviewed; 4 patients refused interview and 1 could not be interviewed because of language difficulty. Of the total eligible cases, 661 (82%) were identified and interviewed in the Institute of Oncology, Ljubljana, and 145 (18%) in the regional hospitals outside Ljubljana.

The diagnosis of breast cancer was confirmed by histology in 532 cases (66%), by cytology alone in 225 cases (28%), and on clinical grounds alone in 49 cases (6%). The 49 cases without microscopic confirmation were patients with advanced disease in whom cytologic diagnosis failed and a biopsy was not taken because of unequivocal clinical evidence of cancer. Histological examinations were performed in three institutions only. For the majority of cases histologic diagnoses were made at the Histopathological Department of the Institute of Oncology, Ljubljana, and for the remainder histology was performed at the Pathological Department of the General Hospital of Maribor and at the Institute of Pathology, Medical Faculty, Zagreb (Socialist Republic of Croatia). Slides for study patients examined at the latter two institutions were also reviewed by the pathologist of the Institute of Oncology, Ljubljana.

The cases ascertained in the course of the study were checked against those registered by the Cancer Registry of Slovenia as having been diagnosed during the study period. Twenty-five potentially eligible cases which had not been ascertained by the study staff were found; 8 of these were diagnosed in 1966, and 17 in 1967. The main reason for loss of these cases from the study was that some patients were not reported to the study center by the regional hospitals. These were unusual cases: 15 had been discharged immediately after diagnosis (because the illness was too advanced to be treated, the patient refused treatment, or the patient was referred for treatment to a hospital outside Slovenia), 3 patients died soon after admission, 3 were not residents of Slovenia at the time of diagnosis. Four additional cases were diagnosed in the last days of the study and did not come to the attention of the interviewer.

*The study in Slovenia was organised and conducted until September, 1965 by Dr. Janez Kmet, presently at the Epidemiology Unit, International Agency for Research on Cancer (WHO), Lyon, France.

Control patients

Attempts were made to interview 3 eligible control patients for each breast cancer case interviewed. The controls were female hospital patients who were residents of the study area and had never had cancer of the breast. For breast cancer patients in the regional general hospitals outside Ljubljana, the controls were selected from patients admitted to the same clinical department as the cases; most were therefore surgical patients. For breast cancer patients in the Institute of Oncology, controls were selected from inpatients in the neighboring Surgical and Medical Clinics of the University Hospital of Ljubljana, since if they had been selected from the Institute of Oncology all would have been cancer patients. These Clinics, like the Institute of Oncology, admit patients from all parts of Slovenia. For each case at the Institute of Oncology, two controls were selected from the Surgical Clinic and one from the Medical Clinic.

For breast cancer patients 35 years of age or older, the control patients also had to be 35 years or older, but no further age match was required. For breast cancer patients under 35 years of age, however, the controls were required to be within 2 years of the age of the cancer patient.

The control patients were selected in a systematic way. If possible, they were chosen from patients who were admitted to the hospital on the same day as the breast cancer case. When insufficient eligible control patients were admitted on the same day as the case, the patients who were admitted nearest in time to, but not more than one week before or after, the day of admission of the study case were chosen.

Using the above criteria, it was not always possible to obtain 3 controls for each interviewed case. Two thousand three hundred and thirty-two controls were selected and 2308 (99%) of these were interviewed. Twenty-two controls refused interview, and 2 were too ill to be interviewed. The percentage of patients who refused interview was somewhat higher in the controls than in the cases, and the percentage who were too ill for interview was higher in the cases than in the controls. However, none of these groups is large enough to be of any significance as a potential source of bias.

It was not practical to return to the hospital to ascertain the final diagnoses made in the control patients, but admission diagnoses were recorded. Even though controls were not obtained from the Institute of Oncology, neoplasms were the most frequent admission diagnosis, being recorded for 398 (17·1%) of the controls. Other major categories of admission diagnoses were non-malignant abdominal surgical conditions — 324 (13·9%), cardiovascular disease — 322 (13·8%), diabetes — 127 (5·5%), thyroid disease — 98 (4·2%), infections — 35 (1·5%), and accidental injuries — 29 (1·2%).

Interviews

All interviews were conducted in the hospitals by one full-time interviewer — an experienced visiting nurse. Before being sent to the coordinating center of the international study, the data obtained at interview were edited, translated and transferred to English forms by another nurse.

After completing the interview the interviewer recorded her subjective evaluation of the reliability of the information obtained. She rated 15 (1·9%) interviews with breast cancer cases as unreliable, and 31 (4·0%) as doubtful; for the controls 22 (1·0%) were judged unreliable, and 40 (1·7%) doubtful. The interviews rated as unreliable have been excluded from the analyses.

The time at the beginning and ending of each interview was recorded, and the duration of the interview calculated subsequently. The means and standard deviations of these durations were $24\cdot2\pm8\cdot5$ min for the breast cancer cases, and $22\cdot7\pm8\cdot8$ min for the controls. The difference, $1\cdot5\pm0\cdot4$ min, although small is statistically significant, suggesting that the interviewer spent slightly more time with the cases than with the controls.

Statistical methods

After exclusion of those rated unreliable, there were 757 interviews with breast cancer patients and 2286 with control patients. These form the basis of most of the analyses. The discrepancies between these numbers and the totals in individual tables result either from item non-response, or exclusion of groups specified in the context (e.g. single or non-fertile women).

For the analysis of a particular variable, the observed distribution of the cases is compared with the distribution expected on the basis of findings in the control group. The expected distributions were computed by applying to the total number of interviewed cases in each 10 year age group the percentage distribution of the control group in the same age group, and summing the expected numbers over all age groups. All the expected values shown are adjusted for age differences between cases and controls in this manner. Some items are ad-

Table 1. Estimated female population as of 30 June 1966, and average annual female breast cancer incidence rates according to the data of this study, and according to the data of the Cancer Registry of Slovenia

Age	Female population (1966)	No. of new cases		Annual incidence rate*	
		This study†	Cancer Registry‡	This study†	Cancer Registry‡
Under 25	343,575	—	—	—	—
25–29	60,790	4	3	2·0	1·7
30–34	63,862	17	19	8·1	9·9
35–39	65,367	66	60	31·1	30·6
40–44	61,890	97	94	43·3	50·6
45–49	38,549	81	81	64·6	70·0
50–54	47,178	112	104	73·1	73·3
55–59	50,460	113	115	69·0	75·7
60–64	44,814	89	94	61·1	69·8
65–69	37,595	111	106	91·0	93·6
70–74	24,455	65	68	81·8	92·4
75–79	15,597	31	39	60·9	83·4
80–84	8032	14	23	53·5	103·3
85+	3665	6	11	49·1	98·2
All ages	865,829	806	817	28·6	31·4

*Average annual rates per 100,000 female population.
†39 months, October 1, 1964–December 31, 1967.
‡36 months, January 1, 1965–December 31, 1967.

justed for more than one variable, and are so indicated in the footnotes to the tables.

The χ^2 values are the summary chi-square statistics with one degree of freedom described by Mantel and Haenszel [2] and Mantel [3].

RESULTS

Incidence by age

Using the official female population estimates as of 30 June 1966, issued by the Federal Institute of Statistics [4], the average annual breast cancer incidence rates, based on the 806 cases ascertained during the 39 months of the study period, are given in Table 1. Comparable rates for the 3-year period 1965–1967, based on the data of the Cancer Registry of Slovenia [5], are also given. The rates based on the Registry data are about 10% higher than those based on the study data. A number of factors contribute to this difference:

1. As noted, 25 eligible cases treated in hospitals in Slovenia were not ascertained by the study staff. These cases were reported to the Cancer Registry.
2. In the period 1965–1967 there were 36 cases of breast cancer ascertained by the Registry from death certificates only. These were mostly women of older ages, and account for the fact that the difference between the two sets of rates is especially large in the oldest age groups.
3. The Registry data include 11 cases with a second primary following a first in the other breast. These cases were reported as primary cases to the Registry, but were specifically excluded from this study in accord with the protocol of the international study.

In the younger age groups the incidence rates increase with age — those based on the study series up to the 50–54 age group, and those based on the Registry series up to the 55–59 age group. Thereafter, there are fluctuations between individual age groups in both series, but while the Registry series shows an upward trend of rates in the older ages, the study series shows a tendency for rates to decrease in the older age groups. Since the rates in the Registry series in the older age groups are heavily influenced by the few cases ascertained only from death certificates, the study series probably gives a more accurate indication of the shape of the age curve, in so far as incidence is concerned. Figure 1 illustrates

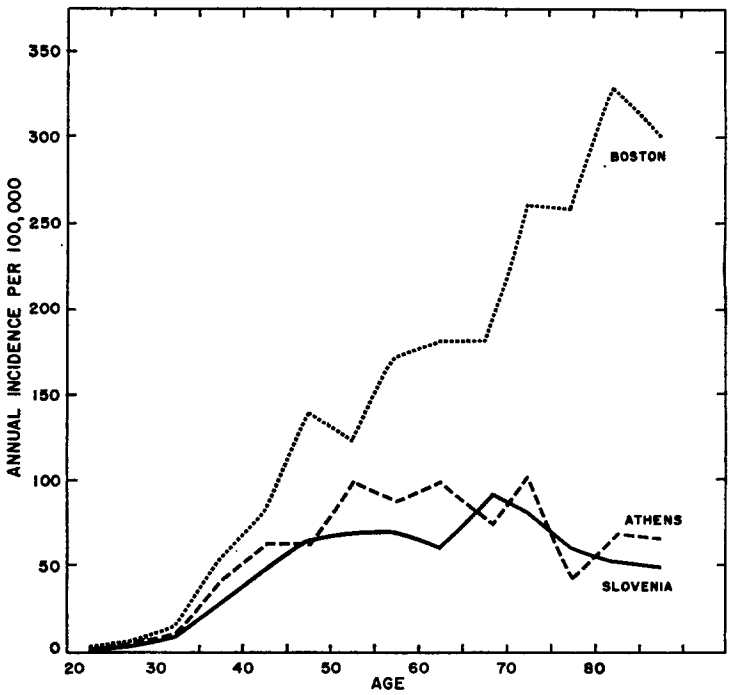


Fig. 1. *Pattern of age-specific breast cancer incidence rates in Slovenia, Athens and Boston.*

how similar the age pattern of the breast cancer cases in Slovenia is to that in Athens, Greece — the other South European center in this study [6] and how it contrasts with that in the United States [7], where incidence rates continue to increase steeply throughout life.

Place of residence

Ninety-three per cent, both of the cases and of the controls, were born in Slovenia. There was also no difference between the cases and controls in the proportions of women born in specific areas outside Slovenia. Ninety-nine per cent, both of the cases and of the controls, had resided in Slovenia for 20 years or more.

However, a substantial difference between cases and controls was found in respect to place of residence within Slovenia. Thirty-three per cent of the controls, but only 19% of the cases, were residents of the City of Ljubljana. This unexpected difference resulted from the fact that the proportion of patients who came from outside Ljubljana was higher at the Institute of Oncology than at the Medical and Surgical Clinics, from which the controls for the Institute of Oncology cases were drawn. The possible effects of this difference must be considered in connection with the analyses of other variables.

Socio-economic status

In Table 2, the observed and expected

Table 2. *Numbers of cases observed and expected by duration of patient's schooling, and for married women, by duration of husband's schooling*

Duration of schooling (yr)	Patient's schooling		Husband's schooling	
	Obs.	Exp.	Obs.	Exp.
<8	498	494·0	293	288·6
8–11	179	183·9	158	162·2
12–15	61	63·3	50	49·1
16+	7	3·8	24	25·1
Total	745	745·0	525	525·0

Duration of schooling was unknown for 12 cases and 22 control patients, and for husbands of 109 ever-married cases and 296 controls.
$\chi^2=0·01$, $P>0·9$.

distributions by duration of schooling of the patients and of their husbands are compared. For ever-married women, the cases and controls were also compared with respect to the socio-economic classification of the husband's occupation, and, for single women, observed and expected distributions of cases by occupational class of the women themselves were also examined. In Slovenia, an economic differential exists between families in which both husband and wife work, and those in which there is only one wage-earner. Married cases and controls were also, therefore, compared in this respect. In none of these measures of socio-economic status was there any appreciable or statistically significant difference between cases and controls. This finding was surprising. In the other centers in this study [9–12] and in previous studies [10–12], a tendency for breast-cancer patients to be of high socio-economic status has been observed consistently.

However, the difference between cases and controls in the proportion resident in Ljubljana is relevant in this context, since in all the measures used the control patients who were residents of Ljubljana were of substantially higher socio-economic status than those from other parts of the Republic. For example, only 38% of the controls from Ljubljana but 73% of the other control patients had had less than 8 years of schooling; 21% of the Ljubljana residents but only 7% of the residents of other areas had had 12 or more years of schooling. Since the cases came from outside Ljubljana in higher proportion than the controls, a tendency for the cases to have longer schooling than the controls could be concealed by these differences. If the data for patients schooling given in Table 2 are adjusted for the difference between cases and controls in place of residence, as well as age, the expected values for less than 8 years and 12 or more years of schooling become, respectively, 522·4 and 54·6, suggesting a 30% higher risk for women with 12 or more years schooling than for those with less than 8 years. It is quite likely, therefore, that the same economic differential in breast cancer risk that has been seen elsewhere, exists in Slovenia, but is masked in this study by the difference in the sources of cases and controls.

Marital status

Among 757 cases, 122 were single. The expected number on the basis of the control distribution was 106·1. The excess number of cases among single women is in the direction anticipated from previous studies, but is not statistically significant ($\chi^2=1·7$, $P\sim0·2$). There was no significant difference in distribution of marital status between residents of Ljubljana and residents of other areas.

Table 3. Observed and expected distributions by parity (full term deliveries). Ever-married women only

Parity	No. of cases Obs.	Exp.	Relative risk*
0	63	55·9	1·00
1	113	104·1	0·96
2	158	156·0	0·89
3	108	114·4	0·83
4	74	80·3	0·81
5+	118	123·3	0·85
Total	634	634·0	—

*Relative to a risk of 1·00 for non-parous women.
$\chi^2=1·2$, $P>0·3$.

Fertility

Distributions of ever-married women by parity are given in Table 3. In this and subsequent analyses "parity" is defined as the number of full-term deliveries — i.e. stillbirths are included in the computation, while abortions are not. The direction of the parity differences between cases and controls is as anticipated from previous work and from other centers in this study, but the difference is not statistically significant and its size is much less than that noted in other areas. For example, in Athens [6], Boston [7], Wales [8] and Tokyo [9] the risks for women with 5 or more full-term deliveries, compared to those for nulliparous married women, were 50%, 59%, 39% and 38%, respectively. In Slovenia the risk for the para 5+ group was 85% of that for the nulliparous. The observed and expected distributions by parity were compared according to age at the time of interview. No significant or consistent changes with age were noted.

Again, however, the difference between residents of Ljubljana and of other areas is relevant. Among married controls who were Ljubljana residents, 24·5% were nulliparous and 8·8% were of para 5 or more; among the residents of other areas these values were 13·0% and 23·1%, respectively. Adjusting for area of residence as well as age, the expected values in Table 3 for parity 0 and parity 5+ become 52·7 and 137·2, respectively, giving a relative risk for the parity 5+ group of 0·72. This is appreciably lower than the 0·85 shown in the Table, but is still higher than those observed for this parity group in the other centers.

Table 4. Observed and expected distributions of cases and estimates of relative risk, by parity and age at first pregnancy. Single and married patients

Parity	Item	Age at first pregnancy <20	20–24	25–29	30+	Total
1	Observed	9	40	38	49	136
	Expected	6·8	43·2	42·7	43·3	136·0
	Rel. risk	1·2	0·8	0·8	1·0	—
2	Observed	8	60	57	38	163
	Expected	7·4	63·9	59·1	32·7	163·1
	Rel. risk	0·9	0·8	0·8	1·0	—
3	Observed	5	42	40	22	109
	Expected	8·1	43·8	39·6	17·6	109·1
	Rel. risk	0·5	0·8	0·8	1·0	—
4	Observed	5	25	31	13	74
	Expected	7·4	33·2	25·2	8·2	74·0
	Rel. risk	0·4	0·5	0·8	1·0	—
5+	Observed	11	50	46	12	119
	Expected	12·9	63·6	35·1	7·4	119·0
	Rel. risk	0·5	0·5	0·8	1·0	—
All parities	Observed	38	217	212	134	601
	Expected	42·6	247·7	201·7	109·2	601·2
	Rel. risk	0·73	0·71	0·86	1·00	—

Relative risks are expressed relative to a risk of 1·0 for women with first pregnancy at 30 years of age or older.
For all parities, $\chi^2=8\cdot1$, $P<0\cdot01$.

Association of breast cancer risk with age at first pregnancy is examined in Table 4. Single as well as married women are included, since a substantial number of the single women reported pregnancies. The data are shown for each parity, the expected values for "all parities" being adjusted for both parity and age at interview. A significant excess of cases having their first pregnancy over the age of 25 is evident. Computation of the relative risks suggests that by comparison with women first pregnant before the 25th year of age, women first pregnant between 25 and 29 have 20% higher risks, and those first pregnant at 30 or older have 40% higher risks. Again, these differences are in the same direction as those observed in the other centers [6–9] but are smaller.

It is again necessary to enquire whether the fact that this association is less strong than that observed in other studies can be accounted for by the difference between cases and controls in area of residence, since one might expect patients from the more rural areas to have earlier first pregnancies. However, while there is such a tendency, it is quite slight and is almost entirely eliminated if the difference in parity distribution of Ljubljana and non-Ljubljana residents is taken into account. Adjustment of the expected values in Table 4 for area of residence as well as age leaves them virtually unchanged.

The 634 ever-married women had a total of 1809 children of which 51 were stillborn. The expected number of stillbirths based on the rates in the control series was 55·8; the difference is not significant ($\chi^2=0\cdot3$, $P\sim0\cdot6$).

Two hundred and twenty-nine breast-cancer patients (ever-married and single) reported having had one or more abortions; the expected number was 243·1. The difference is not significant ($\chi^2=1\cdot4$, $P\sim0\cdot2$).

There were no differences between cases and controls in the reported use of contraception. Of the cases, 313 (expected 327·3) reported that they have never used contraceptives ($\chi^2=1\cdot1$, $P\sim0\cdot3$). The answers to the question regarding this item are probably not

very reliable, for in addition to the usual uncertainties about such questions, the question was worded in such a way that it is possible that it was interpreted by some women as referring only to contraceptives used by women.

Lactation

The patients in Slovenia reported a relatively extensive lactation experience. Thus, in the control series 94% of all children were breast fed, and the mean duration of breast feeding per child was over 7 months.

There are many possible ways of measuring lactation experience. We have compared cases and controls with respect to the following:

1. Among women who bore at least one live-born child, the proportion who never nursed.
2. Among all live-born children born to patients, the number who were not nursed.
3. The mean duration of nursing for children who were nursed.
4. Among women who nursed at least one child, the proportion who, during their lives, nursed for a total of more than 24 months.

In all analyses adjustments have been made for differences between cases and controls both in age and in parity. The analyses are shown in Tables 5 and 6. In one of the measures there is a significant difference between the cases and controls, the cases having significantly more live-born children who were never nursed. However, the statistical significance of this difference is a consequence of the large number of observations, the difference itself being small. Thus, 7·6% of the children of cases were not breast-fed, in comparison to 6·1% expected. It seems unlikely that this difference has any biological significance in light of the negative findings in the other measures of lactation experience. Table 6, shows that the same negative conclusions are reached when women of high, low, or intermediate parity are considered separately.

Since the question of lactation history was

Table 5. Selected lactation characteristics. Married women who had at least one full-term pregnancy. By age

Characteristic	Age of women 20–39	40–49	50–59	60–69	70–79	80+	All ages*	X^2†
No. of women with live-born children	75	122	156	137	65	9	564	
No. who never nursed								
Observed	5	6	7	10	3	0	31	2·4
Expected	3·3	5·5	5·8	5·0	2·6	0·0	22·3	
No. of live-born children	148	338	521	455	254	43	1759	
No. who were not nursed								
Observed	7	19	49	39	19	0	133	4·5
Expected	9·2	21·4	34·8	23·2	18·1	0·8	107·5	
No. of children nursed	141	319	462	414	219	43	1598	
Mean duration of nursing (months) per child								
Observed	6·4	6·3	7·0	8·5	8·5	8·8	7·4	2·1
Expected	4·9	6·4	7·3	7·9	8·3	6·9	7·2	
No. of women who nursed	70	116	148	126	60	9	529	
No. who nursed more than 24 months								
Observed	8	28	47	47	31	5	166	
Expected	5·7	23·3	49·9	48·7	23·9	4·7	156·2	0·9

*The values for "All ages" are adjusted for both age and parity.

†Summary χ^2 with one degree of freedom, based on observations for all ages. For $P=0·05$, $\chi^2=3·84$.

Table 6. Selected lactation characteristics. Married women who had at least one full-term pregnancy. By parity

Characteristic	Parity of women 1	2	3	4	5+	All parities
No. of women with live-born children	108	157	107	74	118	564
No. who never nursed						
Observed	12	10	6	1	2	31
Expected	12·9	4·8	2·2	0·9	1·5	22·3
No. of live-born children	108	312	313	291	735	1759
No. who were not nursed						
Observed	12	25	25	16	55	133
Expected	12·9	18·6	20·4	17·7	37·9	107·5
No. of children nursed	96	287	288	272	655	1598
Mean duration of nursing (months) per child						
Observed	7·3	6·4	7·4	6·9	8·2	7·4
Expected	6·0	6·0	6·8	7·3	8·0	7·2
No. of women who nursed	96	147	101	72	113	529
No. who nursed more than 24 months						
Observed	1	15	33	34	83	166
Expected	1·2	7·8	31·5	31·1	84·6	156·2

The values for "All parities" are adjusted by both age and parity and are therefore identical to the values for "All ages" given in Table 5.

one of the primary issues in this study, and since differences in lactation experience might well be anticipated between urban and rural residents, it was important to evaluate the possible effect of the difference noted between cases and controls in place of residence. However, it was found that, after allowance for known difference in parity, the lactation experience of patients from Ljubljana and from other areas was virtually identical. Among the control patients from Ljubljana and from other areas the parity-adjusted percentages of women who had at least one live-born child but never lactated were 3·4 and 4·3, and the percentages who lactated for 24 months or more were 30·3 and 28·8, respectively. The average durations of nursing per child nursed were 7·0 months for the Ljubljana residents and 7·4 months for residents of other areas. None of these differences is statistically significant or of sufficient size to warrant concern for their influence on the comparison of cases and controls.

Menstrual history

Distributions by reported age at menarche are given in Table 7. Over most of the distribution there is a distinct trend towards higher breast cancer risk with earlier age at menarche. The exception to the trend is the group with menarche under 13 years of age which shows a lower risk than all but one other group (17 or more). The numbers of patients in the group with menarche under 13 years of age is not sufficient to be confident that the trend is indeed reversed in this age group. In spite of the

Table 7. Observed and expected distributions by reported age at first menstrual period

Age	No. of cases Obs.	Exp.	Relative risk*
<13	76	85·6	1·2
13	116	99·5	1·6
14	190	159·0	1·6
15	130	120·7	1·5
16	110	113·6	1·3
17+	122	165·7	1·0
Total	744	744·1	—

$\chi^2=4·9$, $P>0·03$.

Age at first menstrual period was unknown for 13 cases and 27 controls.

*Relative to a risk of 1·0 for women having their first period at age 17 or older.

Table 8. Observed and expected distributions by age at natural menopause

Measure	Age at menopause <40	40–44	45–49	50–54	55+	Total No.
Observed	7	17	107	198	25	354
Expected	6·7	33·8	103·4	183·0	27·1	354·0

$\chi^2=2\cdot6$, $P\sim0\cdot1$.

The table is restricted to women of 55 years or older who reported having had a natural menopause. It excludes 1 case and 4 comparison patients reported to be still menstruating, and 15 cases and 18 comparison patients for whom age at menopause was not reported.

irregularity of this group, the χ^2 for linear trend in the relationship between breast cancer risk and age at menarche is significant.

There is a distinct, though small, difference in the ages at menarche reported by residents of Ljubljana and of other areas, the median ages in the control series being 14·4 and 14·9, respectively. These differences are in the direction to suggest that, in view of the larger proportion of non-Ljubljana residents in the cases than in the controls, the true difference between cases and controls in age at menarche may be greater than that shown in Table 7. However, the change in the relative risks resulting from adjustment for residence differences is quite small.

The great majority of patients (566 observed, 561·1 expected) reported that they had had regular menstrual periods at intervals of 25–30 days. Regular periods at intervals less than 25 days were reported by 119 cases (expected 122·1) and 46 cases (expected 47·5) reported irregular intervals between periods.

Thirty-eight cases (expected 49·3) reported having had a surgical menopause, and 2 other cases (expected 3·9) reported other forms of artificial menopause. Thirty-three of these artificial menopauses (expected 41·2) were induced after 40 yr of age, and 7 (expected 11·9) prior to that age. Artificial menopause less than 2 yr prior to the current hospitalization, or performed for the condition for which the patient was currently hospitalized, were not included. The deficit of artificial menopauses among breast cancer cases is consistent with earlier studies, but is not statistically significant in this series ($\chi^2=2\cdot6$, $P\sim0\cdot1$).

Age at natural menopause is examined in two ways. First, Table 8 shows the distribution by reported age at menopause for women 55 years of age or more at the time of the interview, who reported having had a natural menopause. There is a deficit of cases among women reporting menopauses prior to age 45, but overall, the observed and expected distributions are not significantly different. Second, among 169 cases between 45 and 54 years of age (excluding those who had reported an artificial menopause) there were 90 who were still menstruating at the time of interview (women having had artificial menopause in the current hospitalization or for their present disease being considered as "still menstruating"). The expected number of such cases, based on the percentage in the control series at each individual year of age, was 75·6. The observed value significantly exceeds the expected ($\chi^2=6\cdot7$, $P\sim0\cdot01$). Thus, both these analyses suggest a tendency towards late menopause among the breast cancer patients.

Height and weight

Distributions of cases by weight and by height are examined in Tables 9 and 10, respectively. In Athens [6], the breast cancer cases were significantly taller and heavier than the controls. There are suggestions of similar trends in Slovenia, although they are not entirely regular,

Table 9. Observed and expected distributions by weight

Weight (kg)	No. of cases Obs.	Exp.	Relative risk*
<55	96	111·9	1·0
55–59	92	90·8	1·2
60–64	155	152·4	1·2
65–69	111	117·3	1·1
70–74	104	115·2	1·1
75–79	83	64·4	1·5
80+	109	98·0	1·3
Total	750	750·0	—

$\chi^2=2\cdot45$, $P\sim0\cdot1$.

Weight was not stated for 7 cases and 14 control patients.

*Relative to a risk of 1·0 for patients weighing under 55 kg.

Table 10. *Observed and expected distributions by height*

Height (cm)	No. of cases Obs.	Exp.	Relative risk*
<155	67	65·8	1·0
155–159	153	187·4	0·8
160–164	291	295·2	1·0
165–169	174	141·6	1·2
170+	60	55·0	1·1
Total	745	745·0	—

$\chi^2=5·14$, $P\sim0·02$.
Height was not stated for 12 cases and 40 comparison patients.
*Relative to a risk of 1·0 for patients under 155 cm, in height.

and only the trend by height is statistically significant.

DISCUSSION

Findings with respect to the major variable under investigation in this study — lactation — require little comment. The absence of any consistent difference between cases and controls in duration of lactation supports the conclusion from the international study as a whole that this variable is not a factor in determining breast cancer risk [1]. Although the cases and controls in Slovenia were not entirely similar with respect to place of residence within the Republic, we have shown that the differences observed do not appreciably influence lactation experience if the differences in parity distribution are taken into account. The fact that the cases and controls in Slovenia were of very similar socio-economic status removes the likelihood of a confounding effect of this variable. Tables 5 and 6 give more detailed analyses than were presented in the summary report of the international study, and show that there is no subgroup of cases — defined either in terms of age at diagnosis of cancer or of high or low parity — in which lactation is protective. There is no evidence in the data that even very prolonged lactation has a protective effect. Thus among the 118 cases and 359 controls of parity 5 or more, the percentages who lactated for a total of 5 years or more were 33·9 and 37·6, respectively, and there were 3 cases and 10 controls (2·5% and 2·8%, respectively) who nursed for a total of 10 years or more. Neither of these differences is statistically significant. In addition, comparison of these percentages does not take account of the fact that even within the para 5+ group the cases tend to be of lower parity than the controls, and adjustment for this difference would bring the percentages even closer together.

There are two unusual aspects of the other findings in the Slovenia study. One of these is the fact that, while at first pregnancy under the age of 25 is associated with decreased breast cancer risk, the extent of this decrease is considerably less than that seen in other centers in this study. For the international study as a whole, analyses have also been conducted considering only full-term births, and these analyses also show substantially less protective effect for first births under 20 years of age in Slovenia than is seen in the other centers [17]. The 30% reduction in risk for women with first pregnancies between 20 and 24 years of age (Table 4), is not markedly out of line with comparable values in the other centers, and the discrepancy is primarily in the group first pregnant under 20 years of age in which reductions in risk (relative to women first pregnant at 30 or older) of 50% and more have been reported [6–9]. We have no explanation for this difference. The number of women in this series who were first pregnant under 20 years of age is not large, and it is possible that chance variation is responsible. On the other hand, the possibility that there are real differences between populations with respect to the protective effect of early pregnancy on breast cancer cannot be ruled out.

The other somewhat unexpected aspect of the data from Slovenia is the high age at menarche reported both by cases and by controls. The relatively late median age at menarche of Japanese women is well known and has been considered of interest for its possible relevance to their low breast cancer risk. However the median age at menarche reported by the controls in Slovenia, 14·7 years, is precisely the same as that reported by the control series in Tokyo [9]. While low in relation to populations in Northern Europe and North America, the breast cancer rate in Slovenia is nevertheless twice as high as that in Japan [18]. The late median age at menarche in Slovenia (14·7 years) is also of interest when contrasted to the median age of 13·0 years reported in the center in Athens [6] where the breast cancer rates were virtually identical with those in Slovenia. It is possible that the early menarche of the population in Athens, if it is associted with high breast cancer risk, is compensated by the higher proportion of early pregnancies. Thus 20% of parous control patients in Athens [6] but only 7% of those in Slovenia, were first pregnant under 20 yr of age. When more information is available on the relationship of

breast cancer risk to age at menarche and to age at first birth, more detailed comparisons of these variables in Athens and Slovenia may be informative.

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The interviews were conducted by Mrs. Slava Saksida, and the data were edited by Mrs. Franka Zupancic, who was also responsible for the administrative work concerning the study. In Boston, Mrs. Hazel Coven, Mrs. Joyce Berlin, and Mrs. Linda Desmond were responsible for the coding, data processing and computer programming, respectively.

Histological slides were provided mainly by the Histopathology Department of the Institute of Oncology, Ljubljana, and by the Pathology Department of the General Hospital, Maribor, and by the Institute of Pathology, Medical Faculty, Zagreb. All slides were reviewed and selected for forwarding to the Boston center by Dr. Pavla Mavec (Mrs), Chief of the Department of Histopathology of the Institute of Oncology, Ljubljana. Dr. Harry Carter, Department of Pathology, New England Deaconess Hospital, conducted the central review of histologic slides for the international study.

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REFERENCES

1. B. MacMahon, T. M. Lin, C. R. Lowe, A. P. Mirra, B. Ravnihar, E. J. Salber, D. Trichopoulos, V. G. Valaoras and S. Yuasa, Lactation and cancer of the breast. Summary of an international study. *Bull. Wld. Hlth. Org.* **42,** 185 (1970).
2. N. Mantel and W. Haenszel, Statistical aspects of the analysis of data from retrospective studies of disease. *J. nat. Cancer Inst.* **22,** 719 (1959).
3. N. Mantel, Chi-square tests with one degree of freedom; extensions of the Mantel-Haenszel procedure. *J. Amer. statist. Ass.* **58,** 690 (1963).
4. Savezni zavod za staiistiku, Demografska statistika 1966, Beograd (1968).
5. The Cancer Registry of Slovenia, unpublished data Ljubljana (July, 1969).
6. V. G. Valaoras, B. MacMahon, D. Trichopoulos and A. Polychronopoulou, Lactation and reproductive histories of breast cancer patients in greater Athens, 1966–67. *Int. J. Cancer* **4,** 350 (1969).
7. E. J. Salber, D. Trichopoulos and B. MacMahon, Lactation and reproductive histories of breast cancer patients in Boston, 1965–66. *J. nat. Cancer Inst.* **43,** 1013 (1969).
8. C. R. Lowe and B. MacMahon, Breast cancer and reproductive history of women in South Wales. *Lancet* **i,** 153 (1970).
9. S. Yuasa and B. MacMahon, Lactation and reproductive histories of breast cancer patients in Tokyo, Japan. *Bull. Wld. Hlth. Org.* **42,** 195 (1970).
10. P. Stocks, Social status in relation to carcinoma of the breast. *Schweiz. Ztschr. Allg. Path.* **18,** 706 (1955).
11. P. Stocks, The epidemiology of carcinoma of the breast. *Practitioner* **179,** 233 (1957).
12. D. Schwartz, P. F. Denoix and C. Rouquette, Enquête sur l'étiologie des cancers génitaux de la femme. 1. Cancer du sein. *Bull. du Cancer* **45,** 476 (1958).
13. A. M. Lilienfeld, The relationship of cancer of the female breast to artificial menopause and marital status. *Cancer* **9,** 927 (1956).
14. B. MacMahon and M. Feinleib, Breast cancer in relation to nursing and menopausal history. *J. nat. Cancer Inst.* **24,** 733 (1960).
15. E. L. Wynder, I. J. Bross and T. Hirayama, A study of the epidemiology of cancer of the breast. *Cancer* **13,** 559 (1960).
16. T. Hirayama and E. L. Wynder, A study of the epidemiology of cancer of the breast. II. The influence of hysterectomy. *Cancer* **15,** 28 (1962).
17. B. MacMahon, P. Cole, T. M. Lin, C. R. Lowe, A. P. Mirra, B. Ravnihar, E. J. Salber, V. G. Valaoras and S. Yuasa, Age at first birth and breast cancer risk. *Bull. Wld. Hlth. Org.* **43,** 209 (1970).
18. R. Doll, P. Payne and J. Waterhouse (Editors), *Cancer Incidence in Five Continents.* Springer-Verlag, Berlin (1966).

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Relation Between the Pathological Nature and the Growth Rate of Human Tumors

A. CHARBIT, E. P. MALAISE and M. TUBIANA
Institut Gustave-Roussy, 94 - Villejuif, France

Abstract — *An analysis of 530 cases reported in the literature over the past 14 years has demonstrated differences in the mean growth rate of various pathological types. This analysis shows that the various types of tumors may be ranked with respect to their increasing D.T.: embryonal carcinomas, lymphomas, malignant mesenchymal tumors, squamous cell carcinomas and adenocarcinomas.*

INTRODUCTION

THE GROWTH rate of human pulmonary metastasis was first studied by Collins in 1956 [1]. This important work stimulated interest in the quantitative study of human tumor growth. Three concepts emerged from Collins' work about the growth rate of human tumor metastasis:

it is exponential;
it is relatively slow; and
it has no apparent relationship to the pathological nature of the tumor.

Numerous investigators have subsequently studied the growth rate of human tumors both primary and metastatic [2–14]. All the studies involved a relatively limited number of patients. The variations of the growth rate in tumors of the same pathological nature were such that no statistical study was possible. However, the cumulative figures reported in the literature are now sufficiently large to justify a statistical analysis to determine whether or not there is a correlation between the pathological nature and the mean growth rate of human tumors.

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MATERIAL AND METHODS

We have collected from the literature 538 cases of tumors well described in terms of pathological type and doubling time (D.T.s). To these, we added 8 unpublished cases of our own, bringing the total to 546 cases (Table 1). Sixteen of these (8 melanomas and 8 primary sarcomas) were excluded because the sample size was not sufficiently large to warrant analysis. The remaining 530 cases included 131 patients with primary tumors and 389 patients with metastasis in the lung. The latter group included 66 patients with multiple metastases of variable D.T.s and 32 patients whose tumors had variable growth rates during period of observations. For these patients, we individually calculated the geometric mean of D.T.s and used that value in subsequent calculations.

The 520 tumors were divided into 5 pathological groups: squamous cell carcinomas (Table 2), adenocarcinomas (Table 2), malignant mesenchymal tumors (Table 3), malignant lymphomas (Table 4), and embryonal carcinomas (Table 5). The squamous cell carcinomas and the adenocarcinomas were further subdivided according to their primary or metastatic site, thus creating a total of 7 groups (Table 6).

Table 1. Breakdown by author and pathological type of the different groups of patients studied

Authors	Squamous cell carcinomas (primary and metastatic)	Adenocarcinomas (primary and metastatic)	Metastatic malignant mesenchymal tumors	Malignant lymphomas	Metastatic embryonal carcinomas	Total	Unclassified tumors
Collins *et al.* (1956) [1]	5	6	2	11	10	34	—
Collins (1962) [2]	—	25	—	—	—	25	—
Schwartz (1961) [3]	11	6	—	1	1	19	—
Garland *et al.* (1963) [4]	34	7	—	—	—	41	—
Spratt (1965) [5]	—	—	6	—	—	6	8
Breur (1966) [6]	11	19	40	3	10	83	4
Weiss *et al.* (1966) [7]	13	5	—	—	—	18	—
Weiss *et al.* (1966) [8]	6	2	—	—	—	8	—
Brenner *et al.* (1967) [9]	12	18	4	—	—	34	—
Gros *et al.* (1967) [10]	—	15	—	—	—	15	—
Combes *et al.* (1968) [11]	21	39	17	6	3	86	4
Rambert *et al.* (1968) [12]	4	5	14	30	16	69	—
Chahinian (1969) [13]	29	17	1	—	3	50	—
Garetta *et al.* (1969) [14]	—	—	1	—	33	34	—
Personal unpublished results	2	4	2	—	—	8	—
Total	148	168	87	51	76	530	16

Table 2. Localization of primary tumors (columns 1 and 2) and tissue of origin of pulmonary metastases (columns 3 and 4) of squamous cell and adenocarcinomas

Localization (primary tumors) or site of origin (metastases)	Squamous cell carcinoma 1	Adenocarcinoma 2	Squamous cell carcinoma 3	Adenocarcinoma 4
Bronchii	97	19	5	—
Breast	—	15	—	20
Ear, nose, throat	—	—	20	11
Ovary	—	—	—	2
Digestive apparatus (oesophagus, colon, rectum, liver)	—	—	6	55
Thyroid	—	—	—	17
Kidney	—	—	—	19
Corpus uterii	—	—	—	10
Cervix uterii	—	—	13	—
Bladder	—	—	7	—
Total	97	34	51	134

Table 3. Tissue of origin of pulmonary metastases of mesenchymal tumors

Site of origin	Bones	Soft tissues	Breast	Uterus	Nerves	Unknown origin	Total
Pathology							
Liposarcomas	—	2	—	—	—	—	2
Osteosarcomas	34	—	—	—	—	—	34
Fibrosarcomas	—	26	1	—	—	—	27
Rhabdomyosarcomas	—	1	—	—	—	—	1
Leiomyosarcomas	—	3	—	2	—	—	5
Hemangiosarcomas	—	5	—	—	—	—	5
Synovial sarcomas	—	4	—	—	—	—	4
Neuro sarcoma	—	—	—	—	1	—	1
Giant cell sarcomas	2	—	—	—	—	—	2
Unclassified	—	—	—	—	—	6	6
Total	36	41	1	2	1	6	87

Table 4. Site and pathology of malignant lymphomas

Site of tumour/ pathology	Lymph node	Lung	Bone	Skin	Total
Malignant lymphoblastic lymphomas	13	2	—	1	16
Hodgkin's disease	12	3	—	—	15
Ewing sarcomas	—	—	4	—	4
Reticular cell sarcoma—Parker and Johnson type	—	—	6	—	6
Total	25	5	10	1	41

Table 5. Pathology of pulmonary metastases of embryonal carcinomas

Seminomas	4
Embryonal carcinomas	31
Choriocarcinomas	32
Malignant teratomas	5
Wilm's tumors	4
Total	76

The growth rates were measured on successive chest X-rays in the case of lung tumors [1–9] and on successive mammographs or lymphographs in the case of primary breast cancer [10] or of hematosarcomas [12]. The skin tumors were measured by calipers [12].

RESULTS

The distribution of individual D.T.s within each group appears to be log-normal (Figs. 1–3); χ^2 analysis is consistent with this interpretation. The individual values are plotted according to Henry's method (probit plot of cumulative frequency versus the logarithms of the D.T.s). Median values and geometric means as well as 95% confidence limits were calculated for all the groups. The median values fell always within confidence limits calculated for the means*; this is consistent with the assumption that the distributions are log-normal.

The mean values of the D.T.s and their confidence limits, as well as the distribution of the individual values of the different groups, are shown in Fig. 3.

While there is a considerable variation among the individual D.T.s (Fig. 3), the great similarity between distributions is remarkable as it is demonstrated by the parallelism of the probit-log curves.

The primary adenocarcinomas (i.e. those of

*All means are geometric means.

Table 6. Growth rates of different pathological groups of human tumors and comparison of these growth rates

Pathology	Geometric mean of the D.T.s (days)	95% confidence interval	Median D.T.s	Pathological groups compared	"Reduced" standard deviation between test groups*	p
1. Lung metastasis of embryonal tumors	27·0	22·4– 32·6	25			
2. Lymphomas	28·9	22·5– 37·2	32	1 and 2	0·43	not significant
3. Malignant mesenchymal tumors	41·4	34·5– 49·7	37	2 and 3	2·33	$2 \cdot 10^{-2}$
4. Lung metastasis of squamous cell carcinomas	58·0	47·8– 70·3	56	3 and 4	2·51	$2 \cdot 10^{-2}$
5. Primary squamous cell carcinoma	81·8	69·2– 95·5	80	4 and 5	2·78	$2 \cdot 10^{-2}$
6. Lung metastasis of adenocarcinoma	82·7	71·9 –95·5	89	5 and 6	2·98	10^{-2}
7. Primary adenocarcinoma	166·3	122·5–225·7	207	6 and 7 5 and 7	4·15 4·15	10^{-4} 10^{-4}

*Formula used to calculate the "reduced" standard deviation: $\frac{(m_A - m_B)}{\sqrt{\frac{s_A^2}{n_A} + \frac{s_B^2}{n_B}}}$.

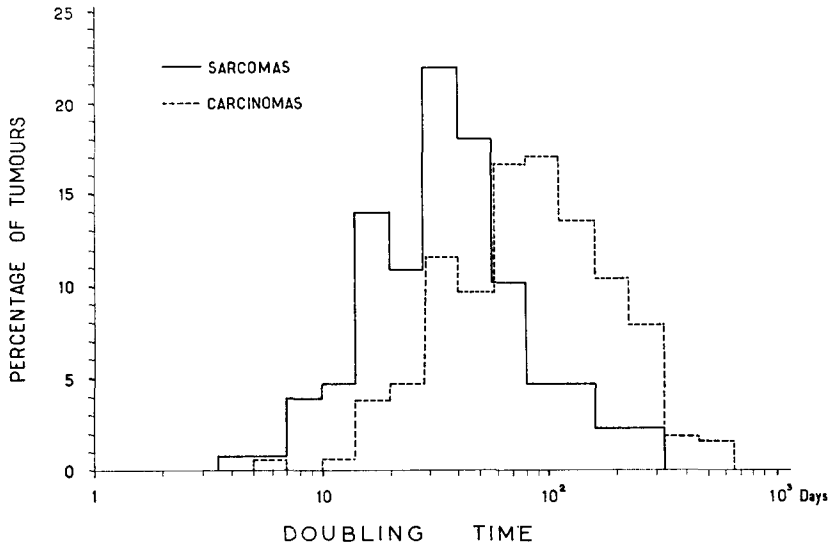


Fig. 1. *Histogram of the percentage distribution of the metastatic sarcomas and of the primary and metastatic carcinomas as a function of the D.T.*

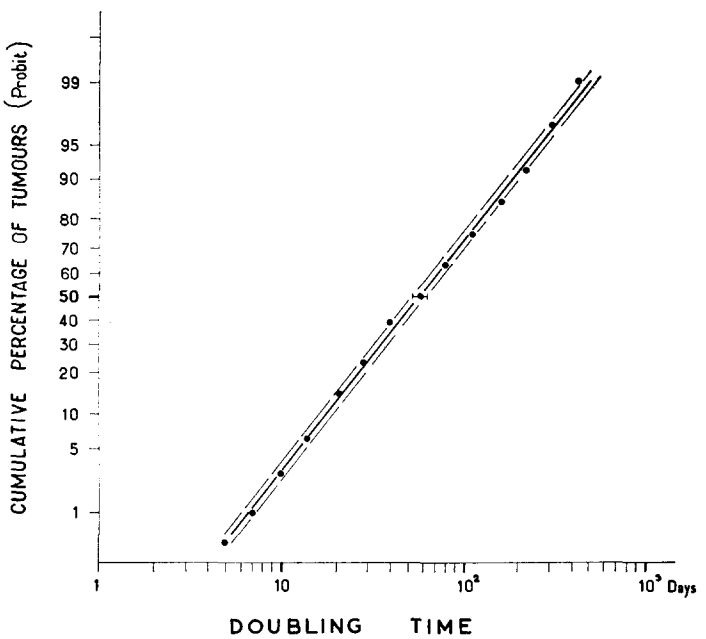


Fig. 2. *Distribution of the cumulative percentages of the total number of tumors as a function of their D.T.s.*

mammary and bronchial origin) exhibited the slowest growth rate (Table 6), followed by the squamous cell carcinomas. In both cases, the primary tumors had lower growth rates than their lung metastases. The metastases of malignant sarcomatous tumors showed a more rapid growth rate than those of squamous cell carcinomas. Finally, malignant lymphomas with localisation in the nodes, lungs and the subcutaneous tissue grew more rapidly than did pulmonary metastases of malignant sarcomatous tumors. The growth rates of embryonal tumors and malignant lymphomas were similar and exceeded those of the other groups (Table 6).

DISCUSSION

1. This study is concerned with clinically measurable tumors, thereby introducing an element of selection which precludes a truly representative sampling of the entire spectrum of human tumors (Table 7). This point is emphasized by a comparison with a recent study by the Connecticut State Department of

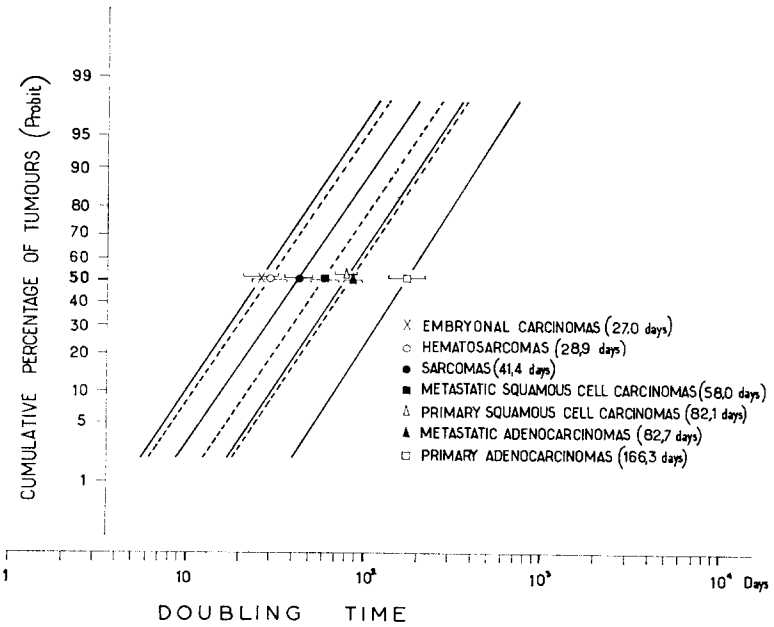


Fig. 3. Distribution of the cumulative percentages of the tumors of the different pathological groups as a function of their D.T.s.

Health [15] in which embryonal tumors and sarcomas accounted respectively for 0·8% and 1·8% of the overall tumor incidence in that State. In this study, these same tumors accounted for 14·6% and 16·7% of the total 530 cases. Moreover, 363 of our 520 cases, that is 69·5%, are pulmonary metastases of various origins.

In classifying the tumors according to pathological type and distinguishing between primary and metastatic tumors, we have endeavoured to minimise this bias. Nevertheless, some of the results should be viewed with circumspection: for example, the entire group of primary tumors is limited to carcinomas of the breast and bronchii. Thus, it would be unwise to extrapolate or generalise these results.

2. Nevertheless, it seems that squamous cell cancers grow more rapidly than adenocarcinomas. This appears to be equally true for primary as well as for metastatic tumors, although the metastatic tumors constitute a less uniform group (Table 2). It is difficult to explain the differences between squamous

Table 7. Relative frequency of the different pathological types of human cancers. Comparison of the tumor incidence in Connecticut [15] *and in the* 530 *patients of the present study*

Pathology	Cancer in Connecticut (1965) Connecticut State Department of Health Hartford-Connecticut 1965			530 patients whose tumors have been included in this study
	Men	Women	Mean	
Carcinomas	82·6	86·7	84·65	60·8
Malignant mesenchymal tumors	1·7	1·9	1·8	16·7
Malignant lymphomas	8·6	5·9	7·25	7·9
Embryonal carcinomas	1·0	0·6	0·8	14·6
Malignant nervous tumors	3·7	3·2	3·45	—
Unclassified	2·3	1·6	1·95	—

cancers and adenocarcinomas. Perhaps, the different rates of cellular proliferation of the tissues of origin play a role.

3. The growth rate of both primary adenocarcinomas and squamous cell carcinomas is exceeded by the growth rate of their metastases. This fact is difficult to interpret but it should be remembered that the tumors included in the metastatic groups are of a different origin from those included in the groups of primary carcinomas.

A possible explanation of this difference may be the purely mechanical factor of exfoliation which occurs to a much greater extent in primary tumors than in metastatic cancers. This explanation is not applicable to primary mammary carcinomas where no difference was found between the growth rate of primary mammary tumors and their lung metastases.

It is likely that the tumor environment plays an important role: pulmonary tissue is probably an ideal medium for supporting metastatic growth, given its lack of mechanical resistance and its abundant blood supply.

On the other hand, it is not possible to exclude the possibility of a selection of metastatic cells although generally accepted observations seem to indicate that there is no morphological difference between primary and metastatic tumors. Pulmonary metastases can be considered as an autologous transplant and it has been shown in experimental systems that a transplanted tumor often shows an accelerated growth rate when compared with the primary tumor [16].

4. The D.T. of the average human tumor has been considered to be about 50 days [19]. In the 530 tumors collected here, the figure is 58 days. As already discussed, this value is probably not representative of the entire spectrum of human tumors.

Two factors must be considered; first the proportion of embryonal carcinomas and mesenchymal tumors in the present series is relatively greater than found in larger samples; second, the group is composed mainly of pulmonary metastatic tumors. These two factors bias our result: it is probable that the D.T. of primary human tumors exceeds 58 days. We have no information about the growth rate of primary sarcomas and primary embryonal carcinomas. On the other hand, the D.T. of primary carcinomas which represent 85% of all human tumors was found to be 82 days for squamous cell cancers and 166 days for adenocarcinomas.

5. In the present state of our knowledge on the kinetics of cell proliferation in human tumors, it is impossible to correlate variations in growth rate with any specific mechanism. The differences between durations of the cell cycle of different tumors are relatively small and cannot explain such large differences [20]. The variations in growth fraction and the rates of cell loss must be taken into consideration.

In a recent discussion on experimental tumors, J. Denekamp suggested that the rate of cell loss is higher in squamous cell carcinomas than in the sarcomas [18]. In this series, the mean D.T. of all carcinomatous metastatic tumors in the lung was found to be 75 days whereas the mean D.T. of all sarcomatous metastatic tumors in the lung was 41·4 days. If Denekamp's conclusions apply to human tumors, they may at least partly explain the present findings.

According to G. G. Steel [19], the difference between the potential and the observed D.T. can be accounted for by a "coefficient of cell loss" which generally exceeds 50%. If the D.T.s were longer than currently accepted, cell loss would be also greater than suggested by Steel. A rate of cell loss of the order of 90% was found in 3 squamous cell carcinomas of the skin [20].

On the other hand, it appears that rapidly-growing tumors such as malignant lymphomas and embryonal tumors are those which generally respond well to chemotherapy. This would suggest that the rapid growth of these tumors is related to a high growth fraction.

6. Breur observed a relationship between the D.T.s of human tumors prior to irradiation and the response to fractionation irradiation [21]. Tumors characterised by a short D.T. were the more radiosensitive.

Among rapidly-growing tumors, there are not only seminomas and lymphomas, the radiosensitivity of which is well known, but also osteogenic sarcomas and fibrosarcomas which are generally considered radioresistant. On the other hand, among the tumors with a slow growth rate, one finds adenocarcinomas of the gastro-intestinal tract and the kidney (radioresistant) as well as adenocarcinomas of the breast (radiosensitive). This apparent discordance with Breur's conclusions may be explained by the spread of the individual D.T.s of the tumors in each group. It should be recalled that Breur studied the D.T. and the response to irradiation in the same tumor. It may be that, within each group, there is a correlation between the radoisensitivity and the growth rate.

7. Spratt has already noted that the distribution of tumors D.T.s is log normal [17].

We have confirmed this for the overall tumor spectrum, as well as for each group. It is remarkable that the distribution of the individual values of the D.T. should be comparable within each group as demonstrated by the paralleling of the slopes in probit-log coordinates. This observation deserves discussion. The pathological type is one of the factors influencing the D.T. but there are certainly other factors. For instance, approximately 10% of embryonal carcinomas (whose average D.T. is the lowest observed) have a D.T. exceeding 70 days, whereas 10% of adenocarcinomas (whose D.T. is the highest) have a D.T. less than 70 days (Fig. 3). It may be then that factors other than pathology exert the same relative influence on the D.T. within each tumor group, and thus are common to all types of tumors. Since all tumors analyzed were solid tumors, these factors may be related to blood supply.

The results of this study emphasize the interest of work on growth rate of human tumors. It should be rewarding to compare the mean growth rate of large groups of tumors of different pathological types and growing in different organs.

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REFERENCES

1. V. P. Collins, R. K. Loeffler and H. Tivey, Observations on growth rates of human tumours. *Amer. J. Roentgenol.* **76,** 988, (1956).
2. V. P. Collins, Time of occurrence of pulmonary metastasis from carcinoma of the colon and rectum. *Cancer* **15,** 387 (1962).
3. M. Schwartz, A bio-mathematical approach to clinical tumour growth. *Cancer* **14,** 1272 (1961).
4. L. Garland, W. Coulson and E. Wollin, The rate of growth and apparent duration of untreated primary bronchial carcinoma. *Cancer* **16,** 694 (1963).
5. J. S. Spratt, The rates of growth of skeletal sarcomas. *Cancer* **18,** 14 (1965).
6. K. Breur, Growth rate and radiosensitivity of human tumours. *Europ. J. Cancer* **2,** 158 (1966).
7. W. Weiss, K. Boucot and D. Cooper, Growth rate in the detection and prognosis of bronchogenic carcinoma. *J.A.M.A.* **198,** 1246 (1966).
8. W. Weiss, K. Boucot and D. Cooper, The survival of men with measurable proved lung cancer in relation to growth rate. *Amer. J. Roentgenol.* **98,** 404 (1966).
9. M. Brenner, L. Holsti and Y. Pertaca, The study by graphical analysis of the growth of human tumours and metastasis of the lung. *Brit. J. Cancer* **21,** 1 (1967).
10. C. M. Gros, Y. Legal et A. Schmeltzer, Evolution spontanée des cancers mammaires. *J. Radiol. Electrol.* (Symposium Européen de Radiologie Mammaire) **48,** 702 (1967).
11. P. F. Combes, J. Douchez, M. Carton et A. Naja, Etude de la croissance des métastases pulmonaires humaines comme argument objectif d'évaluation du pronostic et des effets thérapeutiques. *J. Radiol. Electrol.* **49,** 893 (1968).
12. P. Rambert, E. Malaise, A. Laugier, M. Schlienger et M. Tubiana, Données sur la vitesse de croissance de tumeurs humaines. *Bull. du Cancer* **55,** 323 (1968).
13. P. Chahinian, le temps de doublement des cancers bronchopulmonaires. Thèse de Médecine, Paris (1969).
14. L. Garretta, C. Debonniere, C. Bergiron, R. Josipovici et F. Cioppani, Etude de la vitesse de croissance des métastases pulmonaires des dysembryomes du testicule. *Soc. Méd. Chir. des Hôpitaux et Formations Sanitaires des Armées, Paris* **2,** 93 (1970).
15. Connecticut State Department of Health, Cancer in Connecticut (1965). Hartford, Connecticut (1970).
16. P. J. Cheshire, P. J. Lindop, The influence of intra-cellular recovery and hypoxic cells on the radiation response of mammary tumours and skin in C3H mice. *Brit. J. Radiol.* **42,** 215 (1969).
17. J. S. Spratt and T. S. Spratt, Doubling time of pulmonary metastasis and host survival. *Ann. Surg.* **159,** 161 (1964).

18. J. DENEKAMP, The cellular proliferation kinetics of animal tumors. *Cancer Res.* **30,** 393 (1970).
19. G. G. STEEL, Cell loss as a factor in the growth rate of human tumours. *Europ. J. Cancer* **3,** 381 (1967).
20. E. FRINDEL, E. MALAISE and M. TUBIANA, Cell proliferation kinetics in five human solid tumours. *Cancer* **22,** 611 (1968).
21. K. BREUR, Growth rate and radiosensitivity of human tumours. *Europ. J. Cancer* **2,** 173 (1966).

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Evidence for Sialic Acid–Lecithin Binding at the Plasma Membrane in the Presence of Cations

J. DEMAN*, P. M. VAN VAERENBERGH* and P. JOOS†

**Kliniek voor Radiotherapie en Kerngeneeskunde, and*

†*Laboratorium voor Fysische Biochemie, State University, Ghent, Belgium*

Abstract—*It is demonstrated that interaction exists between a lecithin monolayer and N-acetylneuraminic acid in the presence of calcium, magnesium, potassium and sodium ions. With calcium and potassium the interaction is most pronounced and reaches its peak value at low concentrations. The significance of the results with respect to the malignant behaviour of the cancer cell is discussed.*

INTRODUCTION

IT IS generally accepted that the natural plasma membrane is covered by materials of which proteins and carbohydrates are an essential part.

This is not in contradiction with the view that some proteins also should take part in the structure of the membrane itself. Recently much data have accumulated which strongly suggest that individual cells are surrounded by a sialomucin (sialoglycoprotein) coat [1, 2]. The thickness of the coat varies according to the cell type. Among other things, it becomes apparent that sialic acid plays a rather intriguing part and might well hold a key position in a sense of uniting on a molecular basis, different aspects of the cancer problem.

Sialic acid is a common name given to a group of compounds which are derived from neuraminic acid. Under normal conditions, only *N*-acetylneuraminic acid (NANA) has been found to be present in man [3]. In the glycoprotein coat of the cell wall, NANA appears to be α-ketosidically linked to galactose or *N*-acetyl-galactosamine. The latter molecule, on its turn, is linked to a carboxyl group of a peptide chain. This binding pattern seems to be of general occurrence as it has been found in the mucoprotein of the bovine submaxillary gland [4–7], on the cell surface of the erythrocyte [8], the cell surface of Ehrlich ascites cells [9] and in thyroglobulin [10]. We insist on the fact that NANA stands in terminal position and has a free carboxyl group.

Several authors have proposed that the increased negative surface charge on cancer cells is due to an increased concentration of sialic acid at the hydrodynamic slip plane of the cell. There is however an observation of low total sialic acid content [11] and also of low surface concentration of this molecule [12] in cancer cells. Also the high peripheral sialic acid content of normal rapidly proliferating cells [13–16] and the fact that in solid tumors the charge is not affected by removal of sialic acid [17, 18] raises a serious objection to the simple correlation of sialic acid produced negative peripheral charge and malignancy, although it could be conceivable that weakened cell adhesion in tumor cells and decreased contact inhibition is due to an enhanced electrical repulsion between cells. It has been shown [19] that a positive correlation exists between sialic acid concentration on the

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surface of embryonal polyoma transformed hamster kidney cells and loss of contact inhibition. Treatment of TA3 mouse ascites tumor cells with neuraminidase and subsequent injection into the animal resulted in decreased pulmonary metastases [20].

There are several reasons to believe that the presence of charged polysaccharide chains, and more especially of sialic acid residues, decreases the agglutination tendency of cells [21]. In a recent paper [22], Apffel and Peters present a theory in which they suggest that the increased sialic acid content and the peripheral position of the molecule in the cell coat of tumor cells, could explain the virtual absence of immune response by the host organism. The high charge due to the terminal sialic acid residues, together with the presence of many hydroxyl groups, confer to the coat a high hydrophilic character resulting in a gel-like structure by which tumor specific antigens become masked and humoral antibodies and sensitised cells are excluded.

It has been shown by many that the removal of calcium from tissue cultures weakens mutual adhesion between cells. It is assumed that the ion is bound principally to the carboxyl groups of the glycoprotein coat, and experiments of Weiss [23, 24] indicate that the action of calcium is located in the deeper regions of the coat resulting probably in an enhanced degree of cross-linkage of the peptide chains so that greater compactness to the coat and an increased tendency for stickiness is conferred. Also removal of potassium from the intercellular milieu promotes cell separation [25, 26] although here some doubt remains about the specific role of this ion since the loosening effect of the complexing agent used could probably also be ascribed to its postulated property of breaking tetrahedral water structure [27]. Hause *et al.* [28] detected a potential jump at the periphery of the cell coat of malignant cells. This jump, which was distinct from the potential jump at the membrane, indicates that positive charges are diffusely present in the deeper regions of the coat and do not simply coincide with the negative charges at the hydrodynamic slip plane.

It appeared to us that it was mainly a lack of experimental data that confines us to think of hydrogen bonds and Van Der Waals forces as alone responsible for the attachment of the glycoprotein coat to the lipid layer of the cell membrane. Another possibility seemed worthwhile exploring, namely that of a binding between carboxyl groups and the hydrophilic part of the phospholipids. Because sialic acid is not only an important molecule with respect to cancerology but also contributes a great part to the free carboxylic acid content of the cell wall, measurements were confined to this molecule. As phospholipid we took L-α-dipalmitoyllecithin, a molecule that is a typical representative of the membrane phospholipids. The subject lent itself quite well to an investigation of the role of several physiological cations.

In the Danielli–Davson model [29] for the cell membrane, two lipid layers lay parallel and the hydrophobic parts of the molecules are facing each other. Other models have evolved in the course of time the merits of which have been accentuated in some excellent reviews [30–31]. A common feature of all these models is some hydrophobic/hydrophilic orientation of the molecules taking part in the membrane structure. For this reason we believe that the following experimental model should be close to *in vivo* circumstances: a lecithin film is spread at an air/water boundary. In this way a monolayer is formed of oriented phospholipid molecules. As in the cell membrane, the fatty acid part of the molecules is oriented away from the water phase. Interaction between the monolayer and substances present in aqueous solution gives rise to a lowering of surface tension which is measured. The merit of this model resides in the fact that the phospholipid molecules are in a phase that can be termed neither crystalline nor solubilized and that closely resembles the situation at the natural membrane.

METHOD

The experiments can be simply described as follows: lecithin is spread on aqueous solutions containing a fixed concentration of NANA and varying concentrations of physiological cations. The decrease in surface tension is measured.

The technique is essentially the same as that described previously by Ruyssen and Joos [34] studying molecular interaction in monolayers between cholesterol and saponin. The bulk solution always contains 10^{-4} M *N*-acetylneuraminic acid (Synthetic, type IV, Sigma Chemical Co.), this concentration being in the physiological range. To this bulk solution different inorganic electrolytes were added: $CaCl_2$, $MgCl_2$, KCl and NaCl (Analar grade, purchased from UCB). Since these NANA solutions, with or without electrolytes, were hardly surface active, a lecithin monolayer (L-α-dipalmitoyllecithin, purchased from Sigma Chem. Co., Grade II) could be spread upon it, the spreading condition of Harkins being fulfilled. Immediately before spreading

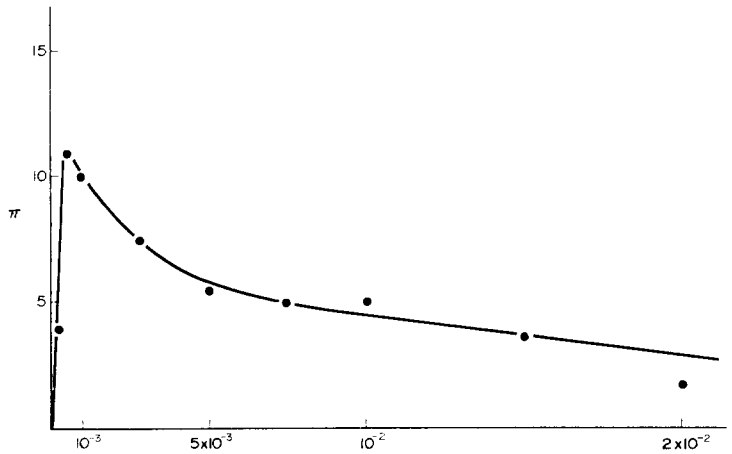


Fig. 1. The effect of calcium concentration on the interaction between a 10^{-4} *M NANA-solution and a lecithin monolayer with surface concentration:* $1 \cdot 32.10^{-10}$ *mole/cm²* (125 Å² *per lecithin molecule).*

the surface was sucked off, whereafter a lecithin monolayer was spread upon it. The molecular area per lecithin molecule always exceeds 100 Å². From the compression curve we know that the surface pressure of such dilute lecithin monolayers is small [35]. To the spreading lecithin solution, in CCL_4, some drops of amylalcohol were added to assist spreading. The lecithin concentration was about 54·5 mg/100 ml and the amount spread never exceeds 8 µl., the area of the surface was 46·54 cm².

The measurements were done at room temperature with a tensiometer with torsion wire (J. Guastalla, Jobin Yvon, Paris-Arcueil). Bidistilled water was used. Each measurement took approximately five hours, this is the time necessary for attainment of equilibrium.

RESULTS

In Figs. 1–4 the effects of the various physiological cations are represented. In ordinate the π-value is plotted i.e. the difference between the surface tension of pure water and that of our system. Additional measurements were made:

1. Lecithin spread on pure water: $\pi = 0$.
2. NANA-solution:
   without lecithin film and without cations: $\pi = 0$; without lecithin film but with addition of the physiological cations: the π-value is negligible.
3. Salt solutions without NANA but with lecithin film.
   10^{-3} M Ca^{2+} : $\pi = 1 \cdot 4$
   10^{-2} M Mg^{2+} : $\pi = 1 \cdot 2$
   10^{-3} M K^{+} : $\pi = 3 \cdot 6$
   $5 \cdot 10^{-4}$ M K : $\pi = 0 \cdot 6$
   10^{-2} M Na^{+} : $\pi = 1 \cdot 0$
4. The NANA-concentration and the Ca-concentration were kept constant and the lecithin concentration was varied. The results are given in Fig. 5.

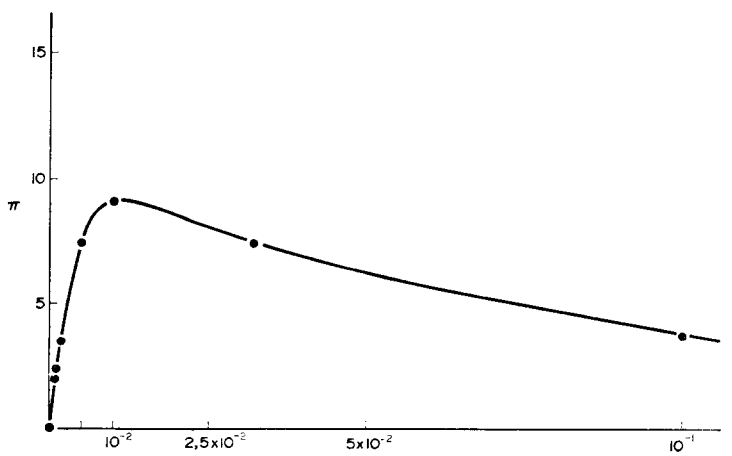


Fig. 2. The effect of magnesium. NANA: 10^{-4} *M; lecithin:* $1 \cdot 32.10^{-10}$ *mole/cm².*

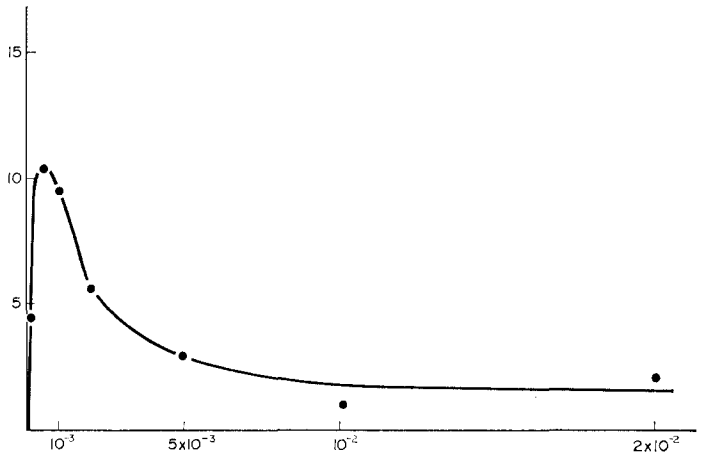


Fig. 3. *The effect of potassium. NANA:* 10^{-4} *M; lecithin:* 1·32. 10^{-10} *mole/cm*^{2}.

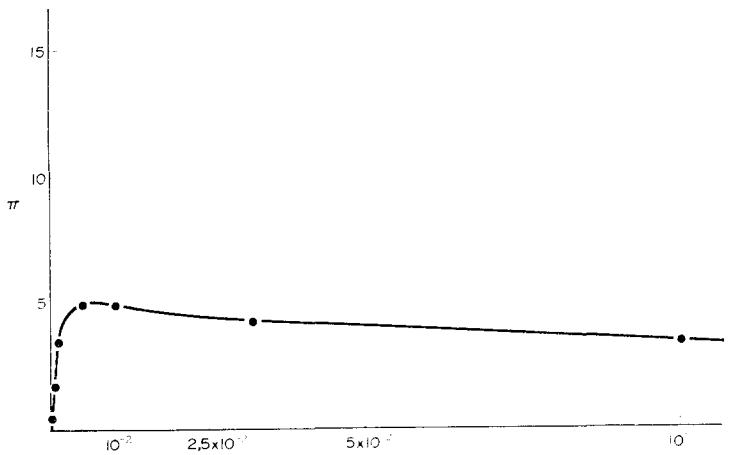


Fig. 4. *The effect of sodium. NANA:* 10^{-4} *M; lecithin:* 1·32. 10^{-10} *mole/cm*^{2}.

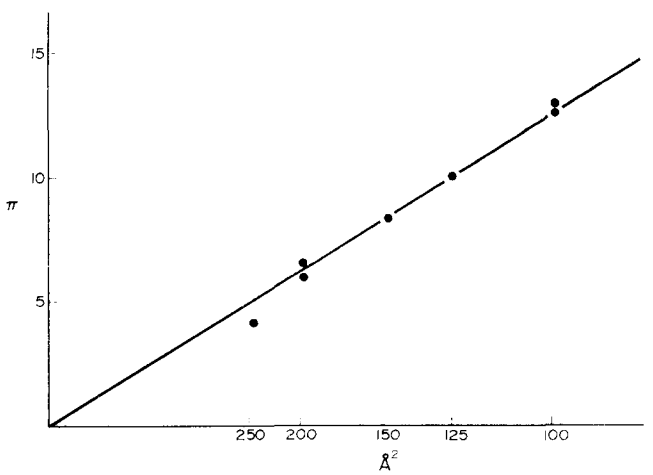


Fig. 5. *Variation of the surface concentration of lecithin expressed in square Ångstrøm available per molecule. NANA:* 10^{-4} *M; Ca:* 10^{-4}*M.*

DISCUSSION

The lowering of surface tension implies some kind of binding between the lecithin film and sialic acid in which the physiological cations play a part. Since there is no interaction in the absence of the cations, their role can be visualized as a bridge between the two organic molecules. Indeed there are reasons to assume that the cations can bind either to lecithin or NANA separately. The phosphate group of lecithin can bind positive ions by Coulomb forces and sialic acid is known to be a weak complexing agent [36].

One can speculate with good right about the possibility that the same interaction occurs at the cell wall. The concentrations of the physiological cations in the intercellular milieu are not exactly known but it can be assumed that they are close to the serum concentrations ($Ca^{2+} = 2 \cdot 5.10^{-3}$ M; $Mg^{2+} = 1 \cdot 10^{-3}$ M; $K^{+} = 4 \cdot 10^{-3}$ M; $Na^{+} = 1 \cdot 4.10^{-1}$ M). It then would follow from our experiments that Ca^{2+} exerts the strongest effect whereas those of the other ions are approximately equally great. However since the interaction under consideration is that between the terminal NANA-group of the glycoproteins of the cell coat and the phospholipids of the cell membrane, the cations have to penetrate across the coat before being able to build up the bridge. There are no data about ionic concentrations at that particular place and it can be doubted if they ever will be available. From our experiments we can only conclude that calcium and potassium have the highest specificity and also reach maximum effectivity at lower concentration than magnesium and sodium. Possibly this can be explained as a question of atomic radius.

It is important to note that the observed specificity of calcium and potassium is in accordance with the already mentioned effects of these ions on cell adhesion [23–26]. We then can propose following model: the NANA-terminal ends of the glycoproteins can be *either* bound to the phospholipids of the cell membrane thereby increasing the force of glycoprotein attachment already achieved by hydrogen bonds and Van Der Waals forces, *or* can be extruded into the intercellular environment. In the latter case there will be less mutual adhesion between cells because the glycoproteins have less attachment to the membrane and sialomucin molecules of adjacent cells undergo increased electrostatic repulsion due to free carboxyl groups. It must be postulated that addition of calcium or potassium ions results in binding the carboxyl groups to the membrane phospholipids rather than binding together the sialomucin molecules of neighbouring cells via these same carboxyls. The experiments of Weiss [23, 24] appear to be in support of this thesis. We cite [24]: "The fact that no calcium-sensitive-aggregation was observed suggests that calcium does not form 'bridges' between the adjacent anionic sites in different cells". The model unites the finding of low calcium content in tumor tissue with the hypothesis of Apffel and Peters [22] on the decreased immunological response due to the extruded NANA-terminal ends of the glycoproteins.

Removal of sialic acid from the surface of ascites tumor cells caused a highly significant reduction in cell mobility whereas sialic acid removal from solid tumor cells caused no mobility change [17, 18]. Our model offers an explanation for this fact, namely in ascites tumor cells the NANA-terminals extrude into the interstitial fluid whereas in solid tumor cells they are phospholipid bound. From the fact that the amount of sialic acid released by neuraminidase treatment is approximately the same for solid tumor cells and ascites tumor cells [17, 18] and moreover the fact that whole normal cells did not show any significant deviation in their sialic acid content from normal cells [37], it may perhaps be concluded that the modification from the solid cell type into the ascites cell type involves a kind of modulation i.e. a reversible transfer of sialic acid residues from the membrane, into an extruded position. Since sialyl-hexose units constitute short side chains on a peptide chain, the modulation requires but a minimum amount of energy because the effect can be produced by a slight axial rotation of peptide chains that lay parallel to the membrane.

It is possible that the present model provides a molecular basis for some interesting speculation. Glick and Githens [38] found that removal of sialic acid inhibited potassium flow across the cell wall either inward or outward depending upon the incubation conditions. Sodium transport was not affected. Sialic acid removal also caused [19] a 40% inhibition of protein release from the cell without effect on the release of nucleotides and sugars. This result seems important with respect to the well-known enhanced leakage of intra-cellular proteins from tumor cells. The authors [39] suggest the existence of a specific "transport protein" which, when bound to sialic acid, would block the secretion of intra-celullar proteins. Our study rather would suggest either a modification of the membrane perme-

ability to proteins by binding of sialic acid to the phospholipids, or a role of the sialomucin modulation in the transport of proteins from the membrane to the extra-cellular region.

REFERENCES

1. E. H. Mercer, The external surface of the cell and intercellular adhesion. *Rec. Progr. Surf. Sci.* **1,** 360 (1964).
2. G. M. W. Cook, Chemistry of membranes. *Brit. med. Bull.* **24,** 118 (1968).
3. R. Barre et J. Labat, Acides nonulosaminiques et nonulosaminidases. *Sem. des Hôpitaux; Path. et Biol.* **24,** 2336 (1961).
4. A. Gottschalk, *The Chemistry and Biology of Sialic Acids and Related Substances.* Cambridge University Press, London (1960).
5. A. Gottschalk and E. R. Graham, 6-α-D-Sialyl-N-acetyl-galactosamine: The neuraminidase-susceptible prosthetic group of bovine salivary mucoprotein. *Biochim. biophys. Acta* **34,** 380 (1959).
6. A. Gottschalk, W. H. Murphy and E. R. B. Graham, Carbohydrate-peptide linkages in glycoproteins and methods for their elucidation. *Nature* **194,** 1051 (1962).
7. W. H. Murphy and A. Gottschalk, Studies on mucoproteins. VII: The linkage of the prosthetic group to aspartic and glutamic acid residues in bovine submaxillary gland mucoprotein. *Biochim. biophys. Acta* **52,** 349 (1961).
8. G. M. W. Cook, Linkage of sialic acid in the human erythrocyte ultra-structure. *Nature* **195,** 15: (1962).
9. O. K. Langley and E. J. Ambrose, The linkage of sialic acid in the Ehrlich ascites-carcinoma cell surface membrane. *Biochem. J.* **102,** 367 (1967).
10. M. J. Spiro and R. G. Spiro, Glycoprotein biosynthesis and thyroid sialyl-transferase. *J. biol. Chem.* **243,** 6520 (1968).
11. N. Ohta, A. B. Pardee, B. R. McAuslan and M. M. Burger, Sialic acid contents and controls of normal and malignant cells. *Biochim. biophys. Acta* **158,** 98 (1968).
12. H. Wu, E. Meezan, P. H. Black and P. W. Robbins, Differences in composition of particulate fractions from mouse fibroblasts 3T3 and SV_{40} transformed 3T3. *Fed. Proc.* **27,** 814 (1968).
13. S. Ben-Or, S. Eisenberg and F. Doljanski, Electrophoretic mobilities of normal and regenerating liver cells. *Nature* **188,** 1200 (1960).
14. D. H. Heard, G. V. F. Seaman and I. Simon-Reuss, Electrophoretic mobility of cultured mesodermal tissue cells. *Nature* **190,** 1009 (1961).
15. G. V. Fuhrmann, Cytopherograms of normal, proliferating and malignant rat liver cells. In *Cell Electrophoresis* (Edited by E. J. Ambrose), p. 92–98. Churchill: London (1965).
16. P. S. Vassar, Electric charge density of human tumor cell surfaces. *Lab. Invest.* **12,** 1072 (1963).
17. D. F. Wallach and M. V. De Perez Esandi, Sialic acid and the electrophoretic mobility of three tumor cell types. *Biochim. biophys. Acta* **83,** 363 (1963).
18. G. M. W. Cook, G. V. F. Seaman and L. Weiss, Physicochemical differences between Ascitic and Solid forms of Sarcoma 37 Cells. *Cancer Res.* **23,** 1813 (1963).
19. V. Defendi and G. Gasic, Surface mucopolysaccharides of polyoma virus transformed cells. *J. cell. comp. Physiol.* **62,** 23 (1963).
20. G. Gasic and T. Gasic, Removal of sialic acid from the cell coat in tumor cells and vascular endothelium and its effects on metastases. *Proc. nat. Acad. Sci.* **48,** 1172 (1962).
21. R. R. Coombs and P. J. Lachmann, Immunological reactions at the cell surface. *Brit. med. Bull.* **24,** 113 (1968).
22. C. A. Apffel and J. H. Peters, Tumors and serum glycoproteins. The "Symbodies". *Progr. exp. Tumor. Res.* **12,** 1 (1969).
23. L. Weiss, Studies on cell deformability. III: Some effects of EDTA on Sarcoma-37 cells. *J. Cell Biol.* **33,** 341 (1967).
24. L. Weiss, Studies on cell deformability. IV: A possible role of calcium in cell contact phenomena. *J. Cell Biol.* **35,** 347 (1967).
25. C. Rappaport and G. B. Howze, Dissociation of adult mouse liver by sodium tetraphenylboron, a potassium complexing agent—Further studies. *Proc. Soc. exp. Biol. Med.* **121,** 1010, 1016 (1966).

26. C. RAPPAPORT, Effect of temperature on the dissociation of adult mouse liver with sodium tetraphenylboron. *Proc. Soc. exp. Biol. Med.* **121,** 1022 (1966).
27. A. C. ALLISON, Some effects of pharmacologically active compounds on membranes. *Brit. med. Bull.* **24,** 135 (1968).
28. L. L. HAUSE, R. A. PATILLO, A. SANCES, JR. and R. F. MATTINGLY, Cell surface coatings and membrane potentials of malignant and non malignant cells. *Science* **169,** 601 (1970).
29. J. F. DANIELLI and H. DAVSON, A contribution to the theory of permeability of thin films. *J. cell. comp. Physiol.* **5,** 495 (1935).
30. A. D. BANGHAM and D. A. HAYDON, Ultrastructure of membranes bimolecular organization. *Brit. med. Bull.* **24,** 124 (1968).
31. J. A. LUCY, Ultrastructure of membranes: Micella organization. *Brit. med. Bull.* **24,** 127 (1968).
32. L. WEISS and E. MAYHEW, The cell periphery. *New Engl. J. Med.* **276,** 1354 (1967).
33. J. W. L. BEAMENT, The insect cuticle and the membrane structure. *Brit. med. Bull.* **24,** 130 (1968).
34. R. RUYSSEN and P. JOOS, Penetration of gaseous cholesterol monolayers by saponins. *Med. Kon. VI. Acad. België, Kl. Wet., Jg* **XXVII,** Nr. 8 (1965).
35. R. A. DEMEL and P. JOOS, Interaction between lecithin and cholesterol at the air/water and oil/water interface. *Chem. Phys. Lipids* **2,** 35 (1968).
36. H. EGGERS-LURA, Investigations into complex formation of tooth calcium. *Dent. Res.* **42,** 1245 (1963).
37. A. KIMURA, Y. NAGAI, K. TURUMI, Y. KAWASHIMA and H. SATO, Hexosamine and sialic acid contents in Cells. *Nature* **191,** 596 (1961).
38. J. L. GLICK and S. GITHENS, The role of sialic acid in potassium transport of L1210 leukemia cells. *Nature* **208,** 88 (1965).
39. J. L. GLICK, A. R. GOLDBERG and A. B. PARDEE, The role of sialic acid in the release of proteins from L1210 leukemia cells. *Cancer Res.* **26,** 1174 (1966).

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Virus-like Particles in L5178Y Murine Leukaemic Cells

H. K. NARANG, T. M. BELL and PATRICIA E. GIBSON

Medical Research Council, Demyelinating Diseases Unit, Newcastle General Hospital, Westgate Road, Newcastle upon Tyne NE4 6BE, Great Britain

Abstract — *Type-A and type-C particles have been demonstrated in three strains of the L5178Y murine leukaemic cell line. Type-A particles were found in 0·5–7% of the cell profiles depending on the strain and method of growth of the cultures. The size and buoyant density of the type-A particles corresponded with that of mouse-mammary carcinoma virus. Negative contrast staining of these particles revealed the presence of external spikes and an indication of a coiled internal structure.*

INTRODUCTION

GOLDENBERG and Thomas [1] described the occurrence of two types of virus-like particles in the L5178Y murine leukaemic cell line. One type occurred in the nucleus and resembled polyoma virus, while the second was only observed extracellularly and was similar to the type-C particles of Bernhard [2]. Only about 0·1% of the cells were affected and Fischer (quoted by Goldenberg and Thomas [1]) failed to find any virus-like particles. As part of a series of investigations involving this cell line (Gibson, Bell and Field, to be published) various aliquots have been examined in the electron microscope. We wish to briefly report the finding of type-A as well as type-C particles in this tumour.

MATERIAL AND METHOD

Three lines of the L5178Y leukaemia have been received in Newcastle from Professor P. Alexander [3, 4]. One (L5178Y-N) was received at passage level 27 and has been maintained for approximately two years by intraperitoneal passage in DBA/2 mice. In addition to copious peritoneal exudate, masses of solid tumour are frequently found, subcutaneously, in the liver and in the mesentery. Pieces of solid tumour from each site, cells pelleted from peritoneal exudate and *in vitro* cultures were taken for examination. The second, standard cell line (L5178Y-E), was received at passage level 29; while the third, aberrant cell line (5178Y-ES), was received at passage level 95. Cells from both these lines were pelleted from the peritoneal exudates and fixed for electron microscopy before passaging in Newcastle.

Cells from the seven different sources were examined by thin-sectioning and negative-staining techniques. Thin slices of solid tumours and pellets of cell suspensions were prefixed in 4% glutaraldehyde in 0·2 M sodium cacodylate solution at pH 7·2 and post-fixed in Dalton's fixative. The material was then embedded in Epon 812, thin sections cut on a LKB ultrotome, and stained with uranyl acetate and lead citrate. They were examined in a Philips 200 electron microscope. For negatively stained preparations unfixed pellets of either whole cell homogenates or ultra-centrifuged homogenates were re-suspended in 2% phosphotungstic acid at pH 6·8.

The buoyant density of the type-A particles was determined by homogenising cells from peritoneal exudate in a Griffith's tube followed

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by sonication and centrifugation at 5000 rev/min for 15 min. The supernatant was re-centrifuged at 20,000 rev/min (46,000 **g** in a Beckman L2 ultracentrifuge for 5 hr. The pellet was redissolved in 10% sucrose solution and layered onto a sucrose gradient which was prepared stepwise from 20% through 60% and buffered to pH 7·5 with 10 mM Tris. The gradient was centrifuged at 35,000 rev/min in a titanium SW41 roto (210,000 **g** max) for 7 hr.

RESULTS

The standard strain (L5178Y-E) grew as a small discrete cell culture in the peritoneal cavity, whereas the other two grew in clumps and the individual cells were much larger. Virus-like particles resembling the type A and type-C particles of Bernhard [2] were observed in every preparation examined (Figs. 1–5).

The intracellular type-A particles had an external diameter of 65–75 mμ (Figs. 1–3). The proportion of cell profiles in each specimen, positive for type-A particles is given in Table 1. Tubules were also found in the mesenteric tumour mass in close association with the type-A particles (Fig. 2). The overall diameter of these tubules was 45–55 mμ with an internal core of 25–30 mμ diameter.

The extracellular type-C particles had an overall diameter of 105–110 mμ and an internal core of 70–75 mμ (Fig. 4). The greatest numbers were seen at the surface of the cells cultured *in vitro*. In some preparations various stages of budding and penetration of the type-C particles were observed (Figs. 5a and 5b).

Cells from the 76th intraperitoneal passage in DBA/2 mice were homogenized in a Griffith's tube and examined by negative contrast staining. Figure 6 shows particles penetrated by stain giving an indication of a coiled internal structure. The overall diameter of these particles was 60–65 mμ and the internal diameter 50–55 mμ. Figure 7 shows the presence of projections approximately 8 mμ long on the surface of intact particles.

In the gradient centrifugation the band containing virus-like particles was obtained at 50% sucrose concentration. This gave a buoyant density of 1·23% for the particles which had the dimensions of type A.

Coiled masses of rough endoplasmic reticulum were observed only in a tumour produced in a scrapie-infected WS mouse inoculated intravenously with cells from the 73rd intraperitoneal passage (Fig. 8).

DISCUSSION

Type-A and type-C particles were present in all three lines of the L5178Y leukaemia. A greater proportion of the cell profiles of the "N" line contained particles and there were also more particles per cell than in the "E" and "ES" lines. In the "N" line the type-A particles occurred most frequently in cells passaged by the intraperitoneal route, without culturing *in vitro*. On the other hand the type-C particles were most prevalent in the *in vitro* culture. These results indicate that the "replication" of the virus-like particles differs under varying conditions of cell growth. The demonstration of budding and entry of the type-C particles suggests that this type, at least, was biologically active.

The size, general appearance and buoyant

Table 1. *Proportion of cell profiles with type-A particles*

Cell line	Passage level*	Tissue specimen	Cell profiles with particles
L5178Y-E	29D	Ascitic fluid	1%
L5178Y-ES	95D	Ascitic fluid	0·5%
L5178Y-N	76D	Ascitic fluid	7%
L5178Y-N	73D; 1X	Subcutaneous tumour	5%
L5178Y-N	73D; 1W†	Liver tumour	5%
L5178Y-N	61D; 19T; 1X	Mesenteric tumour	2–3%
L5178Y-N	76D; 9T	Tissue culture	1%

*D=passage in DBA/2 mice; W=passage in WS (Webster Swiss mice); X=passage in DBA/2 XWS mice; T=tissue culture passage.

†This mouse had early clinical scrapie following i.c. infection 14 weeks previously.

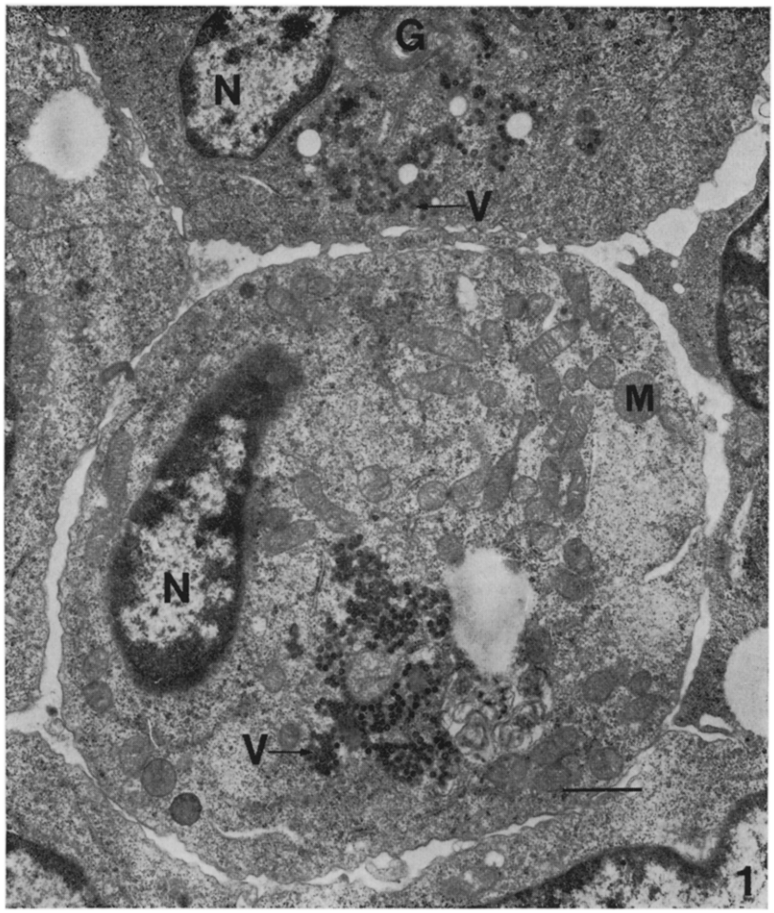


Fig. 1. *Thin section of pelleted cells from ascitic fluid from* 76*th intraperitoneal passage of L*5178*Y-N leukaemia in DBA/*2 *mice. Type-A particles present in the cytoplasm of two adjoining cells. G=Golgi body; M=mitochondrion; N=nucleus; V=virus-like particles. Scale line* =800 mμ.

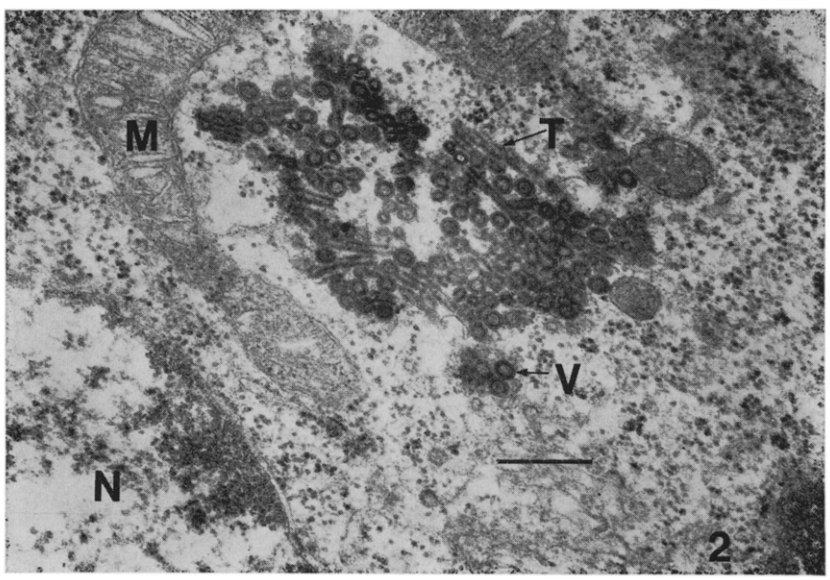


Fig. 2. *Type-A particles in thin section of tumour in mesentery following intraperitoneal inoculation of DBA/*2 *X WS mice with* $1{\cdot}6\times10^5$ *cells cultured for nineteen passages* in vitro. *The tumour occurred* 28 *days post-inoculation. M=mitochondrion; N=nucleus; T=tubules; V=virus-like particles. Scale line=*400 mμ.

(to face p. 326.)

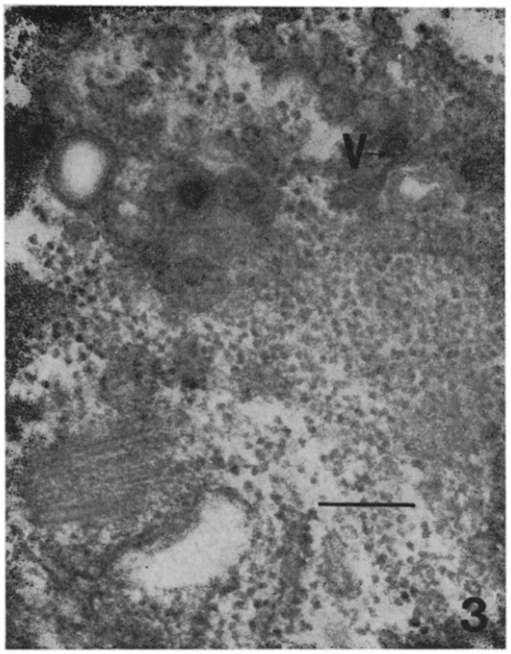


Fig. 3. *Type-A particles in thin section of pellet of cells from ascitic fluid from* 29*th passage of* L5178Y-*E leukaemia.* V=*virus-like particles. Scale line* 200 mμ.

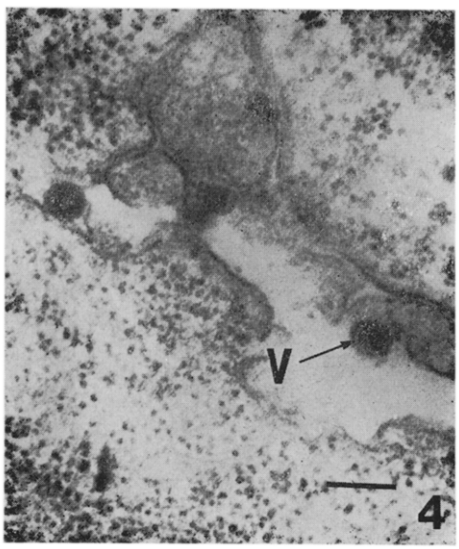


Fig. 4. *Type-C particles in thin section of cells pelleted from* 95*th passage of* L5178Y-*ES leukaemia.* V=*virus-like particles. Scale line*=200 mμ.

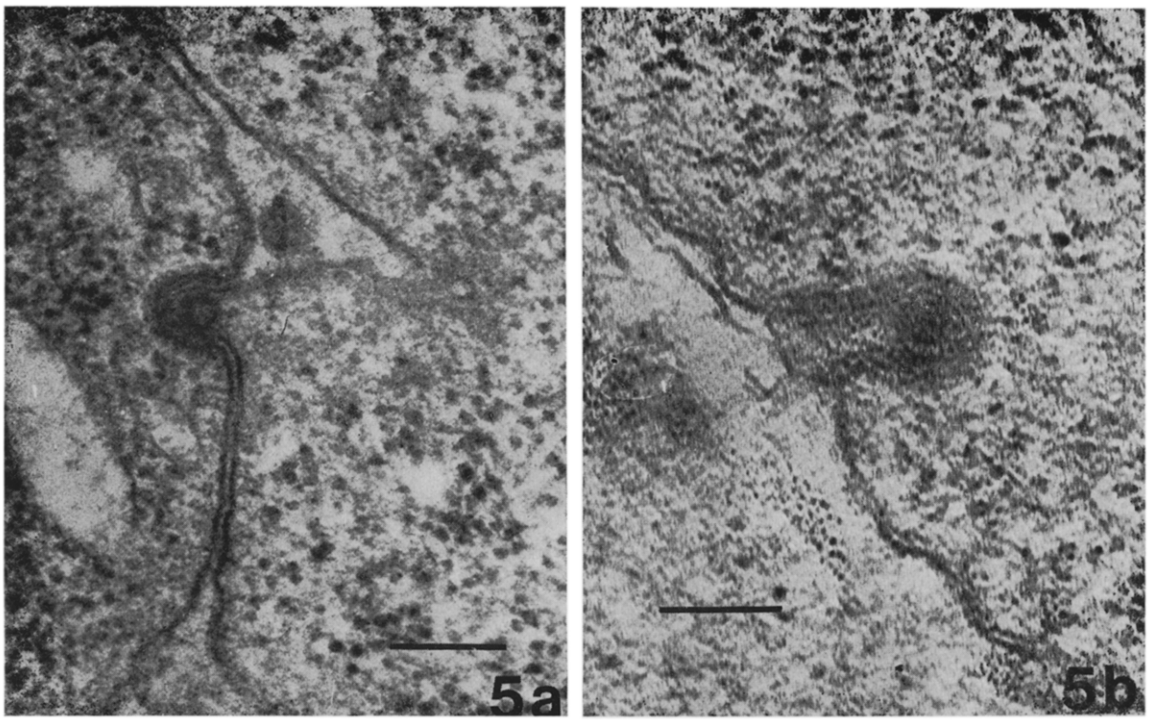


Fig. 5. *Thin section of liver tumour in a scrapie-infected mouse inoculated intravenously with* 3×10^6 *cells from* 73*rd* in vivo *passage.* (*a*) *Type-C particle budding from surface of one cell and entering cytoplasm of a second cell.* (*b*) *Type-C particle deeply embedded in surface of a cell. Scale line* = 200 mμ.

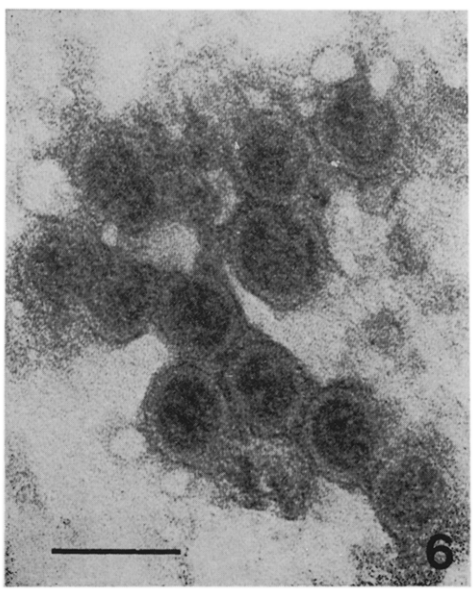


Fig. 6. *Negatively-stained particles in homogenate of cells from* 76*th intraperitoneal passage in DBA/2 mice. Suggestion of coiled internal structure. Scale line* = 100 mμ.

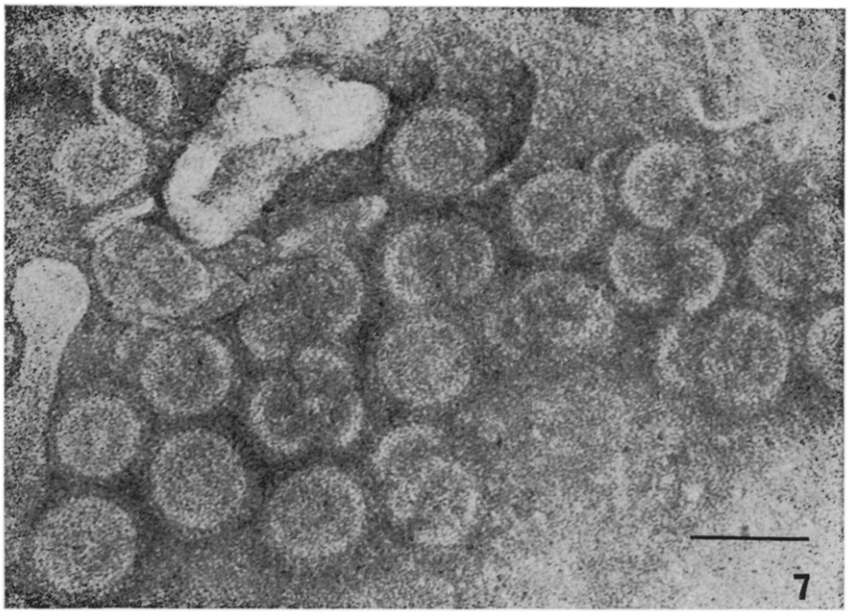


Fig. 7. *Negatively-stained particles in pellet of ultracentrifuged cell homogenate, as Fig.* 6, *showing projections on external coat. Scale line* = 100 mμ.

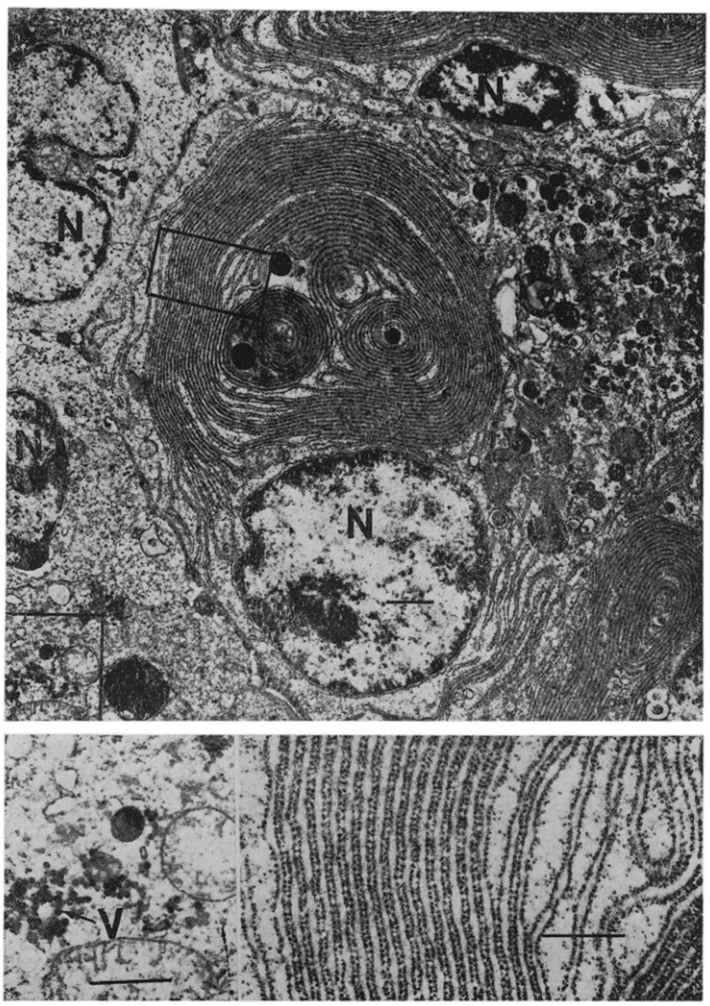


Fig. 8. *Thin section of tumour mass occurring in the liver of a scrapie-infected WS mouse* 17 *days following intravenous inoculation with* 3×10^3 *cells from the* 73*rd* in vivo *passage, showing masses of coiled endoplasmic reticulum in three adjoining cells. Scale line* = 1000 mμ. *Left-hand corner magnified to show A-type particles in a fourth adjoining cell. Scale line* = 800 mμ *Upper rectangle magnified to show ribosomes attached to the surfaces of the endoplasmic reticulum. Scale line* = 400 mμ. *N* = *nucleus; V* = *virus-like particles.*

density of the type-A particles corresponds very closely to that of the mouse mammary carcinoma virus (Bittner Agent) [2, 5, 6]. Negative staining of these particles showed the presence of external spikes and suggested that there was a coiled internal structure. Neither of these findings has been demonstrated previously in connection with type-A particles. Previous reports on the fine structure of partially purified A particles [6, 7] have revealed only the concentric ring structure seen in Fig. 6.

The relationship of the type-A and type-C particles to the L5178Y tumour is uncertain but it appears probable that both types have been "picked up" by the tumour during passage in DBA_2 mice. The intranuclear particles described by Goldenberg and Thomas [1] have not been found in the present material, but their type-C particles do resemble those presented here.

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REFERENCES

1. G. J. Goldenberg and C. E. Thomas, Virus-like particles in murine leukaemia L5178Y. *Nature, Lond.* **214,** 1339 (1967).
2. W. Bernhard, The detection and study of tumour viruses with the electron microscope. *Cancer Res.* **20,** 712 (1960).
3. G. A. Gischer, Studies of the culture of leukemic cells *in vitro*. *Ann. N.Y. Acad. Sci.* **76,** 673 (1958).
4. P. Alexander, A. I. Connell and Z. B. Mikulska, Treatment of a murine leukemia with spleen cells or sera from allogeneic mice immunized against the tumour. *Cancer Res.* **26,** 1508 (1966).
5. C. H. Andrews and H. G. Pereira, *Viruses of Vertebrates*, (2nd Ed.), p. 139. Bailliere, Tindall and Cassell, London (1967).
6. E. L. Kuff, N. A. Wivel and K. K. Lueders, The extraction of intracisternal A-particles from a mouse plasma-cell tumour. *Cancer Res.* **28,** 2137 (1968).
7. J.-P. Kerckaert, J. Montreuil, M.-C. Doyennette, F. Puvion, M.-N. Vlaeminck, A. Verbert, A. Demaille et J. Driessens, Cancérologie — Isolement et purification des particules virales du type A présentes dans le cytoplasme du lymphome médiastinal de la Souris Swiss induit par l'éthyl-uréthane. *C. R. Acad. Sci.* (Paris) **271,** 624 (1970).

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Pneumonia Caused by Gram-Negative Bacilli in Hospitalized Patients Presenting Malignant Disease

JEAN KLASTERSKY*, ROGER CAPPEL*, LOUISE DEBUSSCHER* and MAGDA STILMANT†‡

**Service de Médecine et d'Investigation Clinique (Section des Maladies Infectieuses) et †Service d'Anatomie Pathologique, Institut J. Bordet, Centre des Tumeurs de l'Université Libre de Bruxelles, Bruxelles, Belgium*

Abstract — *Pneumonias due to Gram-negative bacilli are described in* 37 *patients with cancer who were admitted to a specialized center for cancer therapy over a* 6*-month period. Klebsiella and Pseudomonas aeruginosa accounted for most cases. The pneumonias represented* 4·3% *of all hospital admissions and accounted for* 32% *of the deaths occurring in the hospital over the study-period. The disease was much more frequent in men. All the infections, except two, were probably hospital-acquired, but those developing within six days of admission had a lower mortality rate* (40%) *than those developing later* (70%). *The overall mortality rate was* 62% *and mortality correlated best with the clinical severity of the underlying neoplasm.*

On X-ray study lower-lobe bronchopneumonias were characteristic but Klebsiella frequently involved the upper lobes. All three cases of pneumonia caused by Proteus were associated with empyemas. Associated purulent bronchitis was characteristic in Pseudomonas pneumonias. Post mortem examination was performed in 19 *cases and showed interstitial pneumonias with polymorphonuclear infiltrates of alveoli and bronchial mucosa. Hemorrhages and necrosis were frequent in pneumonias caused by Klebsiella.*

Multiple antibiotic resistance was commonplace. Pathogenic strains isolated in early occurring pneumonias were more sensitive to antibiotics than bacteria isolated from pneumonias occurring later. The drug of choice here was gentamicin.

SINCE the introduction of antibiotics, Gram-negative bacilli have become increasingly important in the etiology of human infections [1]. According to Barrett *et al.*, hospital-acquired respiratory infections develop in approximately 5% of patients admitted to a general hospital [2]. Among many factors predisposing to hospital-acquired pneumonia the severity of the underlying disease and previous treatment with antibiotics are important [3, 4]. Primary pneumonia caused by Gram-negative rods is a more rare occurrence, although possibly increasing. It accounted for 0·16% of all admissions in a large general hospital [5]. It is a disease of middle-aged men with underlying alcoholism, diabetes mellitus, cardiac or chronic pulmonary diseases. Both primary and hospital-acquired pneumonias

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*Fellow of the Association "Les Amis de l'Institut Bordet".

‡At present resident in Pathology, Mallory Institute of Pathology, Boston City Hospital, Boston, Mass.

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Please address requests for reprints to Dr. J. Klastersky, Service of Medicine, Institut Jules Bordet, 1 rue Héger-Bordet, 1000 Bruxelles, Belgium.

caused by Gram-negative rods have a very high mortality rate [4, 5].

The study of pneumonias caused by Gram-negative bacilli in patients with cancer provides a unique opportunity to investigate the characteristics of this disease in a relatively homogeneous population as far as the underlying disease is concerned. This study was undertaken in an attempt to discover, if possible, the clinical and bacteriological patterns of pneumonia caused by Gram-negative rods in hospitalized patients presenting a malignant disease.

MATERIAL AND METHODS

The Institut Jules Bordet is a 150-bed hospital, specializing in the medical, surgical and radiological treatment of cancer. During the six-month period of this study (January–June 1970) there were 845 admissions.

Case records of all patients who had one or more sputa reporting predominantly Gram-negative bacteria were reviewed. Only those considered to have clinical infection compatible with the diagnosis of parenchymal involvement of the lung were included here. Criteria for pulmonary infection by Gram-negative rods were:

(1) initial stain of the sputum showing Gram-negative bacilli, and

(2) isolation of the same bacteria from two or more consecutive sputa; or the presence of the same organism in the sputum and in the blood or in the pleural fluid in close temporal proximity. To be considered significant the bacteria had so be present as the only or predominant organism. Moreover, all the patients who survived responded dramatically to specific treatment directed against Gram-negative rods, and histologic and bacteriologic evidence of infection of the respiratory tract was found at autopsy in all autopsied cases (19 out of 23 deaths).

In this laboratory sputum is cultured routinely on blood-agar and eosin-methylene blue (EMB) agar. Specimens of blood are inoculated in trypticase soy broth (Difco) and in thioglycollate broth (Difco). Pleural fluid is cultured on blood and EMB agars and in thioglycollate broth.

Gram-negative bacilli were identified following Edwards and Ewing [6]. Sensitivity studies were performed on nutrient-agar (Difco) containing serial dilutions of antibiotics by the inocula-replicating method of Steers *et al.* [7] using an inoculum containing 10^6–10^7 micro-organisms per ml.

RESULTS

General

During the study-period, infection of the respiratory-tract caused by Gram-negative bacilli was observed in 37 patients and accounted for 4·3% of all hospital admissions as shown in Table 1. The frequency of the disease was much higher in males than in females. The pneumonia was fatal in 23 patients, and thus accounted for 32% (23 out of 71) of the deaths occurring in this hospital during the 6-month period.

Over the same period of time, Gram-negative bacilli were reported in predominant growth in 172 cultures of the sputum; the ratio of clinical pneumonia to bacterial colonization observed here was 1:3·6.

Klebsiella-Enterobacter species (15 Klebsiella and 1 Enterobacter cloacae strain) and *Pseudomonas aeruginosa* were most often responsible for the infections studied here, 16 and 15 cases respecitvely. *Escherichia coli* and *Proteus mirabilis* accounted for 3 cases each. All the pneumonias developed in hospitalized patients

Table 1. *Incidence of pneumonia caused by Gram-negative bacilli among patients admitted to the hospital and relationship to the total number of deaths occurring over the* 6*-month period of the study*

	No. of patients admitted	Patients with pneumonia				Total deaths	
		No.	%	Died	%	No.	Due to pneumonia
Males	364	29	7·9	17	4·6	44	38
Females	481	8	1·6	6	1·2	27	22
Total	845	37	4·3	23	2·7	71	60

(average length of hospitalization prior to the pneumonia was 28·9 days) except in 2 cases, who were admitted with pneumonia caused by Klebsiella. Eight additional cases were diagnosed within 6 days of admission. None followed a pneumonia caused by Gram-positive organisms.

Overall mortality rate was 62% (23 deaths amongst 37 patients). The fatal cases included 10 patients with pneumonia due to Klebsiella (mean survival 5·5 days), 8 due to Pseudomonas (mean survival 11·8 days), 3 caused by Proteus (17·0 days) and 2 by *E. coli* (7·5 days). Mortality was 40% (4 out of 10 patients) in pneumonia which developed within six days of the admission, and 70% when the illness developed later. In the former group one patient was admitted in vasomotor collapse caused by pneumonia and septicemia due to Klebsiella and died within 24 hr of admission to the hospital.

Underlying disease

Malignant disease was present in all the patients studied here and mortality was higher in patients with generalized disease than in those with localized neoplasms, as shown in Table 2. Other underlying diseases were present in 16 patients, but their importance in mortality was presumably overshadowed by the neoplasia itself.

Except for the duration of the hospital stay prior to the onset of pneumonia, which seemed to affect the survival rate as already mentioned, no other condition such as age, sex, previous therapy with antibiotics, administration of corticoids, administration of cytostatic drugs, radiation therapy, surgery or the presence of leukopenia could be correlated to the prognosis.

Table 2. Relationship between various clinical factors and the outcome of pneumonia caused by Gram-negative rods

	Died (No.)	Survived (No.)	Total
Age			
Less than 60 years	8	6	14
Over 60 years	13	10	23
Sex			
Male	17	12	29
Female	6	2	8
Underlying disease			
Leukemia-Lymphoma	7	1	8
Disseminated tumors	15	7	22
Localized tumors	2	5	7
Other diseases*	10	6	16
No other disease	13	8	21
Duration of hospitalization†			
6 days or less	4‡	6	10
Over 6 days	19	8	27

*Congestive heart failure, (4); chronic bronchitis, (4); arterial hypertension, (3); alcoholism, (2); cirrhosis, (2); diabetes mellitus, (1).

†Duration of hospitalization prior to the onset of pneumonia.

‡2 patients were admitted with pneumonia due to Klebsiella.

Table 3. Signs and symptoms observed at the time of diagnosis in patients with pneumonia due to Gram-negative rods

	Organisms (No.)	Infiltrate (X-ray)	Empyema	Fever*	Dyspnea†	Purulent sputum‡	Leukocytosis§	Septicemia‖
Klebisella-Enterobacter sp.¶	16	16	1	14	3	11	8	3
Pseudomonas aeruginosa	15	9	0	15	8	14	8	1
Proteus mirabilis	3	3	3	3	1	3	2	0
Escherichia coli	3	3	1	3	1	2	1	2
Total	37	31	5	35	13	30**	19	6

*Rectal temperature >38°C.

†Associated with bronchial hypersecretion, necessitating tracheostomy.

‡Associated with Gram-negative bacilli on Gram-stained smears.

§Over 10,000 white cells per cubic milliliter.

‖Vasomotor collapse developed in 5 of the patients with septicemia.

¶1 *Enterobacter clocae.*

**All the patients with rare or absent leukocytes in the sputum were leukopenic (WC count <3000 per mm³).

Symptoms and signs

Rales in the lungs were uniformly heard, as shown in Table 3 (37 out of 37 cases), but signs of lobar consolidations were rare. Radiologic infiltrate of the lungs was present in 31 out of 37 cases (83%), most often as a lower lobe bronchopneumonia (10 bilateral, 4 left-sided, 12 right-sided). There were infiltrates in the right upper lobes in three cases (2 Klebsiella, 1 Pseudomonas) and left perihilar infiltrates in two. Pleural effusions were seen in 5 patients and corresponded to empyemas. All the patients with empyema died. Abscesses were not detected by X-ray examination during life.

Sudden onset of fever was observed in 35 out of 37 patients (94%) often with chills. The average initial temperature was 38°C and persisted for an average of 5 days. One patient remained afebrile throughout and survived, and another had an initial temperature of 40·5°C and died in vasomotor collapse within 24 hr of admission. The average initial temperatures in patients who died or survived were respectively 38·6°C and 38·7°C. Tachycardias were out of proportion to fevers. Respiratory symptoms such as cough, and hemoptysis were difficult to evaluate in those patients. Twelve patients had primary neoplastic lesions of the bronchial tract or pulmonary metastasis, and many of them had thrombocytopenia in addition. Severe dyspnea associated with bronchial hypersecretion developed in 13 (35%) patients and tracheostomy was performed in all of them.

Yellow sputum containing numerous white cells was observed in 30 patients (81%); 17 patients were able to produce spontaneously copious amounts of sputum but in 13 other cases transtracheal aspirations had to be performed for diagnostic purposes. The 7 patients with moderate or low leukocyte counts in the sputum had all a depressed blood white cell count (average 1520 granulocytes per mm^3, range 2600–500).

Blood cultures were performed in all patients prior to the administration of antibiotics. Septicemia was associated with the pneumonia in 6 cases (17%); 5 developed vasomotor collapse and died (mean survival duration 6·4 days, range 1–12 days). One patient with *E. coli* pneumonia associated with bacteremia survived.

Laboratory data

Much of the laboratory data was difficult to evaluate in our patients because of the severe underlying neoplastic disease.

Nineteen patients (51%) had initial white-cell counts over 10,000 white-cell per mm^3. The average initial white-cell count was 10,400 WC/mm^3, range 34,000–950 WC/mm^3. Marked shifts to the left were noted in all cases (average 8570 granulocytes/mm^3, i.e. 82% of the average WC count) even in those who did not show leukocytosis or those who were leukopenic as a result of previous radiation therapy or administration of cytotoxic drugs. Pleural fluids were uniformly polymorphonuclear leukocytic exudates with 15,000–60,000 cells per mm^3. Protein concentrations in these fluids ranged from 4·3–7·0 g per 100 ml.

In most patients serum bilirubin (mean 0·7 mg%) and alkaline phosphatases (mean 2·4 Bodansky units/ml) were within the normal range, except in 4 patients all of them having metastases of the liver. Blood urea (mean 35 mg%) was elevated in 4 patients but serum creatinine levels were normal in all of them. Transaminases were slightly elevated in 13 out of 37 patients (mean 20 mU/ml, range 15–50).

The blood levels of immunoglobulins was within the normal range in all patients; respectively the mean values for IgA, IgG and IgM were 296, 1243, and 126 mg%.

Therapy

Antibiotics were administered to all patients as soon as a tentative diagnosis of pneumonia had been made.

Table 4 shows the sensitivity patterns of organisms responsible for the pneumonias in this study, as determined by the inocula-replicating method [7] using an inoculum containing 10^6–10^7 micro-organisms/ml. These results are in general accordance with the sensitivities reported by others for Gram-negative bacilli isolated from the sputum in hospitalized patients [8]. Table 5 shows the percentage of strains susceptible to levels of antibiotics that can be reached in the blood. Tillotson and Finland showed that the use of one of several antibiotics for which a minimal inhibitory concentration of 3·1 μg/ml or less had been demonstrated *in vitro* was effective in eliminating or reducing the Gram-negative bacteria from the sputum [8]. Our data suggest that the organisms isolated from cases of pneumonia occurring within 6 days of admission were more frequently susceptible to these levels of several antibiotics than bacteria isolated in cases occurring later. Since the doses of ampicillin, cephalotin and carbenicillin usually given are higher than those required to produce blood levels of

Table 4. Sensitivity of the Gram-negative rods isolated from cases of pneumonia to 8 commonly used antibiotics (median and range), as determined by the inocula-replicating method using an inoculum of 10^6–10^7 organisms per milliliter

	Klebsiella-Enterobacter sp. (16)	*Pseudomonas aeruginosa* (15)	*Escherichia coli* (3)	*Proteus mirabilis* (3)
Ampicillin	12·5 (6·2–>50)	>50 (>50)	12·5 (12·5–>50)	0·4 (0·4–>50)
Cephalotin	12·5 (0·8–>50)	>50 (>50)	6·2 (6·2–>50)	12·5 (0·8–25)
Carbenicillin	100 (0·8–>200)	50 (25–>200)	12·5 (12·5–>50)	0·8 (0·8–>200)
Tetracyclin	3·1 (0·4–>50)	25 (6·2–>50)	6·2 (0·8–>50)	>50 (>50)
Chloramphenicol	3·1 (<0·4–>50)	>50 (>50)	1·6 (1·6–>50)	3·1 (1·6–>50)
Kanamycin	3·1 (<0·4–>50)	25 (1·5–>50)	1·6 (0·8–>50)	0·8 (0·8–>50)
Gentamicin	3·1 (<0·4–12·5)	6·2 (1·6–12·5)	1·6 (0·4–3·1)	3·1 (0·4–6·2)
Polymyxin B	6·2 (<0·4–>50)	6·2 (3·1–>50)	3·1 (0·4–6·2)	>50 (>50)

Table 5. Percentage of susceptible strains to feasible blood levels of various antibiotics. (3·1 μg/ml for gentamicin, kanamycin, polymyxin B, tetracycline and chloramphenicol; 100 μg/ml for carbenicillin and 12·5 μg/ml for cephalotin and ampicillin)

	No.	Gentamicin	Kanamycin	Polymyxin B	Chloram-phenicol	Tetracycline	Carbenicillin	Cephalotin	Ampicillin
Micro-organisms isolated within 6 days of admission	10	90	70	40	60	40	40	50	40
Micro-organisms isolated later than 6 days after admission	27	44	25	29	14	11	44	14	7
Total	37	56	37	33	26	19	43	24	16

Table 6. Autopsy findings in cases of pneumonia caused by Gram-negative bacilli

Micro-organisms	Purulent material in trachea and bronchi	Empyema	Necrosis abscesses	Hemorrhage	Parenchymal infiltration	Bacteria	
						Histology	Culture
Klebsiella (8)	6	1	6	5	8	3	6
Ps. aeruginosa (7)	7	0	0	2	7	1	7
Proteus mirabilis (2)	2	2	1	1	2	0	2
E. coli (2)	1	1	0	2	2	1	2
Total (19)	16	4	7	10	19	5	17

3·1 μg/ml, the percentage of strains sensitive to 12·5 μg and 100 μg/ml respectively are shown here.

Thirty patients were treated with gentamicin (80 mg given intramuscularly, 3 times daily) and in 22 of them carbenicillin (30 g daily, given as 6 intravenous infusions over a period of one hour) has been added for 2–6 days. Our series is too small to permit a comparison between the results obtained with the combination gentamicin/carbenicillin and those obtained with gentamicin alone. Among 7 patients treated with gentamicin within 6 days of admission, there were 2 deaths (42%), while the mortality of pneumonias treated with the same antibiotic regimen, but later than 6 days after admission was 73% (17 patients out of 23). Polymyxin B (150 mg daily), associated with carbenicillin, was given to 5 patients, 3 of whom died, and kanamycin (1·5 g daily) with ampicillin (8 g daily) was given to 2 patients as shown in Table 6.

Adjunctive therapy included surgical procedures such as thoracocentesis and insertion of pleural tubes (5 cases), tracheostomies (13 cases), blood transfusions, corticoids and vasopressors. Hypertension, congestive heart failure and diabetes mellitus were managed routinely.

Pathological findings

Autopsies were carried out in 19 out of the 23 fatal cases.

The lungs were heavy (average combined weight of 1463 g). Purulent material was found in the trachea and/or in the bronchi in 16 cases (84%) and empyema was present in 4 (21%). On cut section there were diffuse lower-lobe bronchopneumonias in all. Moreover, upper lobe involvement was observed in 7 cases, and in all these cases (6 cases due to Klebsiella and 1 due to Pseudomonas) areas of necrosis and abscess formation were observed both macroscopically and microscopically.

Microscopic study showed leukocytic infiltration of the bronchial mucosa and/or of the alveolar septa in 16 cases (84%) as shown in Table 7. Hemorrhage was present within the pulmonary lesions in 10 cases (52%) with large numbers of macrophages laden with hemosiderin.

Bacteria were seen in the lesions in 5 instances, 3 of which represented infiltration of the blood vessels by Gram-negative rods. Bacteria were recovered from the lesions in pure or predominant cultures in 17 cases (89%). Cultures of the blood and of the spleen were positive in 3 patients (2 Klebsiella and 1 *E. coli*) in whom bacteremia had been documented by pre-mortem blood cultures.

DISCUSSION

Pneumonia due to Gram-negative bacilli are generally found in patients with debilitating underlying diseases [5, 9], but the incidence and characteristics of the disease in patients presenting a neoplastic disease have not been systematically studied.

The disease was prevalent in males (7·9% of all male hospital admissions) and accounted for the death of 4·6% of the males who were admitted during the study-period (38% of all the deaths in males). Overall mortality was 62% and could not be correlated with factors such as age, sex, previous radiation therapy, administration of cytotoxic drugs, surgery or leukopenia, but correlated best with the clinical severity of the neoplastic disease and the duration of the hospital stay (less than or over 6 days) prior to the onset of the pneumonia.

In the patients who presented pneumonia early in the course of their stay in the hospital, the Gram-negative bacilli responsible for it could have represented part of the "normal" respiratory flora [10]. They were more sensitive to several commonly used antibiotics than the Gram-negative pathogens isolated from patients who developed pneumonia later. These resistant bacilli could have been acquired from the environment but it has been shown that Gram-negative pathogens may increase their resistance to antibiotics within the patient in the course of hospitalization and treatment [8].

Patients with leukemia, lymphoma and extensive neoplastic disease had a higher mortality rate than those with localized tumors. The frequency of colonization of the upper respiratory tract by Gram-negative bacilli has been shown also to correlate best with the severity of the underlying illness [11]. Bacterial pneumonias may result from the entry into the lungs of bacteria present in the upper respiratory tract; thus the prevention of the colonization of the oro-pharynx by Gram-negative rods and maintenance of a normal pharyngeal flora would appear to be an effective protection against gram-negative pneumonia. However, at the present time, only early recognition and therapy with effective drugs can prevent death from these infections. Therefore attention should be paid to signs that were frequently found here, however, many of them are not specific and all are difficult to evaluate in patients with Gram-stained smears of the sputum, as recom- disseminated malignant lesions. Repeated mended by Tillotson and Finland [4], will fail to separate between colonization and superinfection in some leukopenic patients.

There were a few distinctive characteristics of these pneumonias, according to the bacteriologic species responsible for the illness. Empyemas were found in all three pneumonias caused by Proteus, but only in 2 other cases (1 *E. coli* and 1 Klebsiella).

Pneumonias caused by Klebsiella had a more acute and shorter course in fatal cases than those due to Pseudomonas. In these latter diseases, severe bronchitis and production of large amounts of sputum were typical. Pseudomonas persisted within the bronchial secretions for prolonged periods. Although no quantitative cultures of the sputum have been performed, it should be noted that Pseudomonas often persisted in the bronchial secretions as the predominant organism, even in patients who ultimately recovered from their infection.

Parenchymal involvement of several lobes was more frequent in pneumonias due to Klebsiella. Necrosis, abscess formation, hemorrhage and septicemia were most often found in pneumonias due to Klebsiella.

On the basis of the sensitivity studies that were performed, all patients received appropriate therapy with antibiotics. Although gentamicin and polymyxin have not always been found very effective in respiratory infections caused by Gram-negative rods [12], the mortality rate observed here, if the severe underlying disease of our patients is taken into account, is not different from that reported by others [4, 5].

Gentamicin was the first choice drug in this series as indicated by *in vitro* sensitivity studies. Moreover, the frequency of Pseudomonas as the organism responsible for pneumonia in our patients, makes the choice of a drug active on Pseudomonas aeruginosa imperative while awaiting species identification reports.

REFERENCES

1. M. Finland, W. F. Jones, Jr. and M. W. Barnes, Occurrence of serious bacterial infections since introduction of antibacterial agents. *J. Amer. med. Ass.* **170,** 2188 (1959).
2. F. F. Barrett, J. I. Casey and M. Finland, Infections and antibiotic use among patients at Boston Hospital, February 1967. *New Engl. J. Med.* **278,** 5 (1967).
3. A. K. Pierce, E. B. Edmonson, G. McGee, J. Ketchersid, R. G. London and J. P. Sandford, An analysis of factors predisposing to Gram-negative bacillary necrotizing pneumonia. *Am. Rev. resp. Dis.* **94,** 309 (1966).
4. J. R. Tillotson and M. Finland, Bacterial colonization and clinical superinfection of the respiratory tract complicating antibiotic treatment of pneumonia. *J. infect. Dis.* **119,** 597 (1969).
5. J. R. Tillotson and A. M. Lerner, Pneumonias caused by Gram-negative bacilli. *Medicine (Balt.)* **45,** 65 (1966).
6. P. R. Edwards and W. H. Ewings. *Identification of Enterobacteriacae* (2nd Edn.), Burgess, Minneapolis, Minn. (1962).

7. E. STEERS, E. L. FOLTZ and B. S. GRAVES, Inocula-replicating apparatus for routine testing of bacterial susceptibility to antibiotics. *Antibiot. and Chemother.* **9,** 307 (1959).
8. J. R. TILLOTSON and M. FINLAND, Patterns of antibiotic susceptibility of Gram-negative bacteria isolated from sputum after chemotherapy. *Antimicrob. Agents Chemother.* **8,** 315 (1968).
9. M. H. LEPPER, Opportunistic Gram-negative rod pulmonary infections. *Dis. Chest* **44,** 18 (1963).
10. A. L. BLOOMFIELD, The mechanism of the carrier state with special reference to carriers of Friedlander's bacillus. *Amer. Rev. Tuberc.* **4,** 847 (1921).
11. W. G. JOHANSON, A. K. PIERCE and J. P. SANFORD, Changing pharyngeal bacterial flora of hospitalized patients. Emergence of Gram-negative bacilli. *New Engl. J. Med.* **281,** 1137 (1969).
12. A. PINES, H. RAAFAT, G. M. SIDDIQUI and J. S. B. GREENFIELD, Treatment of severe pseudomonas infections of the bronchi. *Brit. med. J.* **1,** 663 (1970).

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The Susceptibility to SV40 Virus Transformation of Fibroblasts Obtained from Patients with Down's Syndrome

DAVID YOUNG

MRC Clinical and Population Cytogenetics Unit, Western General Hospital, Crewe Road, Edinburgh, Great Britain

PATIENTS with Down's syndrome have been shown to be more prone to leukaemia (by a factor of 18) and to other solid tumours (by a factor of 2·6) [1]. Various workers have recently suggested that the increased risk of developing cancer associated with certain inherited human disorders, e.g. Fanconi's anaemia [2] and Down's syndrome [3, 4], may be reflected in the *in vitro* susceptibility to SV40 virus transformation of the cultured skin fibroblasts from these patients.

The purpose of this report is to confirm and extend the observations [3, 4] that skin fibroblasts from patients with Down's syndrome are more easily transformed with SV40 virus than those from individuals with a normal chromosome constitution and to discuss the implications.

The fibroblasts used in this work were derived from skin biopsies approximately 3 mm^2 taken from the deltoid region of the upper arm. Biopsies were obtained from four patients with Down's syndrome (two males and two females) who were all trisomic for a G-group autosome. Control biopsies were obtained from healthy, cytogenetically normal people (two males and one female) of similar ages to the patients with Down's syndrome.

Cell cultures were initiated from skin biopsies held down by coverslips in 3 cm plastic dishes, sub-cultured into 5 cm dishes, and then infected with SV40 virus between their 5th and 15th sub-culture. Cells at these passage levels still retain their original karyotypes, and do not show the chromosomal aberrations described by Saksela and Moorhead [5] which arise in aged cultures. The virus used was a strain of SV40 originally isolated by Pfizer and concentrated by dialysis to a final concentration of $10^{11.3}$ tissue culture infective doses (TCID/50) per ml — determined by titration on secondary cultures of African Green Monkey kidney cells. This stock was diluted 1:10 using Dulbecco's saline and used to infect the fibroblasts, and assay the transformation rate of these cells, by the technique described by Todaro *et al.* [2].

Counts of the numbers of colonies of transformed cells in the cultures of normal and Down's syndrome cultures are shown in Table 1.

The results reported here demonstrate a considerably higher number of SV40 transformed colonies in the cultures of fibroblasts from patients with Down's syndrome than in the cultures of normal fibroblasts. The multiplicity of infection was approximately 20,000 infectious units/cell, and this gave a transformation rate, in terms of colonies/cells infected, of approximately 0·008%. This compares with 1000 infectious units/cell and 0·03% respectively, as reported by Todaro and Martin [3]. The difference between these two sets of results may be accounted for by the difference in the strain of SV40 virus used in these experiments, and also by the slightly different culturing techniques. The mean number of transformed colonies found in the cultures of fibroblasts from Down's patients, divided by the mean number of transformed colonies found in

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Table 1. *Transformation of normal and trisonic (Down's syndrome) human skin fibroblast cultures by SV*40 *virus*

Cell strain	Figures represent transformed colonies per dish (5×10^4 cells)						
Normal	Exp. 1*	Exp. 2	Exp. 3	Exp. 4	Exp. 5	Average	
Hu	3·90			4·22		4·06	
Wa		3·57	4·00			3·79	Mean for normals = 3·90
Ca			4·11		3·57	3·84	
Down's syndrome							Mean for Down's syndrome cells / Mean for normals = 2·69
Sti	13·80	12·75				13·28	
Th			9·37	11·37		10·38	
Re			11·25		10·50	10·87	Mean for Down's syndrome cells = 10·50
Ste				7·50		(7·50)	

*These figures represent original number of colonies $\times 3$, since the virus used was one-third as concentrated as that used in the other experiments.
Each count represents the mean of the number of transformed colonies from between 8 and 26 cultures.

cultures of normal cells, was 2·69 which is in good agreement with Todaro and Martin's figure of 3·16. The small discrepancy between these two figures is well within the limits of experimental error using the techniques available. Consequently, these results substantiate the reports that fibroblasts from skin biopsies of patients with Down's syndrome are more susceptible to transformation with SV40 virus than are normal fibroblasts.

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REFERENCES

1. W. W. Holland, R. Doll and C. O. Carter, Mortality for leukaemia and other cancers among patients with Down's syndrome (mongols) and among their parents. *Brit. J. Cancer* **16,** 177 (1962).
2. G. J. Todaro, H. Green and M. R. Swift, Susceptibility of human diploid fibroblasts to transformation by SV40 virus. *Science* **153,** 1252 (1966).
3. G. J. Todaro and G. M. Martin, Down's syndrome: increased sensitivity of fibroblasts in cell culture to transformation by an oncogenic virus. *Proc. Soc. exp. Biol. Med.* **124,** 1232 (1967).
4. C. W. Potter, A. M. Potter and J. S. Oxford, Comparison of T-antigen induction in human cell lines. *J. Virol.* **5,** 293 (1970).
5. E. Saksela and P. S. Moorhead, Aneuploidy in the degenerative phase of serial cultivation of human cell strains. *Proc. nat. Acad. Sci. (Wash.)* **50,** 390 (1963).

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Imbalances in DNA and Histone Synthesis in the Rat Liver during Neonatal Carcinogenesis Induced by Dimethylnitrosamine

MICHAEL GRONOW
Department of Experimental Pathology and Cancer Research, University of Leeds, 171 Woodhouse Lane, Leeds, LS2 3AR, England

Abstract — *Changes in the rates of synthesis of rat liver DNA and histones were investigated after giving a carcinogenic dose of dimethylnitrosamine to newborn rats. Nuclei were isolated from the liver of 3- and 5-day old animals 1 hr after administration of a mixture of ^{14}C-labelled amino acids and ^{3}H-thymidine. The specific activity of the DNA and histone fraction F1, F1a, F2b and F3 were determined. At both time intervals a large decrease in the specific activity of the DNA was evident in the DMN-treated rats as compared to the controls. Histone synthesis was also decreased, but not to such an extent. The most marked effect was that on the F2a fraction, but the F3 also showed a significant decrease in specific activity.*

Similar experiments were performed on treated rats at the post-weanling stage. The liver DNA of these animals exhibited a threefold increase in specific activity over the controls. In contrast, the synthesis of the F1, F2a and F2b histones was slightly inhibited, and the F3 sto ne , was inhibited to a considerable extent.

INTRODUCTION

THE NITROSAMINES are a particularly potent group of carcinogenic chemicals capable of inducing cancer in many types of differentiated cells [1]. Continuous administration of these compounds produces tumours in very high yield in the liver and kidney [2]. Under certain conditions one dose at toxic level is sufficient to produce tumours in the surviving animals. From the biochemical point of view the latter system has the advantage over continuous feeding in that any non-specific toxic effects are limited to a narrow time interval.

Della Porta and Terracini [3] have reported that both the liver and kidneys of Wistar Porton rats were susceptible to the carcinogenic action of one dose of dimethylnitrosamine (DMN) if the compound was given within the first 24 hr of birth. This compound induced renal and liver tumours and for both the tumours the incidence was of the order of 66%; no sex difference was observed. As it has been shown [4] that in the neo-natal animal nearly all the DMN is metabolized within 24 hr of administration it is therefore likely that the initial carcinogenic step had taken place in this time interval. It is known [5] that DMN causes extensive methylation of nucleic acids in adult animals and Lee and Spenser [6] showed that this occurred in both liver and kidney RNA in rats after a neonatal dose of this carcinogen.

Although evidence has been presented to implicate alterations in cellular DNA as one of the primary events in carcinogenesis (e.g. [7]) it is still uncertain to what extent the nuclear proteins are involved in the initiation of malignant transformation. It has been proposed [8, 9] that the group of basic nuclear proteins, the histones, present in the cell nucleus, may be involved in gene regulating activity. Their composition, properties and rates of turnover in neoplastic tissue have been extensively investigated [10]. No gross qualitative or quantitative differences have been found between the histones of normal and tumour tissue [11, 12]. Also, the increased rates of histone synthesis observed in malignant tissue

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probably arise as a result of the high proportion of cells engaged in DNA synthesis.

So far, little has been reported about the effects of carcinogens on histone synthesis, although evidence has been presented for the interaction of several carcinogens with histones *in vivo* [13]. In view of the close relationship of the synthesis of these proteins to that of DNA a study of these events during the initial stages of DMN-induced neonatal carcinogenesis seemed desirable.

MATERIAL AND METHODS

Neonatal dose of the carcinogen

Wistar SPF rats were used, the animals being housed under artificial illumination from 07.00 to 19.00 hr at constant temperature. Treated newborns were given 125 μg of DMN in 50 μl. of water subcutaneously in the dorsal region, within 24 hr of birth [4]. Controls were given 50 μl. of water; wherever possible animals were used which were born within hours of the treated litters. The effects of this carcinogen on the liver were examined 2 and 4 days after the initial dose and also in post-weanling animals.

1. *Experiments on* 3- *and* 5-*day old rats*

In these experiments one litter consisting of 10–12 animals constituted one experimental group. Each animal was given 10 μC of tritiated thymidine (Radiochemical Centre, Amersham TRA 61 specific activity 5 c/m-mole) and 1 μC of ^{14}C labelled amino acids (Amersham algal protein hydrolysate CFB 25 specific activity 52 mC/milliatom of carbon) subcutaneously in 50 μl. of water. All injections were given at 09.30 hr to avoid possible complications due to diurnal variation and animals were killed 60 min later. The livers were removed as completely as possible and put into 0·25 M sucrose at 4°C. Samples were also taken for histology and fixed in formalin-saline.

(*a*) *Preparation of nuclei.* All procedures were carried out at 4°C. Nuclei were prepared by a modification of the Chauveau procedure [14]. The livers of approximately 10 animals were homogenized in 40 ml of 0·25 M sucrose 0·003 M Ca^{++} using a teflon-glass homogenizer with a ten-thousandth of an inch gap. Four complete up and down strokes at 2500 rev/min were used and the mixture filtered through six layers of fine gauze. The liver brei was centrifuged at 1000 **g** for 10 min and the supernatant discarded. The sediment was resuspended in 40 ml of 2·3 M sucrose 0·003 M Ca^{++} in a teflon-glass homogenizer (same type) using one stroke at 1000 rev/min. Fifty ml of the same sucrose solution were added, the whole mixed by shaking and centrifuged at 40,000 **g** for 60 min to sediment the nuclei.

(*b*) *Extraction of histones.* All procedures were carried out at 4°C. Basically the method 2 of Johns [15] was used. After extracting the nuclei three times with 0·14 M NaCl 0·05 M tris 0·01 M citrate pH 7·6, F2a and F3 fractions were extracted with ethanol: 1·25 M HCl. Then F1 and F2b were extracted from the nuclear sediment with 0·25 M HCl.

F3 was separated from F2a by dialysis against absolute ethanol and F1 from F2b by precipitation with acetone. After further acetone precipitation the protein fractions were centrifuged down and dissolved in a small quantity of 0·001 M HCl.

(*c*) *Specific activity of histone fractions.* The protein content was determined by the Folin-Lowry procedure [16], using calf thymus histone (Sigma) as a standard. It should be noted that due to its lower tyrosine content, the F1 fraction gives colour values about 22% lower than the same weight of Sigma whole histone. Suitable aliquots were then taken and sufficient concentrated HCl added to make the mixture 6N. After heating at 110°C for 48 hr the mixture was taken to dryness in a vacuum dessicator. Finally, the amino acids were redissolved in 1 ml of water and 10 ml of Bray's scintillation fluid added. Samples were counted in a Packard Tri-carb liquid scintillation spectrometer and a minimum of 10,000 counts recorded.

(*d*) *Specific activity of the DNA.* A sample of nuclei or the nuclear pellet remaining after extraction of the histone was washed with 5% trichloracetic acid (TCA) at 4°C. Initially the DNA was extracted with perchloric acid, according to the method of Hutchinson *et al.* [40], which extracted all the DNA from isolated rat liver nuclei. However, a more convenient procedure using 10% TCA was found to give similar results.

The DNA was extracted by three 30-min extractions with an equal volume of ether. removed from the combined extracts by three extractions with an equal volume of ether. Samples of up to 200 μl. were mixed with 10 ml of Bray's scintillator and counted. The DNA content of the extract was determined by the method of Dische as modified by Burton [12] using highly polymerized calf thymus DNA (Sigma) as a standard.

No cross contamination of ^{14}C with ^{3}H counts was observed in these measurements on

either histones or DNA. Also, experiments with a single label of each precursor confirmed this result.

2. *Experiments on post-weanling animals*

Similar experiments were carried out on 4 to 5 week-old rats treated at birth with DMN. However in this case the radioactive precursors were given i.p. in 0·5 ml of water. The dose consisted of 0·5 μC/g body weight of 3H-TdR (TRA 61) and 0·05 μC/g body weight of ^{14}C labelled amino acids. Four to five animals of the same sex were used per group. The preparation of nuclei and subsequent extracts was carried out by the methods already described.

Single and double label experiments were also carried out.

(*a*) *Polyacrylamide gel electrophoresis of histone fractions.* This was carried out in order to check the reproducibility of the extraction procedure and to ascertain if any changes in the composition of these fractions occurred as a result of DMN treatment. Samples containing 50–100 μg of protein were subjected to gel electrophoresis as described by Reisfeld *et al.* [18]. Electrophoresis was carried out in 15% acrylamide gels using β-alanine-acetic buffer, pH 4·4 for 2·5 hr at 4mA/tube, constant current. Gels were then soaked overnight in a solution of 0·5% (w/v) naphthalene black in 7% (v/v) acetic acid, de-stained electrophoretically, and scanned in a modified Joyce-Loebel Chromoscan.

RESULTS

Three- and 5-day old animals

The specific activities of the histone fractions and of the DNA in control and treated animals killed 60 min after administration of radioactive precursors are given in Table 1. The average value is quoted from three experiments each involving 8–10 siblings. From this Table it is clear that there is a considerable depression of the incorporation of ^{3}H-TdR into the DNA of the liver 48 hr after the administration of DMN. The decrease was still apparent after 96 hr and the specific activity of the treated DNA was approximately the same as at 48 hr. These changes are partly due to the acute cessation of erythropoiesis in the liver following DMN treatment. Histological examination of haematoxylin and eosin-stained sections showed that 33% of the cell population in the controls consisted of darkly staining erythopoietic cells. In the treated liver these had been virtually eliminated, leaving mainly parenchymal cells. A fuller report on these histological changes will be presented in another publication.

A decrease in the rate of histone synthesis

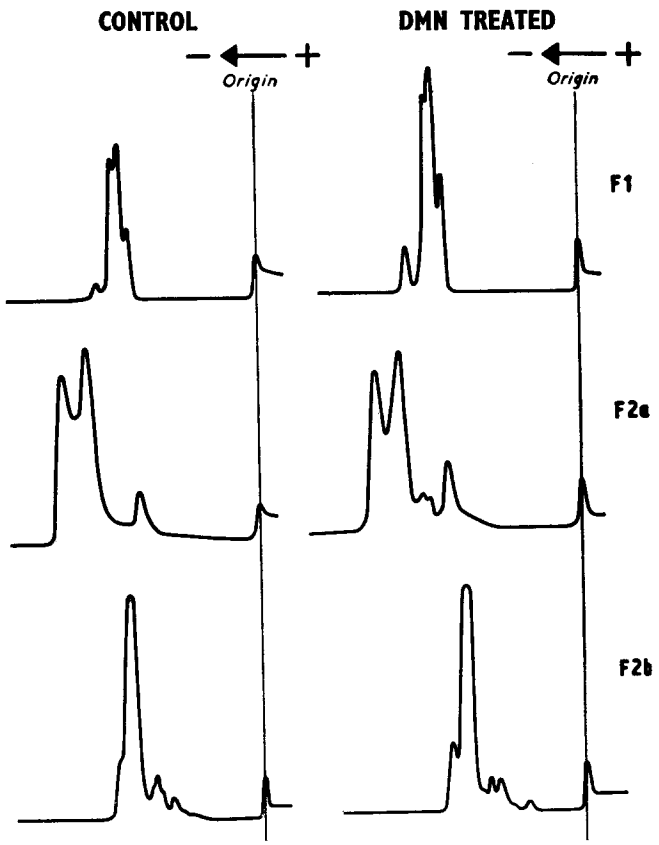


Fig. 1. *Densitometer scans of polyacrylamide gels stained with naphthalene black. Histone from* 3- *and* 5-*day old DMN treated and control animals was applied to the gel and electrophoresis performed at pH* 4·4 *for* 2·5 *hr at* 4*mA/tube constant current.*

Table 1. *Experiments on* 3- *and* 5-*day old rats*

Age	Average specific activity (3 groups)				
	Histones counts/min/mg				
	F1	F2a	F2b	F3	DNA counts/min/mg × 10^{-3}
3 days control	1914 (1570, 1949, 2223)	1548 (1280, 1564, 1800)	1585 (1430, 1589, 1736)	1573 (1456, 1558, 1705)	195 (176, 195, 214)
3 days treated	895 (690, 983, 1012)	319 (275, 288, 394)	721 (530, 804, 831)	487 (382, 447, 632)	30 (30, 30, 31)
5 days control	1453 (1227, 1490, 1642)	723 (672, 688, 810)	931 (826, 949, 1020)	736 (611, 747, 850)	79 (76, 75, 86)
5 days treated	1498 (1425, 1488, 1581)	257 (253, 256, 264)	729 (600, 738, 941)	499 (304, 580, 613)	36 (29, 37, 41)

The average of three groups (results in parentheses) are shown, each group consisting of 8–10 animals.

is indicated by the lower specific activity of the fractions at 3 days, but the effect is not as marked as that shown by the DNA. At 5 days, despite the inhibition of ^{3}H-TdR uptake the specific activity of the F1 fraction is equal to that of the control. However, this could reflect an increased synthesis in the remaining parenchymal cells. F2b and F3 synthesis is still lower than the control values but the inhibition of the F2a fraction is very marked indeed.

Polyacrylamide gel electrophoresis

Densitometer scans of gels containing F1, F2a and F2b proteins from 3- and 5-day old treated and control animals are shown in Fig. 1. From these patterns it is evident that both groups gave virtually identical histone patterns showing that no qualitative change in the composition of the fractions had occurred. Insufficient F3 protein for electrophoresis was obtained in these experiments.

Samples of histones from the post-weanling animals also gave the same gel patterns characteristic of the histone fraction run. Identical gel patterns were obtained from histone fractions isolated from the liver of 3-day to 12-month old animals using the Johns' technique described.

Post-weanling animals

From the data presented in Table 2 an approximately threefold increase in DNA synthesis occurred in the liver of 4 to 5-week old treated animals. However, no parallel increase in the rate of histone synthesis was observed. The same results were obtained with both sexes. In fact most histone fractions show a slight, though probably insignificant, decrease in specific activity. Although the specific activity of the F3 fraction displayed considerable variation in these experiments there is undoubtedly an inhibition of its synthesis in the DMN treated animals.

Histological examination of the livers of treated animals did not reveal any evidence of liver damage but an increased number of mitoses were present. At this stage no erythropoietic cells were present in either treated or control livers.

DISCUSSION

In the eukaryote cell the real function of the histones, a group of basic proteins found exclusively in association with nuclear DNA remains obscure. However, since the suggestion was put forward by the Stedmans [19] that these proteins may act as regulators of gene expression many salient facts have come to light which have been discussed at length in several reviews [8–10, 20–22]. Furthermore, recent findings [23] suggest a considerable degree of conservation of primary structure of the histones during the evolution of higher organisms.

The work of some authors [24–27] has suggested that histone biosynthesis runs almost parallel to DNA replication in cells. Also histones seem to be conserved through mitosis [28, 29]. There is, however, no clear evidence that the synthesis of DNA and histone are mutually dependent (see [21] for discussion). In fact there is evidence that histone synthesis can occur without concomitant DNA synthesis under certain conditions. This has been shown to occur when DNA synthesis is blocked by thymidine [27] 5-Fluorodeoxyuridine or amethopterin [25, 30]; also after a lethal dose of ionizing radiation [31]. In contrast Yarbro *et al.* [32] reported a reduction in histone synthesis in regenerating rat liver in which DNA synthesis had been inhibited with hydroxyurea; a similar result was obtained with an ascites tumour [33].

In studying the regenerating rat liver several workers [34, 35] came to the conclusion that in the preparation of cells for division, histones begin to be synthesised earlier than the DNA. These observations have been confirmed on the basis of alterations in the ratio of the absolute amounts of these substances in the nuclei of regenerating rat liver cells [36]. On the other hand, Takai *et al.* [37] using isotopic techniques deduced that the synthesis of histones and DNA occurs simultaneously in this system and was tightly coupled. Hnilica [21] concluded that studies on the rates of biosynthesis of individual histone fractions in various tissues suggests the possibility that genetic regulation may be

Table 2. Experiments on post-weaning rats (4-5 weeks old)

	Specific activity counts/min/mg				
	F1	F2a	F2b	F3	DNA
Controls	265±31	134±23	221±21	193±23	3100±1000
DMN treated	237±19	111± 9	179±17	116±57	8820±2100

Based on the average of 6 experiments, each involving 4–5 rats±standard deviation.

achieved by tissue specific variations in the amounts of individual histones biosynthesized.

Thus changes in the rates of biosynthesis of DNA and histones and the resulting imbalance observed during these neonatal carcinogenesis experiments could be highly significant. This is especially true with regard to the imbalance observed in the treated post-weanling rats. The slight, probably insignificant, decrease in specific activity in the F1, F2a and F2b is surprising in view of the observed threefold increase in tritiated thymidine uptake. The decrease in the specific activity of the arginine-rich histone fraction F3 is very interesting, despite the large standard deviation obtained. Evidence has indicated that structural changes in this fraction may affect nucleic acid synthesis [38]. Also, some evidence has been put forward [39] which indicates that the proportion of this histone fraction present in rat liver decreases with age.

Differences in the rate of histone synthesis in the 3- and 5-day old rat are a little difficult to interpret. That the extent of the inhibition of histone synthesis in the treated animals is not as great as that of the DNA is apparent, but the F2a shows a definitely lower specific activity than the rest. Whether F2a1 and F2a2 are equally affected is unknown. Polyacrylamide gel electrophoresis of these fractions from the treated animals (and those from the post-weanling animals) were not different from those of the controls. However, it should be pointed out that there is an alteration in the cell population in the 3- and 5-day old treated animals. In the controls 33% of the liver cell population consists of erythropoietic cells. In the DMN treated animals their number had decreased to less than 1%. Autoradiographic studies (unpublished data) showed the parenchymal cells in the treated liver to be well labelled with ^{3}H-TdR. From the results presented it is evident that the ratio of specific activities of the various histone fractions is far from normal for parenchymal cells. This indicates that an imbalance in histone synthesis is occurring in these cells. The two different effects on the very lysine-rich (F1) and arginine-rich (F3) histones in the 5-day old animals could certainly have drastic effects on the structure of the chromatin. Arginine-rich histones are thought to participate in the association of individual DNA molecules rather than the maintenance of the chromosomal superstructure performed by the very lysine-rich fraction.

Further work is required to ascertain the significance of this event. It could be speculated that such drastic alterations in the turnover of the liver cell genetic material at the initial stages of differentiation might result in preferential destruction of morphologically important templates to yield a neoplastic cell.

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REFERENCES

1. H. Druckery, R. Preussmann, S. Ivankovic and D. Schmähl, Organotropic carcinogenic effects of 65 different N-nitroso compounds on BD rats. *Z. für Krebsforsch.* **69,** 103 (1967).
2. P. N. Magee and J. M. Barnes, Carcinogenic nitroso compounds. *Adv. in Cancer Res.* **10,** 163 (1967).
3. G. Della Porta and B. Terracini, Chemical carcinogens in infant animals. *Progr. exp. Tumor Res.* **11,** 334 (1969).
4. B. Terracini and P. N. Magee, Renal tumours in rats following injection of dimethyl-nitrosamine after birth. *Nature (Lond.)* **202,** 502 (1964).
5. P. N. Magee, *In vivo* reactions of nitroso compounds. *Ann. N.Y. Acad. Sci.* **163,** (article 2), 717 (1969).
6. K. Y. Lee and K. Spenser, Methylation of liver and kidney ribonucleic acids in newborn rats treated with H^3 dimethyl-nitrosamine. *J. nat. Cancer Instit.* **33,** 957 (1964).
7. "Biological Effects of Alkylating Agents," *Ann. N.Y. Acad. Sci.* **163,** (article 2), 746 (1969).
8. J. Bonner and P. O. P. Tsò (Editors), *The Nucleohistones.* Holden Day, San Francisco (1964).
9. *Histones: their role in the transfer of genetic information.* Ciba Foundation Study Group, No. 24 (1966).

10. H. Busch, *Histones and Other Nuclear Proteins.* Academic Press, New York (1965).
11. P. F. Davison, Histones from normal and malignant cells. *Biochem. J.* **66,** 703 (1957).
12. J. A. V. Butler, Nuclear proteins of normal and cancer cells. *Exp. Cell Res. Supp.* **9,** 349 (1963).
13. M. Sluyser, Interaction of carcinogens with histones *in vitro. Biochim. biophys. Acta* **154,** 606 (1968).
14. J. Chauveau, Y. Moule and C. Rouiller, Isolation of pure and unaltered liver nuclei, morphology and biochemical composition. *Exp. Cell Res.* **11,** 317 (1956).
15. E. W. Johns, Preparative methods for histone fractions from calf thymus. *Biochem. J.* **92,** 55 (1964).
16. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193,** 265 (1951).
17. K. Burton, A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of DNA. *Biochem. J.* **62,** 315 (1956).
18. R. A. Reisfeld, M. J. Lewis and D. E. Williams, Disk Electrophoresis of basic proteins and peptides on polyacrylamide gels. *Nature (Lond.)* **195,** 281 (1962).
19. E. Stedman and E. Stedman, Cell specificity of histones. *Nature (Lond.)* **166,** 780 (1950).
20. D. M. P. Phillips, The Histones. *Progress in Biophysics and Biophysical Chemistry,* **12,** pp. 211–280 (1962).
21. L. S. Hnilica, Proteins of the cell nucleus. *Progress in Nucleic Acid Research and Molecular Biology* (Edited by J. N. Davidson and W. E. Cohn), Vol 7, p. 25. Academic Press, New York (1967).
22. E. W. Johns, The histones, their interactions with DNA and some aspects of gene control. Ciba Foundation Symposium on Homeostatic Regulators, **128** (1969).
23. E. L. Smith, R. J. Delange and J. Bonner, Chemistry and biology of the histones. *Physiol. Rev.* **50,** 159 (1970).
24. D. M. Prescott, The synthesis of total macro-molecular protein, histone and DNA in the cell cycle in Euplotes Eurystomis. *J. cell. Biol.* **31,** 1 (1966).
25. J. Spalding, K. Kajivara and G. C. Mueller, The metabolism of basic proteins in HeLa cell nuclei. *Proc. Nat. Acad. Sci., U.S.A.* **56,** 1535 (1966).
26. E. Robbins and T. W. Borun, The cytoplasmic synthesis of histones in HeLa cells and its temporal relationship to DNA replication. *Proc. Nat. Acad. Sci., U.S.A.* **57,** 409 (1967).
27. L. R. Gurley and J. M. Harding, The metabolism of histone fractions: I. Synthesis of histone fractions during the life cycle of mammalian cells. *Arch. Biochem. biophys.* **128,** 285 (1968).
28. J. G. Gall, Macronuclear duplication in the ciliated Protozoa Euplotes. *Biochem. biophys. Cytol.* **5,** 295 (1959).
29. R. Hancock, Conservation of histones in chromatin during growth and mitosis *in vitro. J. mol. Biol.* **40,** 457 (1969).
30. G. R. Chalkley and H. R. Maurer, Turnover of template-bound histone. *Proc. nat. Acad. Sci., U.S.A.* **54,** 498 (1965).
31. M. Gronow and P. Todd, The synthesis of DNA and nuclear proteins by rat hepatoma cells immediately after gamma irradiation. *Radiation Res.* **39,** 705 (1969).
32. J. W. Yarbro, W. G. Niehaus and C. P. Barnum, Effect of hydroxyurea on regenerating rat liver. *Biochem. biophys. Res. Commun.* **19,** 592 (1965).
33. J. W. Yarbro, Relationship of histone synthesis to DNA synthesis in mouse ascites tumour 6 C 3 HED. *Biochim. biophys. Acta (Amst.)* **145,** 531 (1967).
34. D. J. Holbrook, J. H. Evans and J. L. Irvin, Incorporation of labelled precursors into proteins and nucleic acids of the nuclei of regenerating liver. *Exp. Cell Res.* **28,** 120 (1962).
35. J. A. V. Butler and P. Cohn, Studies on histones: observations on the biosynthesis of histones and other proteins in regenerating rat liver. *Biochem. J.* **87,** 330 (1963).
36. L. V. Orlova, M. J. Nedelyaeva and V. M. Rodionov, Histone content of the cell nuclei of regenerating rat liver. *Biokhimiya* **34,** 729 (1968).
37. S. Takai, T. W. Borun, J. Muchmore and J. Lieberman, Concurrent synthesis of histone and DNA in liver after partial hepatectomy. *Nature (Lond.)* **219,** 860 (1968).

38. M. G. Ord and L. A. Stocken, Further studies on the phosphorylation and thiol/disulphide ratio of histones in growth and development. *Biochem. J.* **112,** 81 (1969).
39. H. P. Van Hahn, J. Miller and G. L. Eichhorn, Age-related alterations in the structure of nuclear protein. IV: Changes in the composition of whole histone from rat liver. *Gerontologia* **15,** 293 (1699).
40. W. C. Hutchinson, E. D. Downie and H. N. Munro, Factors affecting the Schneider Procedure for estimation of nucleic acids. *Biochim. biophys. Acta (Amst.)* **55,** 561 (1962).

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Autoregulation of Ascites Tumour Growth by Inhibition of the G-1 and the G-2 Phase*

P. BICHEL

Cancer Research Institute, Danish Cancer Society, Radiumstationen, DK 8000 *Aarhus C, Denmark*

Abstract — *The addition to* JB-1 *ascites tumours in exponential growth of cell free ascites fluid obtained from mice bearing* JB-1 *ascites tumours at the plateau was investigated. A reversible block of the cell cycle in both the* G-1 *and the* G-2 *phase was observed. An auto-regulation of the growth of ascites tumours is postulated similar to the homeostatic growth regulation of some normal tissues.*

INTRODUCTION

IT HAS been known for several years that the growth rate of most ascites tumours decreases with time, leading to a steady state as far as the total number of cells is concerned [1–7].

Accumulation of toxic metabolites, O_2-deficiency, increasing competition among the cells for the nutrients, deterioration of the host, and increasing cell loss are the most obvious reasons for this decelerating growth rate. In recent years, however, experimental results have been presented which may be interpreted as support of the hypothesis of an autoregulation of growth in some malignant tissues. Aspiration of most of the tumour from mice bearing ascites tumours at the plateau phase of growth is followed by a considerable increase in the growth rate (the recurrent growth) [8–10], and a marked inhibition of this recurrent growth was seen by repeated injections of acellular ascites fluid obtained from mice bearing the same kind of tumours at the plateau [9–11]. Simultaneous inoculation of two different ascites tumours, the Ehrlich hypotetraploid ascites carcinoma (the E-2 line) and the Crocker ascites sarcoma in the same mouse demonstrated that each tumour grew up independently of the other. The two tumours grew in a completely additive fashion for the first 5 days after inoculation and in a partially additive fashion 9 and 14 days post challenge indicating that accumulation of toxic by-products, O_2-deficiency and insufficient nutrient supply are of minor importance [12].

The data presented here seem to indicate that the inhibition of the recurrent growth of the ascites tumour seen after injection of the cell free ascites fluid apparently is due to inhibition of the tumour cells in both the G-1 and the G-2 phase of the cell cycle.

MATERIAL AND METHODS

The ascites tumour employed in the present work is a hypotetraploid plasmacytoma JB-1 [13], which is maintained syngeneically in our inbred AK-strain of mice by intraperitoneal passage of 5×10^6 tumour cells every ninth or tenth day. For further details see reference 9.

The aspiration of the ascites tumour was performed by very careful percutaneous puncture with a fine needle in order to avoid leakage. The conditions of the experiment include preparation of several smears of the ascites

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tumour cells from the same mouse at different times. In order not to change essentially the amount of ascites tumour present in the mouse these smears were prepared using very small quantities of tumour (less than 0·01 ml). The percentage of mitotic cells (MI) was determined by counting 1000 tumour cells on the smears. In reference 9, MI represents mean values. The counting of mitoses in the present work was limited to smaller but identical areas of the smears leading to somewhat higher, relative but more distinct values of the MI.

Colcemid(R) Analysis

Mice were injected intraperitoneally with 1 mg/kg body weight of N-deacetyl-N-methyl-colchicine (Colcemid(R)) dissolved in sterile, isotonic NaCl. The accumulation of tumour cells in metaphase was determined on smears obtained by hourly aspirations of the mice.

Due to the cytotoxic effect of Colcemid(R) the observation period was limited to 4 or 5 hr. In order to extend the observation time beyond 5 hr the mice were divided into two groups. The first group received the injection of Colcemid(R) at zero hour and the other group 4 hr later. By this means it was possible to follow the accumulation of metaphases for 8–9 hr without any risk of erroneous results due to Colcemid(R) intoxication.

Autoradiography

Generation time and duration of the phases of the mitotic cycle were estimated by means of the labelled mitosis method. Each of the mice received an intraperitoneal pulse label of 1 μC/g body weight of tritiated thymidine (specific activity 2·0 C/mM) purchased from the Radiochemical Centre, Amersham, Bucks, England. Every hour for the following 34 hr smears were made from all of the mice investigated. After fixation with absolute methanol the preparations were extracted for 5 min with a 5% trichloracetic acid at 0°C in order to eliminate non-incorporated precursor and labelled compounds not bound in the DNA. The smears were covered with Ilford K-2 liquid emulsion heated to 42°C and exposed in the dark for 10 days at 4°C. After development the smears were stained with Meyer's haematoxylin and eosin. At least 100 mitotic cells were counted in each preparation to construct the labelled mitosis curve. Each cell with more than 5 grains covering the nucleus or mitotic figure was considered labelled. The labelling index was determined by random counting of 1000 cells, and for the grain count at least 250 cells were analyzed.

RESULTS

In recent years much attention has been paid to the rate of cell loss as an important factor in determining the overall growth rate of malignant tumours [14–17]. In the ascites tumour, however, the cell loss apparently plays no appreciable role during most of the growth [6]. No essential change in the percentage of dead cells was demonstrable in the JB-1 ascites tumour from the second to the tenth day after the inoculation, which is in accordance with data obtained by other investigators [2, 3, 8, 18–21], and no increase of the percentage of dead cells determined with Lissamine Green was seen after intraperitoneal application of the cell-free tumour ascites.

Mice bearing JB-1 ascites tumours in the recurrent phase of growth were at the same time injected intraperitoneally with 2 ml of cell-free JB-1 ascites and with Colcemid(R). The accumulation of mitotic cells was followed for the next 5 hr.

During the first 2½ hr the increase of the MI was the same as in the controls (Fig. 1). For

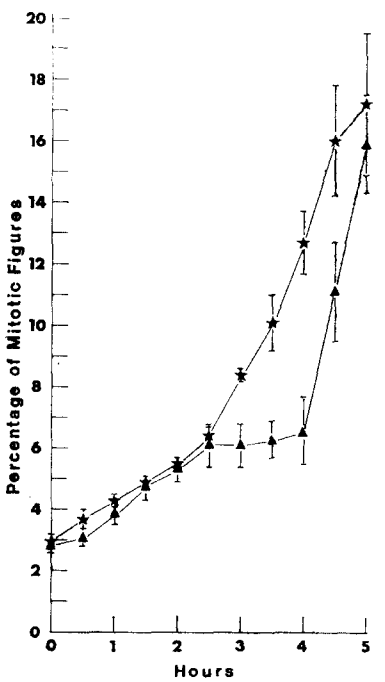


Fig. 1. Accumulation of mitotic tumour cells after intraperitoneal addition of Colcemid(R) at zero hour. ★—★ The recurrent growth (mean of 4 mice ± S.D.). ▲—▲ The recurrent growth and addition of 2 ml of tumour ascites fluid at zero hour (mean of 8 mice ± S.D. (see Table 1).

the following 1½ hr there was a complete cessation in the admission of cells to the mitotic phase indicating that a blockage of the mitotic cycle had taken place about 2½ hr before the midpoint of the mitotic phase. Four hours after

the application of cell-free ascites a burst of mitotic cells was seen probably representing the cells concentrated before the block and indicating that the inhibition of the tumour cells is only temporary.

No break in the accumulation curve was observed by injection of 2 ml of sterile isotonic NaCl or 2 ml of sterile ascites obtained from non-tumour-bearing mice.

The generation time T_g of the tumour cells was estimated by means of the labelled mitosis method and measured as the time interval between the 50% rise in the first and second waves. The duration of the DNA synthesis T_S was measured as the interval between the 50% rise and decline of the first wave of the labelled mitoses. $T_{G-2}+T_{1/2M}$ was measured as the time interval between the injection of the labelled compound and the 50% rise of the first wave.

Twenty-four hours after the aspiration T_g was about 20 hr, T_S about 12 hr and $T_{G-2}+T_{1/2M}$ about 3 to 4 hr. In accordance with earlier observations [6, 22–24] the generation time of the tumour cells of the non-aspirated ascites tumour at the plateau was considerably increased, the main part of the mitotic cycle being occupied by the S-phase.

The length of G-2+1/2M of 3–4 hr in the fast growing tumours indicates that the blockage found with the Colcemid method is localized to the beginning of the G-2 period or at the boundary between S and G-2.

In order to investigate whether it is possible to extend the detention of the tumour cells in the G-2 phase, a group of mice aspirated 24 hr earlier was given 3 injections of 2 ml of the cell-free ascites fluid at intervals of 1½ hr. Colcemid(R) was given 4 hr after the first injection of ascites fluid. From Fig. 2 it appears that no accumulation of tumour cells in metaphase was seen for 4½ hr corresponding to the effect of the 3 injections each lasting 1½ hr.

It is a general assumption that control of growth is a control of the initiation of DNA synthesis [25, 26]. To investigate the possibility that the cell-free ascites also exerts a G-1 inhibitory effect, ascites tumour-bearing mice aspirated 24 hr earlier received an intraperitoneal injection of 2 ml of ascites fluid. At short intervals 2 or 3 of the mice were injected with H^3-thymidine 1 µC/g, and 15 min later smears were prepared of the ascites tumour cells, covered with emulsion for autoradiography, and the labelling index (LI) was determined (Fig. 3). It appears that for the first hour no difference was observed between the uninjected controls and the group treated with ascites fluid. From 1–5 hr after the injection of ascites fluid the percentage of labelled tumour cells was constantly decreasing. Six hours after the injection the LI was again within the range of the controls. Repeated injections of the cell-free ascites fluid every

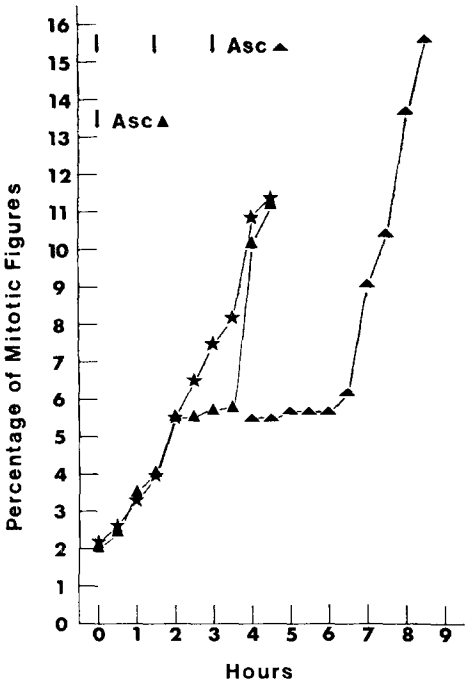


Fig. 2. Accumulation of tumour cells in mitosis determined with Colcemid(R). ★—★ The recurrent growth. Addition of Colcemid(R) at zero hour (mean of 3 mice). ▲—▲ The recurrent growth. Addition of Colcemid(R) and 2 ml of tumour ascites fluid at zero hour (mean of 3 mice). ▲—▲ The recurrent growth. Addition of 2 ml of tumour ascites fluid at 0, 1½, and 3 hr. Addition of Colcemid(R) at 4 hr (mean of 7 mice) (see Table 2).

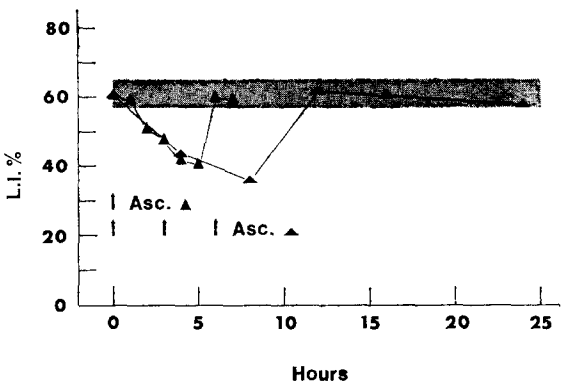


Fig. 3. Labelling index (LI) of JB-1 ascites tumour cells (1) in recurrent growth (▒▒▒ indicates the range), (2) in recurrent growth following one injection of ascitic fluid at zero hour (▲—▲), and (3) in recurrent growth following three injections of ascitic fluid at 0, 3, and 6 hr (▲—▲) (see Table 3).

Table 1. Percentage of mitotic figures following injection of Colcemid(R) at time zero

	No. of animals	Mean value of percentage of mitotic figures ± S.D.										
		0 hr	½ hr	1 hr	1½ hr	2 hr	2½ hr	3 hr	3½ hr	4 hr	4½ hr	5 hr
★—★	4	2·9±0·3	3·7±0·3	4·3±0·2	4·9±0·1	5·5±0·1	6·4±0·3	8·4±0·2	10·1±0·9	12·7±1·0	16·0±1·8	17·2±2·3
▲—▲	8	2·9±0·3	3·1±0·3	3·9±0·4	4·7±0·4	5·3±0·4	6·1±0·7	6·1±0·7	6·3±0·6	6·6±1·1	11·1±1·6	15·9±1·6

S.D. = Standard deviation.
Text and signatures, see Fig. 1.

Table 2. Percentage of mitotic figures following injection of Colcemid(R)

	No. of animals	Mean value of percentage of mitotic figures ± S.D.									
		0 hr	½ hr	1 hr	1½ hr	2 hr	2½ hr	3 hr	3½ hr	4 hr	4½ hr
★—★	3	2·2±0·2	2·6±0·2	3·3±0·2	4·0±0·2	5·5±0·4	6·5±0·5	7·5±0·4	8·2±0·4	10·9±0·6	11·4±0·5
▲—▲	3	2·1±0·4	2·5±0·2	3·6±0·3	4·0±0·3	5·5±0·1	5·5±0·1	5·7±0·1	5·8±0·1	10·2±0·7	11·2±0·4
		4 hr	4½ hr	5 hr	5½ hr	6 hr	6½ hr	7 hr	7½ hr	8 hr	8½ hr
▲—▲	7	5·5±0·6	5·5±0·3	5·7±0·5	5·7±0·4	5·7±0·3	6·2±0·3	9·1±0·7	10·4±2·4	13·7±3·8	15·6±4·4

S.D. = Standard deviation.
Text and signatures, see Fig. 2.

Table 3. Percentage of labelled cells (LI)

	No. of animals	Hr after injection of the ascites fluid	0	½	1	2	2½	3	4	5	6	7	8	12	16	24
⁘	2		61				58±2*		63				63	65	64	62
▲—▲	3			59±3	59±2	51±2		48	42±3	41±2	60±2	59				
▲—▲	3		61±2						44±3				36±3	62±1	61±3	58±1

*Mean of 4 animals.
Mean ± standard deviation of (1) recurrent growth of the JB-1 ascites tumour and (2) recurrent growth of the JB-1 ascites tumour following one (▲—▲) and three (▲—▲) injections of the acellular ascites fluid (see Fig. 3).

third hour, at 0, 3, and 6 hr were followed by a prolonged inhibition of the tumour cells in G-1, and 6 hr after the last injection the LI was again normal (Fig. 3).

No decrease of the mean grain count was seen after injection of the ascites fluid, indicating, although it has to be taken with great caution, because of the unknown size of the endogenous thymidine pool, that once the cell has entered the S-phase no inhibition of DNA synthesis is demonstrable in the single cell following injection of the cell-free ascites fluid.

DISCUSSION

One of the characteristic features which separates the ascites tumour model from most other experimental tumour systems is the plateau phase of growth following after a period with constantly decreasing growth rate.

Although the living conditions for the tumour cells in the peritoneal cavity of mice bearing large, slow-growing ascites tumours evidently are far from optimal, several functions such as the synthesis of RNA and proteins are to a wide degree maintained within normal limits [27, 28], while the DNA synthesis is very low.

The decreasing growth rate is known mainly to be due to increasing cell cycle times and decreasing growth fractions, but it has been found that neither the low oxygen tension nor the lack of glucose can explain the prolongation of the generation time [29].

In a previous work the growth rate of the JB-1 ascites tumour was inhibited by injections twice daily of acellular ascites fluid from fully developed JB-1 ascites tumours [9]. Principally similar results have been obtained with the Ehrlich ascites tumour [10, 11] and, furthermore, as mentioned in the introduction, additive growth has been seen after simultaneous inoculation of two different ascites tumours in the same mouse [12].

All these data may be interpreted as support of a hypothesis postulating self-inhibition of growth by a mechanism which is at least partially independent of simple nutritional exhaustion or accumulation of toxic metabolites.

In the present experiment, an injection of cell free ascites obtained from mice bearing ascites tumours at the plateau to mice with ascites tumours in the recurrent phase of growth was followed by a complete but temporary block of the mitotic cycle localized to both the G-1 and the G-2 phase. Repeated injections of the ascites fluid were succeeded by prolonged inhibition of the cells in G-1 and G-2. It will be noticed that following a single injection of acellular ascites fluid, the cells are blocked in G-1 for about 4 hr and in the G-2 for about 1½ hr, and repeated injections of the ascitic fluid were only followed by inhibition of the cells corresponding to the number of injections. It is quite obvious that the acellular ascites fluid is a complex mixture of several different substances, and it is uncertain whether the inhibitory action on the cells in G-1 and G-2 is due to a single matter or it is a question of two or more substances in combination. So far no attempt has been made to isolate and characterize chemically the inhibiting principle(s) of this system.

Holmberg demonstrated that addition to partially synchronized HeLa cell cultures of a polypeptide isolated from ascites fluid of human patients carrying inoperable ovarial carcinomas was followed by a block in the S-phase [30]. After release of the block the accumulation curve determined with Colcemid(R) revealed that a temporary inhibition of the S-phase had occurred but that part of the cells blocked in S never reached mitosis, probably because they had been destroyed.

Apparently, the loss of cells from G-2 following a single injection of ascites fluid is negligible. However, the increase of the accumulation of cells after repeated injections was less pronounced, and it is uncertain whether this is due to differences in the sensitivity of the cells to the blocking effect of the acellular ascites fluid or it is due to destruction of some of the cells following prolonged inhibition of the cells in the G-2 phase.

In the epidermis Gelfant [31] described two cell populations, one blocked in G-2 ready for division, and another population waiting in the G-1 and within 1 hr after a cutting stimulus beginning to synthesize DNA.

In the last 10 years investigation on the growth regulation in normal epidermis has revealed a tissue specific mitotic inhibitor, the epidermal chalone [32–34] which apparently specifically regulates the proliferative activity of the epidermis, and in addition the chalone has recently been reported to exert a G-1 inhibiting effect also [35]. It is believable that the growth of at least some other normal tissues may be similarly controlled by chalones [36–40].

In 1958 Rose [41] suggested the following: ". . . evidence indicates that some tumours

produce aberrant products. As evidence begins to appear indicating that self-limitation may in general result from the accumulation of specific products, possibly the failures of malignant tumours to achieve self-limitation because of excessive deaths could be compensated for by the addition of sufficient quantities of their aberrant products".

Although the relation between the chalones and the inhibitor(s) or "aberrant products" in the ascites tumour system is unknown, they apparently influence the growth of their tissue of origin in similar ways.

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REFERENCES

1. H. M. Patt, M. E. Blackford and J. L. Drallmeier, Growth characteristics of the Krebs ascites tumor. *Proc. Soc. exp. Biol. (N.Y.)* **83,** 520 (1953).
2. G. Klein and L. Révész, Quantitative studies on the multiplication of neoplastic cells *in vivo* — I. Growth curves of the Ehrlich and MCIM ascites tumors. *J. nat. Cancer Inst.* **14,** 229 (1953).
3. H. M. Patt and R. L. Straube, Measurement and nature of ascites tumor growth. *Ann. N.Y. Acad. Sci.* **63,** 728 (1956).
4. R. Baserga, Mitotic cycle of ascites tumor cells. *Arch. Path.* **75,** 156 (1963).
5. H. M. Patt and M. E. Blackford, Quantitative studies of the growth response of the Krebs ascites tumor. *Cancer Res.* **14,** 391 (1963).
6. P. K. Lala and H. M. Patt, Cytokinetic analysis of tumor growth. *Proc. nat. Acad. Sci. U.S.A.* **56,** 1735 (1966).
7. E. Frindel and M. Tubiana, Durée du cycle cellulaire au cours de la croissance d'une ascite expérimentale de la souris C_3H. *C.R. Acad. Sci. (Paris)* **265,** 829 (1967).
8. E. R. Burns, Initiation of DNA synthesis in Ehrlich ascites tumor cells in their plateau phase of growth. *Cancer Res.* **28,** 1191 (1968).
9. P. Bichel, Tumor growth inhibiting effect of JB-1 ascitic fluid — I. An *in vivo* investigation. *Europ. J. Cancer* **6,** 291 (1970).
10. E. R. Burns, On the failure of self-inhibition of growth in tumors. *Growth* **33,** 25 (1969).
11. E. R. Burns and B. L. Soloff, Further studies on the recurrent growth of the Ehrlich ascites tumor. *Anat. Rec.* **166,** 285 (1970).
12. H. R. Brown, The growth of Ehrlich carcinoma and Crocker sarcoma ascitic cells in the same host. *Anat. Rec.* **166,** 283 (1970).
13. J. Bichel, A transplantable plasma cell leukemia in mice. *Acta path. microbiol. scand.* **29,** 464 (1951).
14. O. H. Iversen, Kinetics of cellular proliferation and cell loss in human carcinomas: A discussion of methods available for *in vivo* studies. *Europ. J. Cancer* **3,** 389 (1967).
15. S. B. Refsum and P. Berdal, Cell loss in malignant tumours in man. *Europ. J. Cancer* **3,** 235 (1967).
16. G. G. Steel, Cell loss as a factor in the growth rate of human tumours. *Europ. J. Cancer* **3,** 381 (1967).
17. G. G. Steel, Cell loss from experimental tumours. *Cell Tiss. Kinet.* **1,** 193 (1968).
18. T. S. Hauschka and S. T. Grinnell, Comparison of the growth curves of a diploid mouse ascites tumor and a tetraploid subline of the same tumor. *Proc. Amer. Ass. Cancer Res.* **2,** 22 (1955).
19. T. S. Hauschka, S. T. Grinnell, L. Révész and G. Klein, Quantitative studies on the multiplication of neoplastic cells *in vivo*. IV. Influence of doubled chromosome number on growth rate and final population size. *J. nat. Cancer Inst.* **19,** 13 (1957).
20. A. K. Laird, Dynamics of tumor growth. *Brit. J. Cancer* **18,** 490 (1964).
21. S. Tolnai, An analysis of the life cycle of Ehrlich ascites tumor cells. *Lab. Invest.* **14,** 701 (1965).
22. F. Wiebel and R. Baserga, Cell proliferation in newly transplanted Ehrlich ascites tumor cells. *Cell. Tiss. Kinet.* **1,** 273 (1968).
23. I. F. Tannock, Comparison of cell proliferation parameters in solid and ascites Ehrlich tumours. *Cancer Res.* **29,** 1527 (1969).
24. S. Peel and P. A. Fletcher, Changes occurring during the growth of Ehrlich ascites cells *in vivo*. *Europ. J. Cancer* **5,** 581 (1969).

25. G. C. Mueller, K. Kajiwara, E. Stubblefield and R. R. Rueckert, Molecular events in the reproduction of animal cells. I. *Cancer Res.* **22,** 1084 (1962).
26. R. Baserga, Relationship of the cell cycle to tumor growth and control of cell division: A review. *Cancer Res.* **25,** 581 (1965).
27. R. Baserga, A study of the nucleic acid synthesis in ascites tumor cells by two emulsion radioautography. *J. cell Biol.* **12,** 633 (1962).
28. R. Baserga, Uptake of radioactive thymidine and cytidine by Ehrlich ascites tumor cells in different stages of growth. *Z. Zellforsch. Microskop. Anat. Abt. Histochem.* **64,** 1 (1964).
29. E. Frindel, M. C. Blayo and J. J. Pocidallo, Modifications métaboliques *ex vivo* des cellules tumorales au cours de la croissance d'une ascite expérimentale de la souris C_3H. *Europ. J. Cancer* **5,** 85 (1969).
30. B. Holmberg, A blockage in the S-phase of HeLa cells produced by a human tumor polypeptide. *Europ. J. Cancer* **4,** 475 (1968).
31. S. Gelfant, Initiation of mitosis in relation to the cell division cycle. *Exp. Cell Res.* **26,** 395 (1962).
32. W. S. Bullough, The control of mitotic activity in adult mammalian tissues. *Biol. Rev.* **37,** 307 (1962).
33. W. S. Bullough and E. B. Laurence, Mitotic control by internal secretion: The role of the chalone-adrenalin complex. *Exp. Cell Res.* **33,** 176 (1964).
34. W. S. Bullough, E. B. Laurence, O. H. Iversen and K. Elgjo, The vertebrate epidermal chalone. *Nature (Lond.)* **214,** 578 (1967).
35. H. Hennings, K. Elgjo and O. H. Iversen, Delayed inhibition of epidermal DNA synthesis after injection of an aqueous extract (chalone). *Virchows Arch. Abt. B.* **4,** 45 (1969).
36. H. Saetren, A principle of autoregulation of growth. Production of organ specific mitose-inhibitors in kidney and liver. *Exp. Cell Res.* **11,** 229 (1956).
37. T. Rytömaa and K. Kiviniemi, Control of granulocyte production. I. Chalone and antichalone, two specific humoral regulators. *Cell Tiss. Kinet.* **1,** 341 (1968).
38. D. P. Chopra and J. D. Simnett, Demonstration of an organ-specific mitotic inhibitor in amphibian kidney. *Exp. Cell Res.* **58,** 319 (1969).
39. J. D. Simnett, J. M. Fisher and A. G. Heppleston, Tissue-specific inhibition of lung alveolar cell mitosis in organ culture. *Nature (Lond.)* **223,** 944 (1969).
40. M. Wolm, K. Wayss and H. Hinderer, A new model of growth regulation. *Naturwissenschaften* **11,** 566 (1969).
41. S. M. Rose, Failure of self inhibition in tumors. *J. nat. Cancer Inst.* **20,** 653 (1958).

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Letter to the Editor

Incomplete Sedimentation of Rauscher Leukaemia Virus Particles During Ultracentrifugation

SYLVIA OFFERS and P. BENTVELZEN
Radiobiological Institute TNO,
Lange Kleiweg 151, *Rijswijk* (*Z.H.*), *The Netherlands*

AFTER ultracentrifugation for isolation of the Rauscher leukaemia virus, a considerable loss in infectivity has repeatedly been observed, sometimes up to 90% [1]. This may be due either to aggregation or disruption of the particles during pelletting and subsequent resuspending, or to incomplete sedimentation.

Chenaille *et al.* [2] reported no loss of infectivity using a Tris-sucrose buffer. In our experience, however, such a buffer does not prevent a decrease in infectivity. This drop seems to be due to incomplete sedimentation of C-type particles (Rauscher virions) as to be presented below.

Plasma was collected from leukaemic BALB/c mice, infected with a crude Rauscher virus preparation. Plasma was centrifuged for 20 min at 2300 rev/min (1200 × **g** in a PR6 International Equipment Centrifuge; the supernatant was spun for 20 min at 11,000 rev/min (10,000 × **g** in a 40 rotor of a Spinco ultracentrifuge. All operations were executed at 4°C. To the supernatant was then added an equal volume of a buffer (15 × 10^{-4}M Tris sucrose pH 7·2). After this procedure such material, indicated as *S*, was subjected to several different treatments.

In one experiment *S* was spun for 30 min at 50,000 rev/min (226,000 × **g** in a Ti 50 rotor. The pellet was resuspended in the original volume of buffer (15 × 10^{-4}M Tris + 0·25 M sucrose pH 7·2). *S*, the pellet and the supernatant were assayed for biological activity with the spleen-weight method of Rowe and Brodsky [3]. Groups of 10 BALB/c male mice, 3 weeks of age, were inoculated with 0·5 ml of the various materials. After two weeks the mice were killed, the spleens taken and weighed. A spleen weight of 150 mg or less was considered as a negative result [1].

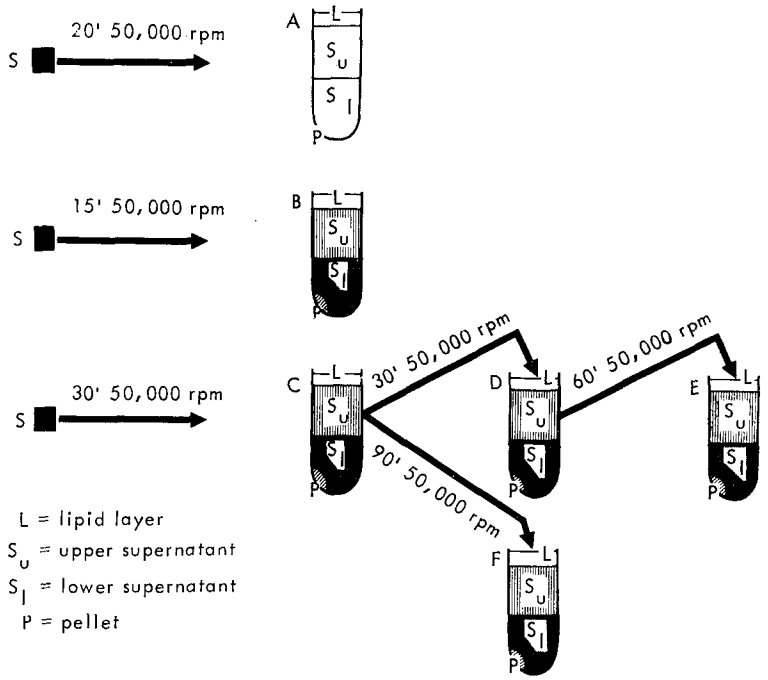
We have repeatedly observed, like in Table 1, that, in contrast to our expectations, not all infectivity is pelletted this way. Considerable variation in the loss of infectivity can be observed between the four tubes in the same rotor. In our experience this may vary between 1 and 50%. The activity of the supernatant varies between 0·5% and 9% of *S*. It seems that, in some cases, considerable inactivation of the virus occurs due to the various procedures.

Table 1. *Biological activity of supernatants and pellets from S after* 30 *min ultracentrifugation at* 50,000 *rev/min*

Fraction	Mean spleen weight in mg ± s.d.
S 10^{0}	1180 ± 290
10^{-1}	610 ± 140
10^{-2}	370 ± 90
S tube 1	340 ± 40
P tube 1	765 ± 290
S tube 2	370 ± 60
P tube 2	1170 ± 440
S tube 3	360 ± 60
P tube 3	1140 ± 360
S tube 4	530 ± 70
P tube 4	1085 ± 330

The remaining infectivity in the supernatant might either be due to subviral particles as postulated for the mouse-mammary tumour virus [4] or to a few C-type particles, which

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SCHEME OF VARIOUS ULTRACENTRIFUGATIONS OF S

Fig. 1. Scheme of various ultracentrifugations of S (for details see text).

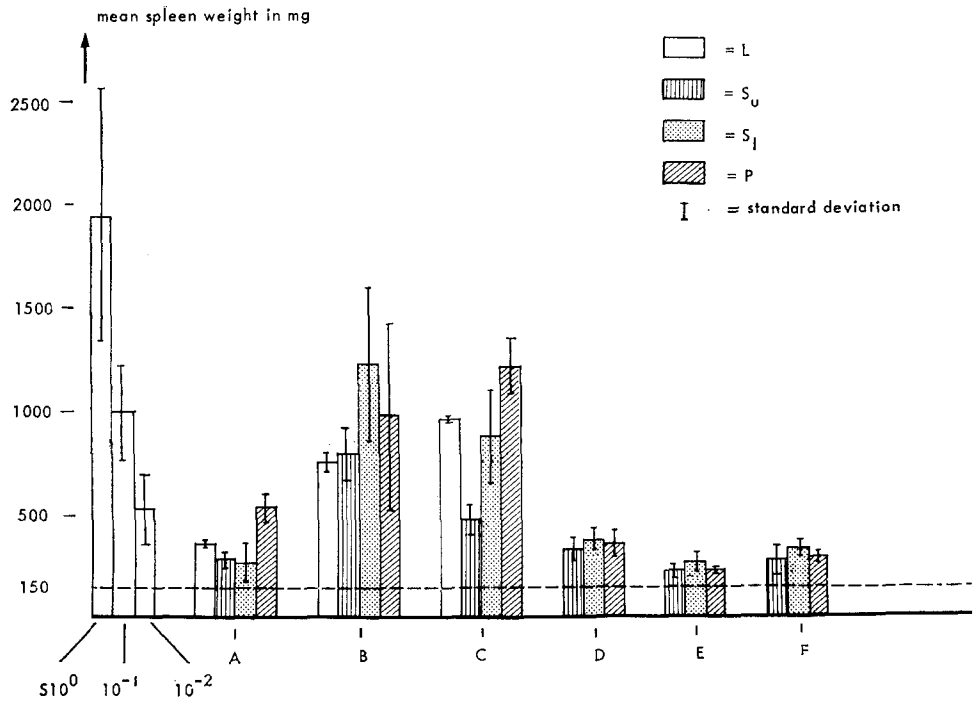


Fig. 2. Results of bio-assay of fractions collected from S subjected to various ultracentrifugation (see Fig. 1).

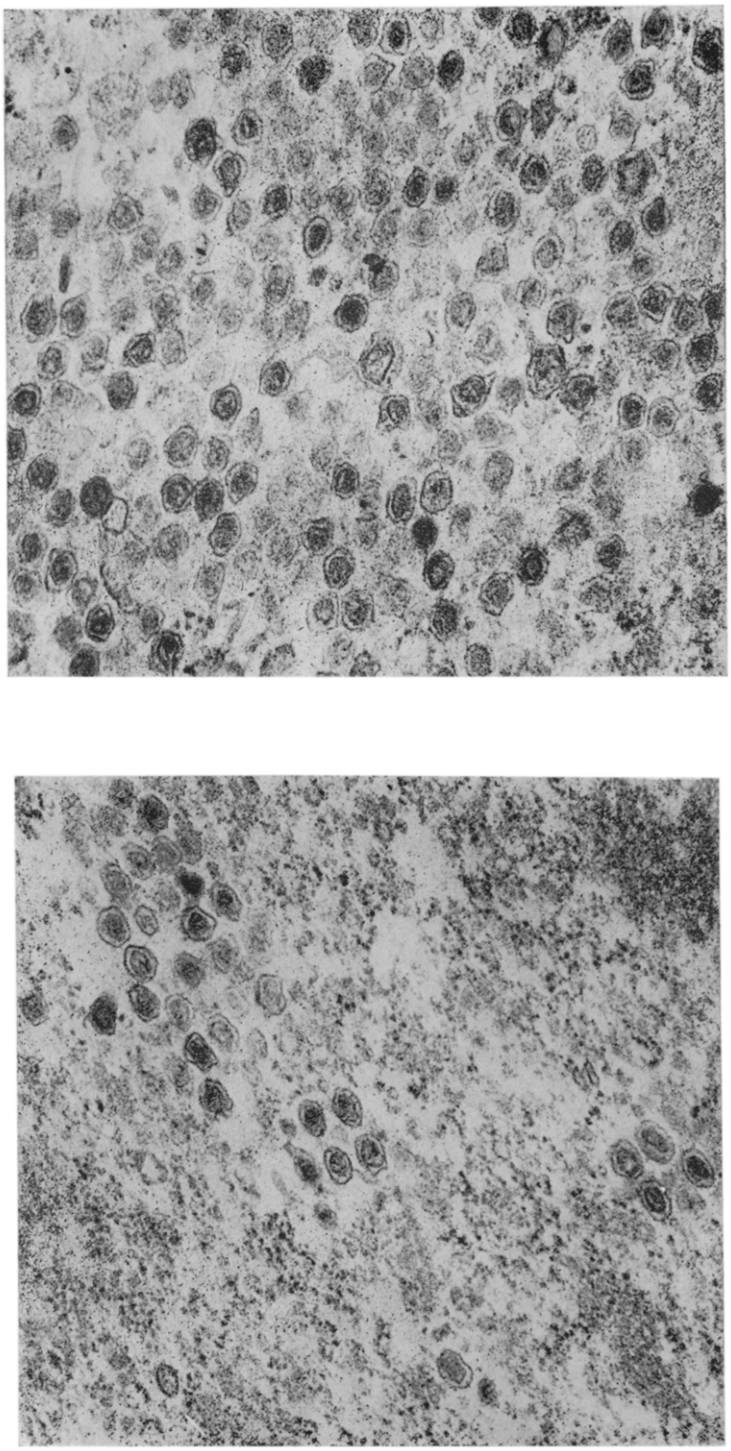


Fig. 3. *Electron micrographs* (62,500) *of thin sections of pellets produced by ultracentrifugation. A.* 20 *min* 50,000 *rev/min. B.* 30 *min* 50,000 *rev/min, followed by* 30 *min* 50,000 *rev/min of the supernatant.*

(to face p. 358)

escaped pelletting despite the extremely high forces produced by ultracentrifugation.

In order to investigate both possibilities supernatants of the various tubes of *S*, after spinning at 50,000 rev/min, were collected and recentrifuged at 50,000 rev/min. This procedure was repeated once more except for centrifugation time being 60 min (see Fig. 1). For comparison *S* was centrifuged at 50,000 rev/min for 15 and 120 min. In an additional experiment the supernatant of *S* spun at 50,000 rev/min for 30 min was recentrifuged for 90 min at the same speed.

Pellets were either used for bio-assay or electron microscopy in which case they were fixed for one night in cacodylate buffer pH 7·2 +1½% glutardialdehyde, followed by 1 hr fixation in Millonigs phosphate buffer + 1% OsO_4 (pH 7·4). The pellets were then embedded in Epon. Ultrathin sections were stained with uranylacetate + lead citrate and then examined with a Philips EM 300 electron microscope.

As can be concluded from Fig. 2 which presents the results of the bio-assays, even after 120 min at 50,000 rev/min (A, E, F) not all infectivity is being spun down. However, during the various recentrifugations, infectivity is pelletted from the upper supernatant of C (D, E, F). In this experiment considerable activity is retained in the lipid layer produced after one cycle of high speed ultracentrifugation as has been observed before [5]. Prolonged centrifugation (2 hr) seems to produce inactivation of biological activity, since not only in supernatant but also in the pellet (A) much less activity is found than in the pellets of B and C.

As expected the pellets of A, B and C contained many C-type particles (Fig. 3A). Using negative staining Zeigel and Rauscher [5] could not detect C-type particles in various supernatants but our thin sectioning of the pellets D and F produced from such a supernatant by recentrifugation demonstrated the presence of C-type particles (Fig. 3B).

It has to be concluded that the activity retained in the supernatant after ultracentrifugation is due to complete C-type particles. We are extremely sceptical of claims about subviral particles which are based on ultracentrifugation data [4].

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REFERENCES

1. J. P. Levy, S. Oppenheim, Ph. Chenaille, D. Silvestre, A. Tavitian and M. Boiron, Quantitative study of Rauscher virus inactivation by various physical and chemical agents. *J. Nat. Cancer Inst.* **38,** 553 (1967).
2. Ph. Chenaille, A. Tavitian and M. Boiron, Preservation of the leukemogenic activity and elimination of contaminants during purification of Rauscher virus. *Europ. J. Cancer* **3,** 511 (1968).
3. W. P. Rowe and J. Brodsky, A graded response assay for the Friend leukemia virus. *J. Nat. Cancer Inst.* **23,** 1239 (1959).
4. S. Nandi, Host-virus interactions in the mouse mammary tumour system. In *Carcinogenesis: A Broad Critique*, pp. 295. Williams and Wilkins, Baltimore (1967).
5. R. F. Zeigel and F. J. Rauscher, Electron microscopic and bio-assay studies on a murine leukemia virus (Rauscher): Preliminary report. *J. Nat. Cancer Inst.* **30,** 207 (1963).

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Letter to the Editor

Drug Metabolism After Repeated Treatments with Cytotoxic Agents

M. G. DONELLI and S. GARATTINI
Istituto di Ricerche Farmacologiche "Mario Negri", Via Eritrea 62, 20157 *Milano, Italy*

PREVIOUS investigation showed that the metabolism of several drugs is reduced in tumor-bearing mice as compared to controls [1–3]. It was thought that one of the possible reasons for this effect was the decrease of liver microsomal oxygenases activity [1], which are important enzymatic systems for the metabolism of drugs [4, 5]. In further studies Tardiff and Dubois [6], as well as workers of this laboratory [7], observed that some antitumoral drugs after a single administration may impair for several days the activity of liver microsomal enzymes.

This note reports the effect of several drugs, given at therapeutical doses, on liver microsomal O-demethylation and p-hydroxylation.

Male Sprague–Dawley rats of the average weight of 150±10 g received intraperitoneally, daily, for 6 days, cyclophosphamide, nitrogen mustard, chlorambucil, 5-fluorouracil, methotrexate, daunomycin, actinimycin D or hydrocortisone at a dose corresponding for all drugs, with the exception of hydrocortisone, to one-third of the LD_{50} evaluated after 6 days of treatment. The animals were sacrificed 3, 6, 9 or 12 days after the end of the treatment and their livers were homogenized with 4 volumes of ice-cold 1·15% KCl solution in a Potter-Type Teflon-homogenizer. The homogenates were centrifuged at 9000×**g** for 20 min; 3 ml of 9000×**g** supernatant equivalent to about 600 mg of liver, were mixed with 2 ml of a solution containing NADP (1·5 μM, glucose-6-phosphate (50 μM, magnesium chloride (25 μM), nicotinamide (100 μM), potassium phosphate buffer (280 μM, pH 7·4) and the substrate to be metabolized (3 μM of para-nitroanisole or 5 μM of aniline). The mixture was then incubated for 30 min at 37°C. O-demethylation of para-nitro-anisole and para-hyroxylation of aniline were determined by measuring respectively the formation of para-nitro-phenol and para-amino-phenol according to the method of Gilbert and Goldberg [8]. Protein determinations were performed according to Lowry *et al.* [9].

As shown in Table 1, the metabolism of p-nitro-anisole and aniline is significantly decreased 3 or 6 days after the end of the treatment for nitrogen mustard, chloroambucil, 5-fluorouracil, methotrexate and daunomycin. The degree of inhibition was however lower than after a single treatment [7]. It is also interesting that, at longer intervals after the end of the treatment, a stimulating effect is taking place. This stimulating activity is statistically significant for chlorambucil, methotrexate cyclophosphamide and hydrocortisone and it is particularly striking for 5-fluorouracil at 9 days after the end of the treatment.

Table 2 reports data on body weight, liver weight and liver proteins in similar experimental conditions. However, no clear correlation exists between these parameters and the impairment or the stimulation of liver microsomal enzymatic activity.

These findings indicate that the administra-

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*Table 1. p-NO_2anisole and aniline hepatic (9000 × **g** liver fraction) metabolism after 6 days of treatment with cytotoxic agents*

Treatment	Dose (mg/kg/i.p. × 6 days)	mμM metabolite produced after treatment (Days) 3		6		9		12	
		p-NO_2P	p-NH_2P	p-NO_2P	p-NH_2P	p-NO_2P	p-NH_2P	p-NO_2P	p-NH_2P
Controls	—	100	100	100	100	100	100	100	100
Cyclophosphamide	16·0	74	80	81	82	133†	123†	133†	107
Nitrogen mustard	0·3	119	93	83†	85	106	93	97	92
Chlorambucil	4·0	73*	82	106	87	122†	122†	114	122
5-Fluorouracil	20·0	101	92	77†	74†	224†	182*	141†	137†
Methotrexate	0·8	46*	56*	73*	57*	112†	102	—	—
Daunomycin	2·0	80†	84	122	83†	95	86	88	85
Actinomycin D	0·03	88	94	86	89	120	118	93	87
Hydrocortisone	50·0	102	104	88	80	119†	120*	123*	120†

In control rats the formation of p-nitro-phenol (p-NO_2P — metabolite of p-nitro-anisol) was ranging from 339±17–464±30 mμM/g liver/30 min while the formation of p-amino-phenol (p-NH_2P — metabolite of aniline) was ranging from 391±40 to 531±22 mμM/g liver/30 min. 10 animals per group were used.
Controls = 100.
*$P<0·01$.
†$P<0·05$.

*Table 2. Body wt. (B.W. g) liver wt. (L.W.-% wt.) and 9000×**g** liver protein content (Pr.-mg/g liver) after 6 days treatment with cytotoxic agents*

Treatment	Dose (mg/kg/i.p. ×6 days)	Days after treatment 3 B.W.	3 L.W.	3 Pr.	6 B.W.	6 L.W.	6 Pr.	9 B.W.	9 L.W.	9 Pr.	12 B.W.	12 L.W.	12 Pr.
Cyclophosphamide	16·0	87*	102	100	83†	90	101	89	98	99	67*	95	120*
Nitrogen mustard	0·3	67*	97	97	72*	104	92	88	109†	104	76*	103	96
Chlorambucil	4·0	89*	104	82	93†	98	95	82*	107	109*	84*	97	107
5-Fluorouracil	20·0	81*	104	106†	86	114	99	97	95	103	83	91	124*
Methotrexate	0·8	90	88*	—	75*	98	98	75*	82*	100	—	—	—
Daunomycin	3·0	91†	96	102	83*	89*	123*	73*	97	106	80	93	98
Actinomycin D	0·03	90*	93	94	82*	104	91†	101	102	94	91	100	102
Hydrocortisone	50·0	100	96	—	92	94	110	88*	95	102	97	93	100

Body wt. and liver wt. of control rats were respectively after 3 days, 179·0±5·0 g and 4·9% body wt., after 6 days 194·5±3·9 g and 4·9%, after 9 days 239·0±3·6 g and 4·6%, after 12 days 244·5±8·7 g and 4·5%.
The protein content of the 9000×**g** liver fraction in control animals was after 3 days 134±4 mg, after 6 days 132±4 mg, after 9 days 137±5 mg, after 12 days 135±3 mg/g liver.
Ten animals per group were used.
Controls=100.
*$P<0·01$.
†$P<0·05$.

tion of antitumoral agents may change in both directions, depending on the schedule of treatment and the activity of liver microsomal enzymes which are considered important in affecting the toxicity and the pharmacological activity of many drugs. This may be relevant also in cancer patients who are frequently submitted to a multiple therapy [10].

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REFERENCES

1. G. Franchi and R. Rosso, Metabolic fate of zoxazolamine in tumor bearing rats. *Biochem. Pharmacol.* **18,** 326 (1969).
2. R. Rosso, E. Dolfini and M. G. Donelli, Prolonged effect of pentobarbital in tumor bearing rats. *Europ. J. Cancer* **4,** 133 (1968).
3. R. Kato, A. Takanaka, A. Takahashi and K. Onoda, Drug metabolism in tumor bearing rats. I. Activities of NADPH- linked electron transport and drug-metabolizing enzyme systems in liver microsomes of tumor bearing rats. *Jap. J. Pharmacol.* **18,** 224 (1968).
4. A. H. Conney, Enzyme induction and drug toxicity . In *Drugs and Enzymes,* (Edited by B. B. Brodie and J. R. Gillette), p. 277. Pergamon Press, Oxford (1965).
5. J. R. Fouts, Impairment of drug metabolism in drug toxicity. In *Drugs and Enzymes,* (Edited by B. B. Brodie and J. R. Gillette), p. 261. Pergamon Press, Oxford (1965).
6. R. G. Tardiff and K. P. Dubois, Inhibition of hepatic microsomal enzymes by alkylating agents. *Arch. int. Pharmacodyn.* **177,** 445 (1969).
7. M. G. Donelli, G. Franchi and R. Rosso. The effect of cytotoxic agents on drug metabolism. *Europ. J. Cancer* **6,** 125 (1970).
8. D. Gilbert and L. Goldberg, Liver response tests. III. Liver enlargement and stimulation of microsomal processing enzyme activity. *Food Cosmet. Toxicol.* **3,** 417 (1965).
9. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, Protein measurement with the folin phenol reagent. *J. biol. Chem.* **193,** 265 (1951).
10. D. P. Rall, Pharmacologic aspects of selective chemotherapy of leukemia and Burkitt's tumor. Combination chemotherapy: advertent and inadvertent. *Cancer Res.* **27,** 2650 (1970).

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Letter to the Editor

Preliminary Clinical Screening with Adriamycin* in Lung Cancer

G. BONADONNA, M. DE LENA and G. BERETTA
Clinical Chemotherapy Unit, National Cancer Institute, Milan, Italy

IN THE April–May issue of this Journal [1] Y. Kenis and G. Brulé presented the results of a preliminary screening with daunorubicin in lung cancer. The clinical trial included 14 patients with epidermoid, oat-cell and glandular carcinomas. The results were unsatisfactory. A radiological improvement was noted only in 1 case while toxicity (mainly myelosuppression) with the doses and with the regimen used in that study was found to be prohibitive. Because of the limited effectiveness associated with a high incidence of severe toxicity, the authors came to the conclusion to discard daunorubicin in the routine treatment of far-advanced lung cancer.

During the past two years, as part of the phase-II evaluation, we tested adriamycin, a new antitumor antibiotic structurally related to daunorubicin [2] in some patients with far-advanced lung cancer. Some of these cases have been already reported [3–7]. Recently, the drug was administered to a few more patients, ending so far to a total of 14 cases, an equal number to that treated with daunorubicin by Kenis and Brulé. Since favourable results were observed in an appreciable number of cases with an acceptable degree of toxicity, we think it is worthwhile reporting briefly our experience with adriamycin in lung cancer.

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*Adriamycin was kindly supplied by Farmitalia, Milano (Italy).

RESULTS

Table 1 summarizes the essential findings concerning the histology and the type of response, as measured by using Karnofsky's scale [8], in the 14 patients treated with adriamycin. All cases were considered adequate to detect an antineoplastic effect of the drug. The patients were randomly selected and they were all beyond the possibilities of radiotherapy. In particular, before receiving adriamycin, 2 patients were already treated with radiation therapy and respectively 3 with conventional chemotherapy. In 4 cases the histologic type could not be determined through cytological examination of the sputum. In pretreated patients, the dose was that of 0·4 mg/kg, while the other patients were given 0·6 mg/kg. In most cases adriamycin was administered with an intermittent schedule (the drug was injected daily for 3 consecutive days. After a 7-day rest period this course was repeated. After another 7-day rest period adriamycin was restarted every 4 days). This was found to be, in our experience, the less toxic regimen without affecting the therapeutic results [3, 4, 6]. A total of 4/14 patients had a category-1 response (1-A and 1-B), i.e. objective regression in all measurable criteria for 1 month or more. In particular, 2 patients had a 1-B response, i.e. objective regression for at least 50 per cent in bulk, as detected through chest X-ray. The actual duration of response cannot yet be determined since 3/4 patients are still on maintenance treatment. In 6 cases a less

Table 1. *Response to adriamycin in far-advanced lung cancer*

Histological type	Evaluable patients	Response (Karnofsky's categories)					
		O-O	O-A	O-B	O-C	1-A	1-B
Epidermoid	4				2	2	
Glandular	3	2		1			
Oat cell	2				1		1
Undifferentiated	1	1					
Unknown	4	1	1		1		1
Total	14	4	1	1	4	2	2

prominent degree of response was obtained and the duration was less than 1 month (categories O-A, O-B and O-C).

As far as toxicity is concerned, alopecia was noted in 10/14 cases, stomatitis in 11/14 and respectively marrow depression in 7/14. Electrocardiographic abnormalities (tachycardia, depression of S–T segment and flattening or inversion of T waves) without concomitant clinical symptoms of cardio-pulmonary insufficiency were observed in 3 patients. All signs of toxicity were reversible within a short period of time.

COMMENT

This preliminary trial, as opposed to what has been reported with daunorubicin, reveals that adriamycin is effective also in different histologic aspects of lung cancer. Therefore, it is worthy of further trials in this type of disease. Toxicity, by administering the drug with an intermittent schedule and with a lower dose in pretreated patients, is usually mild and reversible within a relatively short period of time. Although E.C.G. abnormalities are not rare, definite cardiac toxicity seems to be exceptional [3, 4, 6]. Since regressions are short-lived in most cases, even with maintenance treatment, we re-emphasize what was stated in previous publications [3–7] i.e. adriamycin appears to be more useful to induce the first remission rather than to maintain it. For this reason, when the drug has been proved to be effective in a statistically significant number of patients with a given type of tumor, such as in acute leukemia, malignant lymphomas and neuroblastomas [3, 5, 6], it should be properly tried in combination with other chemotherapeutic agents.

The fact that adriamycin appears to be more active than daunorubicin in lung cancer is on the line of our previous observations on the spectrum of activity of this drug [3–7]. In more than 320 treated patients (adults and children) with various forms of leukemia and cancer, adriamycin at about half of the dose used for daunorubicin was producing consistent objective regressions in 11 different types of neoplastic disease [4–6]. Daunorubicin on the contrary, appears from the literature to be effective only in acute leukemia and in some solid tumors of children. This discrepancy in tumor responses could possibly find, in part, an explanation in the results of experiments carried out in animals [9] where adriamycin was found to have a higher therapeutic index (1·25) than daunorubicin (0·67).

REFERENCES

1. Y. Kenis and G. Brulé, Preliminary clinical screening with daunorubicin in lung cancer. *Europ. J. Cancer* **6,** 155 (1970).
2. F. Arcamone, G. Franceschi, S. Tenco and A. Selva, Adriamycin (14-hydroxydaunomycin) a novel antitumor antibiotic. *Tetrahedron Letters* **13,** 1007 (1969).
3. G. Bonadonna and S. Monfardini, Therapeutic effects of adriamycin in the neoplastic disease of children and adults. *Proc. Amer. Ass. Cancer Res.* **11,** 10 (1970).
4. G. Bonadonna, S. Monfardini, M. De Lena and F. Fossati-Bellani, Clinical evaluation of adriamycin, a new antitumor antibiotic. *Brit. med. J.* **3,** 503 (1969).

5. G. Bonadonna, S. Monfardini, M. De Lena, F. Fossati-Bellani and G. Beretta, Phase I and preliminary phase II evaluation of adriamycin (NSC-123, 127). *Cancer Res.* In press, October (1970).
6. G. Bonadonna, S. Monfardini and S. Di Pietro, Clinical evaluation of adriamycin. *Proc. Int. Cancer Congress* Houston, 22–29 May (1970), Abstr. 774, p. 478.
7. S. Monfardini, G. Bonadonna, S. Di Pietro, A. Guindani, F. Fossati-Bellani and M. De Lena, Clinical trials with adriamycin in leukemia and in solid tumors. *Tumori* **55,** 197 (1969).
8. D. A. Karnofsky, Meaningful clinical classification of therapeutic responses to anticancer drugs. *Clin. Pharmacol. Ther.* **2,** 709 (1961).
9. A. Di Marco, M. Gaetani and B. Scarpinato, Adriamycin, a new antibiotic with antitumor activity. *Cancer Chemother. Rep.* **53,** 33 (1969).

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Letter to the Editor

Probable Clonal Genesis of Cellular Islands Induced in Rat Liver by Diethylnitrosamine

E. SCHERER* and M. HOFFMANN†

Max-Planck-Institut für Virusforschung, Abteilung für Physikalische Biologie, Spemannstrasse 35/1, 74 *Tübingen, Germany*

CONTINUOUS administration of certain nitrosamines (e.g. diethylnitrosamine, DENA) to rats results in a rapid development of liver cell tumours [1, 2]. The resulting carcinoma cells show a loss of histochemically demonstrable glucose 6-phosphatase and/or nucleosiol-5′-polyphosphatase [3, 4] activities [5].

The first irreversible change, found some weeks after the beginning of the DENA treatment, consists of the appearance of clearly defined regions (so called "islands" = "Inseln" [6]) which differ from the surrounding liver in their more or less complete loss of the above enzyme activities [3, 7]. Number and size of the "islands" are dependent on the dose of DENA administered. Indirect evidence was presented [8] that these regions may differ from normal adult parenchymal cells in their antigenic properties. Although no conclusive evidence is available, it is probable that tumours arise from some of these regions [7].

Enzymic deficiency can be detected histochemically in regions consisting of more than about 20 cells. The present experiment was designed to answer the question whether the "islands" were formed from single DENA transformed parenchymal cells or not. The alternative to the possibility of clonal origin could be that locally, clusters of cells were enzymatically damaged, or that randomly formed enzyme-deficient cells migrated to form "islands".

Enzyme-deficient regions were induced in female Sprague–Dawley rats (weighing about 200 g) by a single oral administration of DENA (10 mg/kg) 21 hr after hepatectomy, by which two-thirds of the liver was removed [9]. 125 μc ^{3}H-methyl-thymidine (15 c/mmol) was injected intraperitoneally at the same time. Earlier experiments [10] had shown that a single dose of DENA was more effective in the induction of "islands" when given during the regeneration after partial hepatectomy, than when administered to untreated animals. The animals were sacrificed 6 weeks later. Frozen sections of the liver (10 μ) were stained for nucleosid-5′-polyphosphatase using ATP as substrate (ATPase) [11] or covered for autoradiography with emulsion.

The mean number of cells per "island" 6 weeks after initiation amounted to approximately 100, whereas the grain count of the labelled parenchymal cells directly after ^{3}H-thymidine administration (before cell division) amounted to about 300. Thus, in case the enzyme-deficient regions had been formed by cell division from single transformed labelled cells each, the labelling of these cells should have been diluted out. However, if the "islands" had been formed by transition to enzyme deficiency of larger areas or by migration together of enzyme-deficient cells, the labelling of the "island"-cells should correspond to that of the surrounding tissue.

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*Present address: The Netherlands Cancer Institute, Amsterdam, Netherlands.

†Present address: University of California, San Diego, Department of Biology, La Jolla, California 92037.

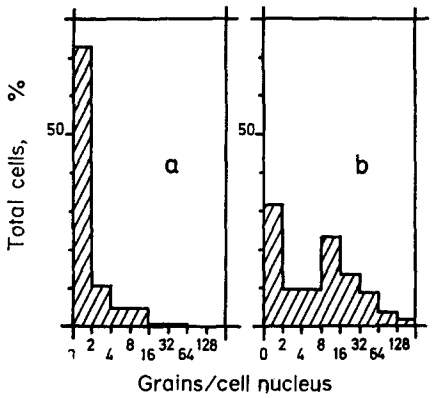


Fig. 1. Distribution of label in parenchymal cells of ATPase-deficient "islands" (a) and of surrounding tissue (b). Evaluation of 20 enzyme-deficient regions. The animals were sacrificed 6 weeks after injection of ^3H-thymidine. Autoradiography with emulsion K5 from Ilford. Time of exposure 105 days.

Figure 1 shows the grain count for the nuclei of "island"-cells (1a) and of normal surrounding parenchymal cells. Counting has been done on 20 enzyme-deficient regions. The labelling was significantly lower in the "islands" than in the normal surroundings. The grain count for "island"-cell nuclei was only slightly higher than the background labelling of a corresponding area (0·5 g). "Island"-cells with a higher number of grains were very rare.

Figure 2 shows the tracing of a photograph of an autoradiogram containing an "island". The outline of the enzyme-deficient region was taken from the parallel section stained by the ATPase reaction. Only some of the nuclei of the "island"-cells are slightly labelled.

We believe that the present result provides reasonable evidence for the view that the enzyme-deficient "islands", which probably represent a first stage in the liver carcinogenesis due to DENA treatment, are formed by cell division from liver parenchymal cells transformed by DENA. The fact that mitoses are regularly found during the early development of "islands" (4 weeks after initiation [10]) is consistent with this interpretation.

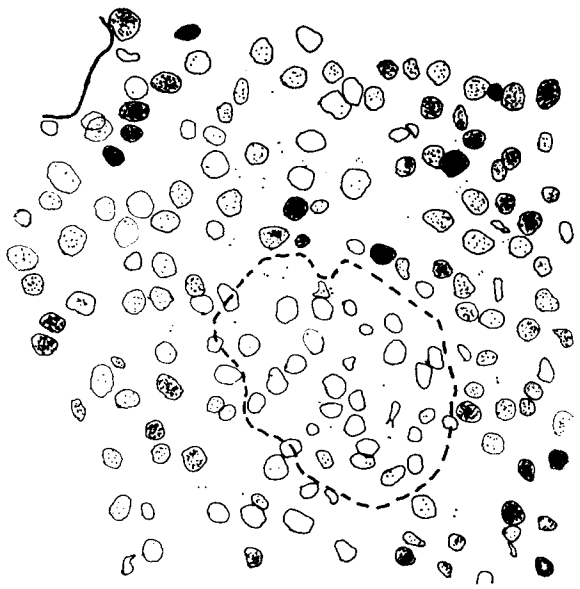


Fig. 2. Distribution of labelled cells after autoradiography (data as in Fig. 1). Only the cell nuclei and silver grains are shown in this tracing of a photograph. The outline of an "island" is taken from a parallel section stained for ATPase. The nuclei of the "island" are nearly unlabelled.

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REFERENCES

1. P. N. Magee and J. M. Barnes, The production of malignant primary hepatic tumours in the rat by feeding dimethylnitrosamine. *Brit. J. Cancer* **10,** 114 (1956).
2. H. Druckrey, R. Preussmann, S. Ivankovic and D. Schmähl, Organotrope carcinogene Wirkungen bei 65 verschiedenen N-Nitroso-Verbindungen an BD-Ratten. *Z. Krebsforsch.* **69,** 103 (1967).
3. A. Schauer and E. Kunze, Enzymhistochemische und autoradiographische Untersuchungen während der Concerisierung der Rattenleber mit Diäthylnitrosamin. *Z. Krebsforsch.* **70,** 252 (1968).
4. B. Sandström, On the specificity of histochemically demonstrable bile canalicular phosphatase activities. *Histochemie* **22,** 316 (1970).
5. G. Papadopulu, Histochemische Untersuchungen an der Rattenleber nach Verabfolgung von Nitrosaminen. Thesis, Universität Tübingen (1967).
6. W. Gössner and H. Friedrich-Freksa, Histochemische Untersuchungen über die Glucose-6-phosphatase in der Rattenleber während der Kanzerisierung durch Nitrosamine. *Z. Naturforsch.* **19b,** 862 (1964).
7. H. Friedrich-Freksa, W. Gössner and P. Börner, Histochemische Untersuchungen der Cancerogenese in der Rattenleber nach Dauergaben von Diäthylnitrosamin. *Z. Krebsforsch* **72,** 226 (1969).
8. H. Friedrich-Freksa and M. Hoffmann, Immunological defense against preneoplastic stages of diethylnitrosamine. Induced carcinomas in rat liver. *Nature, Lond.* **223,** 1162 (1969).

9. G. M. Higgins and R. M. Anderson, Experimental pathology of the liver. I. Restoration of the liver of the white rat following partial surgical removal. *Arch. Path.* **12,** 186 (1931).
10. E. Scherer and M. Hoffmann, Unpublished observations.
11. M. Wachstein and E. Meisel, Histochemistry of hepatic phosphatases at a physiologic pH. Modification: prefixation in 0·5% glutaraldehyde for 5 sec. Incubation time 40 min. *Amer. J. clin. Path.* **37,** 13 (1957).

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Induction of Hyperplasia in Mouse Salivary Gland Isografts*†

KAZUMASA HOSHINO and CHING DER LIN‡

Department of Anatomy, Health Sciences Centre, University of Western Ontario, London, Ontario, Canada

Abstract—*Single submandibular glands, single parotid glands or the combined three major salivary glands, were intraperitoneally isografted from female BALB/c donor mice. Without further treatment, surviving graft tissues consisted predominantly of hyperplastic duct-like structures throughout the 12-month investigations. Hyperplastic proliferation declined in intensity after showing strong features one week after grafting but instead of disappearing, it became again more active 12 months after transplantation. In any of the above-mentioned grafts, isoproterenol administration, prior to and following isografting, demonstrated that remarkable hyperplastic changes, some hardly microscopically distinguishable from an adenocarcinoma, could be induced. Post-transplantational treatments with either testosterone enanthate (20 mg fortnightly) or estradiol valerate (1 mg or less fortnightly) could accelerate hyperplastic alterations of duct-like structures surviving in the grafts and cause changes at 6 months after grafting which resembled those observed in untreated controls at the 12-month interval. In surviving duct-like structures, acini and secretory tubules reappeared following administration of isoproterenol and testosterone, respectively. However, these reappearing cells have never undergone hyperplasia. These phenomena suggest that only de-differentiated duct-like structures are capable of undergoing mitoses and becoming hyperplastic but, after re-differentiation, cells lose their capability.*

INTRODUCTION

FOLLOWING intraperitoneal isografting, mouse salivary glands were found to lose their characteristic histological features and consist predominantly of duct-like structures, which without any further treatment soon become hyperplastic [1]. The selective effects of isoproterenol and testosterone upon the reappearance of acinar cells and secretory tubules respectively, regenerating from duct-like structures in mouse salivary gland isografts, were also reported [2,3].

In *in situ* rat salivary glands, Selye *et al.* [4] observed a sialotrophic action of isoproterenol and attributed it to mitotic proliferation and hypertrophy of the parenchymatous cells. Although Brown-Grant [5] denied increased mitoses by isoproterenol in the mouse salivary gland, stimulatory effects of isoproterenol on DNA synthesis in the salivary gland in rats [6–8] and in mice [9] have also been reported.

Selye *et al.* [4] attempted to induce true neoplasia in *in situ* salivary glands by a protracted treatment with gradually increasing doses of isoproterenol in rats, but failed to observe any evidence of true neoplasia.

Although our postulation that the hyperplasia-inducing factor is present in salivary gland isograft tissues in mice may be debatable, we obtained further experimental data which demonstrate the protracted persistence of hyperplasia in those isografts for 12 months. These data will be briefly presented in this communication.

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†Reprint requests should be sent to: Dr. K. Hoshino, Department of Anatomy, University of Manitoba, Winnipeg 3, Manitoba, Canada.

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MATERIAL AND METHODS

Only adult female BALB/c mice [1] were used.

Single submandibular glands or single parotid glands or a combination of the 3 major salivary glands were isografted intraperitoneally under Nembutal anesthesia immediately after removal of the glands from the donors. In control groups, no further treatment was given to the donors or the hosts. In isoproterenol-treated groups, intraperitoneal injections of 0·25 mg isoproterenol-HCl per g of body weight in normal saline solution were given daily: some donors were treated with isoproterenol-HCl for one week prior to the donation of the grafts but all the hosts received treatments immediately following transplantation for either one or two weeks. In androgen-treated groups, 20 mg of testosterone enanthate in 0·1 ml of sesame oil per mouse was dorsal-subcutaneously injected fortnightly. In estrogen treated groups, for the first 6 weeks, the experimental mice received 1 mg of estradiol valerate in 0·1 ml of sesame oil each fortnightly, and thereafter, due to its toxic effect, the dosage was reduced to 0·2 mg per mouse fortnightly.

In all groups, the grafts were removed from at least five hosts at each of several different intervals following transplantation, fixed in Susa fixative, and prepared for histological observations. The grafts were removed only on the day following the last injection to the host mice in isoproterenol groups; in both androgen- and estrogen-treated groups, at 1, 2, 4, 12 weeks, or 6 months; and in control groups, at 1, 2, 4, 8, 12 weeks, 6, 9 or 12 months after transplantation. The tissues were examined after staining with hematoxylin and eosin, or in some grafts with periodic-acid-Schiff reagent, orange G or light green, with or without nuclear staining with hematoxylin.

RESULTS

The grafts were recovered from all the host mice regardless of the treatments and the periods of transplantation, with some exceptions. A few grafts were not recovered from untreated control hosts at 9 and 12 months following transplantation. Many grafts, recovered at 1 and 2 weeks after transplantation, contained varying amounts of necrotic masses, particularly in the central portion of the grafts. As the periods of transplantation were prolonged, the necrotic portions were replaced by regenerating tissue as will be described below.

1. *Untreated controls*

The histological changes occurring in the isografts were comparable among the three different transplants, that is, the submandibular gland alone, the parotid gland alone, and the three major salivary glands together.

The grafts recovered from untreated control host mice consisted only of duct-like structures which often appeared to be hyperplastic even 1 week after transplantation (Fig. 1). Hyperplastic changes continued to be observed throughout the 12 months following transplantation (Fig. 2). The acinar cells reappeared occasionally in some grafts when maintained for longer than 4 weeks. Histologically, the acinar cells are PAS-positive and the secretory tubules are Orange G-positive [3]. Reappearance of the secretory tubules was only rarely observed in the isografts consisting either of the submandibular gland alone or of the three major glands together. Mitotic figures were never observed in these reappearing acinar cells and secretory tubules. Mitoses were observed only in hyperplastic duct-like structures throughout the investigations.

2. *Isoproterenol-HCl treated group*

In this group, the grafts of either the submandibular gland or the parotid gland or the combined three major glands consisted mainly of duct-like structures undergoing hyperplasia. The acinar cells often reappeared particularly from the peripheral to the central regions of the grafts (Fig. 3).

More actively proliferating hyperplasia, with frequent mitotic figures, were observed in the grafts which had been treated with isoproterenol for one week prior to transplantation (Figs. 4, 5). When the isoproterenol pretreatment was omitted, hyperplastic changes in the surviving duct-like structures were less. When post-transplantational isoproterenol treatment was prolonged from one week to two weeks, the reaction was much less. When the one-week pretreatment with isoproterenol was added to the latter, hyperplastic changes became still less (Fig. 6).

3. *Hormone-treated groups*

In the host mice treated with either testosterone or estrogen, both submandibular and parotid grafts consisted predominantly of hyperplastic duct-like structures. However, in the submandibular grafts of the hosts treated with testosterone, secretory tubules often reappeared amongst duct-like structures (Fig. 7). Mitotic figures were observed only in hyperplastic duct-like structures. Other than the presence of reappearing secretory tubules in

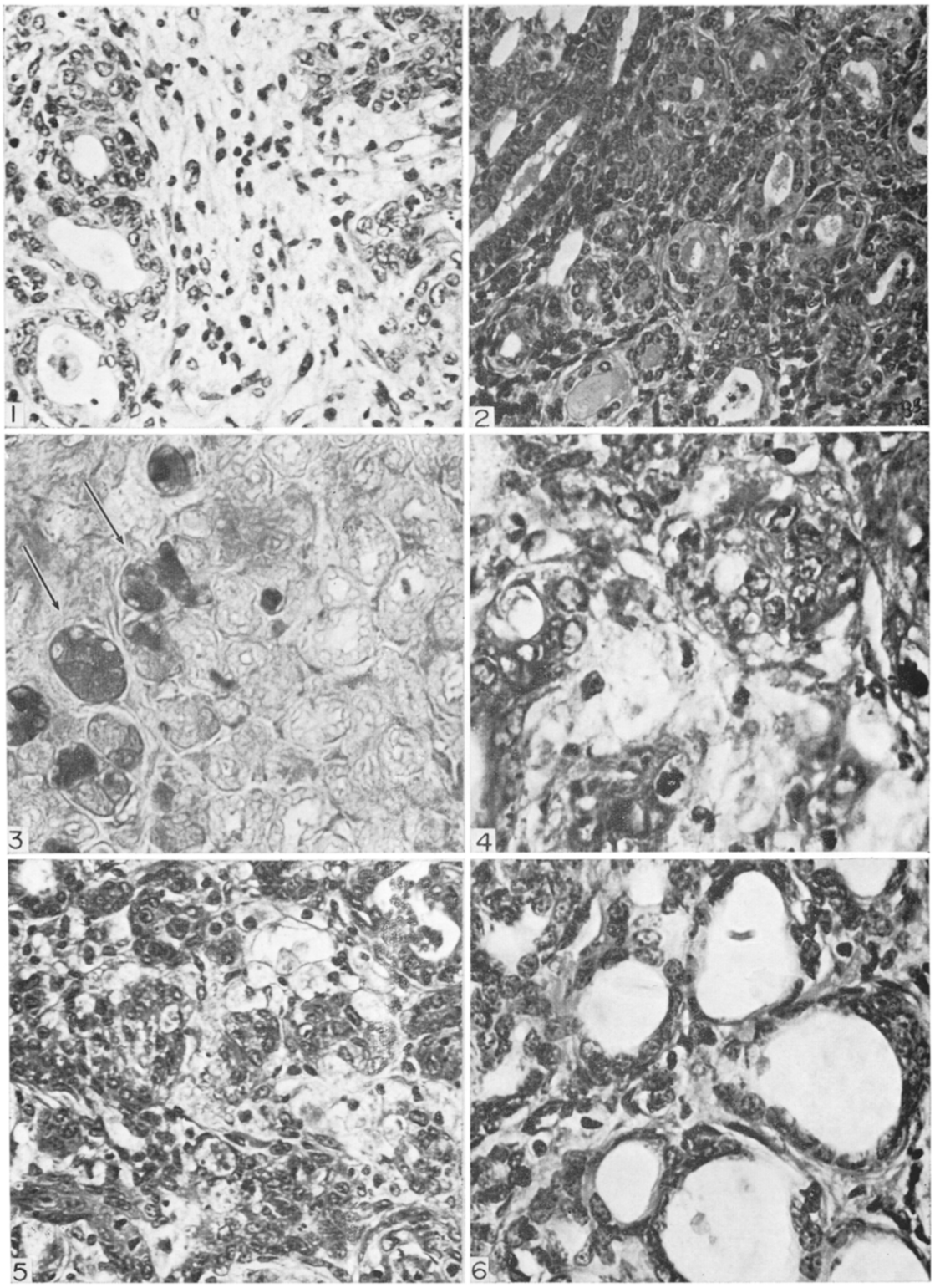


Fig. 1. *Photomicrograph of a parotid gland isograft transplanted intraperitoneally from a* 141*-day old normal female donor to a* 149*-day old normal female BALB/c host mouse and recovered one week after transplantation. H & E.* ×250.

Fig. 2. *Photomicrograph of a parotid gland isograft transplanted intraperitoneally from a* 292*-day old normal female donor to a* 143*-day old normal female BALB/c host mouse and recovered* 12 *months after transplantation. H & E.* ×250.

Fig. 3. *Photomicrograph of a submandibular gland isograft recovered* 2 *weeks after intraperitoneal transplantation from a* 358*-day old female mouse which subsequently received isoproterenol treatment for* 2 *weeks. Arrows indicate reappearing acinar cells. Light green and PAS. (without nuclear staining).* ×250.

Fig. 4. *Photomicrograph of a three salivary gland isograft transplanted intraperitoneally from a* 239*-day old female BALB/c donor mouse, which had received isoproterenol treatment for one week, to a* 216*-day old normal female host which subsequently received isoproterenol treatment for one week. Note very strong hyperplastic features which are hardly distinguishable histologically from an adenocarcinoma. H & E.* ×400.

Fig. 5. *Photomicrograph of a three-salivary gland isograft in the same experimental group as in Fig.* 4. *H & E.* ×250.

Fig. 6. *Photomicrograph of a three-salivary gland isograft transplanted intraperitoneally from a* 172*-day old female BALB/c donor mouse, which had received isoproterenol treatment for one week, to a* 167*-day old normal female host which subsequently received isoproterenol treatment for two weeks. Note much weaker hyperplastic changes as compared with those in Figs.* 8 *and* 10. *H & E.* ×400.

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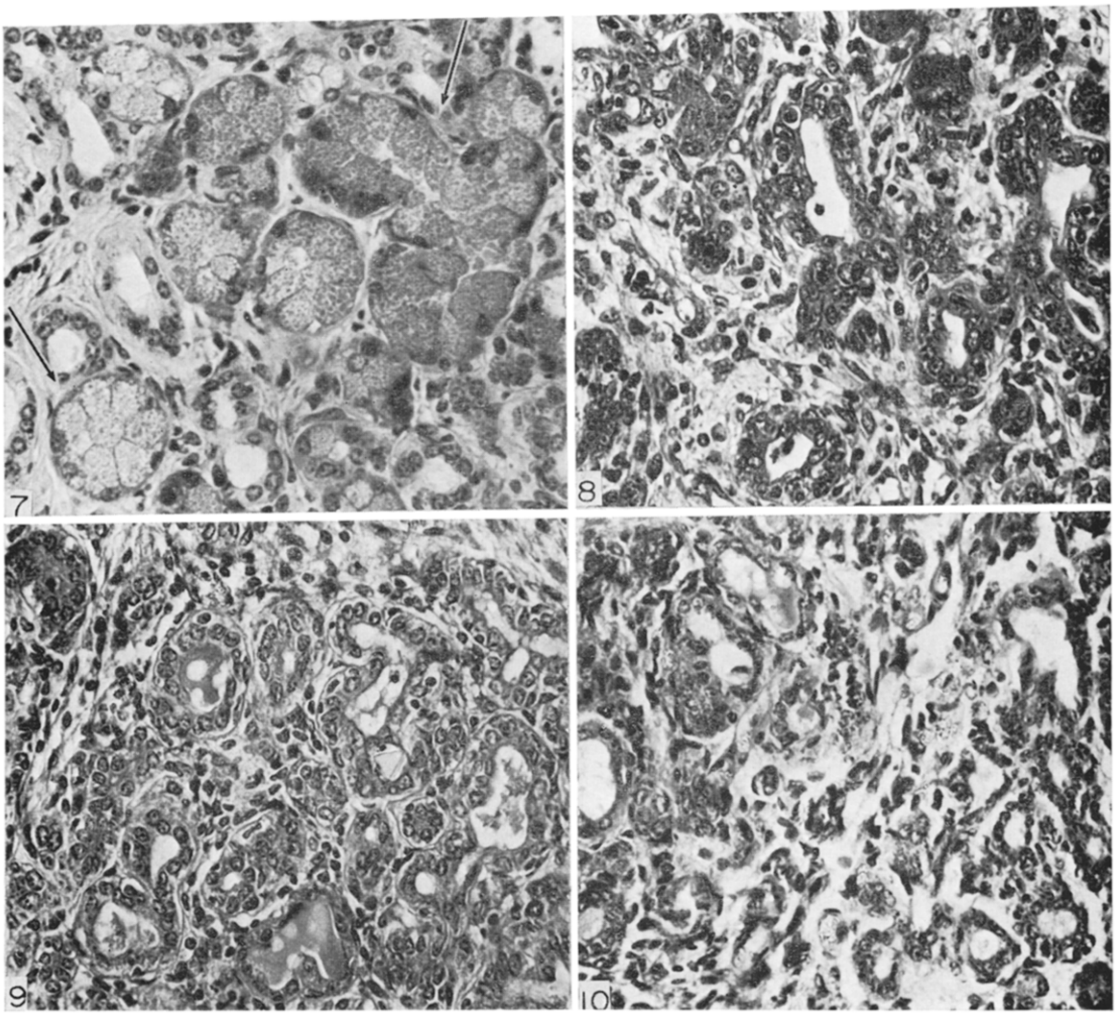


Fig. 7. *Photomicrograph of a submandibular gland isograft transplanted intraperitoneally from a* 276*-day old normal female BALB/c mouse to a* 158*-day old normal female host which subsequently received two separate injections of* 20 *mg of testosterone enanthate given fortnightly, and removed from the host* 4 *weeks after transplantation. Arrows indicate reappearing secretory tubules amongst hyperplastic ductal cells. PAS-Orange G-hematoxylin.* × 250.

Fig. 8. *Photomicrograph of a parotid gland isograft transplanted intraperitoneally from a* 297*-day old normal female BALB/c mouse to a* 117*-day old normal female host which subsequently received one injection of* 20 *mg testosterone enanthate and removed from the host one week after transplantation. H & E.* × 250.

Fig. 9. *Photomicrograph of a parotid gland isograft transplanted intraperitoneally from a* 284*-day old normal female BALB/c mouse to a* 101*-day old normal female host which subsequently received one injection of* 20 *mg testosterone enanthate fortnightly, and removed from the host* 6 *months after transplantation. H & E.* × 250.

Fig. 10. *Photomicrograph of a parotid gland isograft transplanted intraperitoneally from a* 275*-day old normal female BALB/c mouse to a* 156*-day old normal female host which subsequently received one injection of* 1 *mg estradiol valerate fortnightly for* 6 *weeks and then the dosage of estradiol valerate was reduced to* 0·2 *mg fortnightly, and removed from the host* 6 *months after transplantation. H & E.* × 250.

testosterone treated grafts, histological features were not really distinguishable among the grafts derived either from the submandibular gland or the parotid gland in the hosts treated either with testosterone or with estrogen.

Hyperplastic proliferation in duct-like structures was more active when observed at one week after transplantation (Fig. 8) than that observed at 2–12 week intervals, but at 6 months after transplantation, the structures resembled one week grafts (Figs. 8, 9, 10).

DISCUSSION

The present experiments confirm the persistence of hyperplastic changes in intraperitoneal isografts of the salivary glands for 12 months, which is much longer than that observed previously [1, 3]. The long-term investigations revealed additional facts; that hyperplastic proliferation declined in intensity after showing more active features one week after grafting and, instead of disappearing, became again active 12 months after transplantation in an untreated control host environment. Either testosterone or estrogen treatments could accelerate hyperplastic alterations and cause changes at 6 months after grafting which resemble those observed in controls at the 12-month interval. However, none of the grafts in hormone-treated and control hosts showed hyperplasia which resembled malignant neoplasia.

Hyperplastic changes occurring in either submandibular or parotid gland isografts or in grafts consisting of the three major salivary glands, were often hardly microscopically distinguishable from an adenocarcinoma, although they were functionally non-malignant. Such features were induced in the grafts which had been treated with isoproterenol for one week prior to transplantation and one week following transplantation. Although the length of treatment was the same, hyperplastic changes were not as active with two week post-transplantational treatment. With only one week of post-transplantational treatment, hyperplastic proliferation was more active than that observed after treatments for two weeks. Schneyer *et al.* [10] stated that mitotic rates sharply increased 2–3 days after the initiation of isoproterenol, and thereafter dropped. Selye *et al.* [11] failed to induce true neoplasia by protracted administration of isoproterenol for 180 days. Barka [6–8] observed by tritiated thymidine autoradiography that isoproterenol markedly stimulated the incorporation of tritiated thymidine into the nuclei of acinar cells of the rat submandibular glands, the maximum incorporation occurring within 40 hours after administration. He also observed that continuous isoproterenol administration produced lesser peaks of labeling by the 7th and 21st day. Baserga [9] confirmed Barka's findings in the mouse submandibular gland. We observed the reappearance of acinar cells and secretory tubules among surviving duct-like structures after administration of isoproterenol and testosterone, respectively, but no mitoses nor hyperplastic changes were observed in these reappearing cells. Barka [8] observed in the rat submandibular gland, by autoradiography, that 89·5% of the total number of nuclei labeled with tritiated thymidine were in the acinar cells and none in the duct cells, even when following injections with testosterone and isoproterenol. In the present experiments, reappearing secretory tubules amongst persistent duct-like structures in mouse submandibular gland isografts were never hyperplastic. However, both testosterone and estrogen acted unspecifically upon surviving graft tissues and accelerated hyperplastic changes of duct-like structures at 6 months after isografting. The specific effect of testosterone upon secretory tubules of the mouse submandibular gland has been well established [3, 12, 13]. Therefore, it seems that these steroids could unspecifically stimulate metabolism of surviving graft tissues.

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REFERENCES

1. K. Hoshino and C. D. Lin, Hyperplasia-inducing factor in mouse salivary gland isografts. *Cancer Res.* **28,** 2556 (1968).
2. K. Hoshino and C. D. Lin, Comparative effects of testosterone and isoproterenol upon regenerating submandibular gland isografts in female BALB/c mice. *Anat. Rec.* **163,** 200 (1969).
3. K. Hoshino and C. D. Lin, Selective effects of testosterone and isoproterenol upon regenerating submandibular gland isografts in BALB/c. *Anat. Rec.* **167,** 489 (1970).

4. H. Selye, R. Veilleux and M. Cantin, Excessive stimulation of salivary growth by isoproterenol. *Science* **133,** 44 (1961).
5. K. Brown-Grant, Enlargement of salivary gland in mice treated with isopropylnoradrenaline. *Nature, (Lond.)* **191,** 1076 (1961).
6. T. Barka, Induced cell proliferation: the effect of isoproterenol. *Exp. Cell Res.* **37,** 662 (1965).
7. T. Barka, Stimulation of DNA synthesis by isoproterenol in the salivary gland. *Exp. Cell Res.* **39,** 355 (1965).
8. T. Barka, Stimulation of deoxyribonucleic acid synthesis in the salivary gland by isoproterenol. The effect of sex. *Exp. Cell Res.* **48,** 53 (1967).
9. R. Baserga, Inhibition of stimulation of DNA synthesis by isoproterenol in submandibular glands of mice. *Life Sciences* **5,** 2033 (1966).
10. C. A. Schneyer, W. H. Finley and S. C. Finey, Increased chromosome number of rat parotid cells after isoproterenol. *Proc. Soc. exp. Biol. Med. (N.Y.)* **125,** 722 (1967).
11. H. Selye, M. Cantin and R. Veilleux, Abnormal growth and sclerosis of the salivary glands induced by chronic treatment with isoproterenol. *Growth* **25,** 243 (1961).
12. A. Lacassagne, Dimorphism sexuel de la glande sous-maxillaire chez la souris. *C. R. Soc. Biol. (Paris)* **133,** 180 (1940).
13. C. D. Lin and K. Hoshino, Testosterone dependency of the lethal factor in mouse submandibular gland isografts. *Canad. J. Physiol. Pharm.* **47,** 335 (1969).

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Effects of Bacillus Calmette-Guérin (BCG) Infection on Residual Disease of the Rat Mammary Tumor After Ovariectomy*

WILLY F. PIESSENS†‡, RODOLPHE HEIMANN§, NICOLE LEGROS† and JEAN-CLAUDE HEUSON†

†*Service de Médecine et d'Investigation Clinique*‖, *et* §*Service d'Anatomie Pathologique, Institut Jules Bordet, Centre des Tumeurs de l'Université Libre de Bruxelles*, 1, *rue Héger-Bordet*, 1000 *Brussels, Belgium*

Abstract—*The effect of non-specific stimulation of the reticulo-endothelial system by Bacillus Calmette-Guérin (BCG) on growth of the DMBA-induced rat mammary tumor was studied after ovariectomy. BCG was administered at the nadir of tumor regression following ablation of the ovaries, either once as a single injection or at weekly intervals. Resumption of tumor growth, that occurred as expected in the untreated control group, was delayed at least three weeks after the single BCG injection and completely prevented when BCG was given weekly. These findings are discussed in relation to previous observations on the effect of BCG on mammary tumor growth in non-ovariectomized rats.*

INTRODUCTION

EXPERIMENTAL trials of non-specific immunotherapy for malignant tumors both in animals and in human beings indicate that this kind of treatment is effective only when small numbers of tumor cells are present [1–4]. Preliminary reduction of the tumor size can be obtained by surgery or chemotherapy; in the case of hormone-dependent tumors this goal can also be achieved by endocrine therapy. The present report deals with the effects of non-specific stimulation of the reticulo-endothelial system (RES) by Bacillus Calmette-Guérin (BCG) on rat mammary tumors, after their partial regression is induced by bilateral ovariectomy.

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‡Present address: Department of Hematology, Peter Bent Brigham Hospital, Boston, Mass. 02115, U.S.A.

‖This Service is affiliated with the European Organization for Research on Treatment of Cancer (EORTC).

MATERIAL AND METHODS

Virgin rats from a random bred Sprague–Dawley strain were used in this study. To induce mammary tumors the animals received a single intragastric instillation of 20 mg 7,12-dimethylbenz(a)anthracene (DMBA) dissolved in 1 ml of sesame oil at age 50 days. Tumors started developing 6 weeks after the feeding of DMBA and continued to appear for several weeks thereafter.

The animals were submitted to a first study, the results of which have been reported

elsewhere [5]. The experimental design of this first study will nevertheless be briefly outlined here, in order to give information on the past history of the rats and to explain why they were divided into two lots as follows: the first lot (A) was treated with a single subcutaneous administration of BCG (1 mg wet weight of living bacilli in 0·5 ml of culture medium obtained from the Institut Pasteur du Brabant, Brussels) in the interscapular area, 1, 3 or 6 weeks after the DMBA feeding, in an attempt, which by the way proved unsuccessful at these particular times, to prevent the induction of mammary tumors. The second lot of animals (lot B) received one injection of 0·5 ml of the culture medium for BCG, serving as control to the previous lot.

In the present study, all the animals were submitted to bilateral ovariectomy, 3 to 4 months after DMBA administration.

Three weeks later, at the time of maximum tumor regression, the animals were allotted to several treatment groups. Since the effect of a prior administration of BCG on the tumor evolution after ovariectomy was unknown at this point, previously treated and untreated animals were considered separately. The animals of lot A were divided into 3 groups: a control group, receiving weekly injections of 0·5 ml of the culture medium for BCG (14 rats), a group receiving weekly injections of BCG, 1 mg wet weight of living bacilli in 0·5 ml of culture medium (14 rats), and a group receiving a single BCG treatment (12 rats). At the onset of the treatment with BCG, the 3 groups were similar according to the following criteria: mean number of tumors and mean total tumor surface per animal, both before and 3 weeks after ovariectomy, and number of rats having received the single BCG treatment at 1, 3 and 6 weeks after DMBA feeding. The animals from lot B (previously untreated with BCG) were divided at random into 2 groups of 12 rats each: a control group and a group treated weekly with BCG as above.

The animals were observed at weekly intervals for changes in body weight and tumor surface (product of two perpendicular diameters), for a total period of 12 weeks after ovariectomy. At the end of this period the animals were killed and submitted to autopsy: special attention was given to the histological appearance of the tumors and to possible differences in the endocrine glands of the different groups. Only typical carcinomata were taken into consideration in the present experiments; occasional fibroadenomata discovered at autopsy were excluded from the calculations because they are markedly different tumors and especially because their growth is usually not influenced by ovariectomy [6].

RESULTS

Groups of BCG treated and untreated rats did not differ as to their body-weight curves, incidence of autopsy-proven fibroadenomata, and weight and histological findings in endocrine glands at autopsy.

The evolution of the mean tumor surface per rat after ovariectomy is given in Table 1. As in previous studies the maximum tumor regression in untreated animals was obtained 3 weeks after ovariectomy; thereafter the tumors resumed their growth. A similar pattern was seen whether or not the animals had been given BCG prior to ovariectomy.

Table 1. *Effect of BCG on mammary-tumor growth after ovariectomy*

		Weeks after ovariectomy				
	No. of rats	0	3	6	10	12
Lot A						
Control	14	732	281	545	972	1258
BCG once	12			182 (0·05)	507 (n.s.)	935 (n.s.)
BCG weekly	14			166 (0·05)	159 (<0·01)	225 (<0·01)
Lot B						
Control	12	897	227	392	390	598
BCG weekly	12			200 (n.s.)	133 (0·01)	101 (<0·01)

BCG was administered once or weekly, starting 3 weeks after ovariectomy. The results are expressed as the mean tumor surface per rat (mm^2). The differences between the experimental and control groups were analyzed for statistical significance by the Mann-Whitney U test; the probability levels are given in the brackets.
n.s.: not significant.

When BCG was given at weekly intervals, starting 3 weeks after ovariectomy, the mean tumor surface per rat remained unchanged at the size it had reached when this treatment was started. The slight apparent decrease in size was statistically not significant.

The tumors of the rats that received BCG treatment only once, 3 weeks after ovariectomy, showed no progressive growth for another three weeks after immunotherapy; thereafter they resumed growth and reached their initial size between 10 and 12 weeks after ovariectomy, namely about 4 weeks later than the controls.

Some of the tumors still present 3 weeks after ovariectomy became progressively smaller and finally non-palpable during the BCG treatment, but their number was not significantly different from that in the control group.

Since the previous BCG treatment, given before ovariectomy, had no demonstrable effect on the evolution of tumor surface after ovariectomy, whether or not followed by BCG vaccination, the results obtained in both lots A and B were pooled and are graphically represented in Fig. 1.

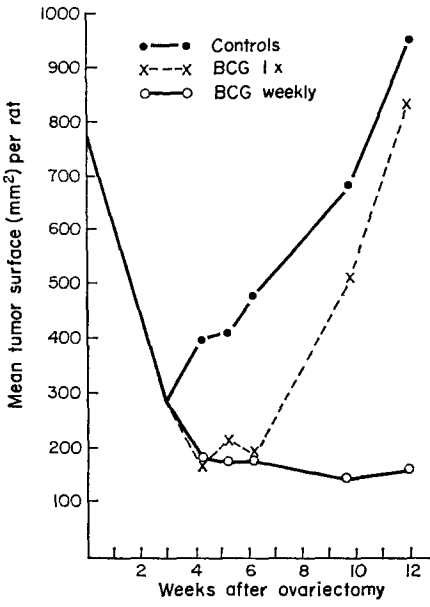


Fig. 1. Effect of BCG on mammary-tumor growth after ovariectomy. BCG was administered as a single dose or at weekly intervals, starting 3 weeks after ovariectomy (nadir of tumor regression). The graph was constructed from the values of Table 1. Because no significant difference was observed between groups until 3 weeks, all results relating to that period were pooled together. Likewise, because no significant differences were detected after 3 weeks between corresponding groups in lots A and B, their values were combined. At 6 weeks, both BCG-treated groups differ significantly from the control group ($P<0{\cdot}02$). At 12 weeks, only the weekly-treated group significantly differs from the control one ($P<0{\cdot}01$).

DISCUSSION

The administration of BCG to small rodents is followed by both an expansion of the RES and an increase of its functional activity, as judged by the clearance of carbon particles [7] and the formation of antibodies against unrelated viral or bacterial antigens [7]. BCG treatment accelerates the rejection of incompatible grafts [8] or tumor transplants [2] and delays the appearance of primary induced tumors when given before or shortly after the tumor induction [9]. BCG can also inhibit the depressive effects of carcinogens on cellularity [5] and function of the RES [10].

Using the transplantable L1210 leukemia, Mathé [3] showed that BCG treatment given 24 hr after the graft delays and reduces the mortality in the treated animals, but only if the number of grafted cells does not exceed 10^5. To be effective in this system BCG must be given repeatedly and late relapses occur in animals thought to be "cured". In the experiments described here, the combined treatment with ovariectomy and weekly vaccinations did not cure the animals, even though some of the tumors disappeared during the phase of vaccination. Under the conditions of the present experiments, weekly BCG vaccinations inhibited resumption of growth of the tumors that regressed after ovariectomy.

Whereas a single BCG treatment had no effect on the morbidity and mortality of the grafted L1210 leukemia, a single BCG treatment given when the mammary-tumor surface reaches its nadir 3 weeks after ovariectomy delayed resumption of tumor growth for at least 3 weeks.

Since the rat mammary tumors induced by DMBA are hormone dependent [12], modification of the hormonal environment by BCG was considered as a possible explanation of the present findings. However, to the best of our knowledge, such hormonal effects of BCG have never been reported. Moreover, the weight and the histological picture of the various endocrine glands at autopsy did not significantly differ in the 3 treatment groups. Finally, in a separate experiment, BCG had no demonstrable effect on the estrous cycle, suggesting a normal function of the ovaries and the pituitary gland. These findings provide evidence that the experimental data presented here are not explained by endocrine mechanisms.

Finally, since mycobacteriae contain variable amounts of asparaginase activity, the possibility was considered that the observed effects were due to chronic asparagine deficiency induced

by BCG. A similar hypothesis to explain the effect of BCG in human leukemia could not be substantiated [13]. None of the usual asparaginase-induced toxic manifestations were observed in the experiments reported here, and the absence of any significant differences in total body weight between treated and untreated rats suggest that depletion of an essential amino-acid did not contribute to the observed differences. We therefore favor enhancement of the RES activity by repeated BCG vaccinations as the probable cause of the inhibition of tumor growth after ovariectomy.

The present results further illustrate the concept that non-specific stimulation of the RES by BCG can delay tumor growth or recurrence when given at a time when only small numbers of tumor cells are present, and suggest that non-specific immunotherapy may be a valuable complement to conventional treatment of disseminated malignant disease under certain circumstances.

However, this type of treatment can also stimulate tumor growth under other circumstances [11], and unless the mechanisms of immunological tumor enhancement are further clarified, its use in human beings should be submitted to careful evaluation.

REFERENCES

1. M. F. A. Woodruff and J. L. Boak, Inhibitory effect of injection of Corynebacterium Parvum on the growth of tumour transplants in isogenic hosts. *Brit. J. Cancer* **20,** 345 (1966).
2. L. J. Old, D. A. Clarke and B. Benacerraf, Effect of Bacillus Calmette-Guérin (BCG) infection on transplanted tumour in the mouse. *Nature (Lond.)* **184,** 291 (1959).
3. G. Mathe and P. Pouillart, Active Immunotherapy of L1210 leukaemia applied after the graft of tumor cells. *Recent Results in Cancer Research* **30,** 64 (1970).
4. G. Mathe, J. L. Amiel, L. Schwarzenberg, M. Schneider, A. Cattan, J. R. Schlumberger, M. Hayat and F. de Vassal, Active immunotherapy for acute lymphoblastic leukaemia. *Lancet* **i,** 697 (1969).
5. W. F. Piessens, R. Heimann, N. Legros and J. C. Heuson, Effects of Bacillus Calmette-Guérin (BCG) on mammary tumor formation and cellular immunity in Dimethylbenz(a)anthracene-treated rats. *Cancer Res.* (in press).
6. P. M. Daniel and M. M. L. Prichard, Three types of mammary tumour induced in rats by feeding with DMBA. *Brit. J. Cancer* **18,** 513 (1964).
7. L. J. Old, B. Benacerraf, D. A. Clarke, E. A. Carswell and E. Stockert, The role of the reticuloendothelial system in the host reaction to neoplasia. *Cancer Res.* **21,** 1281 (1961).
8. B. Vitale and N. Allegretti, Influence of Bacillus Calmette-Guérin infection on the intensity of homograft reaction in rats. *Nature (Lond.)* **199,** 507 (1963).
9. A. Nilsson, L. Revesz and J. Stjernswärd, Suppression of Strontium^{90}-induced development of bone tumors by infection with Bacillus Calmette-Guérin (BCG). *Radiation Res.* **26,** 378 (1965).
10. J. Stjernswärd, Effect of Bacillus Calmette-Guérin and/or methylcholanthrene on the antibody-forming cells measured at the cellular level by a hemolytic plaque test. *Cancer Res.* **26,** 1591 (1966).
11. W. F. Piessens, F. L. Lachapelle, N. Legros and J. C. Heuson, Facilitation of rat mammary tumour growth by BCG. *Nature, (Lond.)* **228,** 1210 (1970).
12. C. Huggins, L. C. Grand and F. P. Brillantes, Mammary cancer induced by a single feeding of polynuclear hydrocarbons and its suppression. *Nature (Lond.)* **189,** 204 (1961).
13. T. Ohnuma, J. F. Holland, J. Yates and S. Grew, B.C.G. vaccination and asparaginase. *Lancet* **ii,** 1310 (1969).

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Adrenal Function in Cancer: Relation to the Evolution

SIMONE SAEZ

Service de Biologie Médicale, Centre Léon Bérard, 28, rue Laënnec, Lyon 8e, France

Abstract—*In 64 patients with cancer, at different stages of the disease, the cortisol production rate was successively calculated under basal conditions, under administration of 20 μg/kg per day of dexamethasone, and of 8 mg per day of dexamethasone.*

Average production rate was more elevated than in normal subjects and the elevation seemed correlated with the stage of the disease. The response to the lower dosage of dexamethasone was less marked in patients whose response to treatment of the cancer was less successful.

The decrease in production rate exceeding 50% after administration of 8 mg per day of dexamethasone suggests that the production rate depends on an alteration of the level of the central regulation, and is not caused by an ectopic ACTH secretion.

In the course of these variations, there was a significant correlation between the urinary excretion of the 17 OHCS and the cortisol production rate.

DURING the previous years, many cases of hypercorticism have been described throughout the course of several types of cancer, which are related to an ectopic ACTH-like product secreted by the tumor [1, 2]. Other workers have disclosed the occurrence of elevated 17 OHCS in the plasma of patients with tumor of the lung [3–8], of the breast [9, 10, 14] or of other localizations [11, 12,] without any clinical sign of hypercorticism [12–16].

Theoretically, this fact could be explained by a defect in the transport and degradation of the cortisol, or could be the result of an increase in its production rate [17–20].

To put some light over this problem, the cortisol production rate has been investigated in patients with different types of cancer and at different stages of the course of the disease.

MATERIAL AND METHODS

The cortisol production rate has been measured by the simple isotopic dilution method [21, 22]. About 2μCi of tritiated cortisol are injected i.v. at 9 a.m.; the urine is collected during the following 48 hours and the excreted radioactivity is checked in each 24-hr specimen. The excretion of the 17 OHCS during the same period of time is estimated by a modification of the method of Glenn and Nelson [23, 24].

When possible, 3 tests have successively been performed in the same patients: under basal conditions, the third day of administration of 20μg/kg per 24 hours of dexamethasone (first dexamethasone suppression test); the third day of administration of 8 mg per day of dexamethasone (second dexamethasone suppression test).

The results of the 17 OHCS excretion and of the cortisol production rates are given in mg/m^2 of body surface area per 24 hours.

Sixty-four cortisol production rates have been estimated under basal conditions, 43 under dexamethasone suppression test with 20 μg/kg, 33 under 8 mg/per day The patients were 25 up to 65 years old. They were 21 males and 12 females bearing different types of tumors with miscellaneous histological patterns, and 31 females with mammary carcinoma.

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The different stages of the evolution of the breast cancer were defined as:

1. primary tumor
2. chest wall recurrence and pleural metastasis
3. bone metastasis with or without lesions of 2
4. visceral localizations, with or without lesions of 3;

these last 3 stages being separated according to the classification suggested by Taylor for the advanced breast cancer [27].

In the patients presented in this study, there was no clinical sign of hypercorticism or of electrolyte disturbance. Such subjects as had undergone recent or present X-ray, chemotherapeutic or hormonal treatment were discarded.

RESULTS

1. *Overall results*

Under basal conditions, the mean of the 64 calculated cortisol production rates was $15{\cdot}88 \pm 3{\cdot}81$ mg/m^2 per day. The mean of the urinary excretion of the 17 OHCS was $4{\cdot}47 \pm 0{\cdot}93$ mg/m^2 day. Both are significantly above the values found in normal subjects [25, 26].

Under administration of 20 μg/kg per day of dexamethasone, the mean of the cortisol production rate measured in 43 of the patients was $8{\cdot}77 \pm 3{\cdot}84$ mg/m^2/24 hr. This value is significantly higher than in normal subjects and the same observation can be made for the urinary excretion [26] of 17 OHCS $2{\cdot}49 \pm 1{\cdot}18$ mg/m^2 per day.

In both conditions, basal and dexamethasone suppression test, the correlation is significant between the cortisol production rate and the urinary excretion of the 17 OHCS (Fig. 1).

The analysis of the results discloses that the cortisol production under dexamethasone suppression test with 20 μg/kg per day is more elevated in patients whose values are elevated under basal conditions: for the 43 pairs of results, there is a significant correlation between the level under dexamethasone test and the level under basal conditions (Fig. 2).

The results of the administration of 8 mg per day of dexamethasone were evaluated in 33 patients subsequently to the evaluation of the cortisol production rate under basal conditions and during the administration of 20 μg/kg of dexamethasone (Fig. 3).

Attention is called to the fact that in nearly all the cases, the high levels of the basal cortisol production rate, which did not decrease under the low dosage of dexamethasone decreased by 3 mg per day when 8 mg of dexamethasone per day was given. Only 6 cases showed values between 4 and 13 mg but these cases showed the most elevated basal rates, and under these conditions their decrease was more than 50%.

In the same way the urinary excretion of the 17 OHCS showed a sharp decrease under 8 mg of dexamethasone, more than 50%, when compared to the level under basal conditions.

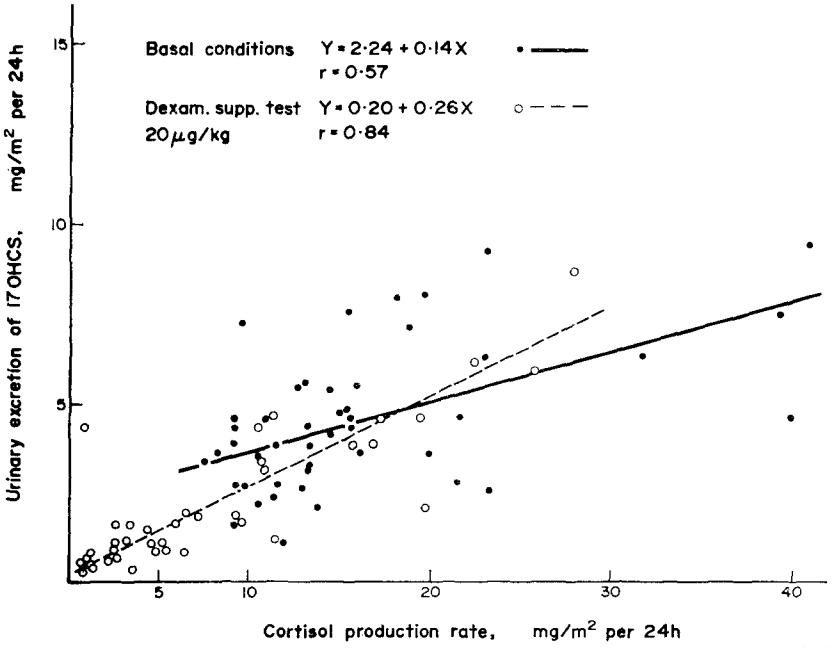


Fig. 1. Correlation between the cortisol production rate and the urinary excretion of 17 OHCS in 43 cancer patients. Results under basal conditions and after dexamethasone suppression test with 20 μg/kg per day.

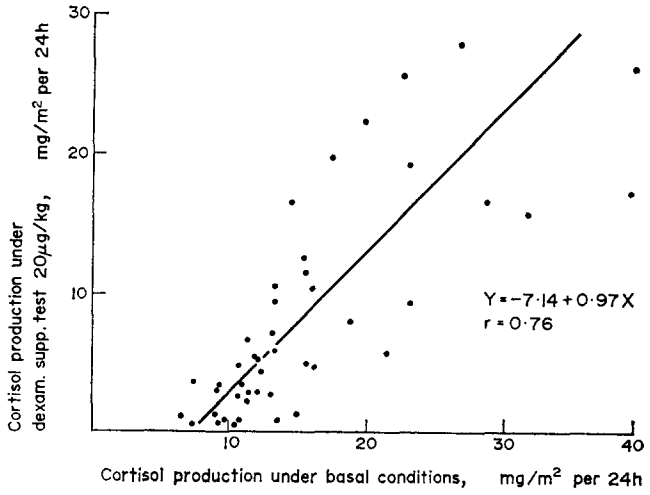


Fig. 2. Correlation between the cortisol production rate under basal conditions and dexamethasone suppression test with 20 μg/kg per day.

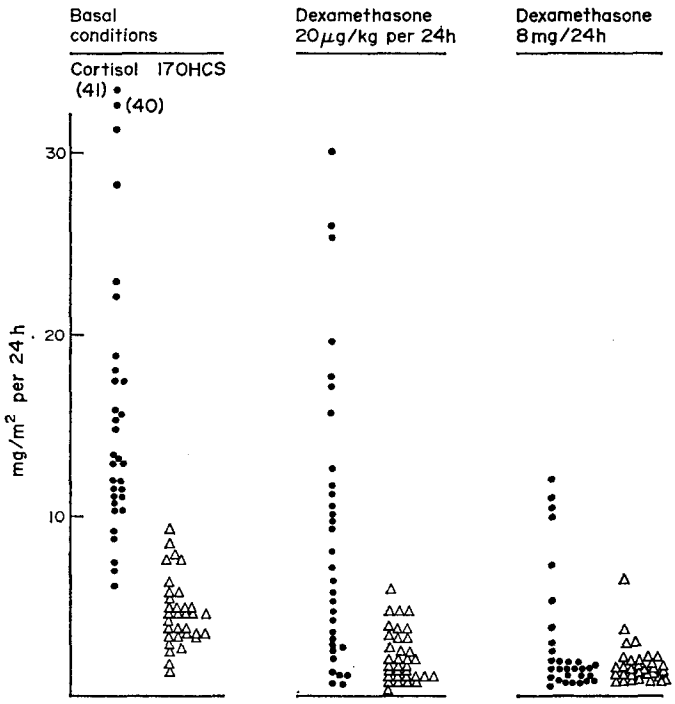


Fig. 3. Cortisol production rate and urinary excretion of 17 OHCS in cancer patients under basal conditions, dexamethasone suppression test with 20μg/kg per day and 8 mg per day.

2. *Results in relation to the stage of the disease*

We tried to investigate a possible relationship between the stage of the disease and the increase of the cortisol production rate incompletely suppressed by the administration of the standard doses of dexamethasone. This was performed in patients with breast cancer, representing the largest group of our patients and where the different stages of the disease are most easily recognized. The cases were separated in groups according to the classification of Taylor (Fig. 4).

It appeared that under basal conditions, the cortisol production rate was in the normal range for the patients with primary tumor and for patients without any recurrence and it was more and more elevated in the most advanced stages in the groups 2, 3, 4. Under administration of 20 μg/kg of dexamethasone the same observation could be made.

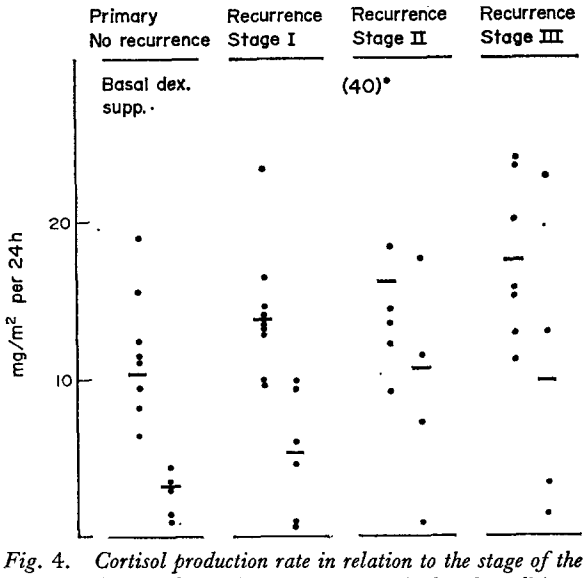


Fig. 4. Cortisol production rate in relation to the stage of the disease in 31 females with breast cancer under basal conditions, and administration of 20 μg/kg per 24 hr of dexamethasone.

But there were few cases in each group and the scatter of the individual values was very wide; even if the means of the values were different in those groups, this classification failed to demonstrate a satisfactory correlation between the stage of disease and the cortisol production rate under basal conditions and dexamethasone suppression test.

It is well known that the behaviour of a malignant tumor cannot be foreseen on the basis of the clinical features only; and, when treatment is undertaken, the results do not depend solely on the extension of the disease at this given time; other factors must be taken into consideration such as the nature of the tumor, and for some kinds of cancer like breast carcinoma, the possibility of hormone dependency.

3. *Results in relation to the evolution*

The previous findings suggested to us to divide our series of patients into two groups according to their evolution during the months following the tests and the treatment. They were separated as follows:

(1) a group including the patients with primary tumor, who did not show any recurrence within the first 6 months after treatment and the patients with advanced tumor who were improved for more than 6 months after the treatment; in both cases, the test was performed just before undertaking the treatment.
(2) a group composed of the patients with early recurrence (within 6 months) or unimproved by the treatment.

For an improvement to be recorded [28], it must be demonstrated that there is a regression of more than 50% of all the lesions. The administration of the different classes of treatment (surgical, hormonal, X-ray or chemotherapeutic) was the same in both groups.

The breast cancer cases, which present in certain conditions a special behaviour related to hormone dependency, were at first separated from the miscellaneous-tumor group: under the low dosage of dexamethasone, the decrease of the cortisol production was small in the patients with bad response to the treatment; it was more marked in the patients with good response to the treatment (Table 1).

Likewise, the 33 cases of miscellaneous tumors were submitted to the classification in relation to the result of the treatment within the first 6 months: likewise, the cortisol production rate was higher in the unimproved patients, and less decreased under the dexamethasone suppression test (Table 1).

Since an hormone dependency did not alter the results, we grouped all the cases of our

Table 1. *Cortisol production* ($mg/m^2/24$ *hr*) *and evolution of the disease*

Remission	>6 months	<6 months	p
Breast cancer			
Basal rate	13·21 ± 3·9 (20)*	16·89 ± 3·2 (11)	NS
Dexamethasone 20 μg/kg per day	4·55 ± 3·2 (13)	11·04 ± 7·3 (6)	$0{\cdot}01 < p < 0{\cdot}001$
Miscellaneous cancer			
Basal rate	11·99 ± 3·9 (17)	21·13 ± 4·8 (16)	$0{\cdot}01 < p < 0{\cdot}001$
Dexamethasone 20 μg/kg per day	6·06 ± 2·9 (11)	15·26 ± 1·8 (13)	$0{\cdot}01 < p < 0{\cdot}001$
Overall results			
Basal rate	13·1 ± 2·3 (37)	19·18 ± 3·5 (27)	$0{\cdot}01 < p < 0{\cdot}001$
Dexamethasone 20 μg/kg per day	5·24 ± 1·9 (24)	15·93 ± 4·3 (19)	$0{\cdot}01 < p < 0{\cdot}001$

*Numbers in parentheses are the number of subjects in the mean.

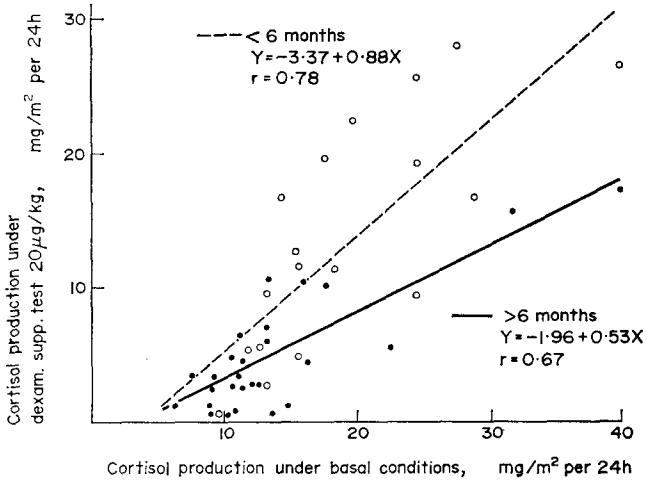


Fig. 5. *Cortisol production rate in relation to the evolution of the disease in* 43 *cancer patients. Correlation between the results under basal conditions and dexamethasone suppression test with* 20 *μg/kg per* 24 *hr.*

series in relation to the evolution after the treatment owing to the behaviour of the tumor within the 6 months delay: under both conditions, basal and dexamethasone suppression test, the cortisol production was more elevated in the patients with poor response to treatment (Table 1).

For the 43 pairs of results (Fig. 5) the regression line showing the cortisol production rate under dexamethasone suppression in relation to the basal rate has been drawn separately for the 2 groups. As shown above the correlation is significant but the slopes of the 2 regression lines are different.

DISCUSSION

All our patients with cancer presented a cortisol production rate significantly higher than normal subjects.

The correlation was significant between the response to the dexamethasone suppression test with 20 μg/kg per 24 hr and the production rate under basal conditions (Fig. 2).

Nevertheless, an ectopic secretion of an ACTH-like substance by the tumor or its metastasis seems to be ruled out because, in all cases (Fig. 3), the production rate decreased to less than 50% of its basal level following the administration of 8 mg of dexamethasone per day [20, 29]. The occurrence of metastases in the adrenals could theoretically be involved. It is well known that they can be encountered in 10 to 40% of advanced cancer [30, 31], the percentage showing some difference according to the conditions in which the study had been performed. In a review concerning 60 patients with advanced breast cancer and treated by adrenal surgery we described 33% of adrenal metastases [11]. However, in those patients investigated some days before the surgery in whom the plasma 17 OHCS levels were more elevated than in the controls, there was no relation with the presence of adrenal metastases. Therefore, it seemed that this hypothesis could be discarded. On the contrary, the fact that we met no clinical sign of hypercorticism suggests that there exists an alteration in the feedback regulation that might be ensured at a level higher than in normal subjects. This phenomenon is regular in acute or chronic diseases accompanied by stress [32, 33]; the importance of the adrenal response varies with the severity of the illness [33, 34]. Given the specific character of the neoplasic evolution as underlined above, the logic is that we should not compare the clinical stage at the time of the examination but the course of the disease during the following months after the treatment with the cortisol production rate. Under these conditions, it is "a posteriori" observed that a high basal rate and an incomplete response to the administration of the low dosage of dexamethasone suggests a poorer prognosis.

The rate of cortisol production seems to express the malignancy of the tumor better than the extension of the lesions, and it parallels the clinical course.

As far as the excretion of the urinary 17 OHCS is concerned, its fluctuation follows the cortisol production rate. The correlation between both is more significant under adrenal suppression with 20 μg per day of dexamethasone than under basal conditions.

REFERENCES

1. C. K. Meador and G. W. Liddle, Cause of Cushing's syndrome in patients with tumors arising from "non endocrine tissue". *J. clin. Endocr.* **22,** 693 (1962).
2. M. B. Lipsett, X. D. Odell, L. E. Rosenberg and T. A. Waldmann, Hormonal syndromes associated with non endocrine tumors. *Ann. intern. Med.* **61,** 733 (1964).
3. E. N. Allott and M. O. Skelton, Increased adrenocortical activity associated with malignant disease. *Cancer* **2,** 278 (1960).
4. J. L. Belsky and L. J. Marks, Plasma 17 OHCS responsiveness to ACTH in bronchogenic carcinoma. *Metabolism* **11,** 435 (1962).
5. A. Borkowski, S. Levin, A. Mahler and V. Verhas, A study of adrenal function in seventy six consecutive cases of bronchial carcinoma. *Europ. J. Cancer* **2,** 263 (1966).
6. J. J. McNamara, Steroid hormone abnormalities in patients with carcinoma of the lung. *J. thorac. cardiovasc. Surg.* **56,** 371 (1968).
7. J. Marmoston, J. M. Weiner, C. E. Hopkins and E. Stern, Abnormalities in urinary hormone patterns in lung cancer and emphysema. *Cancer* **19,** 7, 985 (1966).
8. R. I. Lichter and N. E. Street, Plasma cortisol level in lung cancer. *Brit. med. J.* **2,** 154 (1968).
9. F. A. de la Balze, V. Goldberg, C. Di Paola, J. C. De Paoli, R. Solar, R. Dendukes and J. C. Funes, Urinary excretion of free solvolysable and glucorono conjugated 17 OHCS in normal, obese and carcinomatous females. *Acta. Endocr. suppl.* **119,** 240 (1967).
10. V. Jensen, N. Desphande, R. D. Bulbrook and T. W. Douss, Adrenal function in breast cancer: production and metabolic clearance rate of cortisol in patients with early or advanced breast cancer and in normal women. *J. Endocr.* **42,** 425 (1968).
11. S. Saez-Poulain, E. Pommatau, M. Dargent and M. Mayer, Modifications des 17 OHCS plasmatiques libres au cours d'une perfusion d'ACTH chez 62 malades atteintes d'un cancer mammaire évolué avec ou sans métastases surrénaliennes. *Annales d'Endocr.* **26,** 513 (1965).
12. A. C. Hymes and R. P. Doe, Adrenal function in cancer of the lung with and without Cushing's syndrome. *Amer. J. Med.* **33,** 398 (1962).
13. W. van Hove and A. Vermeulen, Plasma cortisol levels in bronchial carcinoma, influence of cytostatic treatment. *Rev. europ. Etud. clin. et biol.* **25,** 96 (1970).
14. E. E. Werck, Jr. and L. J. Sholiton, Adrenocortical function in carcinoma of the lung. *Cancer* **13,** 469 (1960).
15. R. L. Noble, Tumors and hormones. In *The Hormones* (Edited by G. Pincus), p. 559. Academic Press, New York (1964).
16. A. Kwai, S. Tanimoto, H. Honma and N. Kuzuya, Studies on adrenal function in patients with lung cancer. *Metabolism* **18,** 609 (1969).
17. A. A. Sandberg, W. R. Slaunwhite and A. Carter, Transcortin: a corticosteroïd binding protein of plasma. III. Effects of various steroïds. *J. clin. Invest.* **39,** 1914 (1960).
18. E. Bell, R. D. Bulbrook and N. Desphande, Transcortin in plasma of patients with breast cancer. *Lancet* **ii,** 395 (1967).
19. A. A. Sandberg, K. Eik-Nes, C. J. Migeon and L. T. Samuels, Metabolism of adrenal steroïds in dying patients. *J. clin. Endocr.* **16,** 1001 (1956).
20. G. W. Liddle, W. E. Nicholson, D. P. Island, D. N. Orth, K. Abe and S. C. Lowder, Clinical and laboratory studies of ectopic syndromes. In *Recent Progress in Hormone Research,* Vol. 25, p. 283. Academic Press, New York (1969).
21. F. M. Kenny, C. Preeyasombat and Cl. J. Migeon, Cortisol secretion rate. II. Normal infants, children and adults. *Pediatrics* **37,** 34 (1966).
22. F. M. Kenny, C. Preeyasombat, J. S. Spaulding and Cl. J. Migeon, Cortisol production rate. IV. Infants born of steroïd treated mother and of diabetic mother. Infants with trisomy syndrome and with anencephaly. *Pediatrics* **37,** 960 (1966).
23. E. M. Glenn and M. D. Nelson, Chemical method for the determination of 17 hydrocorticosteroïds and ketosteroïds in urine following hydrolysis with β glucuronidase. *J. clin. Endocr.* **13,** 911 (1953).

24. F. M. KENNY, P. MALVAUX and C. J. MIGEON, Cortisol production rate in newborn babies, older infants and children. *Pediatrics* **31,** 360 (1963).
25. J. BERTRAND, B. LORAS, R. GILLY and B. CAUTENET, Contribution à l'étude de la sécrétion et du métabolisme du cortisol chez le nouveau-né et le nourrisson. *Path. et Biol.* **11,** 997 (1963).
26. G. W. LIDDLE, Test of pituitary suppressibility in diagnosis of Cushing's syndrome. *J. clin. Endocr.* **20,** 1539 (1960).
27. S. G. TAYLOR and C. P. PERLIA, Evaluation of endocrine ablative surgery in the treatment of mammary carcinoma: a preliminary study on survival. In *Biological Activities of Steroids in Relation to Cancer* (Edited by G. PINCUS and E. WOLLMER), Academic Press, New York (1960).
28. C. B. ROSOFF, Hormonal therapy of breast cancer. In *Biological Activities of Steroids in Relation to Cancer* (Edited by G. PINCUS et E. WOLLMER), p. 363, Academic Press, New York (1960).
29. C. M. GOODALL, On para-endocrine cancer syndromes. *Int. J. Cancer* **4,** 1 (1969).
30. H. T. KARSNER, Tumors of the adrenal. In *Atlas of Pathology.* Armed Forces Institute of Pathology.
31. O. SAPHIR and M. L. PARKER, Metastasis of primary carcinoma of the breast with special reference to spleen, adrenal glands and ovaries. *Arch. Surg.* **42,** 1002 (1941).
32. M. MILLER and A. M. MOSES, Effects of temperature and dexamethasone on the plasma 17 OHCS and GH responses to pyrogen. *J. clin. Endocr.* **28,** 1056 (1968).
33. W. R. BEISEL and M. I. RAPOPORT, Adrenocortical functions and infectious illness. *New. Engl. J. Med.* **280,** 541 (1969).
34. CL. J. MIGEON, F. M. KENNY, W. HUNG and M. L. VOORHESS, Study of adrenal function in children with meningitis. *Pediatrics* **40,** 163 (1967).

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Cell Free Transmission of a Plasmocytoma to Various Strains of Adult Mice

G. PEDIO and J. R. RUTTNER
Institute of Pathological Anatomy University of Zurich, Switzerland

Abstract—*To enrich the virus presumably present in HIPA plasmocytoma of BALB/c mice a "double inoculation method" was devised. It consisted of an initial intraperitoneal transplantation of tumor cells followed by intraperitoneal injection of cell free ascitic tumor ultracentrifugate. This procedure was repeated in 7 steps. It resulted in an increased oncogenic potency of the ultracentrifugates in isogenic BALB/c mice.*

Tumor ultracentrifugate of the "double inoculated" mice served as origin for two serial cell free transmission lines; mesenteric plasmocytoma developed after a latency time of 2–4 weeks in 62–85% (UZ/B and UZ/G).

Type A and type C particles were found in ultracentrifugates and in induced plasmocytoma. The ultracentrifugates retained their oncogenic effect after freezing and thawing. By millipore filtration a loss of oncogenicity occurred. With ultracentrifugates of the cell free transmission lines the induction of mesenteric plasmocytomas in allogeneic adult mice of the following strains: $C_{57}Bl$, ICR, C_3H, DBA, AKR was successful. Xenogeneic transmission (rats and hamsters) failed.

THE TRANSPLANTABLE mesenterial HIPA-plasmocytoma was induced in BALB/c mice by injection of spleen homogenate originating from a non-tumor bearing, isogenic mouse pretreated with mineral oil [1]. An attempt to induce plasmocytoma in isogenic mice with ultracentrifugates of HIPA-tumor ascitic fluid of the 19th transplantation generation was successful. Both the ultracentrifugate and the induced tumor contained C-type particles [2]. Serial cell free passages of the tumor were impossible owing to insufficient quantities of ascitic fluid. Moreover, subsequent attempts to repeat the induction of plasmocytoma with ultracentrifugates of ascitic fluid of the 29th, 33rd, 42nd, 51st, 53rd and 58th cellular transplantation generation were not successful. Electron microscopic evaluation revealed a marked decrease in numbers of C-type particles in the plasmocytomas of the 32nd to the 37th transplantation generation, and no virus-like particles were demonstrated in the tumors of mice from the 42nd to the 70th generation. However, C-type virus particles were found again in tumor cells of the 71st transplantation generation.

In this paper we describe a new experimental method for the production of a potent oncogenic cell free extract capable of transmitting HIPA-plasmocytoma. It is based on the assumption that HIPA ascites tumor might be a more susceptible substrate for virus replication than normal tissue. Consequently, a double inoculation technique was developed. It consisted of an initial cellular transplantation of the plasmocytoma followed by an intraperitoneal injection of cell free ascites tumor ultracentrifugate. This procedure was repeated over 7 passages. It resulted in production of an ascites plasmocytoma which now can be transmitted regularly and reproducibly with cell free ultracentrifugate.

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MATERIAL AND METHODS

(1) *Animals.* Inbred mice of the BALB/c, DBA, $C_{57}Bl$, C_3H and AKR strain and random-bred ICR mice maintained at the Biological Central Laboratory of the Kantonsspital Zurich were used. Some experiments were performed with CFN-strain rats and Syrian hamsters.

(2) *Tumor ascites.* The 75th to the 81st transplantation generation of HIPA plasmocytoma, carried in BALB/c mice of both sexes, were used.

(3) *Differential ultracentrifugation of tumor ascites.* The method of Chenaille [3] was used. Ascitic fluid obtained aseptically was diluted with an equal volume of sterile distilled water containing heparin at a final concentration of 10 to 15 IU/ml. After centrifugation for 20 min at 4°C and 1200 **g** and followed by 20 min at 2400 **g**, the intermediate layer of the supernatant was gently collected and centrifuged for 2 min at 10,000 **g**. The supernatant was mixed slowly with an equal volume of 0·5 M sucrose in 0·00075 M tris buffer, pH 7·2. This mixture was centrifuged in a Spinco L 50 ultracentrifuge, rotor 50, for 90 min at 56,000 **g** (0° temperature). The pellets were resuspended in 0·25 M sucrose in 0·00075 M tris buffer, pH 7·2 with a Teflon pestle homogenizer. This suspension was used for animal inoculation. The dose was expressed as ascites-equivalents (AE), one ascites-equivalent being defined as the quantity contained in 1 ml of ascitic tumor.

(4) *Electron microscopy.* The methods used for examination of ultracentrifugate pellets and solid tumors were described in a previous paper [2].

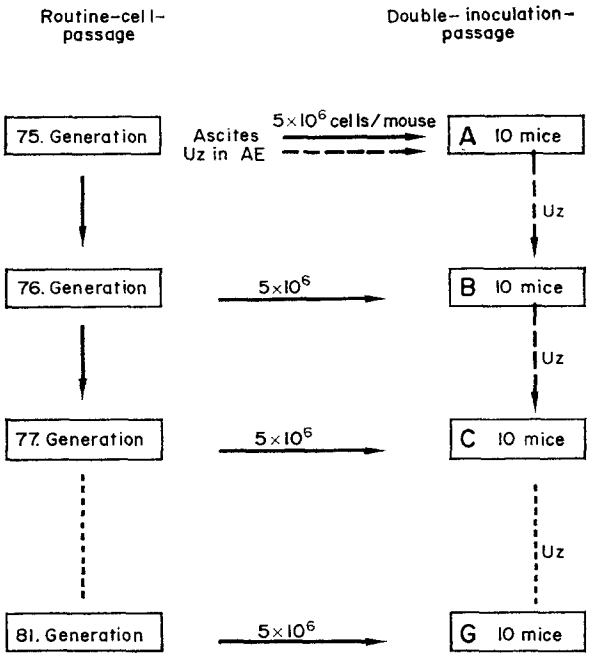
(5) *Histology.* Mesenteric tumors, spleen, lymph nodes and other organs were fixed in 4% formalin, embedded in paraffin, sectioned and stained with haematoxylin-eosin or other stains as required. Blood and ascites smears were stained with Giemsa.

(6) *Electrophoresis.* Blood samples were taken by heart puncture and centrifuged for 10 min at 2000 **g**. Serum electrophoresis was carried out on cellulose acetate strips with a Beckman microzone cell model R-101.

RESULTS

First experiment: double inoculation method

In an attempt to enrich the virus presumably present in ultracentrifugate, a "double inoculation method" was devised according to the following scheme (see also scheme 1):



Scheme 1

1. Step:

   (*a*) 5×10^6 unwashed HIPA ascites cells from the 75th generation were transplanted i.p. into 10 adult BALB/c mice of both sexes (group A).

   (*b*) After 2 days each mouse of group A was inoculated with resuspended ultracentrifugate (5 AE) obtained from ascites (16 days) of tumor bearing mice of the 75th generation.

2. Step:

   (*a*) 5×10^6 HIPA ascites cells of the 76th generation were transplanted i.p. into 10 adult BALB/c mice (group B).

   (*b*) Two days later each mouse of group B was inoculated with resuspended ultracentrifugate (5 AE per mouse), obtained from ascites of mice of group A. Ascites was gained 14 days after double inoculation.

This procedure was repeated five more times with groups of 10 mice designated as group C, D, E, F, G.

Double inoculation resulted in tumor growth in all mice of groups A–G. Ascites developed between the 5th and 7th day. Growth characteristics and morphology of the plasmocytomas did not differ from those seen after routine cellular transplantations.

Table 1. Cell free transmission in isogenic mice. Line UZ/B

Transmission/ Generation	No. of mice/tumor	Age of the mice (days)	Dosis ultracentrifugate AE/mouse	Survival time (average, days)
UZ/B_1	7/ 5	6	7·20	35
UZ/B_2	5/ 5	4	7·00	23
UZ/B_3	2/ 2	49	4·00	24
UZ/B_4	7/ 5	15	3·50	25
UZ/B_5	15/ 3	49	2·50	27
UZ/B_6	16/ 7	49	2·50	34
UZ/B_7	23/ 14	49	2·00	32
UZ/B_8	28/ 19	49	1·50	37
UZ/B_9	22/ 14	49	0·80	31
UZ/B_{10}	15/ 8	49	0·80	32
UZ/B_{11}	10/ 7	49	0·70	35
UZ/B_{12}	8/ 5	49	0·80	32
UZ/B_{13}	6/ 5	49	1·00	29
UZ/B_{14}	8/ 7	49	1·00	26
Total	174/106			29·6

Second experiment: cell free tumor transmission

To test the oncogenic potency of "enriched" ascites plasmocytoma ultracentrifugate, cell free transmissions were performed as follows:

(*a*) Seven BALB/c mice, 6 days old (UZ/B_1), were inoculated i.p. with resuspended ascites ultracentrifugate (7·2 AE per mouse) obtained from doubly inoculated mice of group B. These mice are the origin of a tumor line designated *UZ/B line* which was maintained by cell free transmission.

In 5 of the 7 animals UZ/B_1 ascites developed with a latency period of 22 to 34 days. The animals were sacrificed at an average of 35 days. Ascites fluids were harvested for the preparation of an ultracentrifugate. At autopsy a hemorrhagic ascites of 4 to 9 ml as well as extensively necrotic tumors of the mesenterium were found. The tumor tissue consisted essentially of mature and immature plasmocytes, as usually seen in HIPA plasmocytoma [4].

Lymph nodes, spleen, kidney, liver and other organs were unchanged. The serum electrophoresis did not reveal abnormal proteins, neither did the serum of the donor animals, since abnormal proteins have not been observed in transplanted HIPA tumor beyond the 26th passage [4].

The ascites ultracentrifugate of these mice (UZ/B_1) served as starting material for serial cell free transmission as summarized in Table 1. In 14 passages (UZ/B line) a total of 174 mice of both sexes and of varying ages were inoculated. In the course of the experiments progressively smaller amounts of ultracentrifugate were used. A mesenterial plasmocytoma developed in 106 animals giving a successful tumor transmission

Table 2. Cell free transmission in isogenic mice. Line UZ/G

Transmission/ Generation	No. of mice/tumor	Age of the mice (days)	Dosis ultracentrifugate AE/mouse	Survival time (average, days)
UZ/G_1	7/ 6	49	2·70	36
UZ/G_2	15/ 13	49	2·70	29
UZ/G_3	15/ 13	49	2·00	27
UZ/G_4	27/ 27	49	1·50	25
UZ/G_5	37/ 36	49	1·00	26
UZ/G_6	38/ 33	49	0·75	28
UZ/G_7	40/ 30	49	0·60	26
UZ/G_8	20/ 14	49	0·75	26
UZ/G_9	26/ 20	49	0·60	27
UZ/G_{10}	35/ 29	49	0·55	26
	260/221 (85%)			27

Table 3. Minimal lethal dosis of ascites ultracentrifugate in AE

No. of adult BALB/c mice inoculated with UZ/G 10 ultracentrifugate/ No. of mice with tumor	Dosis ultracentrifugate AE/mouse	Survival time (average, days)
12/10	0·50	30
5/ 5	0·40	28
8/ 5	0·30	31
18/11	0·20	33
14/ 5	0·15	29
8/ 3	0·10	34
6/ 2	0·05	33

of 62%. Tumor incidence was not related to the amount of ultracentrifugate injected. Tumor histology was unchanged and the serum electrophoresis remained normal.

(*b*) Seven *adult* BALB/c mice (UZ/G_1) were inoculated i.p. with resuspended ascites ultracentrifugate (2·5 AE per mouse) originating from doubly inoculated plasmocytoma bearing mice of group G (see first experiment). These mice are the origin of a cell-free tumor line designated as *UZ/G*. Of these (UZ/G_1), six mice developed ascites tumor after a latency period of 22 to 26 days. Autopsy findings, histology of the tumors and serum electrophoresis were the same as those described for mice of group UZ/B_1. The ultracentrifugate of ascites of these animals served as starting material for serial cell-free passages (line UZ/G). So far 11 generations can be evaluated.

A total of 260 adult mice were inoculated. Of these 221 animals (85%) developed the tumor. The results are summarized in Table 2.

From Table 2 it is evident that the decrease in the amount of ultracentrifugate injected did not affect plasmocytoma incidence. In the course of the experiment the latency period appeared to become shortened.

With the ascites ultracentrifugate of the UZ/G line, 10th passage (UZ/G_{10}), an attempt was made to estimate the minimal lethal dosis. Results are shown on Table 3. It is evident that tumors could be induced with minute amounts of ultracentrifugate.

Third experiment: controls for the presence of cells and virus particles

A. *Screening for cell contamination of ultracentrifugate pellets*

To exclude the presence of cells in ascites ultracentrifugates the following experiments were performed:

(*a*) *Morphological control.* Ascites ultracentrifugate pellets of tumor bearing mice from the UZ/B line, 3rd, 4th and 7th cell free passage and from the UZ/G line, 3rd, 4th, 7th and 10th cell free passage were examined in serial ultrathin sections with the electron microscope and by phase contrast microscopy.

This examination revealed no intact cells, larger cell fragments or recognizable cellular organelles. Agglomerations of virus-like structures resembling type-C particles as described by Bernhard [5] were frequently seen (Fig. 1). They were scattered within a fine granular matrix. These virus-like particles were round, with a diameter of 75–86 mμ and exhibited an outer and an inner membrane. They possessed an electron-dense nucleoid with a diameter of 42–53 mμ.

(*b*) *Biological control.* (1) Millipore filtration: ultracentrifugate pellets of UZ/B and UZ/G line were resuspended in 0·25 M sucrose in 0·00075 M tris buffer pH 7·2 and filtered through millipore filter of 1·2 μ average pore diameter (RA 1·2 mikro, Millipore Filter Corp. Bedford, Mass.) and inoculated i.p. into isogenic mice (Table 4).

Twenty-three adult BALB/c mice were injected i.p. with ultrafiltrate of ascites fluid originating from both cell free transmission lines (UZ/B and UZ/G) (Table 4).

Ascites was demonstrated in 3 mice 64, 73 and 82 days after inoculation. All three animals died a few weeks after the appearance of the

Table 4. Oncogenicity of ascites tumor filtrates

Donor mice	Ultrafiltrate dosis AE/mouse	No. of mice/tumor
UZ/B_4	6	4/0
UZ/B_7	4	2/0
UZ/G_2	8	4/2
UZ/G_3	8	4/1
UZ/G_3	8	2/0
UZ/G_5	4	4/0
UZ/G_6	5	3/0
		23/3

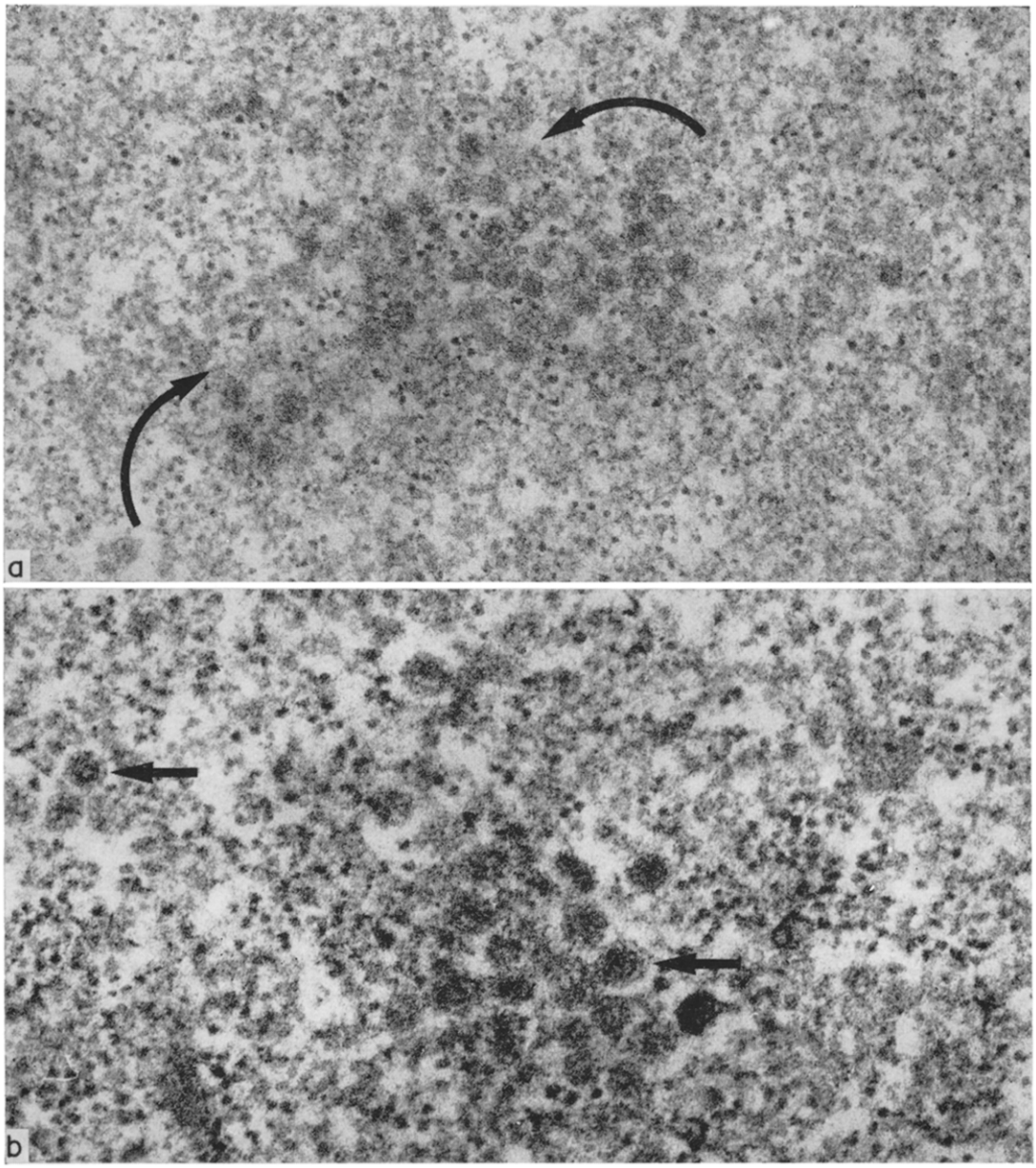


Fig. 1. *Ultracentrifugate pellets from HIPA ascites fluid of the 4th cell-free passage, line UZ/G. Virus-like particles lying in granular matrix. They consist of two concentric membranes (arrow) and an electron-dense nucleoid.*

(a) ca. × 68,000

(b) ca. × 105,000

(to face p. 392)

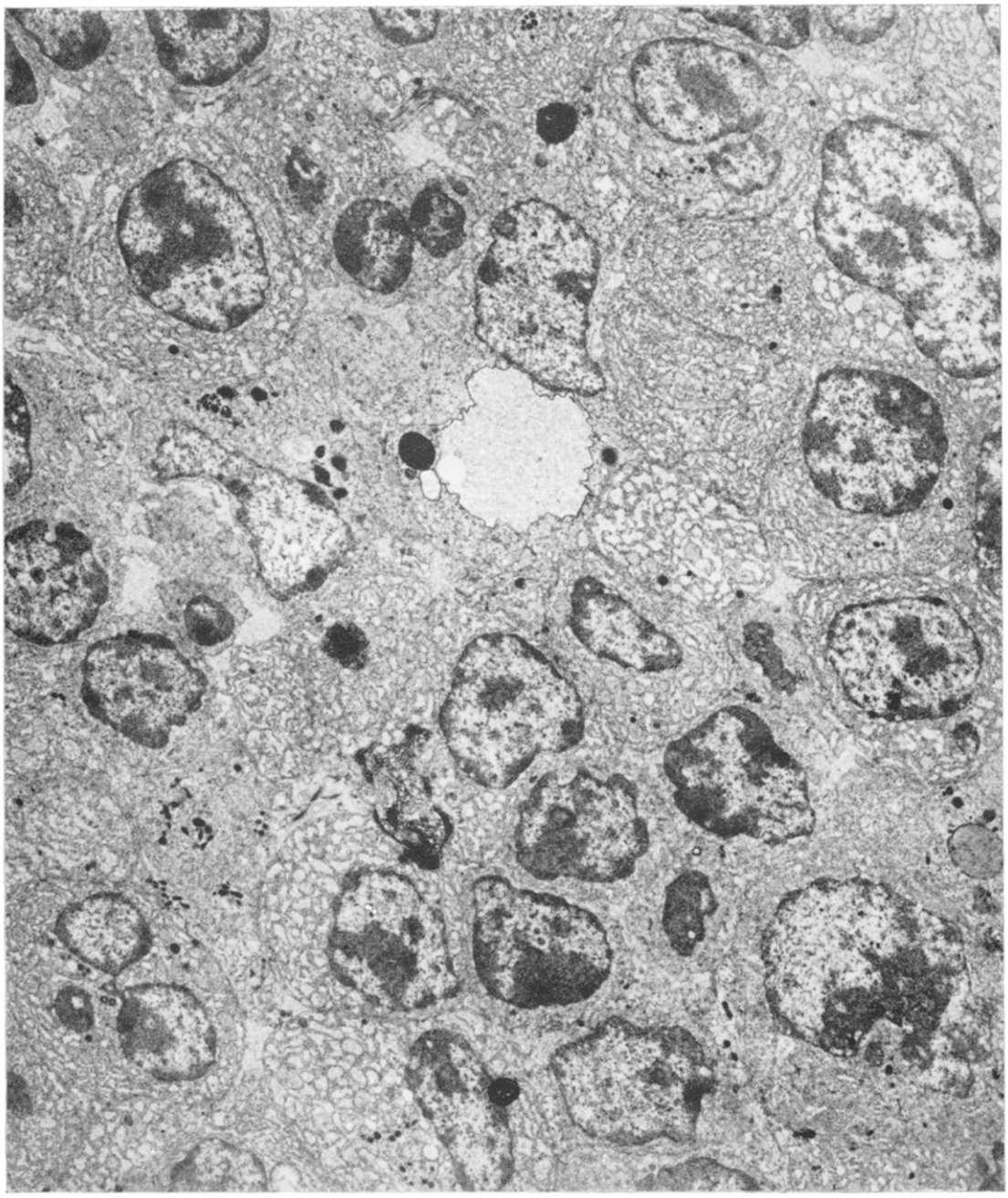


Fig. 2. Mesenteric plasmocytoma in adult $C_{57}B1$ mouse 23 days after i.p. inoculation of cell-free ultracentrifugate from HIPA-ascitic fluid.
The plasma cells are well developed. Observe the empty cisternae. (× 5,000)

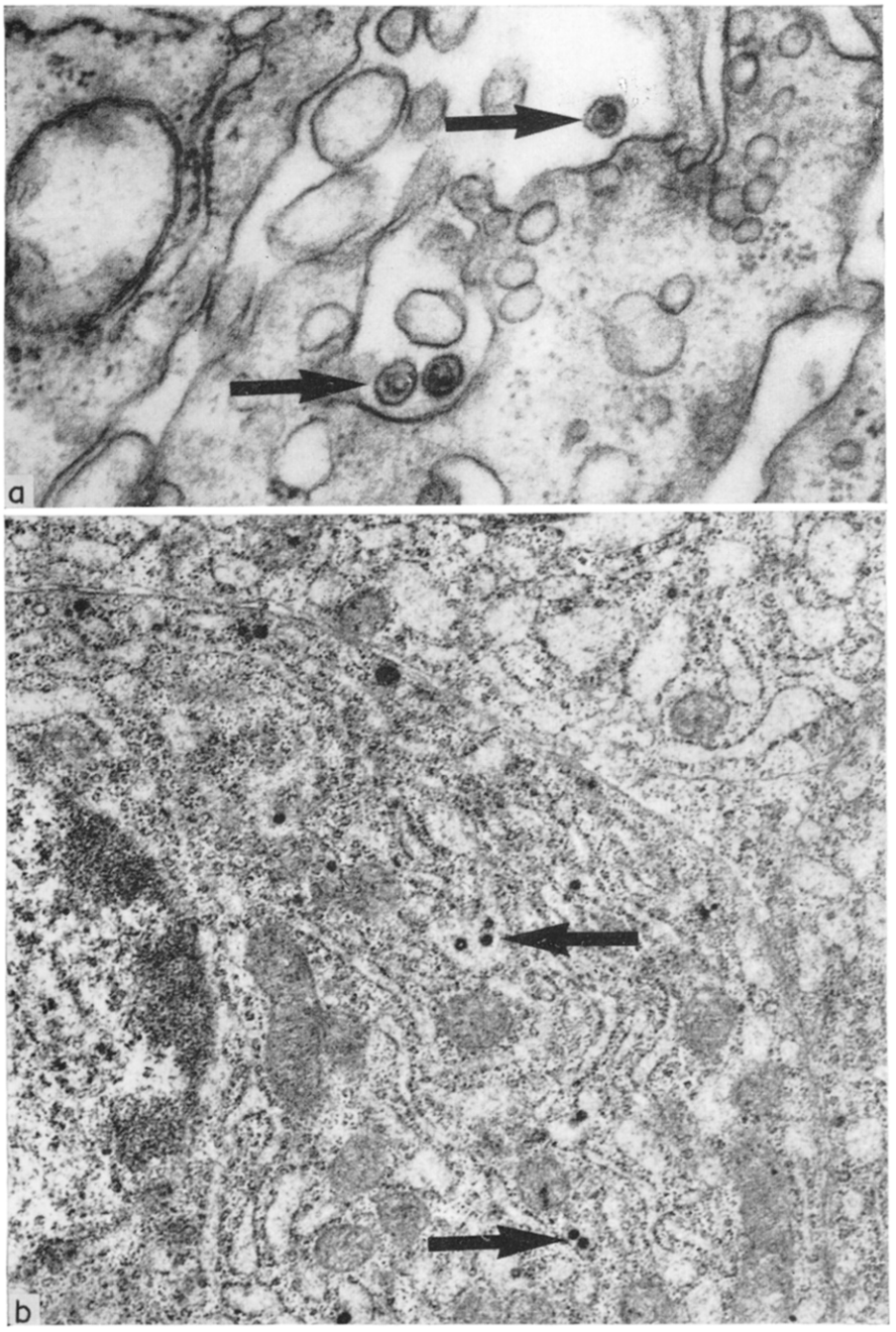


Fig. 3. (a) *Tumor tissue, 4th cell-free passage, line UZ/G. Virus-like particles of type* C *lying free in extra-cellular space (arrows).* ca. × 86,000

(b) *Tumor tissue, 4th cell-free passage, line UZ/G. Virus-like particles of type* A *are visible in numerous cisternae of a well developed plasma cell. (arrows).* ca. × 24,000.

ascites. A mesenterial plasmocytoma was demonstrated at autopsy.*

(2) Freezing and thawing: pellets of the ultracentrifugate of the 6th passage of UZ/G line were frozen in solid CO_2, immediately thawed and inoculated into 8 BALB/c mice (10 to 20 days old) (1·25 AE/animal).

Eight isologous control mice received equal amounts of non-treated ultracentrifugate suspension of the same ascites donor animals.

Freezing and thawing of the pellets of the ultracentrifugates caused no decrease in oncogenic potency. All 16 mice which were inoculated with resuspended ultracentrifugate (UZ/G_6) exhibited a plasmocytoma. Gross examination and histology of the tumors were identical with those found in mice inoculated with untreated ultracentrifugate.

B. *Screening for virus particles in induced tumors*

Samples of solid mesenteric plasmocytoma from animals used for the morphological control of cell contamination (A a) and from HIPA tumor-bearing $C_{57}Bl$ mice were examined by electron microscopy. The tumor cells of the UZ/B and UZ/G lines had maintained the ultrastructural characteristics of plasma cells (Fig. 2). Virus-like particles were observed in the cisternae of the plasma cells (Fig. 3b), or lying free in intercellular spaces (Fig. 3a). The intracisternal particles corresponded to type A according to Bernhard's classification [5]. They were round, about 75 to 82 mμ in diameter and exhibited two concentric membranes limiting a translucent center. The extracellular type C particles are budding from the cellular surface of undifferentiated cells. These particles measured about 85 mμ. They were characterized by an electron-dense centrally located nucleoid measuring 45–55 mμ in diameter and by an outer and an inner membrane.

*In view of the low yield following filtration with 1·2μ millipore filters, passage through finer filters was not attempted.

Fourth experiment: allogeneic and xenogeneic transmission

(*a*) *Allogeneic transmission with HIPA ascites ultracentrifugate.* Ultracentrifugate suspension originating from ascitic plasmocytomas of UZ/G line, 7th and 8th passages were injected i.p. into groups of 10 adult mice of the following strains: $C_{57}Bl$, ICR, C_3H, DBA, AKR (Table 5).

Hemorrhagic ascites and mesenterial plasmocytoma developed after 14 to 23 days. Serum proteins remained unchanged. Survival time varied from a minimum of approximately 20 days ($C^{22}Bl$ strain) to a maximum of 27 days (AKR strain).

(*b*) *Xenogeneic transmission with HIPA ascites ultracentrifugate.* Resuspended ultracentrifugate of UZ/G line, 10th and 11th passages were injected i.p. into [4] newborn Syrian hamsters (7 AE/hamster) and 4 newborn CFN rats (11 AE/rat). This experiment failed to induce plasmocytomas after an observation time of 80 days.

(*c*) *Allogeneic cellular transplantation.* Repeated attempts were made to induce plasmocytoma in adult $C_{57}Bl$ mice with washed tumor cells from ascites of UZ/B and UZ/G line by intraperitoneal inoculation. No tumors developed after application of high dose of tumor cells (see Table 6).

DISCUSSION

The viral induction of neoplastic proliferations of the lymphoid and the myeloid tissue in mice is well recognized [6–10].

On the other hand the viral induction and the cell-free transmission of plasmocytoma in animals has not been shown previously [11–13].

Ebbesen *et al.* [14] described the induction of a plasma cell leukemia in BALB/c mice which also involved spleen and lymph nodes. This tumor could be transferred by i.p. administration of ultracentrifugates of subcellular material. The authors did not demonstrate

Table 5. *Cell free transmission in allogeneic adult mice*

Donor mice	Dosis AE/mouse	Strain	No. of mice/tumor	Survival time (average in days)
UZ/G_8	6·0	$C_{57}Bl$	10/10	19·6
UZ/G_7	2·5	ICR	10/10	21·6
UZ/G_7	2·5	C_3H	10/10	23·8
UZ/G_8	5·1	DBA	10/10	26·2
UZ/G_8	3·1	AKR	10/10	26·6

viruses in the ultracentrifugate nor did they attempt serial cell free transmission of the tumor. However, presumably type A particles were described in spleen and lymph nodes. The latency period from inoculation to manifestation of the plasma cell leukemia was 19 months and the leukemia incidence was 50%.

As a starting material for the cell-free transmission with ultracentrifugal supernatants we chose the morphologically well defined HIPA plasmocytoma of BALB/c mice [1, 4, 15, 16, 17]. Isogenic as well as allogeneic transfers proved successful. In spite of the characteristic plasmocytoma morphology no abnormal serum proteins were found. This is not unusual, since other murine plasmocytomas induced by Bayol F secrete immunoglobulins in only 66% of cases, 34% of murine plasmocytomas either secrete subunits or show no recognizable secretion products in the serum [12]. It is conceivable that the HIPA plasmocytoma also synthesizes paraproteins which are, however, not secreted. We have not tested for such proteins, neither have we looked for specific plasma cell antigens, since the primary goal of our investigations was to show that cell-free transmission of the HIPA plasmocytoma can be successfully performed.

Our experiments demonstrate that the oncogenic potency of ascites ultracentrifugates of the HIPA plasmocytoma could be increased dramatically by the "double inoculation technique" using tumor cells and ultracentrifugates of tumors. The latency period for appearance of ascitic tumor was reduced to 2–4 weeks and the tumor incidence reached 70–100%. These results also indicate that the oncogenic potency of ultracentrifugates increased with the number of "double inoculations". Thus, the ultracentrifugates of the 7th double inoculation passage (UZ/G line) were considerably more potent than those of the 2nd double inoculation passage (UZ/B line).

A somewhat similar procedure was also used by Nordenskjöld [18] who achieved a 100 fold enrichment of the virus yield by injection of polyoma virus into polyoma virus-induced ascites tumors.

A considerable effort was made to prove that the ultracentrifugates used for the transmissions of the plasmocytoma were indeed cell free. Repeated electron microscopic examinations of serial sections of ultracentrifugate pellets of ascitic plasmocytomas failed to show any intact cells or larger cell fragments. Furthermore, the tumor ultracentrifugates retained their oncogenic effect after freezing and thawing. It is unlikely, therefore, that transmission of our plasmocytoma depended on the presence of intact cells. Furthermore, a plasmocytoma was induced in 3 out of 23 isogenic BALB/c mice treated with ultracentrifugate after passage through a 1·2 μ millipore filter. The loss of infectivity in this experiment is difficult to evaluate since some oncogenic and non-oncogenic RNA viruses are not or only poorly filtrable [16–18]. The reason for this technical difficulty may be explained partly by an adsorption of the viruses to the filter membrane [19]. The observation that a few mice did develop a plasmocytoma after inoculation with the filtrate suggests also the viral origin of this tumor.

A strong evidence for the viral origin of HIPA plasmocytoma is derived from the electron microscopic findings of virus-like particles of type C and A in sections of solid tumors as well as in the ascites ultracentrifugate pellets from the same tumors. Another important argument for the viral origin is the induction of plasmocytomas in adult allogeneic mice by inoculation of cell free ascites ultracentrifugates.

The susceptibility of the different allogeneic mouse strains to plasmocytoma induction by ultracentrifugates could not be assessed, since

Table 6. Transplantation of cell-free induced plasmocytoma from BALB/c mice into adult $C_{57}Bl$ and adult BALB/c mice*

Donor mice	Recipient mice	No. of injected cells/mouse (%10^6)	No. of mice/tumor	Survival time (average in days)
UZ/G_{13}	$C_{57}Bl$	2·6	5/ 0	—
UZ/B_{18}	$C_{57}Bl$	18	10/ 0	—
	BALB/c	18	10/10	12

*$C_{57}Bl$ mice were observed for about 80 days.

the doses employed were not uniform: in all cases the amount of ultracentrifugate used for allogeneic transfer was considerably larger than that used for syngeneic transfer (see Table 5). In contrast to the ease with which the plasmocytoma could be induced in allogeneic mice by cell free centrifugate, washed tumor cells even in the enormous dose of 18×10^6 cells per mouse were unable to induce tumors in allogeneic animals (Table 6). This suggests that the tumor in allogeneic mice had been induced by an agent present in the ultracentrifugate and not transplanted by cellular contamination. Considering the above data and in analogy to other reports which consider type-C particles responsible for murine leukemias [5, 22] it appears likely that the type C particles were responsible for plasmocytoma induction, and not the intracisternal type A particles which were also present.

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REFERENCES

1. G. Pedio, E. Grieshaber and J. R. Rüttner, Zur Plasmozytom-Onkogenese. Durch Milzhomogenat einer mit Mineralöl behandelten BALB/c-Maus induzierte transplantable Neoplasie (HIPA) mit plasmazellulärer Evolution. I. Biologisch-histologischer Teil. *Path. et Microbiol. (Basel)* **31,** 278 (1968).
2. G. Pedio, E. Grieshaber and J. R. Rüttner, On plasmocytoma oncogenesis of mice. II. Differential ultracentrifugate which contains RNA virus-like particles from HIPA tumor inducting a plasmocytoid neoplasia in BALB/c mice. *Path. et Microbiol. (Basel)* **32,** 241 (1968).
3. P. Chenaille, J. P. Levy, A. Tavitan and M. Boiron, Concentration and partial purification of a murine leukemia virus (Rauscher). *Nature, (Lond.)* **213,** 5071 (1967).
4. G. Pedio, E. Grieshaber and J. R. Rüttner, On plasmocytoma oncogenesis of mice. Evolution of a poorly differentiated sarcoma (HIPA Tumor of BALB/c mice) into a plasmocytoma. IV. A comparative histological and electron microscopic study. *Path. et Microbiol. (Basel)* **34,** 65, (1969).
5. W. Bernhard, The detection and study of tumor viruses with the electron microscope. *Cancer Res.* **20,** 712 (1960).
6. L. Gross, "Spontaneous" leukemia developing in C_3H mice following inoculation, in infancy, with AK-leukemic extracts, or AK-embryo. *Proc. Soc. exp. Biol. Med.* **76,** 27 (1951).
7. J. B. Moloney, Biological studies on a lymphoid leukemia virus extracted from Sarcoma 37—I. Origin and introductory investigations. *J. nat. Cancer Inst.* **24,** 933 (1960).
8. C. Friend, Cell-free transmission in adult Swiss mice of a disease having the character of a leukemia. *J. exp. Med.* **105,** 307 (1957).
9. F. J. Rauscher, A virus-induced disease of mice characterized by erythrocytopoiesis and lymphoid leukemia. *J. nat. Cancer Inst.* **29,** 515 (1962).
10. A. Graffi, H. Bielka, F. Fey, F. Scharsach and R. Weiss, Gehauftes Auftreten von Leukaemien nach Injektion von Sarkom-Filtraten. *Naturwiss.* **41,** 503 (1954).
11. A. J. Dalton, M. Potter and R. M. Merwin, Some ultrastructural characteristics of a series of primary transplanted plasma cell tumors of the mouse. *J. nat. Cancer Inst.* **26,** 1221 (1961).
12. M. Cohn, "Natural history of Myeloma". Cold Spring Harbor Symposia on Quantitative Biology. Antibodies Vol. XXXII, 211 (1967).
13. E. L. Kuff, N. A. Wivel and K. K. Lueders, The extraction of intracisternal A-particles from a mouse-plasma cell tumor. *Cancer Res.* **28,** 2137 (1968).
14. P. Ebbesen, R. Rask-Nielsen and K. R. McIntire, Plasma cell leukemia in BALB/c mice inoculated with subcellular material. I. Incidence and morphology. *J. nat. Cancer Inst.* **41,** 473 (1968).
15. E. Grieshaber, G. Pedio and J. R. Rüttner, On plasmocytoma-oncogenesis of mice. III. Annulate lamellae with unusual morphology in a plasmocytoid neoplasia of mice (HIPA tumor). *Path. et Microbiol. (Basel)* **33,** 1 (1969).

16. E. Grieshaber, G. Pedio and J. R. Rüttner, On plasmocytoma-oncogenesis of mice. V. Viruses and virus-related structures in HIPA tumor of BALB/c mice. *Path. et Microbiol. (Basel)* **34,** 86 (1969).
17. E. Büsser, G. Pedio and J. R. Rüttner, Knochenlaesionen und Metastasierungsmuster in einem experimentellen Plasmozytom der BALB/c-Maus (HIPA-Tumor). *Path. et Microbiol. (Basel)* **36,** 243 (1970).
18. B. Nordenskjöld, Large-scale production of polyoma virus in mouse ascites tumor cells *in vivo*. *Unio Nordica Contra Cancrum. Symp. on Virus and Cancer.* (Edited by H. Bergstrand and K. E. Hellström), Saltsjöbaden, June 8–9 (1964).
19. D. O. Cliver, Virus interactions with membrane filters. *Biotech. Bioengng* **10,** 877 (1968).
20. V. K. Jenkins and A. C. Upton, Cell-free transmission of radiogenic myeloid leukemia in the mouse. *Cancer Res.* **23,** 1748 (1963).
21. T. Tanaka and A. W. Craig, Cell-free transmission of murine myeloid leukemia. *Europ. J. Cancer,* **6,** 329 (1970).
22. J. B. Moloney, The rodent leukemias, virus-induced murine leukemias. *Ann. Rev. Med.* **15,** 338 (1964).

Europ. J. Cancer Vol. 7, pp. 397–410. Pergamon Press 1971. Printed in Great Britain.

Nucleic Acids and Acid-Soluble Derivatives Released by Isotonic Solutions of Cryoprotective Agents from Ehrlich-Lettré Ascites Carcinoma and Mouse Spleen Cells*

D. G. R. BLAIR,† C. P. ENG‡ and J. F. MORGAN§

Department of Cancer Research, University of Saskatchewan, Saskatoon, Saskatchewan, Canada

INTRODUCTION

DIMETHYLSULFOXIDE (DMSO)‖ is widely used as a cryoprotective agent [1–3] and as a solvent for carcinogenic polycyclic hydrocarbons [4, 5] which are relatively insoluble in aqueous media. *Myo*-inositol also acts as a cryoprotective agent for mammalian cells [6, 7]. Both DMSO and inositol maintain the viability of cells during low-temperature preservation, although alterations in cell physiology, including the loss of mouse tumor cell, mouse-strain specificity [7] and release of nucleic acids and protein [6–10], result from the use of the agents.

The protective action of inositol against the partial desiccation of bacterial and mammalian cells has been studied extensively by Webb [6], and Webb *et al.* [9], and Bather *et al.* [10] reported the release of nucleic acid from bacterial and mammalian cells in the presence of 5% inositol. Eng and Morgan [8] studied the release of nucleic acids and protein from Ehrlich and 6C3HED ascites tumor cells during incubation with glycerol, DMSO or inositol under various experimental conditions. They found that incubation of the cells for 1 hr at 37°C in isotonic solutions of DMSO or glycerol caused the release of large amounts of both nucleic acids and protein, whereas incubation with isotonic inositol caused the release of large amounts of nucleic acids, but of little more protein than that released in the control medium, Hanks' balanced salt solution (HBSS) [11]. Since the release of nucleic acids from the Ehrlich cells in the presence of inositol occurred without a concomitant loss of cellular viability or tumorigenicity, the loss of 1·7–1·9% of the cellular nucleic acid (about 80% RNA) did not seem to be crucial. An interesting observation [8] was that the release of nucleic acids in the presence of inositol could be inhibited by NaCl. An incubation medium com-

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†Research Associate of the National Cancer Institute of Canada and the person to whom reprint requests should be addressed.

‡Present address: Bionetics Research Laboratory, Kensington, Maryland 20795, U.S.A.

§Present address: Department of Biochemistry, University of Saskatchewan, Saskatoon, Saskatchewan.

‖Non-standard abbreviations used: DMSO, dimethylsulfoxide; HBSS, Hanks' balanced salt solution; DNase, deoxyribonuclease; Nucleic acid bases: A, adenine; C, cytosine; G, quanine; U, uracil; and T, thymine; RNase, ribonuclease; rRNA, ribosomal RNA; sRNA, "soluble" RNA; UV, ultraviolet.

posed of 1 part isotonic NaCl and 3 parts isotonic inositol released one-third as much nucleic acid as inositol alone, indicating an inhibition of about 80% when the contributions of inositol and NaCl alone were taken into account.

A recent report by Caldwell and Chan [12] indicates that the loss of perchloric acid-insoluble and -soluble materials from Ehrlich cells in the presence of isotonic salts solutions is a general phenomenon. Materials were released from cells incubated with HBSS, Krebs-Ringer phosphate solution, Tyrode's solution, and various other media at 37°C for up to 3 hr.

The present studies were undertaken to identify the nucleic acids and acid-soluble derivatives which are released from Ehrlich-Lettré ascites carcinoma cells in the presence of inositol, DMSO, or HBSS and, in order to find whether the phenomena described for the tumor cells were unique, the investigations were extended to a normal cell, the mouse spleen cell. A comparison of the effects of inositol and HBSS on the two cell types was emphasized. A differential effect of a physiological substance such as inositol on tumor and normal cells may have many important consequences.

MATERIAL AND METHODS

Ehrlich-Lettré ascites carcinoma cells. The Ehrlich-Lettré hypotetraploid ascites tumor (modal chromosome number of 74)* was obtained originally from Dr. M. J. Fraser, Department of Biochemistry, University of Manitoba, Winnipeg, in November 1963, and has been maintained in the authors' laboratory by weekly serial passage in mice. In this study each female Swiss mouse† was inoculated intraperitoneally with 0·25 ml of ascites tumor fluid containing approximately $5{\cdot}0 \times 10^6$ tumor cells. After 12 to 14 days the ascites tumor was harvested with a syringe and the fluid was centrifuged at 2000 rev/min for 15 min in an International clinical centrifuge, model CL. The packed cells were washed twice with HBSS before further use. The viability of the cells as estimated by the dye-exclusion test with erythrosin B, according to the method of Phillips and Terryberry [13], ranged from 93 to 98%.

Mouse spleen cells. Adult Swiss mice were killed by cervical dislocation, and the spleens were removed and cut into small pieces which were suspended in HBSS and forced through a wire screen with a 43-micron mesh to yield a cell suspension. The cells were washed twice with HBSS and then suspended in hypotonic (0·06 M) NaCl for 10 min to lyse erythrocytes without disrupting spleen cells. The surviving spleen cells were sedimented by centrifugation and washed 3 times by centrifugation in HBSS at 1000 rev/min for 10 min before further use. The viability of 3 preparations of spleen cells ranged from 78·4 to 85·3% with a mean of 83%. Spleens were obtained from 100 mice for one preparation of spleen cells, and from 200 mice for each of the others.

Incubation of cells. One volume of Ehrlich-Lettré or mouse spleen cells was suspended in 5 volumes of isotonic medium (HBSS, 0·85% NaCl, 5·4% of inositol [8], 2·1% DMSO [8], or a 3:1 mixture of 5·4% inositol: 0·85% NaCl) in a 50 ml Erlenmeyer flask, and incubated for 1 hr at 37°C with air as the gas phase, in a modified Dubnoff metabolic water bath with an oscillation of 58 strokes per min. At the end of the incubation period the cell suspension was centrifuged at 2000 rev/min for 20 min. The supernatant fluid was carefully syphoned and recentrifuged at 5000 rev/min for 30 min in head No. 870 in a model B20 International refrigerated centrifuge. The supernatant fluid was carefully withdrawn and analyzed immediately as described below.

Analysis of cell supernatants

A. *Ultraviolet (UV)—absorption spectra.* A UV-absorption spectrum (190–350 mμ) was obtained for each cell supernatant with a Unicam, model SP800, recording spectrophotometer. The appropriate cell incubation medium was used as the reference solution. The spectra indicated the nature and also the quantity (A_{260} mμ units) of material in the "extracts".

B. *Sucrose density sedimentation analysis.* An initial sucrose density sedimentation profile was obtained for each "cell extract". For this purpose a 0·5 or 1·0 ml aliquot of the supernatant was layered on top of a 30 ml linear 5%–25% sucrose gradient in a 1×3 inch cellulose nitrate centrifuge tube. The gradients were spun in head No. SW 25·1 in a model L Spinco centrifuge for 16 hr at approximately 6°C, and were then fractionated by the collection of 12 drops (22 gauge needle) per fraction from the bottom of the tube [14] with the aid of an ISCO, model 270, fraction collector. The absorbance of each fraction was

*Kirk, H. D. Personal communication.

†Connaught Laboratories strain Swiss mice were bred under barrier-sustained conditions at the University of Saskatchewan colony. Food and water were supplied *ad libitum*.

determined with a Beckman, model DB, spectrophotometer and 1 ml cuvettes.

Linear sucrose gradients were made with a Lucite apparatus which was constructed as described by McConkey [14] and stored at 4°C for about 5 hr before use. Sucrose solutions were freshly prepared and were composed of 5% or 25% (weight/volume) sucrose (RNase free; density gradient grade; Mann Research Laboratories, New York) dissolved in 0·01 M sodium acetate buffer, pH 5·0, containing 0·1 M NaCl and 0·001 M EDTA [15].

C. *Isolation and analysis of total RNA.* A method described by Steele and Busch [15] for the isolation of whole nuclear and nucleolar RNA of rat liver and Walker tumor, and modified for use with our material and equipment, was used to isolate the total RNA from the 70% ethanol-insoluble portion of the cell supernatant.

One volume of cell supernatant was combined with 2·5 volumes of absolute ethanol and the mixture was stored at −20°C for at least 24 hr. The precipitate was sedimented by centrifugation (2500 rev/min for 15 min) and suspended in 1 ml of "SDS medium" (0·3% sodium dodecylsulfate (SDS), 0·1 M NaCl, 0·05 M sodium acetate buffer, pH 5·0) by homogenization with a Teflon pestle at 4°C. An equal volume of aqueous phenol-*m*-cresol mixture (4:1) [16] containing 0·1% 8-hydroxyquinoline [17] was added, and the fluid was mixed thoroughly. The rest of the RNA extraction procedure, involving heating at 65°C for a total of 12 min, was carried out according to the procedure of Steele and Busch [15]. The aqueous phase was re-extracted once, and the phenol phases were back extracted in sequence. The pellet of RNA which was isolated was subjected to 5 precipitations from double-distilled water by the addition of 2·5 volumes of absolute ethanol containing 2% potassium acetate and storage for 16 hr at −20°C. All traces of phenol and cresol were removed as judged by the absorption of the supernatant fluids at 272 mμ. RNA was dissolved in double-distilled water for future analysis and stored at −20°C.

RNA was measured spectrophotometrically using yeast "soluble" RNA (Type III, Sigma Chemical Co., St. Louis) as a standard and $E_{260\ m\mu}$ 1 mg/ml = 20 [18], or by the orcinol reaction [19] according to the procedure of Hurlbert *et al.* [20]. DNA contamination was tested for by the diphenylamine reaction [21], and when it was present the RNA preparation was treated with RNase-free DNase (Worthington Biochemical Corp., Freehold, N.J.) by a modification of the procedure described by Steele and Busch [22]. The RNA was incubated with 105 units of DNase in 0·001 M $MgCl_2$ and 0·25 M Tris-HCl buffer, pH 7·5, at 20°C for at least 6 hr. The reaction was terminated by the addition of 2·5 volumes of ethanol containing 2% potassium acetate and storage in an ice bath for 1 hr. The precipitate was sedimented by centrifugation and dissolved in 0·5 ml of double-distilled water, 2 volumes of 4 M NaCl, and absolute ethanol to a concentration of 20%, were added in sequence and the mixture was placed in an ice bath for 30 min [23]. The resulting precipitate was sedimented by centrifugation, washed by resuspension in 0·5 ml of 2·7 M NaCl—20% ethanol and centrifugation, and finally dissolved in double-distilled water. Protein was not detected in any of the RNA preparations by means of the method of Lowry *et al.* [24].

The "phenol" RNA was analyzed by sucrose density sedimentation as described for the cell supernatant. Reference sucrose density sedimentation profiles were also obtained for yeast "soluble" RNA (sRNA) (Type III, Sigma Chemical Co.) alone and combined with bovine liver ribosomal RNA (18S + 28S rRNA) (Mann Research Laboratories).

D. *Isolation and analysis of nucleic acids and protein.* The acid-insoluble material in the cell supernatant was precipitated on treatment with 0·6 M perchloric acid [25] at 0°C and recovered by centrifugation in the refrigerated centrifuge. The supernatant ("acid-soluble") fraction was neutralized with KOH at 0°C and the $KClO_4$ was sedimented by centrifugation in the cold. The resultant supernatant fraction was analyzed as described in the next subsection.

Nucleic acids were extracted from the perchloric acid-insoluble material, purified, and separated according to a modification of the procedure of Herbert *et al.* [26]. Nucleic acids were extracted with 2·5 volumes of 10% NaCl (pH 7·0) at 100°C for 30 min (repeated once for 15 min with cooling in ice after each heating period), precipitated from the NaCl extract upon the addition of 2·5 volumes of 95% ethanol and cooling at −20°C for several days, and recovered by centrifugation. The mixed nucleic acids were heated with a 2:1 mixture of 95% ethanol–ether at 50°C for 30 min to remove any residual lipids, recovered by centrifugation after cooling in ice, and washed twice with ice-cold 95% ethanol. RNA was converted into ribonucleoside 2′(3′)-monophosphates by hydrolysis in 0·1 M NaOH

at 37°C for 18 hr [25, 27]. DNA was precipitated from the hydrolysate on acidification and subsequent refrigeration. Incubation of the nucleic acid residue with 0·1 M NaOH was repeated twice, and the DNA was finally dissolved in 0·01 M NaOH for chemical and sucrose density sedimentation analyses. The latter analysis was carried out in linear 5% to 25% sucrose gradients as described above for the cell extracts and "phenol" RNA. DNA was determined by the diphenylamine reaction [21] with highly polymerized calf thymus DNA (Worthington) as a standard, or spectrophotometrically [28], and RNA was determined by the orcinol method [19, 20] as described for "phenol" RNA. The residue from the extraction of the acid-insoluble material with hot 10% NaCl was dissolved in 1 M NaOH for determination of protein by the method of Lowry *et al.* [24]. Bovine serum albumin, fraction V (The Armour Laboratories, Kankakee, Ill.), was used as a standard.

In order to determine the major-base composition of DNA and RNA, aliquots of the DNA, the RNA hydrolysate, and the "phenol" RNA were hydrolyzed in 70% perchloric acid (DNA) [29] or 1 M HCl (RNA) [30] at 100°C for 1 hr. The DNA hydrolysate was neutralized with KOH in the cold, the $KClO_4$ precipitate was sedimented by centrifugation, and the supernatant fluid was chromatographed as described below. The RNA HCl hydrolysates were dried *in vacuo* and the residues were dissolved in small volumes of water for chromatography. Separation of DNA bases, and of RNA purine bases and pyrimidine nucleotides, was attained by chromatography (descending) on Whatman No. 1 paper strips with the HCl-isopropanol solvent of Wyatt [31]. A blank paper strip was run for each sample as well. Bases and nucleotides were eluted from the chromatograms and determined spectrophotometrically as described by Blair and Hall [32].

E. *Analysis of the acid-soluble fraction.* The acid-soluble fraction of the cell supernatant was analyzed spectrophotometrically and by chromatography. UV-absorption spectra (190–350 mμ) were obtained with the Unicam spectrophotometer and the amount of material was estimated in $A_{260\ m\mu}$ units. The rest of the fraction was applied (pH 6·8–7·0) to a 5 × 1 cm Dowex-1 (formate) resin* column which was operated manually as described by Blair and Rainnie [33] and Blair [34]. Elution was carried out in 10 ml fractions with increasing concentrations of formic acid and of formic acid and ammonium formate or sodium formate. The absorbance of each eluate was determined against the appropriate eluent blank at 260 and 280 mμ with the Beckman spectrophotometer, and UV-absorption spectra of most fractions were later obtained with the Unicam recording spectrophotometer. Substances were tentatively identified by their chromatographic mobilities and the spectral data.

The fractions constituting a major peak on the resin chromatogram were pooled and lyophilized and the residues were dissolved in small volumes of distilled water for analysis by paper chromatography which was carried out with Whatman No. 1 paper strips in the 5% Na_2HPO_4-isoamyl alcohol solvent of Carter [35] (ascending) and in isobutyric acid-water-conc. NH_4OH (661 : 329 : 10, v/v) [36] (descending). Appropriate standards were chromatographed at the same time. UV-absorbing substances were eluted from the chromatograms with dilute HCl or with water, and UV-absorption spectra were obtained for the eluates.

RESULTS

Quantitative analysis of the cell supernatants. In Table 1 are shown the amounts of acid-soluble substances, nucleic acids, and protein present in the supernatants from Ehrlich-Lettré and mouse spleen cells. It is readily apparent that, on the basis of 10^9 cells, 7–8 times as much acid-soluble material was released in HBSS or isotonic inositol from Ehrlich-Lettré cells than from the spleen cells. The smallest amounts were released in the control solution, HBSS, from both types of cells. In the case of the spleen cells, NaCl caused the release of about twice as much acid-soluble material as HBSS, and the 3:1 mixture of isotonic inositol–isotonic NaCl caused the release of almost as much as isotonic inositol alone. Isotonic DMSO was less efficient than isotonic inositol in causing the release of acid-soluble material from Ehrlich-Lettré cells.

DNA was found in all the cell supernatants, although it was barely detectable in the HBSS supernatant of the Ehrlich-Lettré cells (Table 1). In the case of the Ehrlich-Lettré cell supernatants, the ratio of total RNA to DNA was approximately 5:1. The amounts of total nucleic acids released from the tumor cells by inositol, DMSO, or HBSS were similar to those previously released from Ehrlich cells under the

*Dowex-1 (formate) resin was prepared from a purified form of Dowex-1 (chloride) resin (AG × 10(chloride), 200–400 mesh) which was purchased from Calbiochem, Los Angeles, California.

Table 1. *Composition of cell supernatants*

Cells	Incubation medium*	$A_{260m\mu}$ Units per 10^9 cells Total	Acid-sol.	RNA (μg/10^9 cells)	DNA (μg/10^9 cells)	Protein (mg/10^9 cells)
Ehrlich-Lettré ascites tumor	HBSS (2)†	59·5	43·8	194·0	48·6	3·12
	5·4% inositol (2)	127·4	97·4	545·0	118·0	7·67
	2·1% DMSO (1)	155·0	74·3	1413·0	304·0	15·0
Swiss mouse spleen	HBSS (1)	7·1	5·2	50·2	451·0	0·60
	5·4% inositol (3)	42·9	14·4	156·0	2188·0	2·45
	0·85% NaCl (1)	13·9	9·6	22·8	267·0	0·68
	5·4% inositol: 0·85% NaCl (3:1) (1)	30·8	13·5	49·3	1310·0	1·31

*Cell supernatants were prepared by incubating intact cells in the isotonic medium at 37°C for 1 hr as described in the text.

†Numbers in parentheses indicate the number of extracts examined. Determinations were performed on duplicate samples, and the values presented are means for all the samples.

same conditions [8]. The amounts of protein released from the Ehrlich-Lettré cells also were similar to those reported previously for Ehrlich cells [8], but in the present study there was a greater difference between the amounts released by inositol and HBSS.

In contrast to the results for Ehrlich-Lettré cells, approximately 9–27 times as much DNA as RNA was released from the spleen cells (Table 1), and the amounts of RNA released by HBSS, isotonic NaCl, or isotonic inositol: isotonic NaCl (3:1) were very small. Smaller amounts of protein were released from 10^9 spleen cells than from the corresponding number of Ehrlich-Lettré cells by inositol or HBSS.

NaCl inhibited the release of both nucleic acids and protein from spleen cells by inositol (Table 1). The presence of 25% NaCl in the isotonic mixture of inositol and NaCl inhibited* the release of total nucleic acid and of protein from spleen cells by 30·6 and 49·7% respectively (Table 1), whereas the release of nucleic acids from Ehrlich cells was inhibited by about 80% and there was no effect on the release of protein [8]. Isotonic NaCl released 28% as much protein as isotonic inositol from spleen cells (Table 1), whereas the two media released approximately equal amounts from Ehrlich cells [8].

*The percentage inhibition was calculated according to the formula: $((X-Y)/X) \times 100$, where X = percentage of isotonic inositol solution x (amount of nucleic acids or protein released in 100% isotonic inositol solution—amount of nucleic acids or protein released in 100% isotonic NaCl solution), and Y = amount of nucleic acids or protein released in the inositol plus saline solution—amount of nucleic acids or protein released in 100% isotonic NaCl solution.

Analysis of the acid-soluble fractions of the cell supernatants. The UV-absorption spectra of the perchloric acid-soluble fractions of the Ehrlich-Lettré cell supernatants (max = 253–255 mμ) suggested the presence of hypoxanthine, whereas the spectra for the acid-soluble fractions of the spleen cell supernatants exhibited absorption maxima of 255–260 mμ.

Figures 1 and 2 show representative Dowex-1 (formate) resin chromatograms of the acid-soluble fractions from the supernatants of Ehrlich-Lettré and mouse spleen cells, respectively. Most of the acid-soluble material detected by UV absorption was eluted from the resin with water or 0·05 M formic acid and, therefore, would be composed mainly of purines, pyrimidines, and/or nucleosides. The only common nucleotides which would be eluted are NAD^+, NADH, CMP and dCMP [20, 33, 34].

In the case of the Ehrlich-Lettré cell acid-soluble fractions (Fig. 1), small amounts of nucleotides were present in the inositol and DMSO supernatants, whereas nucleotides were almost totally absent from the HBSS supernatant, but in all three supernatants the major components were peaks *a* and *b*, which contained mainly hypoxanthine and xanthine respectively, as determined by UV-absorption spectra and by paper chromatography with authentic compounds which might be components of the peaks (Table 2). Peaks *d*, *e*, *f*, and *g* are most likely to be AMP, GMP, UMP and UDP-coenzymes respectively, according to their chromatographic mobilities and UV-absorption spectra. Peak *c* has an adenosine-like UV-absorption spectrum, but has not been identified.

*Table 2. Identification by paper chromatography of peaks a and b from the Dowex resin chromatogram of the acid-soluble fraction of the inositol supernatant of Ehrlich-Lettré cells**

Substance	Solvent 1†	Solvent 2‡
Peak *a*	0·55, 0·54§	0·61, 0·63§
Peak *b*	0·49, 0·47§	0·49, 0·50§
Adenine	0·34	0·94
Cytosine	0·66	0·85
Guanine·HCl	0·38	0·66
Hypoxanthine	0·56	0·63
Uracil	0·67	0·71
Thymine	0·64	0·83
Xanthine	0·48	0·50
Adenosine	0·49	0·89
Cytidine	0·72	0·76
Guanosine	0·65	0·50
Inosine	0·70	0·52
Uridine	0·70	0·59
Thymidine	0·73	0·83
Xanthosine	0·66	0·38
CMP	0·81	—
NAD	0·71	0·45

*Chromatography was carried out on Whatman No. 1 paper strips in 12 × 18 inch cylindrical glass tanks at room temperature.

†Ascending. Five per cent Na_2HPO_4-isoamyl alcohol [35].

‡Descending. Isobutyric acid–water-conc. NH_4OH (661 : 329 : 10, v/v) (36).

§Samples from 2 resin chromatograms.

The acid-soluble fractions of the inositol supernatants of spleen cells also contained small amounts of nucleotides (Fig. 2). Peaks *c*, *d*, *e* and *f* of Fig. 2 are in the positions of AMP, UMP, ADP, and UDP-coenzymes respectively. However, the major components of all the acid-soluble fractions from spleen cells were peaks *a* and *b*, the latter containing mainly xanthine.

Sucrose density sedimentation analysis of the cell supernatants. The reference sucrose density sedimentation profile for a mixture of 28S and 18S bovine liver rRNA and of yeast 4S sRNA is shown in Fig. 3. Under the conditions employed, 28S RNA migrated almost to the bottom of the gradient, while 4S RNA remained near the top. Thus, RNA species heavier than 28S would be expected to be found at the bottom of the gradient. The sedimentation profiles for the freshly-prepared Ehrlich-Lettré (Fig. 4) and spleen (Fig. 5) cell supernatants indicated the absence of major quantities of ribosomes or of free ribosomal, or heavier, RNA, with almost all of the UV-absorbing material, therefore,

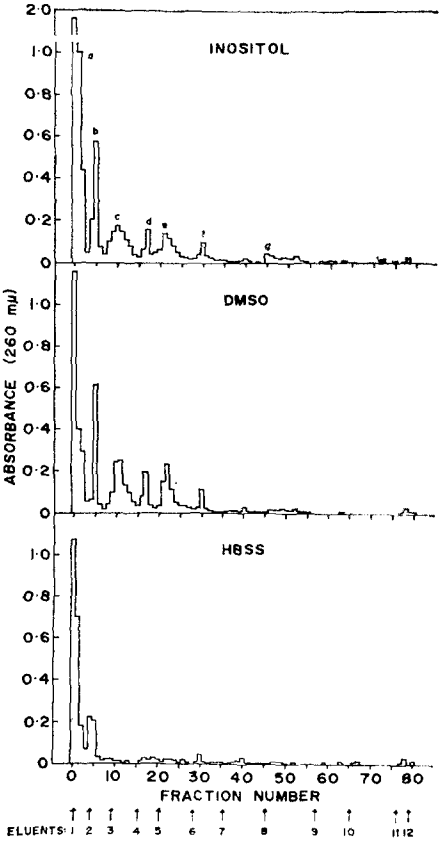


Fig. 1. Dowex-1 (formate) resin chromatograms of the acid-soluble fractions from the 5·4% inositol, 2·1% DMSO, or the HBSS supernatants of Ehrlich-Lettré ascites tumor cells. Eluents, indicated by the numbers (1–12) at the bottom of the chart, were added to the columns in 10 ml fractions at the positions indicated by the arrows. The sample effluent is fraction No. 0. The composition of the eluents was as follows: 1, distilled water; 2, 0·05 M formic acid (FA); 3, 0·1 M FA; 4, 0·4 M FA; 5, 1·0 M FA; 6, 2·0 M FA; 7, 3·0 M FA; 8, 4·0 M FA—0·15 M ammonium formate (AF); 9, 4·0 M FA—0·3 M AF; 10, 4·0 M FA—0·6 M AF; 11, 0·4 M FA—1·0 M AF; 12, 4·0 M FA—1·0 M sodium formate. The resin columns were prepared and operated, and the fractions were analyzed, as described in the text. Peaks a–g are described in the text.

consisting of small molecular weight nucleic acid derivatives, sRNA and DNA, remaining near the tops of the gradients. In the case of the Ehrlich-Lettré DMSO supernatant, negligible amounts of 28S, or heavier, RNA may have been present (fractions 1–4, Fig. 4). Similarly, very small amounts of such "heavy" RNA may have been present in the inositol supernatant of spleen cells (fractions 1–3, Fig. 5). In other respects the profiles for the inositol, HBSS and NaCl supernatants of spleen cells were quite similar, but the profile for the isotonic inositol : isotonic NaCl (3:1) supernatant suggested the presence of heavier material in the sRNA region of the gradient than in the case of the other spleen cell supernatants (fractions 20–33, Fig. 5).

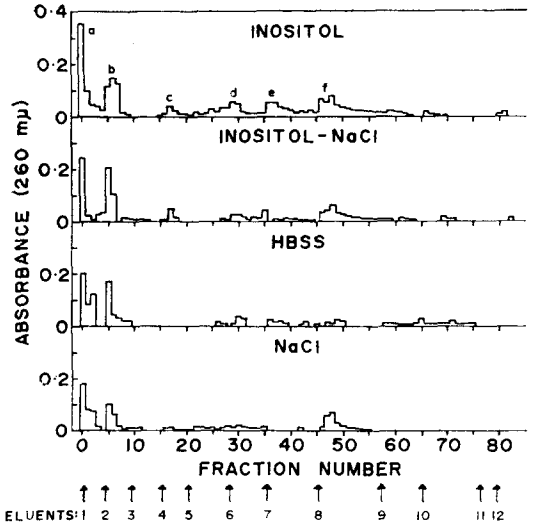


Fig. 2. Dowex-1 (formate) resin chromatograms of the acid-soluble fractions from the 5·4% inositol, 0·85% NaCl, HBSS, or the 5·4% inositol: 0·85% NaCl (3:1) supernatants of mouse spleen cells. Eluents, indicated by the numbers (1–12) at the bottom of the chart, were added to the columns in 10 ml fractions at the positions indicated by the arrows. The composition of the eluents, the preparation and operation of the resin columns and the analysis of the fractions were as for Fig. 1. Peaks a–f are described in the text.

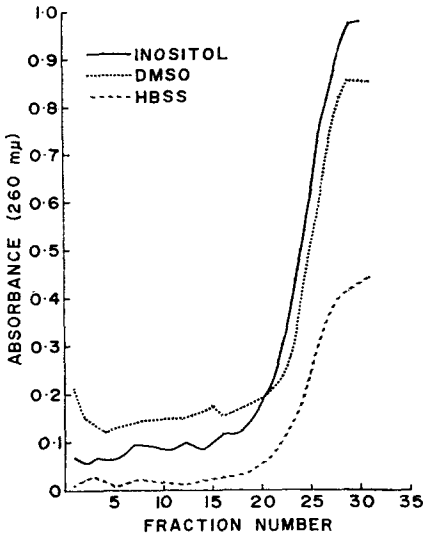


Fig. 4. Composite sucrose density sedimentation profiles of supernatants of Ehrlich-Lettré ascites tumor cells. One ml of the 5·4% inositol, 2·1% DMSO, or the HBSS supernatant was used. Linear 5%–25% sucrose gradients were prepared, centrifuged, fractionated, and analyzed as described for Fig. 3. Fraction No. 1 is the bottom of the gradient.

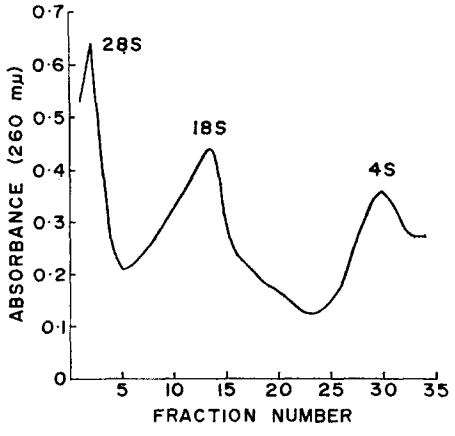


Fig. 3. Sucrose density sedimentation analysis of bovine liver ribosomal RNA (28S+18S; 333 μg) plus yeast "soluble" (4S) RNA (100 μg). The linear 30 ml 5%–25% sucrose gradient, prepared as described in the text, was spun for 16 hr at 25,000 rev/min in head No. 25·1 in a Spinco, Model L, centrifuge at 6°C. The gradient was fractionated by collecting 12 drops per fraction from the bottom (fraction No. 1) of the tube.

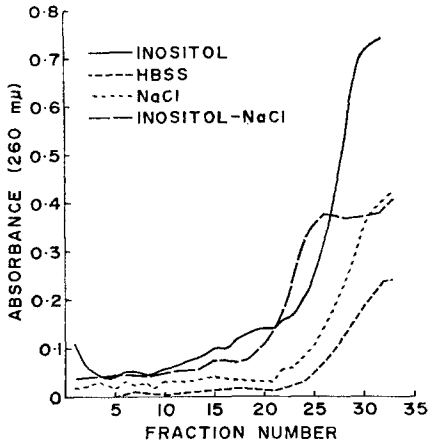


Fig. 5. Composite sucrose density sedimentation profiles of supernatants of mouse spleen cells. One-half ml of the 5·4% inositol, 0·85% NaCl, HBSS, or the 5·4% inositol: 0·85% NaCl (3:1) supernatant was used. Linear 5%–25% sucrose gradients were prepared, centrifuged, fractionated, and analyzed as described for Fig. 3. Fraction No. 1 is the bottom of the gradient.

UV-absorption and sucrose density sedimentation analyses of RNA isolated from cell supernatants. UV-absorption spectra for the total RNA isolated from Ehrlich-Lettré and mouse spleen cell supernatants by the "phenol" method are shown, together with the spectrum for yeast sRNA, in Fig. 6. All the RNA preparations exhibited absorption maxima at 258–259 mμ at pH 5·0, but the RNA isolated from the inositol supernatant of spleen cells exhibited an absorption minimum of 234 mμ compared to 231 mμ for all the other preparations.

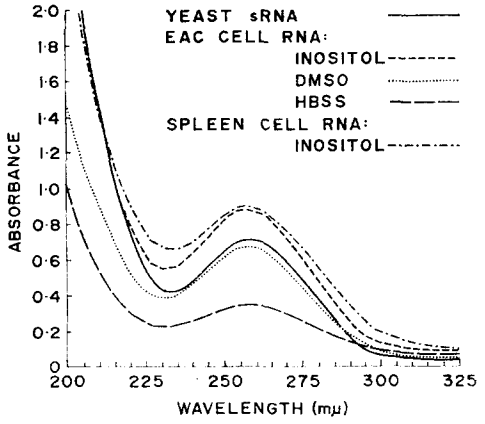


Fig. 6. Absorption spectra at pH 5·0 of yeast sRNA and of RNA isolated by the "phenol" method from supernatants of Ehrlich-Lettré ascites carcinoma (EAC) and mouse spleen cells. Cells were incubated with 5·4% inositol, 2·1% DMSO, or HBSS, and RNA was isolated and purified, as described in the text. RNA was dissolved in 0·01 M sodium acetate buffer, pH 5·0, containing 0·1 M NaCl and 0·001 M EDTA.

The conclusion, based on the sucrose density sedimentation data for crude cell supernatants, that Ehrlich-Lettré cell supernatants contained mainly sRNA was substantiated by the sucrose density sedimentation profiles for the isolated "phenol" RNA (Fig. 7). RNA's from all three supernatants of Ehrlich-Lettré cells migrated in the 5%–25% linear sucrose gradient similarly to the reference yeast sRNA, with UV-absorbance peaks at fraction No. 28 or 29. The profile for RNA from the inositol supernatant was almost identical to that for yeast sRNA, but the profiles for RNA from the HBSS supernatant, and especially from the DMSO supernatant, suggested the presence of some heavier RNA species, but no 28S, or heavier RNA was indicated. The profile for the DMSO RNA changed little when the same amount of RNA was centrifuged in the same sucrose gradient for 24 hr instead of 16 hr, and the same profile was obtained for RNA re-isolated from the first sucrose gradient by precipitation with 70% ethanol at −20°C. It is suggested

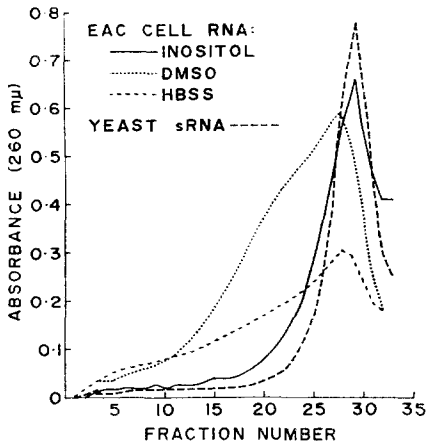


Fig. 7. Composite sucrose density sedimentation profiles of yeast sRNA and of RNA isolated by the "phenol" method from supernatants of Ehrlich-Lettré ascites carcinoma (EAC) cells. Cells were incubated with 5·4% inositol, 2·1% DMSO, or HBSS, and RNA was isolated and purified, as described in the text. RNA was dissolved in 0·01 M sodium acetate buffer, pH 5·0, containing 0·1 M NaCl and 0·001 M EDTA. Linear 5%–25% sucrose gradients were prepared, centrifuged, fractionated, and analyzed as described for Fig. 3. Fraction No. 1 is the bottom of the gradient.

that some form of aggregation of RNA may have occurred in the DMSO and HBSS RNA samples, although the inclusion of 0·001 M EDTA and 0·1 M NaCl in the sucrose gradient should have minimized this [15].

Enough RNA was not isolated from the limited amounts of spleen cell supernatants which were available for adequate sucrose density sedimentation profiles to be obtained, but the data which were procured indicated only sRNA.

UV-absorption and sucrose density sedimentation analyses of DNA isolated from cell supernatants. UV-absorption spectra for the DNA's isolated from Ehrlich-Lettré and spleen cell supernatants compared favourably with the spectrum for "native" bovine thymus DNA at pH 12·0 (Fig. 8). The spectra for the isolated DNA's were characterized by absorption maxima at 259–260 mμ and minima at 238–231 mμ, compared to an absorption maximum of 260 mμ and a minimum of 238 mμ for the reference DNA.

The sucrose density sedimentation profiles obtained for "native" bovine thymus DNA and for the same DNA re-isolated by the "hot 10% NaCl" procedure ("denatured" DNA) are shown in Fig. 9. Absorbance of the "native" DNA was maximal at fraction No. 28, whereas an absorption plateau, rather than a sharp peak, was obtained for the "denatured" DNA.

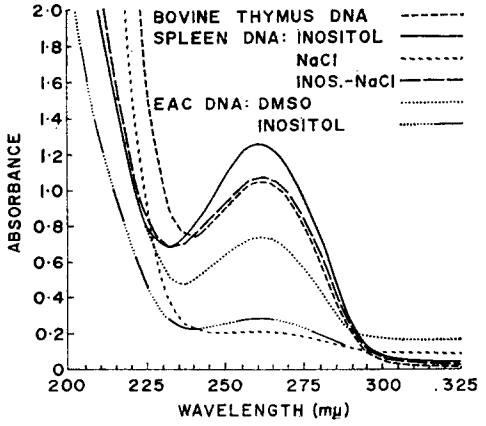


Fig. 8. Absorption spectra at pH 12·0 of bovine thymus DNA (Worthington) and of DNA isolated from supernatants of Ehrlich-Lettré ascites carcinoma (EAC) and mouse spleen cells. Cells were incubated with 5·4% inositol, 2·1% DMSO, 0·85% NaCl, or 5·4% inositol : 0·85% NaCl (3:1), and DNA was isolated and purified, as described in the text. DNA was dissolved in 0·01 M NaOH.

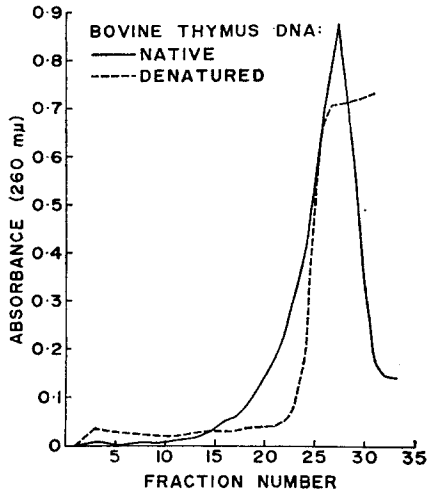


Fig. 9. Composite sucrose density sedimentation profiles of "native" and "denatured" calf thymus DNA. Highly polymerized ("native") "Worthington" calf thymus DNA was dissolved (1 mg/ml) in distilled water and "denatured" by re-extraction, re-isolation, and re-purification according to the "hot 10% NaCl" method which was used to isolate nucleic acids from Ehrlich-Lettré ascites tumor and mouse spleen cells, and to separate DNA from RNA, as described in the text. The procedure involves heating of the DNA at 100°C for 30- and 15-min periods, followed by rapid cooling in ice each time. The DNA samples were dissolved in distilled water and 0·35 ml of each was applied to a linear 5% to 25% sucrose gradient which was prepared, centrifuged, fractionated, and analyzed as described for Fig. 3. Fraction No. 1 is the bottom of the gradient.

DNA released from mouse spleen cells by inositol and by isotonic inositol : isotonic NaCl (3:1) (Fig. 10) sedimented in the sucrose gradient similarly to "native" bovine thymus DNA (cf. Fig. 9), with the exception that the maximum UV-absorbance occurred at fraction No. 30 in the former case rather than at fraction No. 28. Not enough DNA was isolated from the NaCl and HBSS supernatants of the spleen cells to provide satisfactory sucrose density sedimentation profiles (Fig. 10).

The amounts of DNA isolated from the supernatants of Ehrlich-Lettré cells were also limited, and only in the case of the DMSO supernatant DNA was an acceptable sucrose density sedimentation profile obtained (Fig. 11), with the peak of UV absorption occurring at fraction No. 30.

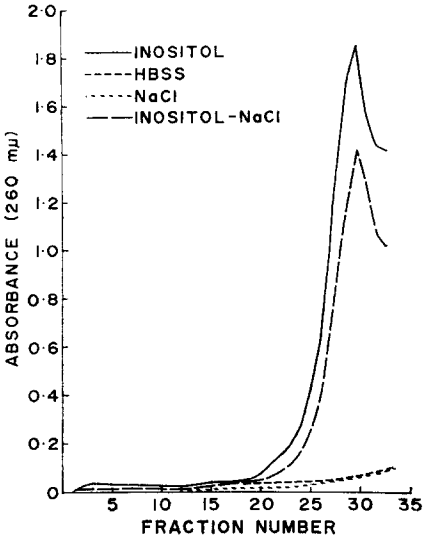


Fig. 10. Composite sucrose density sedimentation profiles of DNA isolated from supernatants of mouse spleen cells. Spleen cells were incubated with 5·4% inositol, 0·85% NaCl, HBSS, or 5·4% inositol : 0·85% NaCl (3:1), and DNA was isolated and purified, as described in the text. DNA was dissolved in 0·01 M NaOH, and 0·3 ml was applied to a linear 5%–25% sucrose gradient which was prepared, centrifuged, fractionated, and analyzed as described for Fig. 3. Fraction No. 1 is the bottom of the gradient.

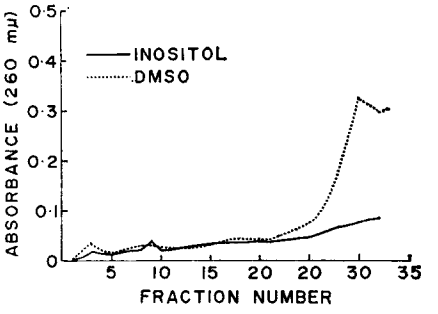


Fig. 11. Composite sucrose density sedimentation profiles of DNA isolated from supernatants of Ehrlich-Lettré ascites tumor cells. The cells were incubated with 5·4% inositol or 2·1% DMSO, and DNA was isolated and purified, as described in the text. DNA was dissolved in 0·01 M NaOH and 0·3 ml was applied to a linear 5%–25% sucrose gradient which was prepared, centrifuged, fractionated, and analyzed as described for Fig. 3. Fraction No. 1 is the bottom of the gradient.

Base compositions of nucleic acids isolated from supernatants of Ehrlich-Lettré and mouse spleen cells. The major-base compositions of the RNA and DNA isolated from the cell supernatants were determined for all samples which were large enough to permit accurate measurements.

Table 3 presents data for RNA isolated from Ehrlich-Lettré cell supernatants by both the "hot 10% NaCl" and the "phenol" methods. All the samples contained an excess of G+C over A+U, the G+C content ranging from 60·1 to 66·0 moles per cent. The RNA isolated from the three different supernatants of the tumor cells by the same isolation procedure contained similar amounts of any one of the four major bases, indicating that the cell incubation media caused the release of similar RNA species. However, the RNA isolated from the supernatants by the "phenol" method contained more C, and less G, than the RNA isolated from the same supernatant by the "hot 10% NaCl" method, but the G+C contents of the RNA's were similar regardless of the isolation method.

Hoagland [37] has presented base composition data for sRNA from Ehrlich ascites tumor cells. The composition of A, 19·5; G, 35·5; C, 26·2, and U, 18·8 moles per cent agrees well with that found for the RNA released from the Ehrlich-Lettré cells by isotonic inositol and isolated by the "hot 10% NaCl" method, but does not agree so well with that for RNA isolated by the "phenol" method (Table 3). The base composition for the "phenol" RNA agrees somewhat better with the molar base ratios which Kit [38] found for the total RNA from Ehrlich tetraploid carcinoma cells. The ratios were 2·17, 1·98, and 1·12 for G, C, and U respectively, relative to A = 1·00.

Enough DNA was obtained only from the inositol supernatant of the spleen cells for accurate DNA base analyses to be carried out by the methods employed. The other samples were sufficient only to be used qualitatively for DNA identification. Four major bases were separated from all DNA's and there was no evidence of uracil. The DNA isolated from the inositol supernatants of spleen cells contained the following amounts (moles per cent) of bases: A, 29·0; T, 26·9; G, 20·8; and C, 23·3. Purines/pyrimidines = 0·99, G/C = 0·89 and A/T = 1·08. These data are means of values obtained for 3 DNA preparations from 3 supernatants prepared at different times. The base composition data agree well with published values. Kleinschmidt [39], using methods of DNA base analysis similar to those employed in the present study, found 42·3 moles per cent G+C in Swiss mouse spleen DNA and Kit [40] quotes a value of 42% G+C for DNA of mouse tissues determined by density gradient sedimentation analysis. However, Kit [41] also found 44·1 and 44·6 moles per cent G+C in spleen DNA from C3H and AKr mice, respectively. Ehrlich tumor DNA contained 45·3 moles per cent G+C [41]. The density of the major band of Ehrlich-Lettré DNA is reported to be identical to that from spleen and other tissues of the C3H mouse [42].

DISCUSSION

It is evident from the results of the present investigation and of previous work [8] that isotonic solutions of *myo*-inositol or DMSO, as well as HBSS, cause the release of various

Table 3. Base composition of RNA released from Ehrlich-Lettré ascites tumor cells

Method of isolation of RNA from cell supernatant	Cell incubation medium	Base composition (moles per cent)* A	U	G	C	$\frac{A+U}{G+C}$
Hot 10% NaCl	Inositol (4)†	20·5 ± 2·42‡	15·9 ± 2·13‡	38·9 ± 0·58‡	24·7 ± 1·00‡	0·57 ± 0·031‡
	DMSO (2)	23·0	14·1	38·2	24·7	0·59
	HBSS (2)	22·0	13·6	37·8	26·6	0·55
Phenol	Inositol (2)	19·1	15·2	35·3	30·7	0·52
	DMSO (2)	22·2	17·7	31·8	28·3	0·66
	HBSS (2)	19·6	17·0	32·0	31·6	0·58

*Values are means for the number of samples indicated.

†Numbers in parentheses indicate the number of samples.

‡Standard deviation of the mean.

amounts of RNA, DNA, protein and acid-soluble nucleic acid derivatives from intact Ehrlich and Ehrlich-Lettré ascites carcinoma cells at 37°C. The results of the present study confirm the previous work of Eng and Morgan [8] regarding the amounts of total nucleic acids and protein released from Ehrlich ascites tumor cells in the three incubation media, but the difference between the amounts of protein extracted by HBSS and isotonic inositol was greater in the present investigation than in the former. The present work also shows that both DNA and RNA are released, and that the RNA is primarily in a "soluble" RNA form.

The present studies have also shown that DNA, RNA, protein and acid-soluble mucleic acid derivatives are also released from intact mouse spleen cells in isotonic solutions of inositol or NaCl, or by HBSS or a solution composed of 3 parts isotonic inositol and 1 part isotonic NaCl.

Previous work by Bather *et al.* [10] showed that 5% inositol caused the release of a larger amount of UV-absorbing material from chicken or Swiss mouse spleen cells or from Ehrlich ascites tumor cells than did HBSS. However, the UV-absorbing material was not definitely characterized, although RNA and alcohol-soluble nucleic acid derivatives were indicated.

The present studies on the 0·6 M perchloric acid-soluble portion of the cell supernatants have shown that, although small amounts of nucleotides are present, the major components are xanthine and hypoxanthine. The origin of these purines is not clear; they could arise via the catabolism of free purines, purine nucleosides, and/or purine nucleotides, or from the catabolism of nucleic acids. Likewise, it is not known whether they arise intracellularly or extracellularly during incubation of the cells in isotonic media. Since special precautions were taken to avoid degradation of constituents of the cell supernatants during handling of the preparations, the purines can not be considered artefacts of the experimental procedure. Bather *et al.* [10] found that the alcohol-soluble material obtained from 5% inositol supernatants of of Ehrlich ascites tumor and chicken and mouse spleen cells contained adenine and guanine, and traces of CMP and UMP, after hydrolysis in 1 M HCl and chromatography of the hydrolysates in isopropanol–HCl [31]. The isopropanol–HCl solvent [31] does not separate adenine from hypoxanthine, nor guanine from xanthine [42]. Thus, the two major spots reported by Bather *et al.* [10] could have been hypozanthine and xanthine rather than adenine and guanine. In the present studies, hypoxanthine and xanthine were separated and tentatively identified by anion-exchange resin chromatography, and the identification was confirmed by chromatograhy on paper in solvents which adequately separate the four purines.

Bather *et al.* [10] concluded, on the basis of results of the diphenylamine reaction on the 10% NaCl extracts of the alcohol-insoluble material obtained from inositol supernatants of Ehrlich ascites tumor and chicken and mouse spleen cells, that only traces of deoxyribose were present. However, NaCl interferes substantially with the diphenylamine determination carried out by the Dische [21] method. We have found that when DNA is dissolved in 10% NaCl and the diphenylamine reaction [21] is performed on the solution, the absorbance at 595 mμ is approxiamtely 30% of that obtained with the same amount of DNA alone. In the present investigation, substantial amounts of DNA were found in the cell supernatants with methods which included the isolation of DNA from interfering substances, thus confirming the previous observation [8] that DNA was released from Ehrlich ascites cells in the presence of isotonic inositol. The identification of the material designated "DNA" has been based on several characteristics of DNA, leaving little doubt that it is DNA. The material was isolated by a complicated procedure which is widely used to isolate DNA. It had the physical properties—white color, fibrous nature, high viscosity—associated with DNA, it gave a highly positive reaction for deoxyribose, it exhibited a UV-absorption spectrum similar to that of authentic DNA, it sedimented similarly to authentic DNA in a sucrose gradient, and the base composition was that expected for mouse DNA.

The cellular origin of the DNA is not known, but the base composition data for the mouse spleen cell DNA, which are similar to those for total DNA from mouse spleen [39–41], suggest that the spleen cell DNA is nuclear rather than mitochondrial, but do not preclude the presence of the latter DNA also. The quantity of DNA released from 10^9 spleen cells greatly exceeds the total amount of cellular mitochondrial DNA which probably constitutes 0·1 to 1·5% [43–46] of the total cellular DNA (8·3 mg/10^9 cells [41]). However, the small quantity of DNA released from the Ehrlich-Lettré cells is compatible with a mitochondrial as well as a nuclear origin.

The characteristics of the RNA released from Ehrlich-Lettré cells by isotonic inositol or DMSO, or by HBSS, are compatible with its designation as "soluble" (4S?) RNA, but it

must be emphasized that its cellular origin is unknown. Likewise, it is not known if the RNA has amino acid transferring activity, and, therefore, the term "transfer RNA" has been avoided. Since the major base compositions of the RNA's isolated by the "hot 10% NaCl" method from supernatants of the Ehrlich-Lettré cells were very similar to those for Ehrlich cell "soluble" RNA, it seems unlikely that the RNA released from Ehrlich-Lettré cells by the isotonic media would be degradation products of heavier RNA's. The relative scarcity of RNA-nucleotides in the acid-soluble fractions of the cell supernatants also supports this view. However, extensive time-sequence studies would be required to establish this point unequivocally.

A major factor underlying the present investigation is the extent to which cell damage may have contributed to the release of intracellular materials. By dye exclusion tests the spleen cell suspensions contained 17% non-viable cells at the beginning of the incubation period, while the ascites cell suspensions contained only 5% non-viable cells. No significant decrease has been found in the viability of the Ehrlich ascites cells or the mouse spleen cells following incubation in the isotonic media for one hour, and the viability of the Ehrlich cells has been found to be stable for up to 5 hr (J. F. Morgan and C. P. Eng. Manuscript in preparation). Bather *et al.* [10] also observed that the viability of Ehrlich ascites cells and of mouse spleen cells was not altered appreciably by incubation for up to 3 hr in inositol. As reported previously [8], cell size distribution studies made on Ehrlich cells with a Coulter electronic counter and automatic plotter showed that the average cell volume decreased slightly during exposure of the cells to isotonic DMSO or glycerol, but remained essentially unchanged during exposure to HBSS or isotonic inositol. Furthermore, the numbers of cells counted (Coulter counter) following the incubation of Ehrlich-Lettré and of mouse spleen cells in the isotonic media for 1 hr were not significantly different from the initial numbers (J. F. Morgan and C. P. Eng. Manuscript in preparation). These results suggest very strongly that release of materials from the cells is related neither to cell viability nor to cell disruption, but is governed by the type of cell and the nature of the suspending media.

The imbalance between the amounts of RNA and DNA released from the Ehrlich-Lettré cells also suggests that sRNA is selectively released. Four times as much RNA as DNA was released in all three isotonic media, whereas the ratio of total RNA to total DNA in Ehrlich cells is very close to 1·0 [47]. As demonstrated previously [8], the release of nucleic acids from Ehrlich cells in the presence of isotonic inositol is not accompanied by a significant loss of viability or by diminution of tumorigenicity of the treated cells. Webb *et al.* [9] and Bather *et al.* [10] have previously emphasized the non-deleterious effect of the loss of RNA from bacterial and mammalian cells exposed to inositol.

The effect of isotonic *myo*-inositol on the Ehrlich-Lettré tumor cells is clearly different from that on the normal spleen cells, as shown by the very different ratios of released RNA to DNA for the two cell types. Isotonic *myo*-inositol also appears to exert a distinctly different effect on both normal and tumor cells than do the other cryoprotective agents tested. Further studies with *myo*-inositol appear warranted to study the mechanism of its cryoprotective action and to establish whether a major difference exists between normal and tumor cells in response to this physiological compound.

SUMMARY

Ribonucleic acid, deoxyribonucleic acid, protein and acid-soluble nucleic acid derivatives were isolated from supernatants of Ehrlich-Lettré ascites carcinoma cells or mouse spleen cells prepared by incubating the intact cells in isotonic *myo*-inositol, dimethylsulfoxide (DMSO) or NaCl, or in Hanks' balanced salt solution (HBSS) (control) or a 3:1 mixture of isotonic inositol : NaCl, at 37°C. The isolated substances were analyzed by chemical, spectrophotometric, chromatographic, or sucrose density sedimentation methods.

On the basis of 10^9 cells, isotonic inositol caused the release of larger amounts of acid-soluble nucleic acid derivatives from Ehrlich-Lettré cells than did DMSO or HBSS, and 7 to 8 times as much acid-soluble material was released from the tumor cells in inositol or HBSS as from the spleen cells. The major components of the acid-soluble fractions were xanthine and hypoxanthine.

Protein, RNA and DNA were detected in all suspending fluids, but HBSS or isotonic NaCl caused the release of much smaller amounts of these substances than the other isotonic incubation media. The ratio of RNA to DNA in supernatants of Ehrlich-Lettré cells was approximately 5:1, whereas in spleen cell supernatants it was 0·038–0·11:1. Total RNA isolated by the "phenol" method from supernatants of Ehrlich-Lettré cells, was shown by sucrose density sedimentation analysis to be mainly "soluble"

RNA, and this identity was confirmed by base composition analyses. The identity of DNA was also confirmed by sucrose density sedimentation and base composition analyses.

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REFERENCES

1. B. A. Bouronela, Preservation of living cells at −79°C with dimethyl sulfoxide. *Proc. Soc. exp. Biol. (N.Y.)* **119,** 958 (1965).
2. L. L. Coriell, A. E. Greene and R. K. Silver, Historical developments of cell and tissue culture freezing. *Cryobiology* **1,** 72, (1964).
3. F. Miya, G. A. Hill and S. Marcus, Preservation of Ehrlich ascites tumor cells with dimethyl sulfoxide. *Appl. Microbiol.* **15,** 451 (1967).
4. L. Diamond, The effect of carcinogenic hydrocarbons on rodent and primate cells *in vitro*. *J. cell. comp. Physiol.* **66**, 183, (1965).
5. T. T. Chen and C. Heidelberger, Quantitative studies on the malignant transformation of mouse prostate cells by carcinogenic hydrocarbons *in vitro*. *Int. J. Cancer*, **4,** 166 (1969).
6. S. J. Webb, *Bound Water in Biological Integrity*. Charles C. Thomas, Springfield, Illinois (1965).
7. J. F. Morgan, M. D. Heuchert and H. D. Kirk, The effect of storage at low temperatures on the mouse strain specificity of the 6C3HED ascites tumor. *Cancer Res.* **29,** 855 (1969).
8. C. P. Eng and J. F. Morgan, The release of nucleic acids and protein from ascites tumor cells incubated with glycerol, dimethylsulfoxide, and inositol. *Canad. J. Biochem.* **47,** 871 (1969).
9. S. J. Webb, C. A. M. Cook and R. Bather, Inositol and the protection of cells. I. The release of nucleic acids and viruses from bacterial and mammalian cells. *Canad. J. Biochem.* **42,** 157 (1964).
10. R. Bather, I. Sebastian and S. J. Webb, Inositol and the protection of cells. II. The release of nucleic acids from normal and malignant cells. *Canad. J. Biochem.* **42,** 167 (1964).
11. J. H. Hanks and R. E. Wallace, Relation of oxygen and temperature in the preservation of tissues by refrigeration. *Proc. Soc. exp. Biol. (N.Y.)* **71,** 196 (1949).
12. I. C. Caldwell and M. F. Chan, Tumor cell metabolism *in vitro*: a study of incubation media. *Canad. J. Biochem.* **48,** 517 (1970).
13. H. J. Phillips and J. E. Terryberry, Counting actively metabolizing tissue cultured cells. *Exp. Cell Res.* **13,** 341 (1957).
14. E. H. McConkey, The fractionation of RNA's by sucrose gradient sedimentation. In *Methods in Enzymology* (Edited by L. Grossman and K. Moldave), Vol. 12, Part A, p. 620. Academic Press, New York (1967).
15. W. J. Steele and H. Busch, RNA: Isolation and fractionation. In *Methods in Cancer Research* (Edited by H. Busch), Vol. 3, p. 61. Academic Press, New York (1967).
16. K. S. Kirby, Isolation and characterization of ribosomal ribonucleic acid. *Biochem. J.* 96, 266 (1965).
17. K. S. Kirby, Ribonucleic acids—II. Improved preparation of rat-liver ribonucleic acid. *Biochim. biophys. Acta (Amst.)* **55,** 545 (1962).
18. T. Staehelin, F. O. Wettstein, and H. Noll, Breakdown of rat liver ergosomes *in vivo* after actinomycin inhibition of messenger RNA. *Science* **140,** 180 (1963).
19. W. Mejbaum, Über die Bestimmung kleiner Pentosemengen insbesondere in Derivaten der Adenylsäure. *Hoppe-Seylers Z. physiol. Chem.* **258,** 117 (1939).
20. R. B. Hurlbert, H. Schmitz, A. F. Brumm and V. R. Potter, Nucleotide metabolism—II. Chromatographic separation of acid-soluble nucleotides. *J. biol. Chem.* **209,** 23 (1954).
21. Z. Dische, Some new color reactions of the thymonucleic acids and their application to their determination. *Mikrochemie* **8,** 4 (1930).
22. W. J. Steele and H. Busch, RNA: Isolation and fractionation. In *Methods in Cancer Research* (Edited by H. Busch), Vol. 3, p. 68. Academic Press, New York (1967).

23. W. J. Steele and H. Busch, Studies on the RNA components of the nuclear ribonucleoprotein network. *Biochim. biophys. Acta (Amst.)* **129,** 54 (1966).
24. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193,** 265 (1951).
25. L. I. Hecht and V. R. Potter, Nucleic acid metabolism in regenerating rat liver. I. The rate of deoxyribonucleic acid synthesis *in vivo*. *Cancer Res.* **16,** 988 (1956).
26. E. Hebert, V. R. Potter and L. I. Hecht, Nucleotide metabolism—VII. The incorporation of radioactivity from orotic acid-6-C^{14} into ribonucleic acid in cell-free systems from rat liver. *J. biol. Chem.* **225,** 659 (1957).
27. G. Schmidt and S. J. Thannhauser, A method for the determination of deoxyribonucleic acid, ribonucleic acid, and phosphoproteins in animal tissues. *J. biol. Chem.* **161,** 83 (1945).
28. G. H. Beavan, E. R. Holiday and E. A. Johnson, Optical properties of nucleic acids and their components. In *The Nucleic Acids* (Edited by E. Chargaff and J. N. Davidson), Vol. 1, pp. 493. Academic Press, New York (1955).
29. A. Marshak and H. J. Vogel, Microdetermination of purines and pyrimidines in biological materials. *J. biol. Chem.* **189,** 597 (1951).
30. J. D. Smith and R. Markham, Chromatographic studies on nucleic acids—II. The quantitative analysis of ribonucleic acids. *Biochem. J.* **46,** 509 (1950).
31. G. R. Wyatt, The purine and pyrimidine composition of deoxypentose nucleic acids. *Biochem. J.* **48,** 584 (1951).
32. D. G. R. Blair and A. D. Hall, The metabolism of 2,6-diaminopurine by diaminopurine-sensitive and diaminopurine-resistant L-strain mouse fibroblasts. *Canad. J. Biochem.* **43,** 1857 (1965).
33. D. G. R. Blair and D. J. Rainnie, Acid-soluble metabolites of 2,6-diaminopurine in L strain mouse cells. *Canad. J. Biochem.* **45,** 347 (1967).
34. D. G. R. Blair, Acid-soluble metabolites of adenine-2-^{14}C and adenine-8-^{14}C in 2,6-diaminopurine-sensitive and 2,6-diaminopurine-resistant L strain mouse cells. *Canad. J. Biochem.* **46,** 217 (1968).
35. C. E. Carter, Paper chromatography of purine and pyrimidine derivatives of yeast nucleic acid. *J. Amer. chem. Soc.* **72,** 1466 (1950).
36. R. J. Block, E. L. Durrum and G. Zweig, *A Manual of Paper Chromatography and Paper Electrophoresis*, 2nd edition, p. 287. Academic Press, New York (1958).
37. M. B. Hoagland, The relationship of nucleic acid and protein synthesis as revealed by studies in cell-free systems. In *The Nucleic Acids* (Edited by E. Chargaff and J. N. Davidson), Vol. 3, p. 349. Academic Press, New York (1960).
38. S. Kit, Base composition of the ribonucleic acids of normal tissues, tumors and tissue subfractions. *Arch. Biochem.* **88,** 1 (1960).
39. W. J. Kleinschmidt, The composition of ribonucleic acid and deoxyribonucleic acid of normal and neoplastic tissue. *Cancer Res.* **19,** 966 (1959).
40. S. Kit, Coding by purine and pyrimidine moieties in animals, plants and bacteria. In *Symposium on Information Storage and Neural Control* (Edited by W. S. Fields and W. Abbott), p. 76. Charles C. Thomas, Springfield, Illinois (1963).
41. S. Kit, Investigations of the DNA content, base composition and chromatography on ECTEOLA-cellulose of normal and tumor DNA preparations. *Arch. Biochem.* **87,** 330 (1960).
42. S. Kit, Equilibrium sedimentation in density gradients of DNA preparations from animal tissues. *J. molec. Biol.* **3,** 711 (1961).
43. W. C. Schneider and E. L. Kuff, The isolation and some properties of rat liver mitochondrial deoxyribonucleic acid. *Proc. nat. Acad. Sci. (Wash.)* **54,** 1650 (1965).
44. B. Hudson and J. Vinograd, Catenated DNA molecules in HeLa cell mitochondria. *Nature (Lond.)* **216,** 647 (1967).
45. G. Attardi and B. Attardi, Mitochondrial origin of membrane-associated heterogeneous RNA in HeLa cells. *Proc. nat. Acad. Sci. (Wash.)* **61,** 261 (1968).
46. M. M. K. Nass, The circularity of mitochondrial DNA. *Proc. nat. Acad. Sci. (Wash.)* **56,** 1215 (1966).
47. S. Kit, The nucleic acids of normal tissues and tumors. In *Amino Acids, Proteins and Cancer Biochemistry* (Edited by J. T. Edsall), p. 147. Academic Press, New York (1960).

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Sensitivity to Antibiotics of Gram-Negative Pathogens Isolated From Hospitalized Patients having Disseminated Malignant Disease

JEAN KLASTERSKY

Service de Médecine et Laboratoire d'Investigation Clinique (Section des Maladies Infectieuses), Institut Jules Bordet, Centre des Tumeurs de l'Université Libre de Bruxelles, Bruxelles, Belgique

Abstract—*Four hundred and seventy-two strains of gram-negative bacilli including Escherichia coli, Klebsiella-Enterobacter, Proteus, Pseudomonas aeruginosa and Paracolon species were isolated from patients with disseminated malignant diseases presenting a clinical infection or a heavy bacterial colonization. Their sensitivity to 8 commonly used antibiotics has been measured.*

Most strains were moderately to highly susceptible to gentamicin and, with the exception of Proteus and Paracolon species, to polymyxin B. Large numbers of strains highly resistant to the other antibiotics, namely ampicillin, cephalotin, carbenicillin, tetracycline, chloramphenicol, and kanamycin, were found among all bacterial species tested.

Pseudomonas aeruginosa was isolated from 25% or more of wound, sputum and blood cultures. Carbenicillin was active, at feasible levels in the blood, on most Pseudomonas aeruginosa strains.

It is therefore concluded from the bacteriological data presented that, while awaiting the results of sensitivity tests, the combination of carbenicillin with gentamicin should be given in suspected severe gram-negative infections, in patients with diminished host resistance.

INTRODUCTION

RECENT reports in the literature have emphasized the increasing importance and frequency of gram-negative bacillary infections, particularly in patients with chronic underlying disease or cancer who are undergoing cytotoxic chemotherapy, radiotherapy or antibiotic therapy [1]. The mortality of severe infections caused by gram-negative micro-organisms has been as high as 40–50% for many years [2]. There are indications that "adequate" antimicrobial therapy, as suggested by *in vitro* tests, is associated with optimal clinical course [3, 4]. It is generally true that the lower the initial minimal inhibiting concentration of an antibiotic for a bacterial isolate is, the greater is the probability of a favourable clinical response to that agent [5].

Antimicrobial therapy in serious infections due to gram-negative rods is usually started before bacterial identification and sensitivity tests of the offending micro-organism have been performed. Therefore surveys of the susceptibility to antibiotics of the various species isolated from cases of gram-negative infections in a particular hospital will suggest what therapy is most likely to be appropriate. Because the underlying clinical condition of the patient has

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been shown to be a determining factor in the prognosis of severe infections [6], the results reported here might have some general significance for clinicians responsible for the management of infections in debilitated patients, especially those with disseminated malignant disease.

This investigation has been undertaken in an attempt to develop a rational approach to the choice of initial treatment of serious infections caused by gram-negative rods until results of *in vitro* sensitivity tests become available.

MATERIAL AND METHODS

Bacteriology

Blood specimens were inoculated in 10-ml amounts into 200 ml each of trypticase-soy broth (Difco) and fluid thioglycollate medium (Difco). Blood cultures were incubated for 2 weeks at 37°C before being discarded as negative.

Sputum specimens were cultured on both blood agar and eosine-methylene-blue (EMB) agar (Difco) media. Urine was cultured routinely, without prior centrifugation, by streaking 0·01 ml of urine on EMB agar, and the technique described by Lorrier and Valkenburg [7] has been used for quantification of the bacterial growth.

Cultures of wounds were performed on blood agar and EMB agar media and a fluid thioglycollate medium was inoculated as well.

All cultures were examined after 24 hr and 48 hr of incubation at 37°C.

For characterization of enterobacteriacae the taxonomic system proposed by Ewing and Edwards [8, 9] was followed.

Although the term paracolon is improper and imprecise taxonomically, it will be used here as a convenient method of referring to strains that might have belonged to the groups Serratia, Citrobacter, Providence, Hafnia, Edwardsiella, Arizona, Herellea, and Mima.

Antibiotic sensitivities

Gram-negative bacteria isolated from various sources were tested for susceptibility to 8 antibiotics, by the inocula-replicating method of Steers *et al.* [10]. The tests were performed on nutrient agar (Difco) using an inoculum containing approximately 10^6–10^7 micro-organisms per ml. The inocula-replicating device delivered approximately 0·001 ml of diluted culture within a 5–6 mm circle of agar containing 2-fold dilutions of various antibiotics. The highest dilution of antibiotic failing to show macroscopic bacterial growth was considered as the bacteriostatic end-point (MIC).

RESULTS

Incidence and significance of the various isolates

Significant bacteremia (2 or more positive blood cultures for the same organism) was observed in 32 patients during the 6-month period of the present study. All were associated with clinical illness suggestive of septicemia; 19 episodes of bacteremia (59%) were caused by gram-negative rods. Pulmonary infection was the source of 5 out of 6 bacteremias caused by Klebsiella and 3 out of 7 due to Pseudomonas. One case of Pseudomonas bacteremia originated from a urinary-tract infection and the 3 others were found in patients with acute leukaemia, but no obvious source has been detected.

Urine cultures studied here yielded at least 10^5 micro-organisms per ml of urine. It can be expected that 85% of those cultures represented significant bacteriuria in the sense that a repeated culture would be positive for more than 10^5 colonies per ml of the same organism [11]. The distribution of the different species among 195 urinary pathogens studied here is shown in Table 1. *E. coli* was the most frequently isolated pathogen.

Table 1. *Frequency of isolation of various gram-negative bacilli from different sources*

	Blood	Urine	Sputum	Wounds
E. coli	2 (11%)	83 (42%)	22 (13%)	13 (15%)
Klebsiella	6 (30%)	25 (12%)	42 (24%)	8 (9%)
Enterobacter	0	6 (3%)	9 (5%)	2 (3%)
Proteus mirabilis	1 (6%)	35 (18%)	34 (19%)	37 (43%)
Proteus (indol-positive)	1 (6%)	15 (8%)	10 (6%)	3 (4%)
Pseudomonas aeruginosa	7 (11%)	17 (9%)	53 (30%)	23 (26%)
Paracolon	2 (6%)	14 (8%)	5 (3%)	0
Total	19	195	175	86

The percentages are relative to the number of a given organism among the total isolates from a given site.

Wound cultures were considered for this study if gram-negative organisms were obtained in pure or predominant and abundant culture, with evidence of leukocytes and gram-negative bacilli on the direct smear from the wound. Most isolates were from surgical wounds, and the others (approximately 20%) from decubitus ulcers, infected tumors, cellulitis, and pleural or peritoneal exudates.

In wound cultures Proteus mirabilis has been the most frequently isolated organism (43%), followed by Pseudomonas (26%) as shown in Table 1.

Although the clinical significance of the pathogens isolated from urine and wounds in our study is not completely established, it should be emphasized that in the very debilitated patient even the saprophytic endogenous flora can be responsible for severe sepsis [12].

Gram-negative bacilli were isolated from the sputum, in 175 instances, in pure or predominant abundant growth. In 40 (22%) cases these findings could be correlated with clinical respiratory infection. In these patients sputum contained large numbers of leukocytes—except in a few severely leukopenic patients—and fever, with radiologic and auscultatory signs of bronchopneumonia were present in all of them. The distribution of the pathogens responsible for respiratory-tract infection is illustrated in Table 2.

Table 2. Incidence of bacterial colonization and superinfection of the respiratory tract

Micro-organisms	Colonization	Superinfection
E. coli	21 (15%)	1 (2·5%)
Klebsiella	29 (21%)	13 (32·5%)
Enterobacter	9 (7%)	0
Proteus mirabilis	28 (21%)	6 (15%)
Proteus (indol-positive)	10 (8%)	0
Pseudomonas aeruginosa	35 (26%)	18 (45%)
Paracolon	3 (2%)	2 (5%)
Total	135	40

The 135 other sputum cultures in which gram-negative bacilli were found are to be considered as reflecting bacterial colonization. The clinical significance of such a colonization hf the respiratory tract in hospitalized patients oas been stressed by Tillotson and Finland [13].

Pseudomonas aeruginosa and Klebsiella were the organisms most commonly isolated from the sputum: they were found respectively in 30% and 24% of the cultures. These organisms were also the most frequent agents in clinical infections: in 45% and 32% of the cases.

Relationship between the sensitivity to antibiotics and the site of isolation of the micro-organisms.

These strains, isolated from blood cultures, were highly resistant to most antibiotics for which sensitivity has been tested. Only gentamicin and polymyxin B were active on most strains. It has been suggested [14] that hospital-acquired gram-negative bacilli in the sputum may become more resistant to antibiotics within the patient during chemotherapy with antibiotics. Because in all our patients septicemia occurred after a prolonged hospital course and multiple treatments with antimicrobial agents, the high resistance pattern observed in bacteria isolated from the blood might be the consequence of a selection of resistant strains by antibiotics.

The susceptibility to antibiotics of micro-organisms isolated from sources other than blood cultures was similar, irrespective of the source of isolation. It has been suggested that bacteria isolated from non-respiratory sources are much more resistant than those obtained from sputum [14]. Such a difference in sensitivity has not been observed here.

The differences in sensitivity pattern of the micro-organisms isolated from urine, wounds and sputum cultures were not considerable. For practical purposes, our results suggest no important differences in the susceptibility to antibiotics of the various strains studied as related to the site of isolation. Similar conclusions have been reached by Petersdorf *et al.* [15].

Antimicrobial susceptibility of gram-negative bacilli to eight antibiotics

The percentage of sensitive strains from all sources to feasible levels of antibiotics in the blood are shown in Table 3.

In spite of its wide use for 2 years within this hospital, gentamicin was the most active drug against the gram-negative bacilli studied here. With the exception of Proteus and Paracolon strains, polymyxin B was effective on most strains studied. The data presented suggest moreover that, with the exception of gentamicin, no single antibiotic can be expected to provide a coverage broad enough for the majority of gram-negative pathogens isolated in this hospital.

Table 3. Percentage of strains of gram-negative bacilli sensitive to levels of antibiotics feasible in the blood

Antibiotics	AMP	AMP	CAR	CAR	CAR	CEP	CEP	TET	CHL	KAN	GEN	POL
μg/ml	12	50	12	50	100	12	50	12	12	25	12	12
E. coli	56	58	54	63	67	54	80	34	56	65	100	83
Klebsiella	12	37	6	16	19	34	42	31	35	39	95	80
Enterobacter	13	37	13	25	50	30	45	34	40	42	93	59
Proteus mirabilis	68	71	71	73	75	79	86	4	60	75	95	5
Proteus (indol-positive)	38	50	56	60	60	40	48	9	54	59	90	3
Pseudomonas	0	2	9	64	83	0	4	28	3	8	56	62
Paracolon	12	27	5	5	5	24	36	22	74	4	50	22

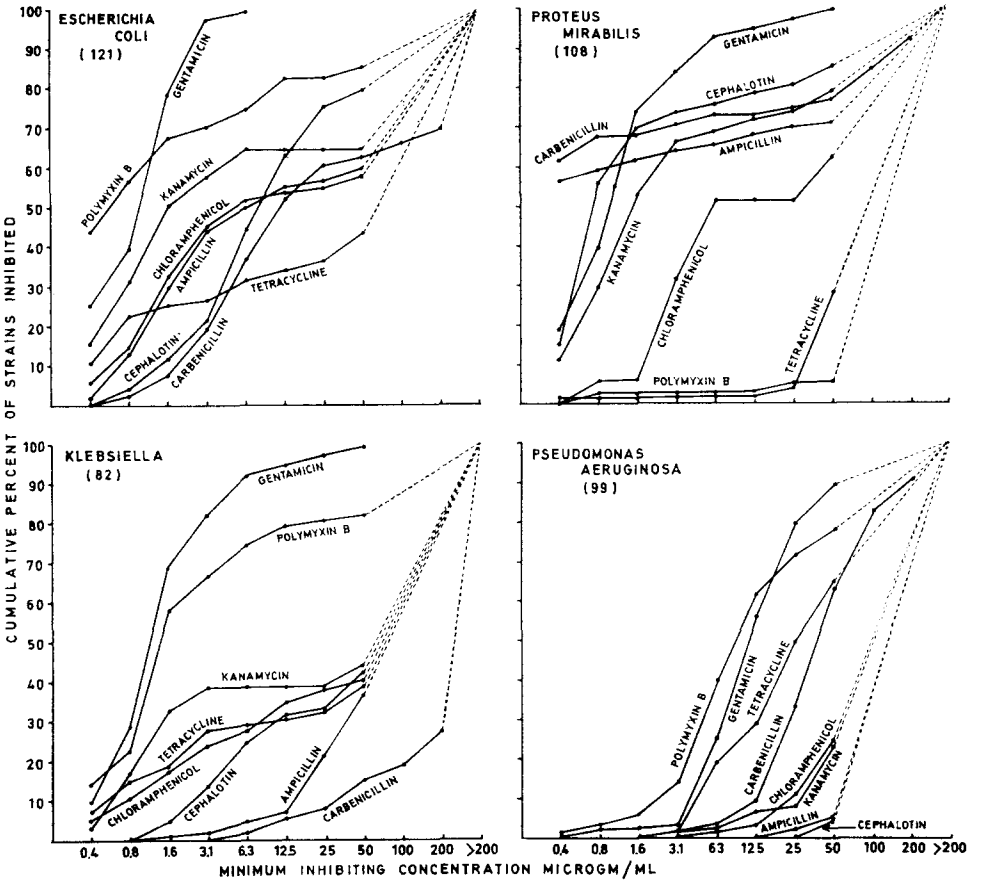


Fig. 1. Sensitivity of pathogens isolated from hospitalized patients to 8 antibiotics.

The susceptibility of various species of gram-negative bacilli is shown in Figs. 1 and 2.

Gentamicin and polymyxin B were active on more strains of *E. coli* than kanamycin; ampicillin, carbenicillin, cephalotin and chloramphenicol inhibited similar numbers of *E. coli* strains.

On Klebsiella, gentamicin and polymyxin B were effective on similar numbers of strains; kanamycin, chloramphenicol, tetracycline and cephalotin were about equally active.

Sensitivity of Enterobacter species was similar to that of Klebsiella, but polymyxin B and cephalotin were active on fewer strains. Ampicillin and carbenicillin were active on only a few strains of the Klebsiella-Enterobacter group. These patterns of sensitivity are in general comparable to other reports in the

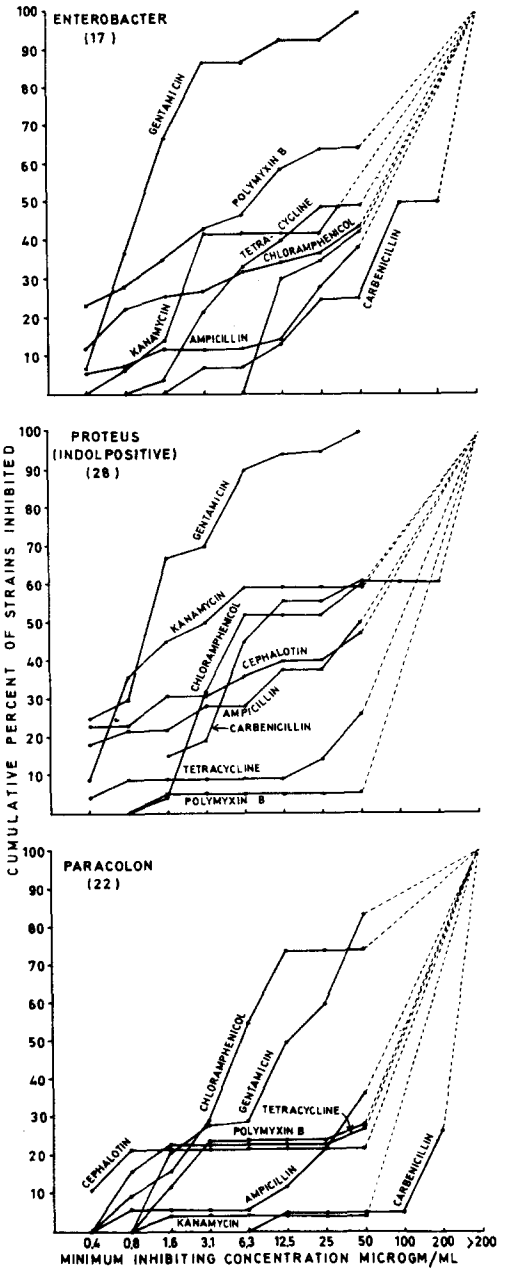


Fig. 2. Sensitivity of pathogens isolated from hospitalized patients to 8 antibiotics.

literature [16], but in our studies polymyxin B was found more active than kanamycin.

Proteus species were almost completely resistant to polymyxin B and tetracycline. Gentamicin was active on more strains of Proteus mirabilis than cephalotin, carbenicillin, ampicillin and kanamycin. Chloramphenicol was equally active on Proteus mirabilis and indol-positive Proteus strains. Indol-positive Proteus strains were more resistant to all antibiotics except gentamicin and chloramphenicol than Proteus mirabilis strains. Carbenicillin, cephalotin and ampicillin were not superior to chloramphenicol for indol-positive Proteus strains.

Chloramphenicol appeared the most active agent on the "Paracolon" bacteria, but all other antibiotics except gentamicin had only a limited effectiveness. The lack of action of kanamycin on many "Paracolon" strains is in opposition to results reported by others [17].

Pseudomonas aeruginosa strains were highly resistant to 4 of the antibiotics tested here: chloramphenicol, kanamycin, ampicillin and cephalotin, as might be expected. Polymyxin B and gentamicin were active on about 60% of the strains when 12·5 μg/ml were used.

Blood levels for carbenicillin with high dosage therapy can be maintained in the range of 100 μg/ml [18]. At this concentration 83% of Pseudomonas strains were inhibited. Thus carbenicillin appears as the antibiotic effective on the largest number of Pseudomonas strains at levels feasible in the blood.

As far as sensitivity of Pseudomonas to carbenicillin, polymyxin B and gentamicin is concerned, our results are in accordance with those reported by Lowbury *et al.* [19]. Unexpectedly, tetracycline was found active on about 30% of Pseudomonas strains when used at 12·5 μg/ml.

DISCUSSION

It is fully realized that the data presented here are essentially valid for the hospital where the study has been performed.

Our data are based on the determination of the minimal inhibitory concentration of several commonly used antibiotics of gram-negative bacilli isolated from clinical sources. Many of these isolates caused clinical diseases and all others were considered to represent a serious threat for the patients with diminished resistance to infection who were studied here. It is recognized that the distinction between colonization and superinfection may be very difficult in those patients.

Among all bacterial species tested here a high proportion of resistant strains (not inhibited by 50 μg/ml) was encountered for all antibiotics tested except gentamicin.

The reason for this high incidence of very resistant strains is not entirely clear. It could be related to prolonged hospitalization and repeated courses of antimicrobial therapy. Both have been shown to increase the resistance to antibiotics of the colonizing bacterial flora [14, 20].

When a tentative diagnosis of gram-negative infection is made on a clinical basis and with the use of gram-stained smears of exudates therapy should be instituted immediately, using

the kind of data reported here, while awaiting results of species identification and of *in vitro* sensitivity tests.

In our hospital Pseudomonas aeruginosa is expected to cause at least 25% of severe infections due to gram-negative rods, except in urinary-tract infections.

Since Pseudomonas aeruginosa species exhibit a high resistance to antibiotics, it is concluded that any infection suspected to be caused by ram-negative rods should be treated as an infection caused by Pseudomonas, when clinical clues for making an estimate of the identity of the organism involved are absent.

Theoretically, the combinations of carbenicillin with gentamicin or polymyxin B can be expected to be particularly effective under these circumstances: the risk of emergence of strains resistant to carbenicillin [19] could probably be reduced and excessive dosage of gentamicin and polymyxin B, both potentially toxic, could be avoided in most cases. Indeed, both combinations have been shown synergistic or at least additive on Pseudomonas *in vitro* [21, 22]. A clinical evaluation of the combination of gentamicin and carbenicillin in this hospital has shown an 85% rate of excellent results in life-threatening infections caused by gram-negative rods (data to be published). The use of the combination of carbenicillin with polymyxin B was associated with less good results (data to be published).

It should be kept in mind that in debilitated patients the infection may be caused by gram-positive as well as gram-negative organisms. However, carbenicillin is active on penicillin-sensitive gram-positive pathogens [18], and the effectiveness of gentamicin on penicillin-resistant staphylococcus is well documented [13].

Emergence of strains resistant to gentamicin might become an important problem if these drugs should become widely used. Therefore we feel that the combination of antibiotics recommended here should be discontinued when *in vitro* tests suggest sensitivity to other drugs. Most urinary pathogens studied here could probably be adequately treated with kanamycin, ampicillin or cephalotin alone.

Infections originating from other sites and caused by *E. coli*, Klebsiella-Enterobacter species or Proteus should probably not be treated with ampicillin or cephalotin alone unless detailed sensitivity studies are available, since many strains are borderline sensitive or resistant to levels of these drugs feasible in the blood. *In vitro* studies suggest that the association of ampicillin with kanamycin could be effective in infections caused by most *E. coli* and Proteus [13]. Similarly the association of cephalotin with kanamycin is active on many Klebsiella *in vitro* [24].

Our results and conclusions should not be interpreted as supporting the concept that all gram-negative infections should be treated with a combination of agents regardless of sensitivity test results, or that commercially available mixtures of antibiotics are needed.

They suggest a form of therapy, based on the known susceptibility of the various bacteria encountered in a particular kind of patient, to be given while awaiting results of *in vitro* sensitivity tests. The efficacy of such regimens should be tested clinically and compared to other therapies already reported [25].

Since the sensitivity of bacterial pathogens within a given hospital may change rapidly, periodic surveys of the susceptibility of pathogenic bacteria isolated from hospitalized patients should be performed.

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REFERENCES

1. F. F. Barret, J. J. Casey and M. Finland, Infections and antibiotic use among patients at Boston City Hospital. *New Engl. J. Med.* **278,** 5 (1967).
2. J. J. Maitzegui, J. Z. Biegeleisen, W. B. Cherry and E. H. Kass, Bacteremia due to gram-negative rods. A clinical, bacteriologic, serologic and immunofluorescent study. *New Engl. J. Med.* **272,** 222 (1965).
3. U. G. Hodgin and P. J. Sandford, Gram-negative rod bacteremia. An analysis of 100 patients. *Am. J. Med.* **39,** 952 (1965).
4. W. R. McCabe and G. G. Jackson, Gram negative bacteremia. II. Clinical, laboratory and therapeutic observations. *Arch. int. Med.* **110,** 856 (1962).
5. J. R. Tillotson and M. Finland, Bacterial colonization and clinical superinfection of the respiratory tract complicating antibiotic treatment of pneumonia. *J. infect. Dis.* **119,** 597 (1969).
6. M. S. Freid and K. L. Vosti, The importance of underlying disease in patients with gram-negative bacteremia. *Arch. int. Med.* **121,** 418 (1968).

7. J. C. Lorrier and H. A. Valkenburg, Quantitative urine culture by surface drop method. *Appl. Microb.* **18,** 57 (1969).
8. P. R. Edwards and W. H. Ewings, *Identification of Entero-bacteriacae.* Burgess, Minneapolis (1962).
9. W. H. Ewings, J. N. Wilfert, L. J. Kunz, M. Dumoff and H. D. Isenberg, How far to go with Enterobacteriacae? *J. infect. Dis.* **119,** 197 (1969).
10. E. Steers, E. L. Foltz and B. S. Graves, Inocula-replicating apparatus for routine testing of bacterial susceptibility to antibiotics. *Antibiot. Chemotherapy* **9,** 307 (1959).
11. C. W. Norden and E. H. Kass, Bacteriuria of pregnancy—a critical appraisal. *Ann. Rev. Med.* **19,** 431 (1968).
12. A. S. Klainer and W. R. Beisel, Opportunistic infection. A review *Am. J. Med. Sci.* **258,** 431 (1969).
13. P. D. Hoeprich, Gentamicin versus Staphylococcus aureus. *J. infect. Dis.* **119,** 391 (1969).
14. J. R. Tillotson and M. Finland, Patterns of antibiotic susceptibility of Gram-negative bateria isolated from sputum after chemotherapy. *Antimicr. Agents Chemother.* **8,** 315 (1968).
15. M. Turck, R. I. Lindemeyer and G. Petersdorf, Comparison of single-disc and tube-dilution techniques in determining antibiotic sensitivities of gram-negative pathogens. *Ann. Int. Med.* **58,** 56 (1963).
16. T. C. Eickoff, B. W. Steinhauer and M. Finland, The Klebsiella-Entero-bacter-Serratia division—Biochemical and serologic characteristics and susceptibility to antibiotics. *Ann. Int. Med.* **65,** 1163 (1966).
17. B. N. Fields, M. M. Uwaydah, J. L. Kunz and M. N. Swartz, The so-called "Paracolon" bacteria. A bacteriologic and clinical reappraisal. *Am. J. Med.* **42,** 89 (1967).
18. G. P. Bodey, V. Rodriguez and D. Stewart, Clinical Pharmacological studies of carbenicillin. *Am. J. med. Sci.* **257,** 185–190 (1969).
19. E. J. L. Lowbury, A. Kidson, H. A. Lilly and G. A. J. Ayliffe, Sensitivity of Pseudomonas aeruginosa to antibiotics: emergence of strains highly resistant to carbenicillin. *Lancet* **ii,** 448 (1969).
20. H. D. Rose and J. Schreier, The effect of hospitalization and antibiotic therapy on the gram negative fecal flora. *Am. J. med. Sci.* **255,** 228 (1968).
21. C. B. Smith, P. E. Dans, I. N. Wilfert and M. Finland, Sue of gentamicin in combination with other antibiotics. *J. infect. Dis.* **119,** 370 (1969).
22. T. C. Eickoff, *In vitro* effects of carbenicillin combined with gentamicin or polymyxin B against Pseudomonas aeruginosa. *Appl. Microb.* **18,** 469 (1969).
23. R. J. Bulger and U. Roosen-Bunge, Bactericidal activity of the ampicillin/kanamycin combination against *Escherichia coli,* Enterobacter-Klebsiella and Proteus. *Am. J. med. Sci.* **258,** 7 (1969).
24. R. J. Bulger, *In vitro* effectiveness of kanamycin and kanamycin/cephalotin against Klebsiella. *Ann. Int. Med.* **67,** 523 (1967).
25. C. M. Martin, A. J. Cuomo, M. J. Geraghty, J. R. Zager and T. C. Mandes, Gram-negative rod bacteremia. *J. infect. Dis.* **119,** 506 (1969).

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Effects of Active Specific and Non-Specific Immunotherapy on E♂G2 Leukaemia According to the Route of Administration and Combination of Immunological Stimulants

J. L. AMIEL and M. BĚRARDET
Institut de Cancérologie et d'Immunogénétique, Hôpital Paul-Brousse, 94-Villejuif, France

Abstract—*The effects of active immunotherapy, specific, using AkR leukaemic cells, and non-specific with BCG, on E♂G2 leukaemia injected intraperitoneally into (DBA/2 × C57B1/6) F1 recipients have been examined. The routes of administration intraperitoneally and intravenously, and the combination of these treatments have been studied.*

Under these conditions the intraperitoneal route appears to be more efficient than the intravenous injection. The combination of BCG and AkR cells can be more effective than either of these treatments given alone (i.e. BCG and AkR cells intravenously), but they can be less effective (i.e. BCG i.p. is more effective if given alone than in combination of AkR cells i.v. or i.p.).

The mortality curves of the treated animals showed a change after a period of time; initially they were very similar to the untreated animals, then the curve became a plateau, the treated animals are either little or unaffected, or cured.

These different points are discussed.

ACTIVE immunotherapy has been shown to be effective in murine [1, 2] and human leukaemias [3]. From the studies in mice several points have been made clear which were shown to be of importance for the application of immunotherapy to clinical practice. These cardinal points are as follows: all the leukaemic cells can be eradicated but only if they are few in number [1]; active immunotherapy given after the reduction of the number of leukaemic cells by chemotherapy is beneficial, but the effects of active immunotherapy are inhibited when it is followed by chemotherapy [4, 5].

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The conditions for active immunotherapy differ in the mouse and man, mainly in the routes employed to give the specific and non-specific stimuli. We have therefore made a more detailed study of the importance of the variations in these routes of giving active specific and non-specific immunotherapy, as well as their combination, upon the effectiveness of the immunotherapy.

MATERIAL AND METHODS

A hundred and eighty adult male (DBA/2 × C57B1/6)F1 mice were divided at random into 9 groups of 20. Each group was then subdivided into two sub-groups "a" and "b".

On day 0 animals in the "a" sub-groups were given 10^4 EG♂2 leukaemic cells intraperitoneally and the animals in the "b" sub-groups received 10^5 of the same cells.

One mg (dry weight) of living BCG from the Institut Pasteur (Paris) was injected on day −4 intravenously into mice of groups I, V and VIII and intraperitoneally into mice of groups III, VI and VII.

10^6 AkR leukaemic cells were injected intravenously on day −4 into mice of groups IV, VI and VIII and intraperitoneally into mice of groups II, V and VII.

When the route of injection was the same the BCG and the leukaemic cells were given in the same injection.

The mice were kept under observation until the 180th day, each mouse that died was autopsied and its spleen examined for the presence of leukaemia.

RESULTS

The results are summarized in Table 1 and Figs. 1 and 2. Several points are illustrated by these experiments.

1. Intraperitoneal BCG is, under these conditions, more effective than intravenous BCG (comparison of the mortalities between the groups I and III: $\chi^2 = 8{\cdot}28$, $P<0{\cdot}01$).

2. The AkR cells appeared to be more effective when given intraperitoneally than intravenously in the "a" sub-groups but the difference is not statistically significant.

Table 1. Results of active specific and non-specific immunotherapy in (DBA/2 × C57B1/6) F1 mice according to the route of administration and combinations of treatments

		Mortality	Mean and range of survival times in days of animals dying of leukaemia		
Group I	*a* 10^4ĒG2 Cells	6	19	31·1	44
BCG i.v.	*b* 10^5ĒG2 Cells	10	19	22·3	33
Group II	*a*	4	19	20·0	21
AkR cells i.p.	*b*	10	19	26·0	28
Group III	*a*	1		30	
BCG i.p.	*b*	6	22	30·1	44
Group IV	*a*	7	22	28·1	34
AkR cells i.v.	*b*	10	21	24·3	30
Group V	*a*	4	23	29·7	34
BCG i.v. +AkR i.p.	*b*	10	19	21·0	26
Group VI	*a*	5	21	24·0	26
BCG i.p. +AkR i.v.	*b*	7	19	25·6	33
Group VII	*a*	5	26	39·2*	91
BCG i.p. +AkR i.p.	*b*	8	20	26·4	35
Group VIII	*a*	4	22	32·0	40
BCG i.v. +AkR i.v.	*b*	6	20	26·0	29
Group IX	*a*	10	21	34·5	44
Control	*b*	10	21	27·0	34

*1 late death, the median survival was 26 days.

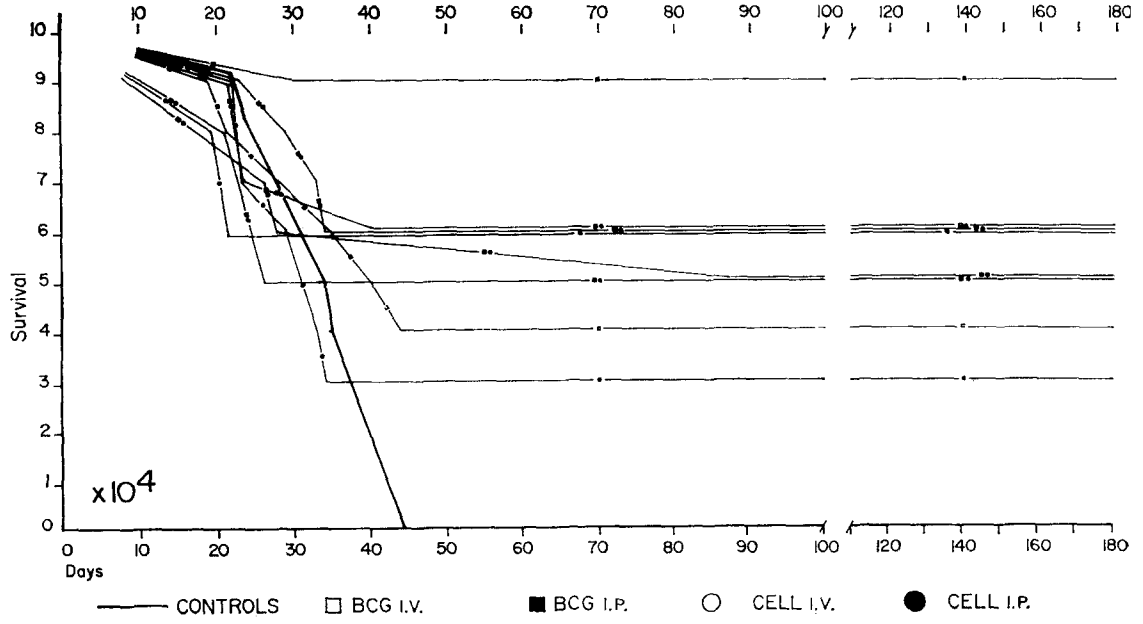


Fig. 1. Active immunotherapy at day −4 of 10^4 E♂G2 leukaemic cells intraperitoneally in (DBA/2 × C57B1/6) F1 recipients.
■ *BCG i.p.* □ *BCG i.v.*
● *AkR cells i.p.* ○ *Akr cells i.v.*

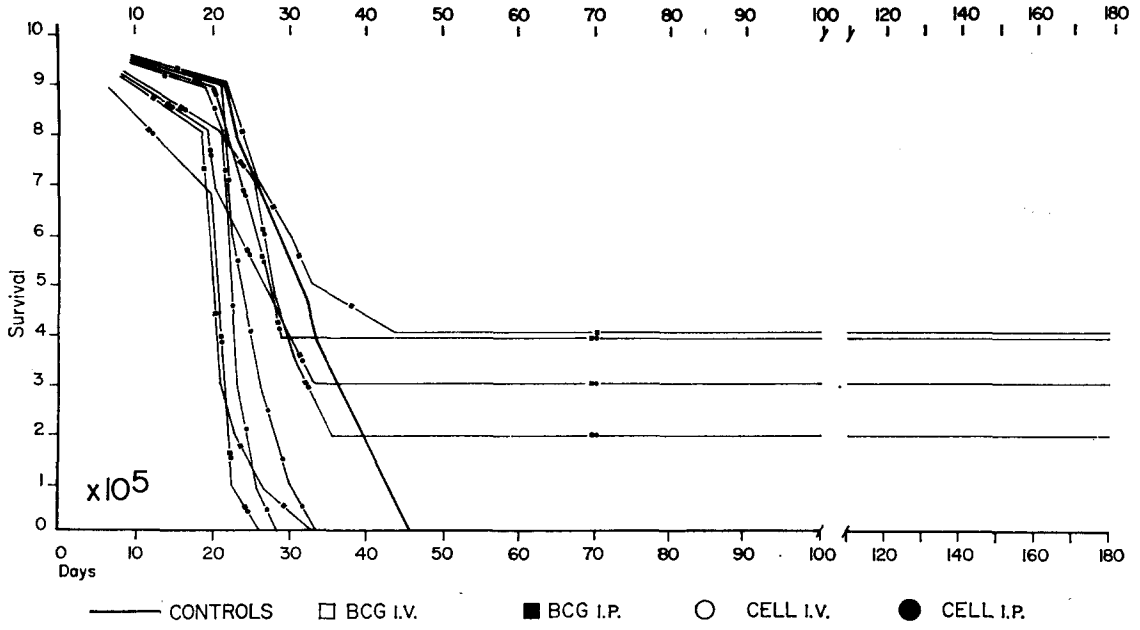


Fig. 2. Active immunotherapy at day −4 of 10^5 E♂G2 leukaemic cells intraperitoneally in (DBA/2 × C57B1/6) F1 recipients.

■ *BCG i.p.* □ *BCG i.v.*
● *AkR cells i.v.* ○ *AkR cells i.v.*

3. Intraperitoneal BCG alone appears to be more effective than intraperitoneal BCG in combination with AkR cells, either given intraperitoneally or intravenously. When the results from intraperitoneal BCG alone are compared to those of intraperitoneal BCG given in addition to AkR cells, there is a significant difference in the mortality that favours BCG alone ($\chi^2 = 4{\cdot}12$, $P < 0{\cdot}05$).

4. Intravenous BCG alone (group I) is less effective than intravenous BCG in combination with intravenous AkR cells (group III) ($\chi^2 = 3{\cdot}96$, $P < 0{\cdot}05$); there was no significant difference between intravenous BCG alone and intravenous BCG combined with intraperitoneal AkR cells.

5. The intraperitoneal AkR cells alone (group II) gave results that were rarely different from those obtained with these cells and the addition of BCG given intravenously or intraperitoneally (groups V and VII).

6. Intravenous AkR cells (group IV) gave less good results than intravenous AkR cells with intravenous BCG (group VIII) ($\chi^2 = 5{\cdot}58$, $P < 0{\cdot}05$), or intraperitoneal BCG (group VI), but the difference between groups IV and VI was not significant.

The mortality curves (Figs. 1 and 2) clearly illustrate the break in the fall of the curve and the development of a plateau that we have previously described in mice [1] and in man [3, 6].

At first the treated animals died with the same frequency as the untreated controls, then, at varying levels, according to the effectiveness of the treatment, the mortality stopped in the treated animals and the curve formed a plateau. The break in the mortality curves was between the 20th and the 45th day, whilst the mortality curves in the untreated animals fell regularly until none survived.

Of the 58 treated animals which were surviving at the 45th day, only one died later with a leukaemia on the 91th day, the remainder were followed to the 180th day.

DISCUSSION

The results of these experiments show the importance of the route of introduction in active immunotherapy. However, it is true to say that these results may only be applicable to the active immunotherapy of the particular leukaemia and the particular conditions we have used. The fact that the compatible E♂G2 leukaemic cells were injected intraperitoneally probably exerts a considerable effect upon the results of therapy.

The timing of the immunotherapy plays an equally important role. We have chosen an active immunotherapy given before the com-

patible leukaemic cells as this gives the most reproducible results. Our previous studies have demonstrated that active immunotherapy can act on a population of leukaemic cells after they have been decreased in number by chemotherapy [4, 7]. As the mechanism of action is the same the distinction between immunotherapy of murine leukaemias made before or after the injection of compatible leukaemic cells seems to be artificial and not justified.

The delay between specific active immunotherapy and the injection of the leukaemic cells is an equally important factor. Our earlier results showed that the optimum time to inject BCG was between the 10th and the 15th day before injecting the leukaemia cells [1, 4]. One of the aims of the present experiments was to examine the effectiveness of combinations of active specific immunotherapy and non-specific immunotherapy; for this reason we have altered the time of injection to the 4th day, which is the optimum time to obtain active immunotherapy from allogeneic leukaemic cells; also we did not wish the maximal therapeutic effect of BCG to mask the potential beneficial action of the combination of BCG with allogeneic leukaemic cells.

The results can not be directly extrapolated to other systems for active immunotherapy of experimental leukaemias, and, for very good reasons, to man. The point that has emerged is the importance of the route of administration of the immunotherapy. The clear superiority of the intraperitoneal BCG compared with intravenous BCG might be due to the fact that it is acting at the site where the leukaemia was inoculated, intraperitoneally.

The study of the combinations of therapy brought to our attention, for the first time, an unexpected fact: not only are certain combinations of active specific and non-specific immunotherapy unable to be more effective than the most effective of the two forms of immunotherapy given alone [4], but they may even be less effective: intraperitoneal BCG alone gave better results than when given in combination with AkR cells. The explanation of this phenomenon is not clear; addition of immunotherapeutic effects and facilitation can be considered, but in that case one would expect a shortening of the survival time of the animals dying of leukaemia in the groups given BCG intraperitoneal + AkR cells compared with the leukaemic mice in the BCG intraperitoneal group; however this difference, if it exists, is small and not significant. Another explanation could be an antigenic competition [8]: a part of the possible immune reaction against the specific leukaemic antigens of the E♂G2, stimulated to maximum activity by the intraperitoneal BCG, could be diverted, in the groups with BCG + AkR cells, to a reaction against the normal allogeneic antigens of the AkR cells. To prove this hypothesis it will be interesting to repeat these experiments and block specifically the reaction against the H-2^k antigens of the AkR cells by injecting anti-H-2^k antibody [9].

The addition of active specific and non-specific immunotherapy does have some advantage; the combination of BCG intravenously and AkR cells intravenously is more effective than each of these stimuli given intravenously alone.

The mortality curves of the treated groups compared with those of the controls can be resolved into two distinct phases. In the first phase the rate of decline of the survivors in the controls and treated falls in parallel, then at variable periods of time the curve of the treated animals is broken and enters a plateau whilst the controls continue to decline unremittently. These curves illustrate the all or none effect of active immunotherapy: the leukaemia is either completely cured (one late relapse in 58 animals) or it is virtually unaffected. This remarkable contrast between the animals unaffected and those cured by active immunotherapy cannot be explained in our experiments by genetic differences in sensitivity to the leukaemia or by possible immune reactions controlled by the genome, as they occurred in genetically identical (DBA/2 × C57B1/6) F1 animals and given a genetically controlled C57B1/6 leukaemia.

It seems that an early event determines in an almost absolute fashion the efficacy of the immunotherapy. This event might be related to the leukaemia, for example to the implantation of leukaemic cells in sites where the immunological reactions can not affect them; or related to the recipient, perhaps its immunological status at the time of active immunotherapy and the injection of leukaemic cells. An immunodepression following an intercurrent infection, or other immunological reactions already in progress, may be capable of exerting an untoward effect, as the result of antigenic competition with the possible reactions against a compatible leukaemia [8].

The factors that can play a role in the efficacity of immunotherapy appear to be numerous, very intricate and of critical importance. These facts emphasize the requirement for large randomized clinical trials to determine the optimum conditions for active immunotherapy of human leukaemias.

REFERENCES

1. J. L. AMIEL, Immunothérapie active non spécifique par le BCG, de la leucémie virale E♂G2 chez des receveurs isogéniques. *Rev. franç. Etud. clin. biol.* **12,** 912 (1967).
2. G. MATHÉ, Immunothérapie active de la leucémie L 1210 appliquée après la greffe tumorale. *Rev. franç. Etud. clin. biol.* **13,** 881 (1968).
3. G. MATHÉ, J. L. AMIEL, L. SCHWARZENBERG, M. SCHNEIDER, A. CATTAN, J. R. SCHLUMBERGER, M. HAYAT and F. DE VASSAL, Active immunotherapy for acute lymphoblastic leukaemia. *Lancet* **i,** 697 (1969).
4. J. L. AMIEL and M. BÉRARDET, Essais de traitement de la leucémie E♂G2 associant chimiothérapie et immunothérapies actives spécifique et non-spécifique. *Rev. franç. Etudes clin. biol.* **14,** 685 (1969).
5. J. L. AMIEL and M. BÉRARDET, Induction d'une insuffisance immunitaire chez la souris adulte par l'utilisation additive d'un stimulant non-spécifique des réactions immunitaires et d'une chimiothérapie immunodépressive. *Rev. franç. Etud. clin. biol.* **14,** 912 (1969).
6. G. MATHÉ, J. L. AMIEL, L. SCHWARZENBERG, M. SCHNEIDER, M. HAYAT and F. DE VASSAL, Preliminary results of a new protocol for the active immunotherapy of acute lymphoblastic leukaemia: inhibition of the immunotherapeutic effect by vincristine or adamantadine. *Europ. J. clin. biol. Res.* In press (1971).
7. J. L. AMIEL and M. BERARDET, An experimental model of active immunotherapy preceded by cytoreductive chemotherapy. *Europ. J. Cancer* **6,** 557 (1970).
8. B. KAYIBANDA and J. L. AMIEL, Effects of heterospecific antilymphocyte serum on mouse spleen cells *in vivo*. *Europ. J. clin. biol. Res.* **15,** 437 (1970).
9. R. MOTTA, The passive immunotherapy of murine leukaemia. The production of antisera against leukaemic neoantigens. *Europ. J. clin. biol. Res.* **15,** 161 (1970).

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Chalone-Induced Mitotic Inhibition in the Hewitt Keratinising Epidermal Carcinoma of the Mouse

W. S. BULLOUGH and JOHANNA U. R. DEOL
Mitosis Research Laboratory, Birkbeck College, University of London, Great Britain

Abstract—*The cells of the Hewitt epidermal keratinising carcinoma synthesize the epidermal chalone so that as the tumour grows the chalone concentration in the body rises and the mitotic activity of the normal epidermis falls. The mitotic activity of the sebaceous glands, which is controlled by a different chalone, remains unaffected.*

The tumour cells are also responsive to the epidermal chalone so that when the chalone concentration is artificially raised the mitotic activity of the tumour is reduced. With the impure chalone preparations at present available, this is not so easily demonstrated in vivo *as* in vitro*; also* in vitro *the importance of the simultaneous presence of adrenalin and a glucocorticoid hormone can be demonstrated.*

These results are closely similar to those previously obtained with 7 other types of tumour. It appears that in all of them the cells suffer from an inadequate chalone content, due possibly to an abnormally high rate of chalone loss into the blood. The question is raised whether an intracellular chalone inadequacy may be typical of tumours in general.

INTRODUCTION

RECENTLY Bullough and Deol [1] have reviewed evidence obtained from a wide range of fast-growing transplantable tumours, and have suggested that a common defect in each case may be an inadequate intracellular concentration of the antimitotic chalone of the tissue from which the tumour originated. So far seven different tumours have been studied from the chalone point of view (two epidermal carcinomata [2], two melanomata [3], two granulocytic leukaemias [4], and a lymphoma [5]); but in all of them it has been found that the cells still synthesize the appropriate tissue chalone and that they still respond by mitotic inhibition when extra chalone is given. In the three cases (two melanomata [6] and a granulocytic leukaemia [7, 8]) in which large enough amounts of pure enough preparations of the appropriate tissue chalones were available, it proved possible to destroy the tumours either temporarily or permanently.

To add to the existing information on tumours and chalones, a study has now been made of the Hewitt epidermal carcinoma of the mouse. This carcinoma, which arose spontaneously in 1965, has been described by Hewitt *et al.* [9], who have also emphasized that it "is indistinguishable from its clinical homologue".

Accepted 24 March 1971.

MATERIAL AND METHODS

The tumour

The Hewitt epidermal keratinizing squamous carcinoma was maintained by subcutaneous inoculation into WHT/Ht albino mice, which was the strain in which the tumour first arose [9]. It was fast-growing and, in the present experiments, the mice, which were all males, either died or had to be destroyed after about a month.

Typically the tumour showed central keratinization and peripheral mitosis [1], and it has never shown spontaneous remission. Hewitt *et al.* [9] have given several reasons for concluding that transplantation immunity is negligible.

The chalone and the stress hormones

The preparations of the epidermal chalone were derived from crude aqueous extracts of macerated pig skin and were partly purified as the 71–80% ethanol precipitate [10, 11]. They were prepared by N. V. Organon (Oss, The Netherlands) and were supplied lyophilized with mannitol. Before use enough distilled water was added to make an isotonic mannitol solution. The tumour extracts, which were tested for their chalone content, were freshly made water extracts of tumour macerates.

The adrenalin was dissolved in distilled water and was in the form of the relatively stable bitartrate; the weights recorded in the tables refer only to the adrenalin part of the molecule. The hydrocortisone was dissolved in a minimal amount of ethanol and then made up to volume with distilled water.

The in vivo *and* in vitro *techniques*

In the *in vivo* experiments the tumours studied ranged between about 10–22 days post-implantation. In all cases 4 hr before death by neck-pulling each mouse received an injection of 0·1 mg colcemid in 0·25 ml water in order to accumulate the developing mitoses in the metaphase. It was considered unlikely that a diurnal mitotic rhythm would exist in such a mitotically active tumour, but since coincident studies were also made of the mitotic activity of the epidermis and sebaceous glands, which both show such a rhythm, all the colcemid injections were made at 11·00 hr and all the mice were killed at 15·00 hr. This was a normal sleep period when both epidermal and sebaceous gland mitosis was maximal. Fixation was in aqueous Bouin, sectioning was at 7 μ, and staining was in Ehrlich haematoxylin and eosin.

In the *in vitro* experiments small tumour fragments, about 2·5 mm cube, were cut from the peripheral mitotic region and were maintained at 38°C in a phosphate-buffered saline medium with 0·02 M glucose and an oxygen gas phase (for details, see Bullough and Laurence [12]). As before, 4 hr before an experiment ended, colcemid was added to a concentration of 4 μg/ml. Each tumour fragment was sectioned completely from side to side.

The assessment of mitotic rates

In the tumour sections the mitotic counts were made under oil on unit section areas of 0·6 sq mm. In the *in vivo* studies the counts were all made within the peripheral tumour zone, which evidently consisted entirely of cells with a mitotic potential. In the *in vitro* studies, although the tumour fragments were taken from this peripheral zone, it was usually found that the highest mitotic activity again tended to be peripheral, which may be taken to indicate the existence, either inwards or outwards, of a diffusion gradient of some important metabolite. In these circumstances, to obtain comparable mitotic counts, it was necessary in each case to search through all the sections until the region of highest mitotic activity was located. The counts were then all made within this region.

In the ear epidermis the mitotic counts were made on unit section lengths of 1 cm. In the sebaceous glands the method devised by Bullough and Laurence [13] was used: the mitoses were counted in unit section areas represented by 50 consecutive gland sections which haphazardly passed centrally or peripherally through the glands.

OBSERVATIONS

The chalone content of the carcinoma

The first experiments were designed to test for the presence of the epidermal chalone in the Hewitt carcinoma. The diagnostic characteristic of this chalone [14] is that it inhibits mitotic activity in mouse ear epidermis during the first 4 hr *in vitro* but not during the second 4 hr when the endogenous adrenalin has been lost, and that its action can be restored during the second 4 hr if the epidermis is briefly washed in a dilute adrenalin solution (0·25 μg/ml). The results in Table 1 show that mouse ear epidermis reacts in this way to the tumour extracts.

A second diagnostic characteristic of the epidermal chalone is the tissue-specificity of its anti-mitotic action. It follows that the mitotic depressions shown in Table 1 should be confined to the epidermis and should not be seen, for instance, in the sebaceous glands which were present in the same pieces of skin and which are known to be controlled by their own tissue-specific chalone [13]. Mitotic counts made on the epidermis and the sebaceous glands are shown in Table 2, from which it is clear that the mitotic inhibition occurred only in the epidermis.

The conclusion must be that water extracts of the Hewitt epidermal carcinoma contain appreciable quantities of the epidermal chalone.

Table 1. *Demonstration of the presence of epidermal chalone in Hewitt epidermal carcinoma*

4 ml culture medium with:	1st 4 hr *in vitro* Mitoses M ± S.E.	Mitotic depression	Adrenalin wash	2nd 4 hr *in vitro* Mitoses M ± S.E.	Mitotic depression
Control	9·3 ± 0·30	—	0	9·6 ± 0·21	—
			+	9·6 ± 0·30	0
0·4 mg chalone extract*	5·1 ± 0·17	45%	0	9·7 ± 0·20	0
			+	5·1 ± 0·30	47%
10 mg tumour extracted†	5·2 ± 0·26	44%	0	9·7 ± 0·26	0
			+	5·0 ± 0·17	48%
100 mg tumour extracted†	4·0 ± 0·38	57%	0	10·0 ± 0·24	0
			+	4·5 ± 0·21	53%
1000 mg tumour extracted†	3·8 ± 0·20	59%	0	10·8 ± 0·21	0

Average numbers of mitoses arrested by colcemid in 4 hr *in vitro* in unit lengths of 1 cm ear epidermis sectioned 7 μ thick. *N*, number of mice = 10.

*0·4 mg was the weight of a 71–80% ethanol fraction of pig skin and was equivalent to 10 mg of the original crude water-soluble pig-skin extract.

†The weight of fresh tumour material from which the crude water extracts added to the culture medium were made.

Table 2. *Tissue-specificity of epidermal carcinoma extracts*

4 ml culture medium with:	Epidermis Mitoses M ± S.E.	Mitotic depression	Sebaceous glands Mitoses M ± S.E.	Mitotic depression
Control	9·3 ± 0·30	—	8·2 ± 0·39	—
100 mg tumour extracted	4·0 ± 0·38	57%	9·4 ± 0·18	0

Average numbers of mitoses arrested by colcemid in 4 hr *in vitro* in unit lengths of 1 cm ear epidermis and in groups of 50 consecutive sections of sebaceous glands sectioned 7 μ thick. *N*, number of mice = 10.

A subsidiary question was whether this epidermal chalone is synthesized within the tumour cells or whether these cells merely accumulate it from the blood. If they synthesize the chalone then a mouse with a growing tumour should contain an ever-increasing chalone concentration, which, via the blood, should depress the mitotic activity of the epidermis but not that of the sebaceous glands. The figures in Table 3 in fact show that in the presence of the Hewitt carcinoma the epidermal mitotic rate was depressed by about 50% while the sebaceous gland mitotic rate was unaffected. The table also shows the progressive fall in the epidermal mitotic rate as the carcinomata became older and larger, although the differences between adjacent figures are not significant.

The conclusions are that the fall in the epidermal mitotic activity of mice with carcinomata could not have been due to a general metabolic deterioration or to an increasing level of stress since the sebaceous glands remained unaffected and that the tissue-specific fall in the epidermal mitotic rate was most probably the result of an increasing serum concentration of the epidermal chalone, which must have been synthesized by the tumour cells themselves.

The carcinoma cell response to the epidermal chalone

The next question concerned the ability of the Hewitt carcinoma cells to react by mitotic inhibition to the epidermal chalone. In normal epidermis the response of the cells to the chalone is complex and it requires the presence of both adrenalin and hydrocortisone. These hormones are not in themselves mitotic inhibitors but when injected they cause a mitotic inhibition due to their cooperation with the endogenous epidermal chalone [14, 15]. Since endogenous chalone exists within the Hewitt carcinoma, the effects of subcutaneous injections of these hormones was determined, but from the results in Table 4, it is clear that with the doses used (and the adrenalin dose was near-lethal) there was no significant mitotic depression.

Table 3. Effect of epidermal carcinoma on skin mitoses

	Epidermis		Sebaceous glands	
	Mitoses M ± S.E.	*N*	Mitoses M ± S.E.	*N*
Control	33·4 ± 0·76	6	11·7 ± 0·77	6
With tumours				
10 days old	20·4 ± 2·10	4	—	
14 days old	18·4 ± 1·64	5	—	
18 days old	13·5 ± 2·91	4	—	
22 days old	11·6 ± 2·63	4	—	
All tumour data	16·1 ± 1·36	17	11·5 ± 0·87	13
Average mitotic depression	52%		0	

Average numbers of mitoses arrested by colcemid in 4 hr *in vivo* in unit lengths of 1 cm ear epidermis and in groups of 50 consecutive sebaceous gland sections sectioned 7 μ thick. *N*, number of mice.

Table 4. Action of stress hormones on epidermal carcinoma in vivo

Dose given	Mitoses M ± S.E.	*N*	Mitotic depression
Control	110·7 ± 2·16	8	—
10 μg adrenalin	111·7 ± 2·59	5	0
10 μg hydrocortisone	112·3 ± 3·77	10	0
10 μg adrenalin + 10 μg hydrocortisone	102·7 ± 2·99	8	7%

Average numbers of mitoses arrested by colcemid in 4 hr in 0·6 sq mm of outer mitotic zone sectioned 7 μ thick. *N*, number of tumours.

The most obvious conclusions are: that the lack of any significant mitotic depression may be due either to some inadequacy in the chalone control mechanism or to an abnormally low chalone concentration within the tumour cells.

If the intracellular chalone concentration is abnormally low then the injection of extra chalone may suppress the mitotic activity of the tumour; the results of experiments to test this are shown in Table 5. For comparison the reaction to the chalone shown by the epidermis of the same mice is also recorded. It may be noted that extra stress hormones were not given coincidentally since it had previously been found with the Vx2 carcinoma [2] and with two melanomata [3] that the endogenous stress hormone concentration is normally high enough to allow the chalone action to develop.

When compared with earlier results from the Vx2 epidermal carcinoma [2] the mitotic depressions shown in the table are small and their significance is not great. However it was not considered feasible to increase the chalone dose greatly since the impurities in the pig-skin extracts could have caused a generalized

Table 5. Action of epidermal chalone on epidermal carcinoma and on epidermis in vivo

	Carcinomata			Ear epidermis		
Colcemid acting for:	Mitoses M ± S.E.	*N*	Mitotic depression	Mitoses M ± S.E.	*N*	Mitotic depression
2 hr:						
Control	59·5 ± 3·86	8	—	—		—
300 mg equivalent of chalone*	51·2 ± 3·58	5	14%	—		—
4 hr:						
Control	141·9 ± 5·27	13	—	18·4 ± 1·64	5	—
300 mg equivalent of chalone*	115·8 ± 2·37	7	18%	9·6 ± 1·49	5	48%

Average numbers of mitoses arrested by colcemid in 2 or 4 hr in 0·6 sq mm of outer mitotic zone of tumour and in 1 cm length of ear epidermis sectioned 7 μ thick. *N*, number of tumours or of mice.

*12 mg of a 71–80% ethanol fraction of pig skin.

metabolic reaction. In some experiments a higher dosage was achieved by means of two chalone injections, one at the beginning of the 4-hr period of colcemid action and the other 1 hr later, but no greater mitotic depression was obtained. The only practicable way to increase the dose *in vivo* would be to use purer preparations of the epidermal chalone, but these, which were in fact used in the case of the Vx2 carcinoma [2], were not available.

To overcome this difficulty the experiments were repeated *in vitro* where higher chalone concentrations can easily be used. In such experiments the endogenous adrenalin is quickly lost, and therefore the effects of the two stress hormones and of the three substances together were also checked, The concentration of the two hormones (2·5 μg/ml) was that found to be effective with several tumours previously studied *in vitro*. The results are in Table 6.

Table 6. *Action of epidermal chalone and stress hormones on epidermal carcinoma* in vitro

4 ml culture medium with:	Mitoses M ± S.E.	*N*	Mitotic depression
Control	16·5 ± 0·72	17	—
10 μg adrenalin	14·8 ± 0·83	9	0
100 μg adrenalin	14·6 ± 0·79	10	0
10 μg hydrocortisone	16·6 ± 0·52	9	0
100 μg hydrocortisone	14·2 ± 0·58	9	0
0·4 mg chalone extract*	16·0 ± 1·43	6	0
4·0 mg chalone extract*	14·0 ± 1·22	6	0
10 μg adrenalin + 10 μg hydrocortisone	11·0 ± 0·61	14	33%
100 μg adrenalin + 100 μg hydrocortisone	5·8 ± 0·60	13	65%
10 μg adrenalin + 10 μg hydrocortisone + 4·0 mg chalone extract*	5·0 ± 0·20	15	70%
100 μg adrenalin + 100 μg hydrocortisone + 4·0 mg chalone extract*	2·1 ± 0·21	14	87%

Average numbers of mitoses arrested by colcemid in 4 hr in 0·6 sq mm of pieces of tumour sectioned 7 μ thick. *N*, numbers of tumours.

*0·4 mg and 4·0 mg were the weights of a 71–80% ethanol fraction of pig skin; they were equivalent respectively to 10 mg and 100 mg of the original crude water-soluble pig-skin extract.

The conclusions are that with adrenalin or hydrocortisone or chalone alone no significant mitotic depressions were seen that when adrenalin and hydrocortisone were used together (in the presence of endogenous chalone) dose-dependent mitotic depressions were obtained and that when the chalone was used together with the two stress hormones the dose-dependent mitotic depressions were even greater. These results *in vitro* are closely similar to those previously obtained with the Vx2 epidermal carcinoma [2], with an amelanotic melanoma [3], and with a lymphoma [5].

General conclusions

The results obtained with the Hewitt epidermal carcinoma are consistent with the following conclusions: that the tumour cells continue to synthesize the epidermal chalone (although possibly at a reduced rate); that this chalone escapes in considerable quantities into the blood to cause a deepening depression of the epidermal mitotic rate; that the tumour cells have a low chalone content (perhaps partly because of an abnormal rate of loss into the blood [4]); and that when in the presence of adrenalin and hydrocortisone the chalone content of the tumour cells is increased their mitotic rate is depressed.

DISCUSSION

These results have a two-fold interest: first, they add one more example to the growing list of tumours which are known to show mitotic inhibition when treated with the chalone of their tissue of origin [1], and second, they relate to a tumour which, although of animal origin, has been described as being "truly representative of a large and important class of clinical malignancy" [9]. Thus the results could have relevance to human tumour chemotherapy.

The tumours which have already been studied for their chalone reactions are: the Vx2 epidermal carcinoma (rabbit) [2], the Chernozemski epidermal carcinoma (hamster) [16, 17], the Harding-Passey melanoma (mouse) [3, 6], an amelanotic melanoma (hamster) [3], the L5178Y lymphoma (mouse) [5], the Shay granulocytic leukaemia (rat) [4, 8], and several cases of spontaneous granulocytic leukaemia (man) studied *in vitro* [18]. In each of these 7 tumour types from 5 animal species it appears that the high mitotic activity may be the result of a reduced intracellular chalone concentration, and in each, when exogenous chalone is administered in the right dose and in the right circumstances, this high mitotic activity can be suppressed. The Hewitt epidermal carcinoma can be added to this list.

The main practical difficulty preventing the active exploitation of these results is that of obtaining large enough quantities of pure enough preparations of the various known chalones. Nevertheless the melanocyte chalone has been used to destroy two types of melanoma

[6] and the granulocytic chalone has been used to destroy a granulocytic leukaemia [7, 8]. In both cases when the dosage was adequate the tumour destruction was permanent: the mice cured of melanoma finally died in old age [19] while the baby rats cured of granulocytic leukaemia are now adult and have bred normally [18]. However, no similar attempts to destroy the Hewitt carcinoma, or any other epidermal carcinoma, can be made until adequate supplies of more highly purified extracts of the epidermal chalone become available.

There remains the important question of whether the results obtained regarding the chalone reactions of the first 8 tumours to be studied are abnormal or whether they are widely applicable to tumours in general. Much more information is needed but Bullough and Deol [1] have already questioned whether a common characteristic of a tumour cell may be an inadequate intracellular chalone concentration due, for instance, to inadequate synthesis or to a defective cell membrane which allows the chalone to escape at too high a rate into the blood. Such a membrane defect is evidently typical of the Shay granulocytic leukaemia [4], and in the Hewitt carcinoma, as in the Vx2 carcinoma [2], the rising chalone concentration in the blood can be measured in terms of the falling epidermal mitotic rate.

Such a rising chalone concentration would also be expected to act back on the tumour itself. In theory the larger the tumour the higher should be the chalone concentration within the limited body space, and the slower the rate of tumour growth. In practice a progressive slowing of tumour growth has often been described, but it has commonly been explained in terms of an inadequate blood supply (which is sometimes certainly an important factor [1]), or of the general metabolic deterioration of the host (which also certainly occurs). However, from surveys of the available evidence, Laird [20, 21] has concluded that the continuous deceleration of tumour growth "is not simply due to failure of the host to support growth" but is dependent on some "feedback control . . . to which the tumours are still responsive to a greater or lesser extent", while, from a similar survey, Burns [22] has concluded "that some tumours may regulate their own growth as normal or non-neoplastic tissues do by the production of a homologous specific mitotic inhibitor".

Such studies suggest strongly that tumours in general do retain in some degree the mitotic control mechanisms of their tissues of origin. The studies already made on tumours and chalones suggest equally strongly that better techniques for the purification and preparation of the various chalones should be urgently sought so that the value of these substances as tumour-specific biotherapeutic agents can be adequately assessed.

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REFERENCES

1. W. S. Bullough and J. U. R. Deol, The pattern of tumour growth. *Symp. Soc. exp. Biol.* **25,** 255 (1971).
2. W. S. Bullough and E. B. Laurence, Control of mitotis in rabbit Vx2 epidermal tumours by means of the epidermal chalone. *Europ. J. Cancer* **4,** 587 (1968).
3. W. S. Bullough and E. B. Laurence, Control of mitotis in mouse and hamster melanomata by means of the melanocyte chalone. *Europ. J. Cancer* **4,** 607 (1968).
4. T. Rytömaa and K. Kiviniemi, Control of DNA duplication in rat chloroleukaemia by means of the granulocyte chalone. *Europ. J. Cancer* **4,** 595 (1968).
5. W. S. Bullough and E. B. Laurence, The lymphocytic chalone and its antimitotic action on a mouse lymphoma *in vitro*. *Europ. J. Cancer* **6,** 525 (1970).
6. U. Mohr, J. Althoff, V. Kinzel, R. Süss and M. Volm, Melanoma regression induced by chalone: a new tumour inhibiting principle acting *in vivo*. *Nature (Lond.)* **220,** 138 (1968).
7. T. Rytömaa and K. Kiviniemi, Chloroma regression induced by the granulocytic chalone. *Nature (Lond.)* **222,** 995 (1969).
8. T. Rytömaa and K. Kiviniemi, Regression of generalised leukaemia in rat induced by the granulocytic chalone. *Europ. J. Cancer* **6,** 401 (1970).
9. H. B. Hewitt, D. P-S. Chan and E. R. Blake, Survival curves for clonogenic cells of a murine keratinizing squamous carcinoma irradiated *in vivo* or under hypoxic conditions. *Int. J. Radiat. Biol.* **12,** 535 (1967).

10. W. S. Bullough, C. L. Hewett and E. B. Laurence, The epidermal chalone: a preliminary attempt at isolation. *Exp. Cell Res.* **36,** 192 (1964).
11. W. Hondius Boldingh and E. B. Laurence, Extraction, purification and preliminary characterisation of the epidermal chalone. *Europ. J. Biochem.* **5,** 191 (1968).
12. W. S. Bullough and E. B. Laurence, The study of mammalian epidermal mitosis *in vitro*. *Exp. Cell Res.* **24,** 289 (1961).
13. W. S. Bullough and E. B. Laurence, Chalone control of mitotic activity in sebaceous glands. *Cell Tiss. Kinet.* **3,** 291 (1970).
14. W. S. Bullough and E. B. Laurence, Mitotic control by internal secretion: the role of the chalone-adrenalin complex. *Exp. Cell Res.* **33,** 176 (1964).
15. W. S. Bullough and E. B. Laurence, The role of glucocorticoid hormones in the control of epidermal mitosis. *Cell Tiss. Kinet.* **1,** 5 (1968).
16. K. Elgjo and H. Hennings, Epidermal chalone and cell proliferation in a transplantable squamous carcinoma in hamsters. I. *In vivo* results. *Virchows Arch. Abt. B Zellpath.* **7,** 1 (1971).
17. E. B. Laurence and K. Elgjo, Epidermal chalone and cell proliferation in a transplantable squamous cell carcinoma in hamsters. II. *In vitro* results. *Virchows Arch. Abt. B Zellpath.* **7,** 8 (1971).
18. T. Rytömaa, personal communication.
19. U. Mohr, personal communication.
20. A. K. Laird, Dynamics of tumor growth. *Brit. J. Cancer* **18,** 490 (1964).
21. A. K. Laird, Dynamics of growth in tumors and in normal organisms. *Nat. Cancer Inst. Monogr.* **30,** 15 (1969).
22. E. R. Burns, On the failure of self-inhibition of growth of tumours. *Growth* **33,** 25 (1969).

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Comparative Ultrastructural Studies of Cells from Tumorigenic and Non-Tumorigenic Cultures Derived from the 6C3HED Mouse Ascites Tumor

W. J. BANKS, Jr.,* M. K. BHATNAGAR† and J. F. MORGAN‡

Department of Veterinary Anatomy, Western College of Veterinary Medicine, and Department of Cancer Research, College of Medicine, University of Saskatchewan, Saskatoon, Saskatchewan, Canada

Abstract—*The ultrastructural features of the* 6C3HED-*mouse ascites tumor cell and its derived tumorigenic and non-tumorigenic cell culture lines are compared. Except for mitochondrial condensed conformations, all other features of the ascites tumor cells* in vivo *were similar to those reported previously. The derived tumorigenic tissue culture cells contained more granular endoplasmic reticulum, a better developed Golgi apparatus and more smooth membranous profiles than the cells harvested* in vivo. *Condensed mitochondria were predominant, but orthodox conformations were encountered in the tumorigenic cell cultures.*

The non-tumorigenic cells were characterized by a better development of cytoplasmic membranes than noted previously. Granular endoplasmic reticulum and Golgi apparatus were well developed and polyribosomes were abundant. Although both condensed and orthodox conformations were encountered, the condensed conformations were predominant. Also, more mitochondria were present in these cells than in the other types. Viral particles (type C) *were observed in the cytoplasm of both* in vivo *and* in vitro *ascites cells.*

The morphological alterations in mitochondrial structure, and the relative increase of endoplasmic reticulum and polyribosomes in the non-tumorigenic tissue cultures, suggest that metabolic and synthetic modifications may be associated with loss of tumorigenicity in vitro.

Tissue culture lines established from presumably normal tissues undergo a spontaneous neoplastic transformation during long-term cultivation *in vitro*, a phenomenon that has received intensive study recently [1–3]. The loss of transplantability by tumor cell cultures during prolonged cultivation *in vitro* has also been reported by several investigators [4–6]. While the mechanism and biological changes associated with this latter phenomenon have not been elucidated, in most instances the loss of transplantability appears to be gradual and not complete [4, 6].

A previous communication from this laboratory described the development of a three-component tumor model system, based on mouse ascites tumors *in vivo*, together with their derived tumorigenic and non-tumorigenic

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*Present address: Department of Anatomy, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins, Colorado.

†Fellow of the Medical Research Council of Canada. Present address: Department of Biomedical Sciences, Ontario Veterinary College, University of Guelph, Guelph, Ontario, Canada.

‡Present address: Department of Biochemistry, University of Saskatchewan, Saskatoon, Canada.

tissue culture lines [7]. Loss of transplantability appeared to be complete [7], the non-tumorigenic tissue culture cells were highly immunoprotective [8] and some change in carbohydrate metabolism appeared to occur during loss of transplantability [9]. The availability of this new tumor model system made possible a comparative study of the ultrastructure of these cells in relation to their tumorigenic potential.

MATERIAL AND METHODS

6C3HED *Ascites tumor*

6C3HED, a near tetraploid mouse-lymphosarcoma ascites cell line was maintained in C3H mice by serial intraperitoneal transplantation at an interval of two weeks.

Ascites tumor cell culture lines

Tumorigenic and non-tumorigenic cell culture lines were derived from 6C3HED ascites tumors by propagating the ascites tumor cells in Blake bottles as monolayer cultures in medium 199 with 5 to 10% calf serum according to the method of Guérin and Morgan [10]. The test of tumorigenicity was conducted at regular intervals by intraperitoneal inoculation of 10^7 cultured cells per mouse. If no tumors developed within 90 days, the cultures were considered as non-tumorigenic [8].

Preparations for electron microscopy

Cell samples from mice and cultures were collected at three different times. 6C3HED ascites cells were collected 9 to 13 days after inoculation in mice. The tumorigenic cell cultures were collected between the 3rd and 6th passage; whereas non-tumorigenic cell culture samples were obtained between the 33rd and 40th passage. The normal passage time for each non-tumorigenic cell line was 7 to 10 days. All samples were washed twice in Hanks' balanced salt solution containing 0·5% calf serum with repeated centrifugation at $900 \times \mathbf{g}$ for 10 min [11]. The cell buttons thus obtained were fixed at 4°C in 6·25% phosphate-buffered glutaraldehyde (pH 7·3) for 2 hr. The glutaraldehyde-fixed cell buttons were washed in a phosphate-buffered sucrose solution (2·5%), pH 7·3 for 1 hr. The glutaraldehyde-fixed and buffered sucrose-washed pellets were rinsed for 1 hr at 4°C in 0·15 M phosphate buffer. Peripheral portions of the pellets were removed, minced into squares approximately 0·1 mm and subjected to post-fixation for $1\frac{1}{2}$ hr in 1% OsO_4 at 4°C [12]. Following a 1-hr rinse in the aforementioned buffer, routine dehydration was undertaken and samples were embedded in Epon-812 [13]. Thick sections (1 μ) were obtained routinely during sectioning with an LKB III ultratome and were stained in 1% toluidine blue. Staining of silver sections was accomplished with uranyl acetate [14] and lead citrate [15]. Unsupported and uncoated specimens were examined with a Zeiss (EM-9A) electron microscope.

RESULTS

Tumorigenic cells, in vivo

Electron microscopic observations indicated that the cell population was composed of two distinct types of viable ascites tumor cells. One was light-staining with a low cytoplasmic density (Fig. 1). Both were extremely pleiomorphic and exhibited a wide variability in size. Other cells present included those in various stages of agonal change, histiocytes and erythrocytes. The following description treats the ascites tumor cells as a single group, since, except in a few specific instances, the dark and light cells had a similar ultrastructural composition.

The most conspicuous features of these cells were their granular cytoplasm and the reduction or total absence of some typical organelles (Fig. 2). Single free ribosomes were abundant, while polyribosomes were observed occasionally. In some instances, the free ribosomes were so plentiful that the resulting contrast between them and other cytoplasmic components was diminished greatly (Fig. 1). Granular endoplasmic reticular profiles were sparse in most cells and absent in others. When present, however, they appeared as single tubular or vesicular units which contained a floccular material of minimal electron opacity. Smooth membranous profiles were scattered throughout these cells and were especially prominent in the large pale-staining cells. These varied from Golgi-like arrangements to large vesicles. Despite the similarity of some of these vesicles to the Golgi apparatus, a distinct Golgi zone was observed infrequently. Multivesicular bodies were also infrequently observed. Fat droplets were present in various numbers in most of these cells. Many were intimately associated with mitochondria (Fig. 2), while others were occasionally associated with a limited lamellar arrangement of membranes.

The mitochondria of these cells were numerous and assumed the condensed con-

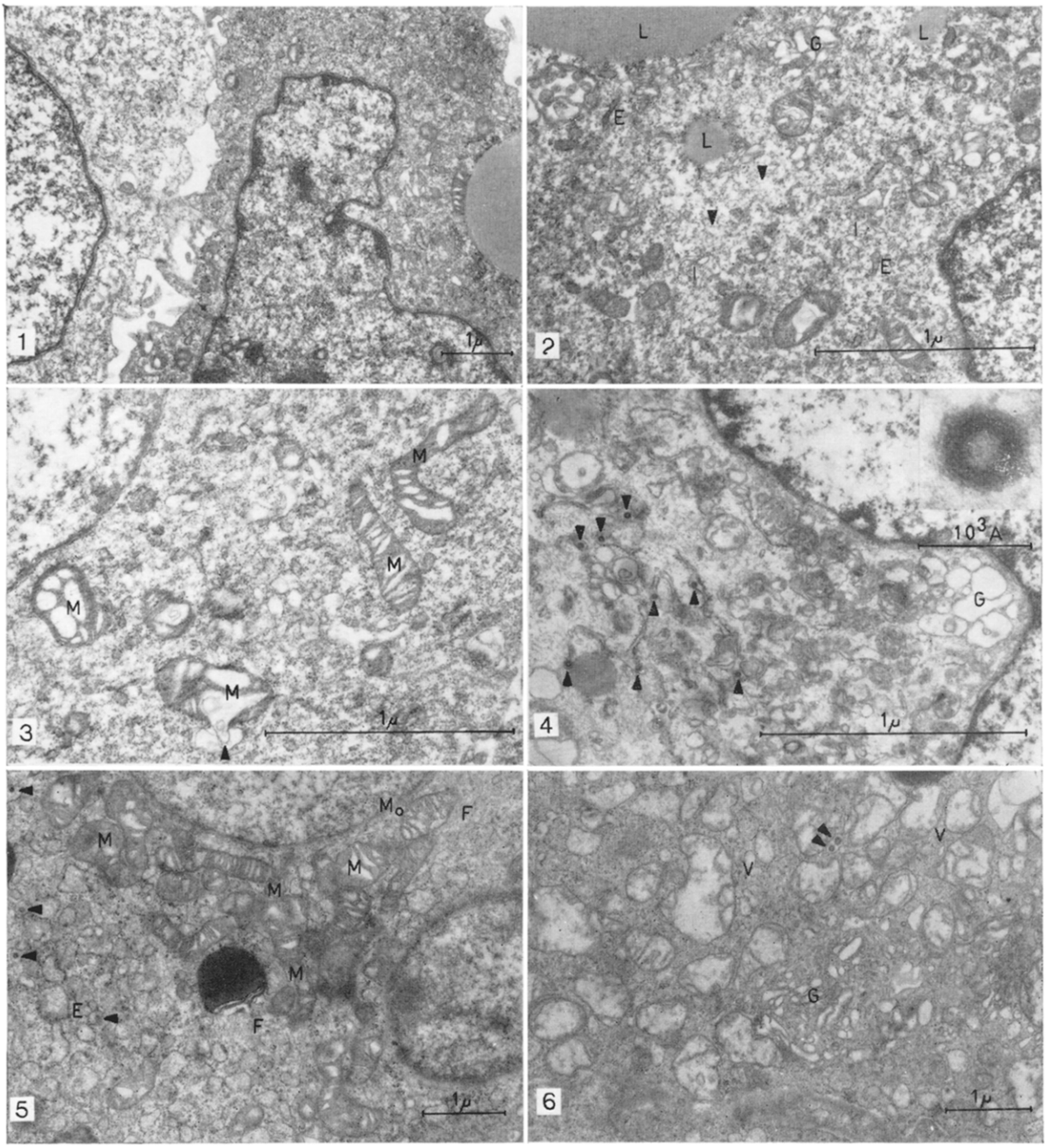


Fig. 1. Two ascites tumor cells from the mouse. Although both these cells are granular and possess a reduced number of cytoplasmic organelles, the cytoplasmic density differences are readily apparent, × 24,000.

Fig. 2. Cytoplasmic components of a low-cytoplasmic density tumor cell from the mouse. Free ribosomes predominate and are interspersed among sparse granular endoplasmic reticular profiles (E). Vesicular portions of the Golgi apparatus (G) and lipid droplets (L) are apparent. Smooth membranous profiles (bars) and cytofilaments (arrows) are scattered throughout the cytoplasm. × 75,600.

Fig. 3. Varied mitochondrial configurations in a tumor cell from the mouse. These mitochondria (M) are typical of those encountered. Note the swelling between the inner and outer mitochondrial membranes (arrow). × 86,400.

Fig. 4. Cytoplasmic constituents of a ruptured tumor cell from the mouse. Cytofilaments are scattered throughout the cytoplasm. The infrequently encountered Golgi apparatus (G) is swollen. Viral particles (arrows) are also present. × 91,800. *Inset: Typical morphology of viral particles that were observed.* × 390,000.

Fig. 5. Cytoplasmic components of tumorigenic-tissue culture cells. Besides numerous mitochondria (M), more granular endoplasmic reticulum (E) is present. Viral particles (arrows) and cytofilaments are also present. Note that one mitochondrion is in the orthodox conformation (Mo). × 29,000.

Fig. 6. Cytoplasm of tumorigenic-tissue culture cells. Smooth vesicles (V), as well as Golgi vesicles (G), are conspicuous in these cells among the numerous enlarged and ruptured mitochondria. Viral particles (arrows) are also present. × 29,000.

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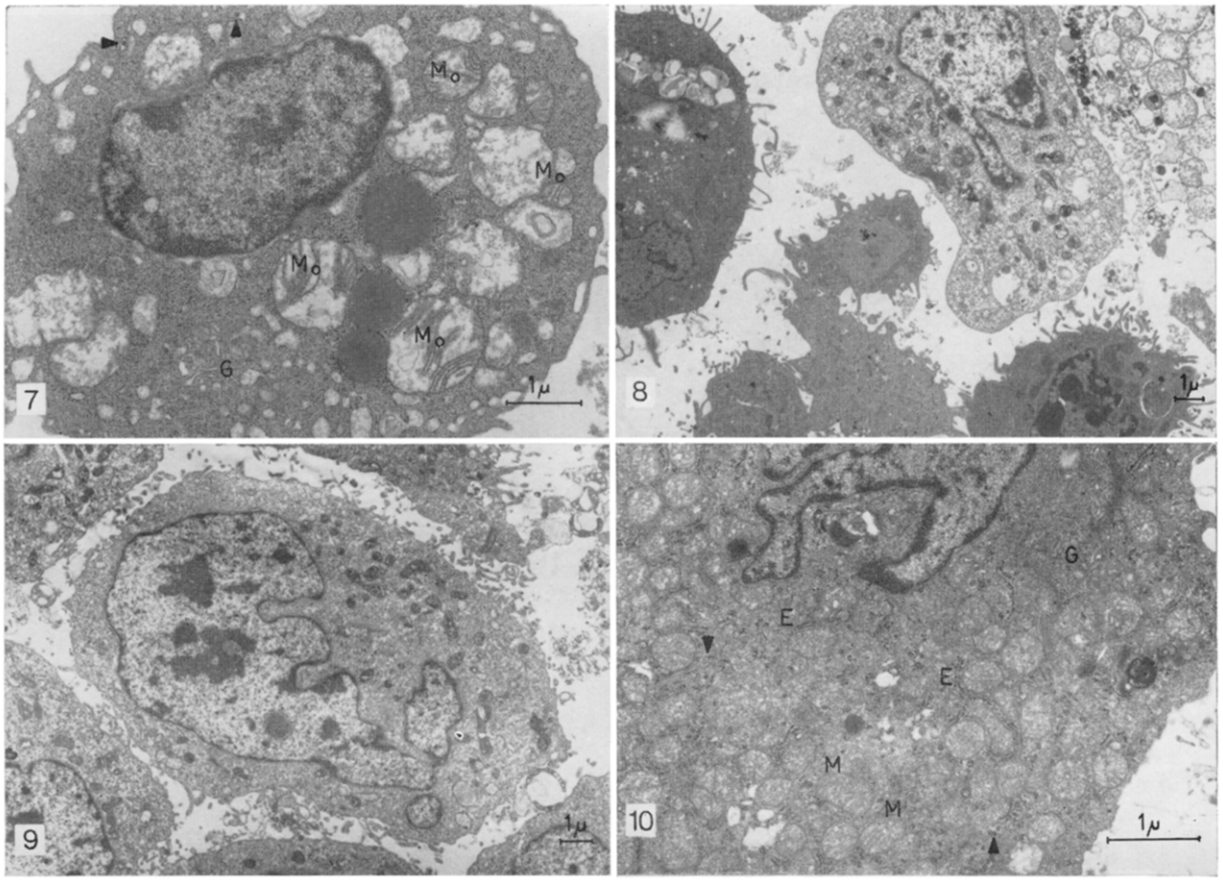


Fig. 7. A tumorigenic-tissue culture cell. The granular cytoplasm contains a distinct Golgi zone (G) and large mitochondria in the orthodox conformation (Mo). Note viral particles (arrows). Glycogen particles are scattered throughout the cell. × 22,000.

Fig. 8. Cells of the non-tumorigenic cell line. The heterogeneity between cells is quite conspicuous. The cytoplasmic components of the dense cells are not readily apparent. × 8000.

Fig. 9. Mitochondria of low cytoplasmic density non-tumorigenic cell. These organelles are quite numerous in this cell line. × 9500.

Fig. 10. Cytoplasmic components of non-tumorigenic cells. Granular endoplasmic reticulum (E) and flattened Golgi lamellae (G) are apparent amidst numerous mitochondria (M) and polyribosomes (arrows) × 27,000.

formation, while those in the orthodox conformation were rarely encountered (Fig. 3). These mitochondria varied from small, rounded structures to large, gourd-like configurations. Although the configuration of the inner membrane of most mitochondria was in the form of cristae, tubules also occurred. Anastomotic and juxtaposed cristae of various sizes and shapes imparted a reticulated pattern to many of these organelles. Swelling of mitochondria was manifested as vesiculations of various sizes between the outer and inner mitochondrial membranes. Various stages of degeneration were also observed, including the formation of intramitochondrial myelin figures and the total disintegration of intramitochondrial components.

Despite the careful attention given the tonicity of all preparatory solutions, a few cells were ruptured. These cells, however, facilitated the observation of otherwise obscure cytoplasmic components. The cytoplasmic matrix was composed of numerous, randomly scattered cytofilaments (Figs. 2 and 4). These occasionally were observed as dense bundles in a juxtanuclear position. More important, viral particles were more readily visible (Fig. 4). These particles, which appeared to be type C, were either free in the cytoplasm or contained within smooth membranes, as well as contained within the confines of the granular endoplasmic reticulum. Some appeared to be budding from these membranes at the time of fixation.

Tumorigenic cells, in vitro

The most conspicuous difference between this group of cells and the *in vivo* cells was that related to the mitochondria. Although the condensed conformational configuration was predominant (Fig. 5), those in the orthodox conformation were encountered more frequently than described in the previous group (Figs. 6 and 7). Not only were the different types observed in adjacent cells, but cells often contained both types (Fig. 5). Both conformations ranged from small, rounded structures to large structures of various shapes. Although they appeared as numerous as the *in vivo* mitochondria, the *in vitro* preparations did not contain condensed mitochondria as large as those previously described.

The type-C virus particles were encountered less frequently in this cell preparation than in tumorigenic cells grown *in vivo* (Figs. 4–7).

Although staining and density characteristics of these cells were similar to those previously described, this cell group was more homogeneous than the *in vivo* cell group. Similarly, these cells were granular, but there was a greater number and variety of organelles present (Fig. 5). Although ribosomes still predominated, there was an increased amount of polyribosomes. Granular endoplasmic reticulum was similar to that previously described; however, there was a slight increase in the amount present. Smooth membranous profiles were numerous and assumed the various shapes described for the *in vivo* cells (Fig. 6). A notable difference, however, was the increased occurrence of a typical Golgi apparatus (Figs. 6 and 7). Multivesicular bodies were occasionally observed. Lipid droplets appeared as described previously; however, lamellar profiles were not observed in association with these inclusions. Glycogen was observed in scattered foci throughout the cell (Fig. 7).

Except for the specific similarities and/or differences discussed, all other features of the *in vitro* tumorigenic cells were similar to the ascites cells *in vivo*.

Non-tumorigenic cells, in vitro

The most conspicuous difference between this cell group and those described previously was related to the mitochondria. Although mitochondria of the orthodox conformation were encountered, the predominant configuration was the condensed conformation. The distribution of these conformations within and among cells, however, was similar to that described for the tumorigenic *in vitro* preparation. Their range of size and shape was similar to that cell group, but, comparatively, the number of mitochondria per cell was higher in this group (Figs. 8 and 9).

The staining and density characteristics of the cells within this population were similar to that described for the *in vivo* specimens (Fig. 8). However, the dense cells of this group were more dense than observed in the *in vivo* population. Notwithstanding the attention given the preparatory and fixative solutions, numerous cells within this group were ruptured or were in various degenerative stages. Except for some notable differences, the non-tumorigenic cells were very similar to the *in vitro* preparation of tumorigenic cells.

These cells were quite granular. Although ribosomes were a dominant feature, polyribosomes were present in greater numbers than noted previously in either of the other preparations. All cytoplasmic membrane systems were as well developed as those observed in the *in vitro* tumorigenic cells (Fig. 10). These included granular endoplasmic reticulum of the tubular and vesicular types, various shapes and sizes

of smooth membranous profiles and a well developed Golgi apparatus with the typical lamellar and vesicular components. Although no quantifications were attempted, the granular endoplasmic reticular profiles may have been slightly better developed than those encountered in the *in vitro* tumorigenic preparations. Lipid droplets were only infrequently encountered and the lamellar profiles associated with them previously were not observed.

An infrequent, but consistent, observation was the occurrence of type-C virus particles. Their distribution within the cell was similar to that described previously. The amount of virus particles that was encountered was appreciably less than that observed *in vivo*.

DISCUSSION

The ultrastructure of 6C3HED-ascites tumor cells observed in this investigation conformed, in most respects, with previous ultrastructural studies of different strains of mouse ascites tumor cells (16–21). However, the salient differences noted in this investigation need elucidation. The presence of dark and light cells among this cell population has not been described previously in mouse ascites tumor cells. A similar heterogeneity among constituent cells has been described for human lung cancer cells [22] and in Yoshida ascites hepatoma cell populations which were fixed by aldehyde-osmium double fixation techniques [23]. Dark and light cells have been demonstrated in Ehrlich-ascites tumor cell populations which were subjected to a 1-min hypotonic treatment [11]. Numerous possibilities exist as to the significance of this cellular heterogeneity. It is possible that the light and dark cells may represent different stages of the cell cycle. This is supported by the observation that ascites fluid usually contains tumor cells in various stages of the mitotic cycle [24]. Another possibility is that the dark cells may represent the end stage of the cell cycle as demonstrated in Yoshida ascites hepatoma [25]. The nuclear cytoplasmic integrity of the dark cells in our study, however, does not lend itself to this interpretation. The cellular heterogeneity and granularity may be related to an increased density of cellular inclusions, which may correspond to permeability differences. The most reasonable explanation of this cellular diversity is that the dark and light cells of 6C3HED ascites tumor cells, as well as the derived tissue culture lines, represent a tumor cell pleiomorphism. This contention is substantiated by earlier light microscopic studies of Ehrlich-ascites tumor cells [26, 27] in which two to three different types of tumor cells were identified, all of which were equally tumorigenic.

Varying quantities of endoplasmic reticulum, free ribosomes and polyribosomes have been associated with different tumors under different culture situations. In mouse sarcoma, the solid form of the tumor possessed more cytomembranes and formed elements than the rapidly-growing ascites form [20]. In several tissue culture lines of human leukemic cells, an increase of endoplasmic reticulum and polyribosomes was not observed; instead, most of the leukemic cell culture lines retained the free ribosomes and possessed sparse cytomembranes [28]. It is generally accepted that rapidly-growing tumors which are not involved in synthesis of secretory proteins have few tubular elements; whereas, slowly growing tumors possess a more differentiated cytoplasm and more endoplasmic reticulum [29]. The gradual increase in the quantity of endoplasmic reticulum and the polyribosomal configuration in the tumorigenic and non-tumorigenic tissue culture lines of the 6C3HED tumor cells probably represents a metabolic and synthetic alteration from the ascites form to the stationary tissue culture form. It is also possible to infer that the loss of tumorigenicity may be linked with metabolic and/or synthetic alterations. A gradual increase in the endoplasmic reticulum and the polyribosomes may be a reflection of this transformation and accompanying morphological alterations. These inferences conform with the findings that the degree of malignancy is directly related to the paucity of endoplasmic reticulum; the less-deviated, slow growing and less malignant hepatomas possess a better developed endoplasmic reticulum [29].

The increase in the polyribosomes and the endoplasmic reticulum in 6C3HED-tissue culture cells observed in this study does not appear to be caused primarily by the tissue culture conditions alone. Lymphoma cells (6C3HED) in ascites or tissue culture form possessed a similar cytoplasmic morphology; sparse endoplasmic reticulum in the vesicular form and a predominance of free ribosomes [30]. It is our opinion, therefore, that the relative increase of endoplasmic reticulum and polyribosomes in the non-tumorigenic tissue culture of 6C3HED lymphosarcoma represented changes in metabolic conditions associated with a loss of tumorigenicity.

The intracytoplasmic virus-like particles in various murine ascites tumors and their various tissue culture lines have been reported by several

workers and have been reviewed by Bernhard [31] and Dalton [32]. Both type A and C viral particles have been shown to be associated with most of the murine tumors. In 6C3HED tumor cells, both in ascites and tissue culture form, Bernhard's type-A virus particles have been reported [30], but most of the RNA viruses reported in murine tumors are now generally believed to be the C-type [33]. The present observation of type C viral particles in ascites cells harvested from the mouse as well as in their derived tumorigenic and non-tumorigenic cell cultures is in agreement with these previous studies. No quantitative relationship between number of viral particles and degree of malignancy in tissue culture has yet been established.

This study is in agreement with most of the previously cited studies on ascites tumor cells with respect to the number and forms of the mitochondria. However, our observations differ from most of the earlier studies in which a predominance of mitochondria in the orthodox conformation was reported. The mitochondria of the 6C3HED ascites cells were in the condensed conformation with moderately to extremely dense mitochondrial matrices. Mitochondria in orthodox conformation were rarely observed in ascites cells. Some swelling and vacuolations were observed. However, some reports on mouse ascites tumors [19, 34] have included observations of mitochondria with dense matrices and somewhat condensed conformations. The conformational differences in mitochondria of murine ascites tumor cells between earlier and recent reports, including this study, may be related to improved handling and fixation techniques.

Mitochondria have been shown to undergo reversible ultrastructural condensation cycles associated with various respiratory rates [35]. These high energy orthodox and low energy condensed conformational states may represent conformational energy capture within the membranes to generate energy-producing processes. Furthermore, a condensed conformation has been linked with active oxidative phosphorylation. Weber and Blair [36], however, concluded from studies of isolated beef heart mitochondria that these conformational changes may not reflect an alteration of energy-producing processes. Since it is generally believed that ascites cells possess a fairly high aerobic and anaerobic respiration, the preponderance of the condensed conformation of mitochondria in the ascites tumor cells seems more likely. The recent work of Recher *et al.* [28] confirms the predominance of mitochondria in the condensed conformation in human leukemic and non-leukemic cell cultures.

A slight increase in the number of mitochondria in the orthodox form, and especially the presence of both the condensed and orthodox conformations in the same cell in the tumorigenic tissue culture cells of 6C3HED ascites tumors, may be associated with a gradual transformation in the neoplastic properties of the cell with a concomitant alteration in its metabolic properties. A further increase in the mitochondria in the orthodox configuration, plus an obvious increase in the number of mitochondria in the non-tumorigenic tissue culture cells of 6C3HED ascites tumor, tends to confirm the aforementioned contentions; i.e. the transformation of the tumorigenic cells to the non-tumorigenic cells is probably reflected by an increase in the formed elements in the cytoplasm which may be associated with an alteration in the metabolic and synthetic behaviour of these cells. Such an alteration does not appear to have been reported previously.

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REFERENCES

1. L. Hayflick, Oncogenesis *in vitro*. *Nat. Cancer Inst. Monogr.* **26,** 355 (1967).
2. K. K. Sanford, "Spontaneous" neoplastic transformation of cells *in vitro*: Some facts and theories. *Nat. Cancer Inst. Monogr.* **26,** 287 (1967).
3. R. Cornell, Spontaneous neoplastic transformation *in vitro:* Ultrastructure of transformed cell strains and tumors produced by injection of cell strains. *J. nat. Cancer Inst.* **43,** 891 (1969).
4. T. C. Hsu and O. Klatt, Loss of malignancy by Novikoff hepatoma cells *in vitro*. *J. nat. Cancer Inst.* **22,** 313 (1959).
5. G. E. Foley and B. P. Drolet, Loss of neoplastic properties *in vitro*. I. Observation with S–180 cell lines. *Cancer Res.* **24,** 1461 (1964).

6. J. Moore, J. Kieler and R. Biczowa, Comparative studies of a near-tetraploid and near-diploid line of Ehrlich ascites tumor propagated *in vivo* and *in vitro*. II. Cytology and transplantability. *Europ. J. Cancer* **4,** 81 (1968).
7. J. F. Morgan, C. P. Eng, M. D. Heuchert and H. D. Kirk, Loss of transplantability and induction of immunoprotection by mouse ascites tumor cells in tissue culture. *Proc. Soc. exp. Biol. Med.* **134,** 305 (1970).
8. C. P. Eng, L. P. Kleine and J. F. Morgan, Tumor specific immunity induced by non-tumorigenic 6C3HED ascites tissue culture cells. *J. nat. Cancer Inst.* **45,** 235 (1970).
9. M. K. Bhatnagar and J. F. Morgan, Hexose-ATP phosphotransferase activity in tumorigenic and non-tumorigenic cell cultures of mouse ascites tumor cells. *Proc. Can. Fed. Biol. Sci.* **13,** 7 (1970).
10. M. M. Guérin and J. F. Morgan, Establishment of the TA3 and Ehrlich ascites tumors as permanent cell strains in tissue culture. *Cancer Res.* **21,** 379 (1961).
11. G. P. Herdson and J. P. Kaltenbach, Fine structural changes in hypotonically treated Ehrlich ascites tumor cells. *Exp. Cell Res.* **42,** 74 (1966).
12. G. Millonig, Advantages of a phosphate buffer for OsO_4 solutions in fixation. *J. appl. Phys.* **32,** 1637 (1961).
13. J. Luft, Improvements in epoxy resin embedding methods. *J. Biophys. Biochem. Cytol.* **9,** 409 (1961).
14. M. L. Watson, Staining of tissue sections for electron microscopy with heavy metals. *J. biophys. and biochem. Cytol.* **4,** 475 (1958).
15. E. S. Reynolds, The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. cell Biol.* **17,** 208 (1963).
16. C. C. Selby, J. J. Biesele and C. E. Grey, Electron microscope studies of ascites tumor cells. *Ann. N.Y. Acad. Sci.* **63,** 748 (1956).
17. G. Yasuzumi and R. Sugihara, A comparative electron microscopic study on Ehrlich ascites tumor cells, Yoshida sarcoma cells, and human cancerous peritonitis ascites cells. *Cancer Res.* **18,** 1167 (1958).
18. V. C. Chambers and R. S. Weiser, An electron microscope study of sarcoma I in a homologous host. I. The cells of the growing tumor. *Cancer Res.* **24,** 693 (1964).
19. D. Brandes, B. Schofield, R. Slusser and E. Anton, Studies of L1210 leukemia. I. Ultrastructure of solid and ascites cells. *J. nat. Cancer Inst.* **37,** 467 (1966).
20. B. Boeryd, P. M. Lundin, K. Norrby and V. Schelin, Ultrastructure of a mouse sarcoma in its solid, enzymatically dissociated and ascites form. *Int. J. Cancer* **3,** 283 (1968).
21. H. W. Beams and R. G. Kessel, Properties of the Ehrlich ascites tumor cells as demonstrated by electron microscopy, ultracentrifugation and hydrostatic pressure. *Cancer Res.* **28,** 1944 (1968).
22. S. Haltori, M. Matsuda, R. Tateishi and T. Terazawa, Electron microscopic studies on human lung cancer cells. *Gann* **58,** 283 (1967).
23. J. Locker, P. J. Goldblatt and J. Leighton, Some ultrastructural features of Yoshida ascites hepatoma. *Cancer Res.* **28,** 2039 (1968).
24. R. Baserga, Mitotic cycle of ascites tumor cells. *Arch. Pathol.* **75,** 156 (1963).
25. M. Hoshino, Submicroscopic characteristics of four strains of Yoshida ascites hepatoma of rats: A comparative study. *Cancer Res.* **23,** 209 (1963).
26. S. Tolnai, Cytological study of "Balloon cells" occurring in Ehrlich ascites carcinoma. *Can. J. Biochem.* **42,** 1375 (1964).
27. N. D. Agnish and S. Fedoroff, Tumor cell population of Ehrlich ascites tumors. *Can. J. Genet. Cytol.* **10,** 723 (1968).
28. L. Recher, J. G. Sinkovics, J. A. Sykes and J. Whitescarver, Electron microscopic studies of suspension cultures derived from human leukemic and non-leukemic sources. *Cancer Res.* **29,** 271 (1969).
29. K. R. Porter and C. Bruni, Comparative fine structure of slow and fast growing hepatoma. *Acta Un. int. Cancr.* **20,** 1271 (1964).
30. L. H. Sobin, Virus-like particles in the cells of lymphoma 6C3HED. *Cancer Res.* **24,** 64 (1964).
31. W. Bernhard, Electron microscopy of tumor cells and tumor virus: A review. *Cancer Res.* **18,** 491 (1958).

32. A. J. DALTON, Micromorphology of murine tumor viruses and of affected cells. *Fred. Proc.* **21,** 936 (1962).
33. R. J. HUEBNER and G. J. TODARO, Oncogenes of RNA tumor virus as determinants of cancer. *Proc. nat. Acad. Sci. U.S.* **64,** 1087 (1969).
34. E. W. DANIELS, P. A. SUSTARSIC and G. B. JACOBS, Ultrastructure of the murine ascites tumor L. 4946. *Cancer Res.* **28,** 1207 (1968).
35. C. R. HACKENBROCK, Ultrastructural bases for metabolically linked mechanical activity in mitochondria. *J. cell Biol.* **30,** 269 (1966).
36. N. E. WEBER and P. V. BLAIR, Ultrastructural studies of beef heart mitochondria. I. Effects of adenosine diphosphate on mitochondrial morphology. *Biochem. biophys. Res. Commun.* **36,** 987 (1969).

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Contribution to the Study of the Ehrlich-Ascites Tumor Cells

A Cytological and Cytochemical Analysis of the Effects of Sarcolysine

ROGER BASSLEER and CLAUDE DESAIVE*

Institut d'Histologie, 20, rue de Pitteurs, Liège;
Institut de Pathologie (Laboratoire d'Anatomie Pathologique), 1, rue des Bonnes-Villes, Liège,
et Centre Anticancéreux, Université de Liège,
Belgium

Abstract—*Three Ehrlich-ascites cell lines propagated* in vivo *have been studied by cytological and quantitative cytochemical methods.*

These lines are hyperdiploid (45 *chromosomes*), *hypotetraploid* (77 *chromosomes*) *or hypertetraploid* (90 *chromosomes*). *They contain marker chromosomes; in each line some of the cells are polyploid* (*double number of chromosomes*).

The line with 77 *chromosomes is tetraploid as far as the* DNA *are concerned. The latter have been measured by cytophotometry after Feulgen reaction. The volume of these cells is about* 1600 μ^3, *as measured with an electronic counter.*

The line with 45 *chromosomes* (ELD) *and the line with* 90 *chromosomes* (ELT) *contain a diploid or a tetraploid amount of* DNA *respectively. For* ELD, *the modal cell volume is about* 880 μ^3; *for* ELT, *it is about* 1600 μ^3. *In these tumor cells, the behaviour of* DNA *during the preparation for mitosis and during mitosis is identical to that observed in normal* (*non-tumor*) *cells.*

Mice which had received hypo- or hypertetraploid Ehrlich cells intraperitoneally have been treated by an alkylating agent, sarcolysine. *This antimitotic agent inhibits cell multiplication by blocking the cells in preprophase* (*post-*S *or* G2); *sometimes, the tumor is killed. However, this blockage is* reversible *under some experimental conditions. In cells blocked in preprophase, the* DNA *content and the volume are doubled. Later on these cells are able to divide again; then, the* DNA *content and the volume are normal. Sometimes the cell volume is* 8 *times higher than in controls though the cells are blocked in preprophase; these cells probably degenerate.*

The electronic Coulter Counter *is very useful to analyze cell populations. However, the results can be interpreted with precision only if the same populations are studied also by cytophotometry. So it is possible to relate the volume variations to the cell cycle.*

Sarcolysine, *as other alkylating agents do, can induce chromosome alterations and abnormal mitoses. The effects of this agent in Ehrlich cells are identical to those produced in normal fibroblasts. Nevertheless, our conclusions concerning the mode of action of sarcolysine could be useful to those who use alkylating agents in cancer chemotherapy.*

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INTRODUCTION

THE EHRLICH-tumor cells have been studied by numerous authors. When injected into the mouse peritoneal cavity, these cells multiply very actively. This ascites tumor comes from a mouse solid tumor which has been cultivated *in vitro* [1].

In previous works, one of us [2–4] has studied hypotetraploid Ehrlich cells (76–78 chromosomes). Their DNA content is tetraploid, when compared to that of leucocytes from the same animal. The leucocytes have been used as a reference for the diploid DNA value. These measurements have been made by cytophotometry after Feulgen reaction. We have also measured (by micro-interferometry) the amount of total proteins in the nucleus of these cells. We have found 70 pg (nucleoli included) in post-mitotic cells but this quantity is doubled, as is DNA, during the "preparation for mitosis". The analysis of our results by various statistical methods [4, 5] has shown that, in this material, total nuclear proteins and DNA syntheses do not occur simultaneously. These observations agree with those from Richards and Davies [6], Seed [7] and Leeman and Ruch [8] concerning ascites tumors of the mouse. However, according to Lederer and Sandritter [9], these syntheses would be simultaneous during the interphase in the Ehrlich cells. From this point of view, we think that the Ehrlich cells we have studied do not differ essentially from normal fibroblasts. In other words, the absence of a close association between these two premitotic syntheses does not seem to be a specific characteristic of cancer cells [4]. According to Mueller [10], the cell cycle is identical in normal or cancer cells. During the mitosis in Ehrlich cells [3], non-chromosomal nuclear proteins migrate into the cytoplasm, but return into the nucleus during the post-telophase. The same results have been obtained for normal fibroblasts [4]. In the hypotetraploid Ehrlich cells, DNA represent about 15% of the fixed nucleus total dry mass, as it does in normal chick, mouse or rat fibroblasts [11]. Concerning the karyotype anomalies, they are not necessarily linked to the fact that the cells are cancer cells [12]. It is not yet known whether these chromosome abnormalities are the cause or consequence of the cancer. Anyway, no specific karyotype alteration has yet been observed in cancerous cells [13].

In the present work, we have studied two other Ehrlich lines with cytological and quantitative cytochemical methods. We have analyzed the effect of an antimitotic agent, sarcolysine, on hypo- or hypertetraploid Ehrlich cells in order to try to find out how this substance exerts its antitumoral action. Some of these results have been published in preliminary notes [2, 3, 14–18].

MATERIAL AND METHODS

1. *Ehrlich cells*

Three Ehrlich lines have been studied. One of them was obtained from Professor Closon (Liège), the others from Professor Betz (Liège). The cells have been injected into the peritoneal cavity of male or female C 57 Bl mice. The mice received 1 million hypotetraploid cells or 10 millions hyperdiploid or hypertetraploid cells.

2. *Histological fixations*

Ehrlich cells smears have been fixed either in Carnoy fluid or by absolute ethanol : acetone (1 : 1), at 4°C during 24 hr.

3. *Histological stainings*

Smears have been stained by von Möllendorff haematoxyline. Other smears have been treated by the Feulgen reaction (see below).

4. *Measurement of the number and volume of Ehrlich cells*

The total number of Ehrlich-ascites cells has been measured after rinsing the peritoneal cavity with Hanks fluid. We used either an haemocytometer or an electronic counter (Coulter counter, Model B). Growth curves have been established. The cell volumes have also been obtained from the Coulter counter.

5. *Karyotypes*

For our three lines, the karyotype has been established by the hypotonic shock method and Unna Blue staining.

6. *Cytophotometric* DNA *measurements*

The DNA content of interphase nuclei and mitotic figures has been measured by cytophotometry after Feulgen reaction, with visible light and in individual cells. We used the classic Feulgen reaction: hydrolysis by HCl N for 20 min at 56°C, the optimal time for our material. The measurements have been performed at 543 mμ with the Barr and Stroud integrating microdensitometer. The results are expressed in arbitrary units.

RESULTS

1. *Cytological and cytochemical analysis of two Ehrlich lines*

Two Ehrlich cell lines (ELD and ELT) have been studied. In smears, ELT cells are larger than ELD cells (Fig. 1).

(*a*) *Karyotypes.* For ELD, the chromosomes modal number is 45 (Fig. 2); for ELT, it is 90 (Fig. 3). Since the karyotype of the normal mouse is composed of 40 acrocentric chromosomes, our ELD cells are hyperdiploid; ELT cells are hypertetraploid. In each line, some cells contain a doubled number of chromosomes. In ELD, we found some marker chromosomes: 1 meta or submediocentric and 2 min chromosomes. In ELT, we observed 1 or 2 meta or submediocentric and usually 4 min chromosomes.

(*b*) *Growth curves.* As shown in Fig. 4, the growth curves for the two lines are nearly identical.

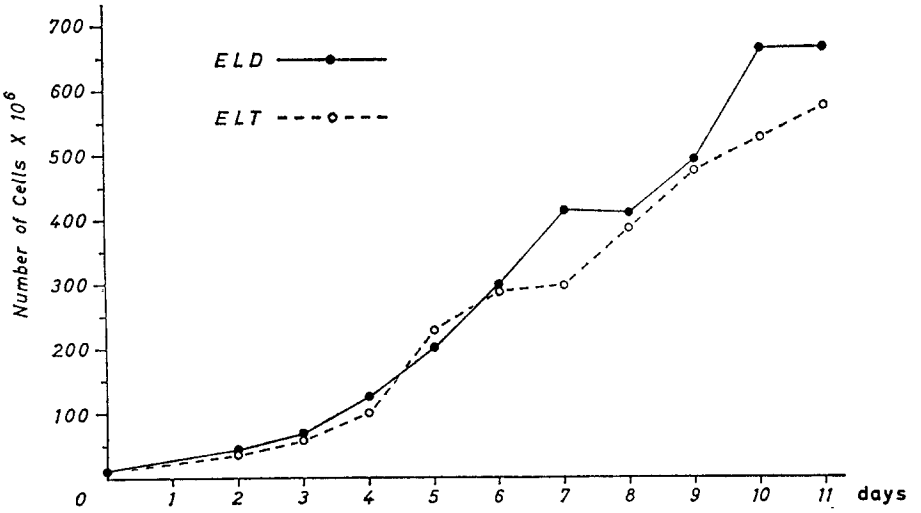


Fig. 4. Growth curve of ELD and ELT propagated in vivo. *In abscissa, the number of days after the inoculation of* 10 *millions tumor cells. In ordinate, the number of cells. Each value is the mean for* 8 *to* 10 *mice.*

(*c*) *DNA content.* Our ELD cells are diploid as far as the DNA content is concerned (Fig. 5); ELT cells are tetraploid. This agrees with the karyotype analysis. In the DNA histograms, we observe cells fixed during premitotic DNA duplication (S period); others are in preprophase (post-S or G2). Some rare cells are polyploid.

(*d*) *Cell volumes.* In the two lines, the cell volumes are grouped around one peak (Fig. 5). The modal volume is around 880 μ^3 for ELD and around 1760 μ^3 for ELT. So, the DNA content and the cell volume are twice as high for ELT as for ELD. The modal cell volume does not change between the first and the seventh day after the inoculation of the cells. In the two volumes histograms (Fig. 5), we see that some cell volumes are twice as high as the modal value: these cells are probably preparing for the next mitosis.

2. *Effects of sarcolysine on a hypotetraploid Ehrlich line*

It is well known that one way to study the mechanisms of cell multiplication is to use cytotoxic chemical or physical agents and to analyze the cell after such a treatment. In previous works [4], we have studied the effects of various antimitotic agents on normal fibroblasts cultivated *in vitro*; among these substances, sarcolysine had been used. Sarcolysine [19, 20] is an alkylating agent [*p*-di- (2 chloroethyl) amino-DL-phenylalanine]. It is an antitumor agent [21].

Mice bearing *hypotetraploid* Ehrlich tumor cells have received intraperitoneal injections of sarcolysine (Sarcoclorine Simes). In th Introduction of the present work, we have recalled the cytological and cytochemical characteristics of this line we had studied earlier. The volume of these cells has been measured by Desaive [22] with the electronic Coulter counter. The modal cell volume is about 1600 μ^3. This volume tends to increase in the peritoneal cavity of the mouse: it is 2240 μ^3 on the seventh day after inoculation. It is possible that this volume increase is due to some cellular

alteration caused by the rapid multiplication of the cells and the high density of the population [23]. We will see later that sarcolysine can provoke a considerable increase of the Ehrlich cell volume.

is 13‰ against 48‰ in the controls, but 48 hr and 72 hr after the beginning of the treatment the mitotic activity is again very high: 83 and 63‰ against 25 and 34‰ respectively in the controls. Usually, the mice bearing this tumor

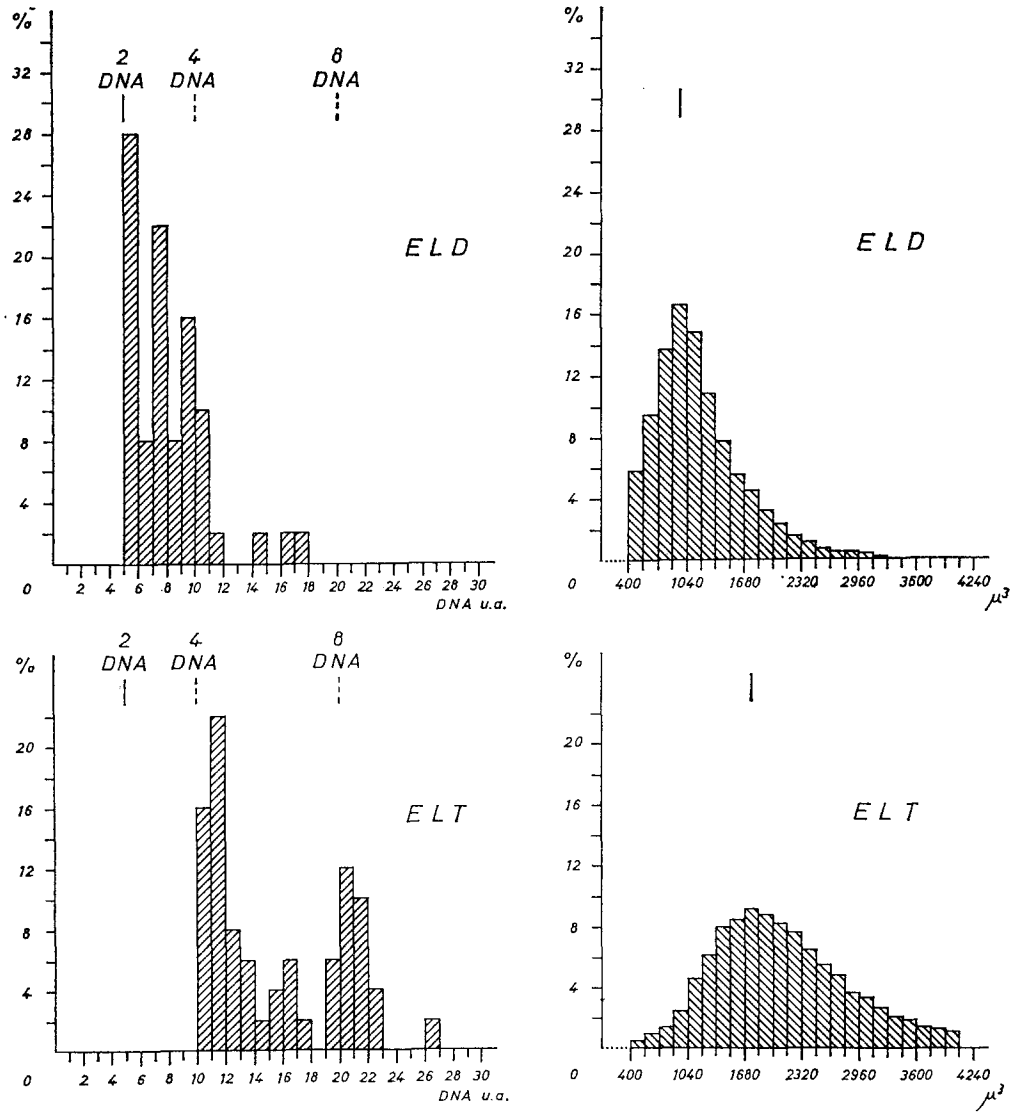


Fig. 5. DNA contents and cell volumes in ELD and ELT. For each tumor, the DNA content and the cell volume have been measured in cells growing in the same animal. The cells have been analyzed 5 days after the inoculation. In abscissae, the cell volumes in cubic microns or the DNA content of interphase nuclei in arbitrary units. In ordinates, the percentages of cells. The volumes of inflammatory cells and red blood cells (volumes under 400 μ^3) are not represented. The line at the top of volume histograms indicates the modal value. At the top of DNA histograms, the full line indicates the mean diploid value found for leucocytes situated on the same slides; the columns relative to the DNA content of the leucocytes have not been represented. The tetraploid (4 DNA) and octoploid (8 DNA) values (dotted lines) have been obtained by doubling or quadrupling the diploid value. For the cell volumes, about 25,000 cells have been measured; for the DNA contents, 50 cells have been studied in each case.

In a first experiment, 1 μg sarcolysine has been injected 24 hr after the inoculation of hypotetraploid tumor cells. The growth of the tumor is inhibited but it becomes again very active after a few days. Twenty-four hours after the injection of sarcolysine, the mitotic index

die between the 9th and the 16th day after the inoculation; when treated by 1 μg sarcolysine, they survive up to the 19th or 23rd day.

One μg sarcolysine has been injected 24 hr after the inoculation of the tumor and then a second dose of 1 μg 72 hr after this inoculation;

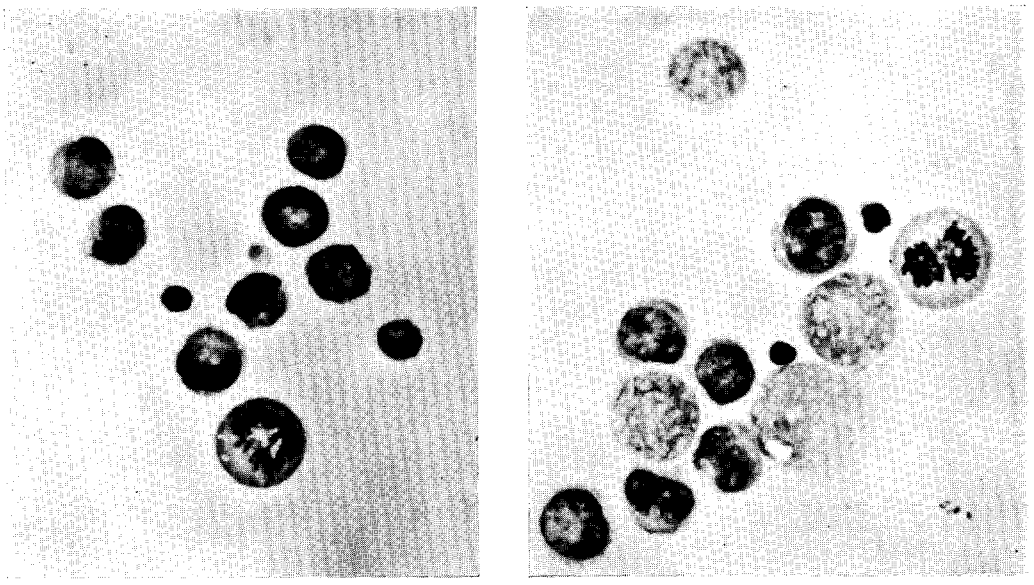


Fig. 1. *Ehrlich ascites cells observed in phase contrast after Feulgen reaction. On the left, ELD cells. On the right, ELT cells.* (*Magn.* 700 ×).

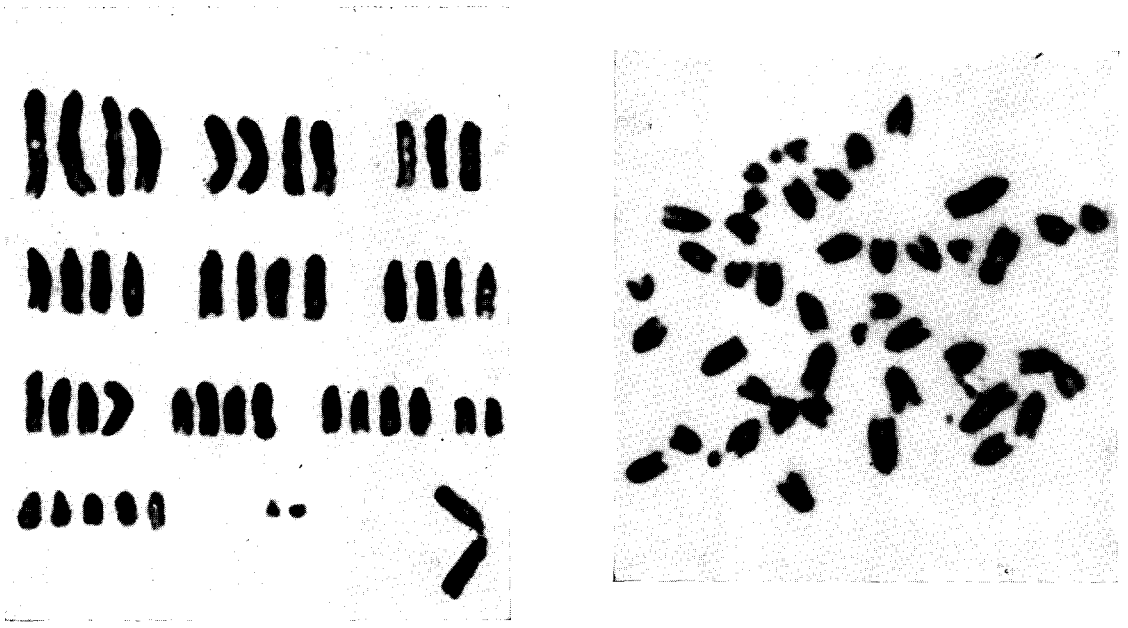


Fig. 2. *Chromosomes of ELD cells. On the left, karyotype with* 45 *chromosomes; among them, one sub-mediocentric and two minute chromosomes.* (*Magn.* 2000 ×). *On the right, chromosomes of another ELD cell.* (*Magn.* 1400 ×).

(to face p. 444)



Fig. 3. *Chromosomes of ELT cells. Above, a cell with* 92 *chromosomes. The other cell contains* 182 *chromosomes.* (*Magn.* 1400 ×).

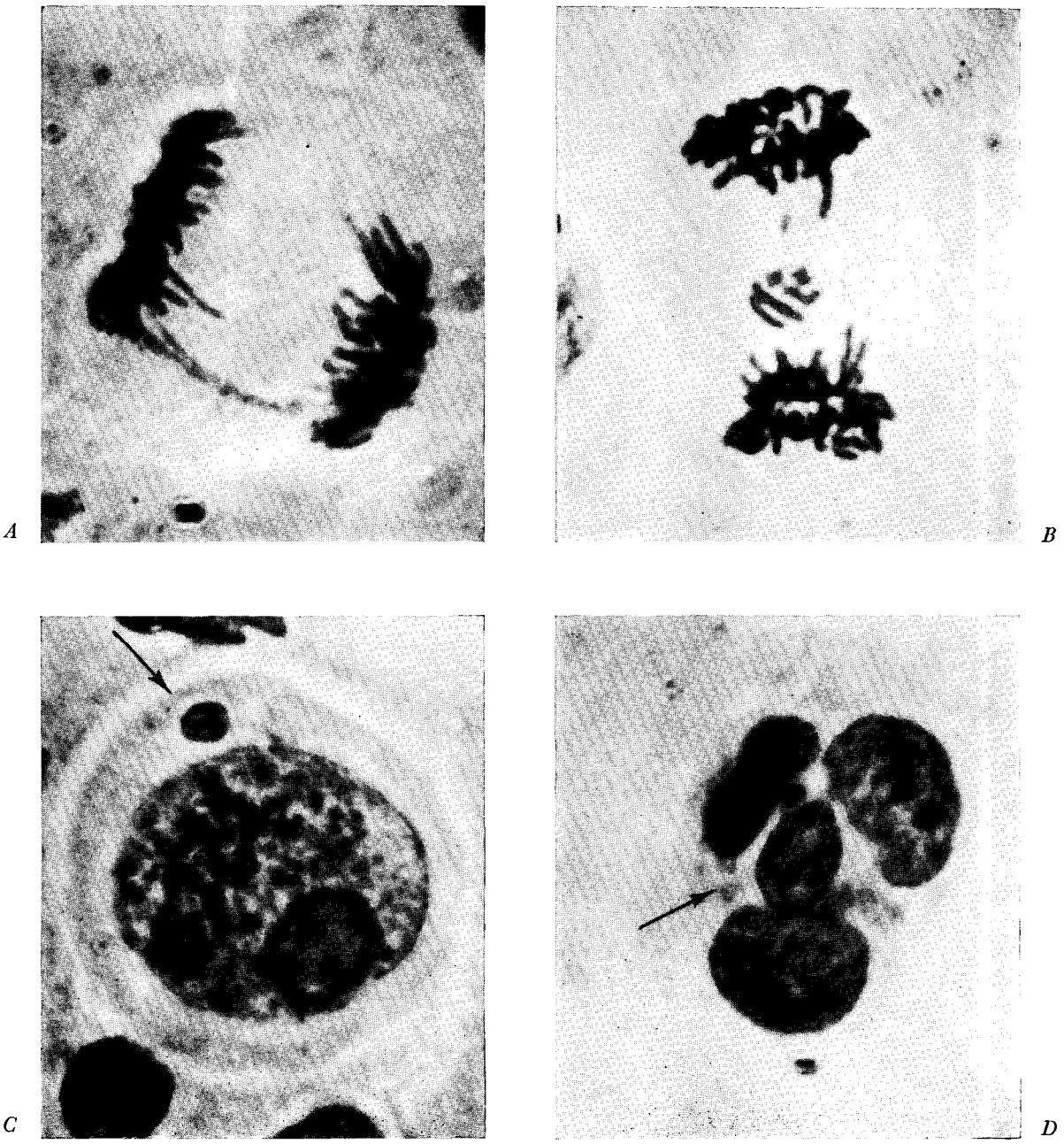


Fig. 6. *Mitotic anomalies and abnormal nuclei in Ehrlich cells treated by sarcolysine. In mitotic cells, one sees a chromosome bridge (A) and lost chromosomes (B). During the interphase, cells often contain one or several micronuclei (arrows in C and D).* (*Magn.* 2500 ×).

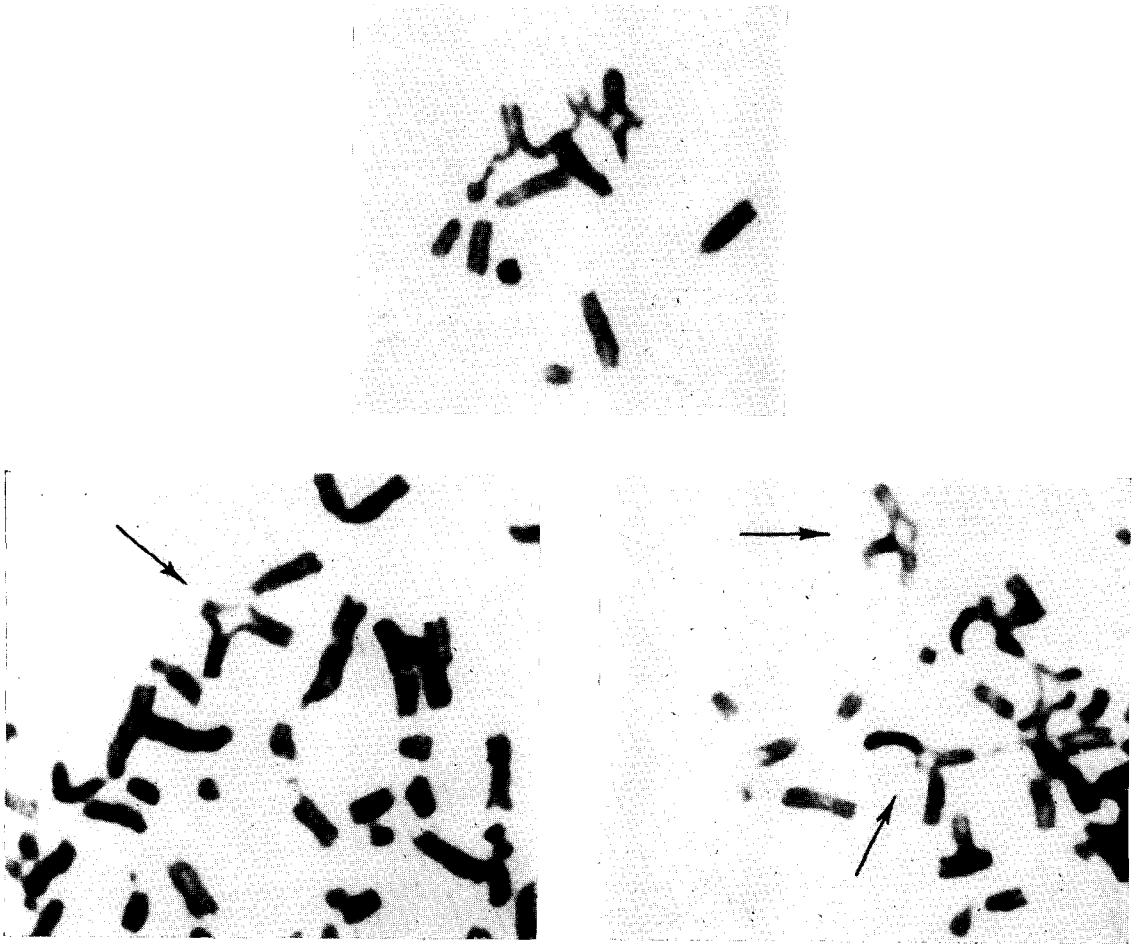


Fig. 9. *Chromosome alterations in ELT cells treated by sarcolysine. The cells have been fixed* 48 *hr after the injection of* 5 μg *sarcolysine. Note tri- or quadriradial figures* (*arrows*) *and various alterations, especially on chromatids.* (*Magn.* 2500 ×).

the mitotic activity is 22‰ against 34‰ in the controls 24 hr after the treatment. Already 24 hr later, the mitotic activity is higher: 57‰ against 33‰ respectively in the controls.

In a third experiment, a unique but higher dose has been injected: 5 μg 72 hr after the inoculation of the ascites cells. One and two days later, the mitotic activity is very low: 15‰ and 9‰ against 34‰ and 33‰ respectively in the controls.

Under our experimental conditions, we have noticed no modification of the percentages of the different phases of the mitosis. So the high mitotic index observed after the period of mitotic inhibition caused by sarcolysine is due to a real increase in the rate of cell multiplication and is not the result of a block of the mitosis, in metaphase for example. The increase of the duration of all the phases of mitosis can be excluded with relative certainty. Many abnormal mitoses can be observed after a treatment by sarcolysine: chromosome bridges at telophase, lost chromosomes, micronuclei at post-telophase (Fig. 6). Some cells with micronuclei enter into mitosis during the next cell cycle.

According to these observations, sarcolysine is able to inhibit the cell multiplication but this effect is *reversible*. However some cellular alterations persist and are visible during the next mitosis. An important question arises now: when does sarcolysine exert its effect during the cell cycle? The answer has been found by performing *cytophotometric DNA measurements* in Ehrlich cells treated by sarcolysine. This line is tetraploid as far as the DNA content is concerned. In the controls (Fig. 7), about 50% of the cells are in preprophase (post-S or G2; 8 DNA); the mitotic index is high: 82‰. Some cells are polyploid (16 DNA). Twenty-four hr after a treatment by sarcolysine (1 μg 24 hr after the inoculation of the tumor cells), the mitotic activity is relatively low: 22‰. The corresponding histogram (S 1, Fig. 7) is strongly modified: 74% of the cells are in preprophase (8 DNA). If 5 μg sarcolysine are injected 72 hr after the inoculation of the tumor, the mitotic activity is inhibited during the next two days. Twenty-four hr after this treatment, (S II, Fig. 7), the mitotic index is only 8‰ and 96% of the cells are in preprophase (8 DNA). Two days after the treatment, the results are identical. Sixteen DNA cells are rare under all our experimental conditions. The blockage in the post-synthesis period (preprophase) caused by sarcolysine is reversible as shown in S III and S IV (Fig. 7). Two and 3 days after the injection of 1 μg sarcolysine, the mitotic activity is very high: 90 and 56‰ against 25 and 34‰ in the controls. The number of premitotic cells diminishes: 58% (S III) and 32% (S IV). Micronuclei are observed in the cells of the different classes (4, 8 or 16 DNA). In S III and S IV (Fig. 7), post-mitotic cells (4 DNA) are numerous and many cells are synthesizing DNA (values between 4 and 8 DNA).

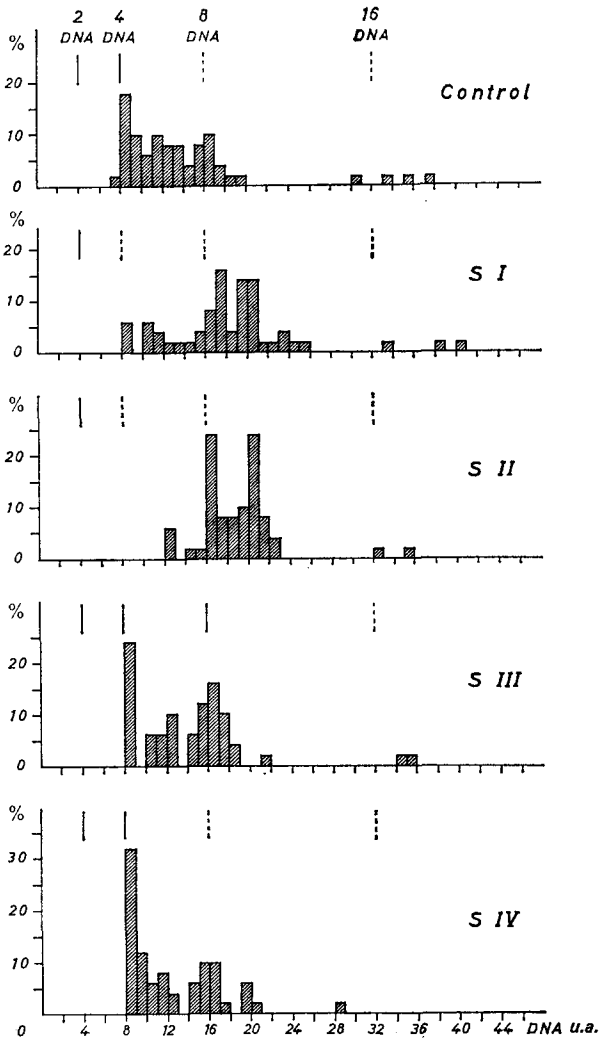


Fig. 7. DNA content measured by cytophotometry after Feulgen reaction in Ehrlich cells treated by sarcolysine and in controls. In abscissae, the DNA content of interphase nuclei in arbitrary units. In ordinates, the percentages of cells. At the top of the histograms, the full lines indicate the observed mean values. The diploid value (2 DNA) has been found in leucocytes situated near the tumor cells. The dotted lines indicate the mean DNA values calculated by multiplying by 2, 4 or 8, the corresponding diploid value. Control: 2 days after the inoculation of the tumor. S I and S II: cells fixed 24 hr after the injection of 1 μg or 5 μg sarcolysine respectively. S III and S IV: cells fixed 48 hr or 72 hr after the injection of 1 μg sarcolysine. In each case, 50 cells have been measured.

From these measurements we conclude that sarcolysine inhibits cell multiplication by blocking the Ehrlich-tumor cells in preprophase (post-S or G2). Under some conditions, this

blockage is reversible and a mitotic wave takes place. It is possible that in these cells some repair of sub-lethal damages due to alkylation has occurred, as demonstrated in other materials [24, 25]. However, when these cells multiply again, some alterations persist and are observed during the next mitosis.

3. *Effects of sarcolysine on hypertetraploid Ehrlich cells*

Mice-bearing ELT cells have received 10 μg sarcolysine intraperitoneally 4 days after the inoculation of the tumor. The survival rate of the mice is slightly increased: 30 days after the inoculation, 14% of the treated mice survive against 8% in the controls.

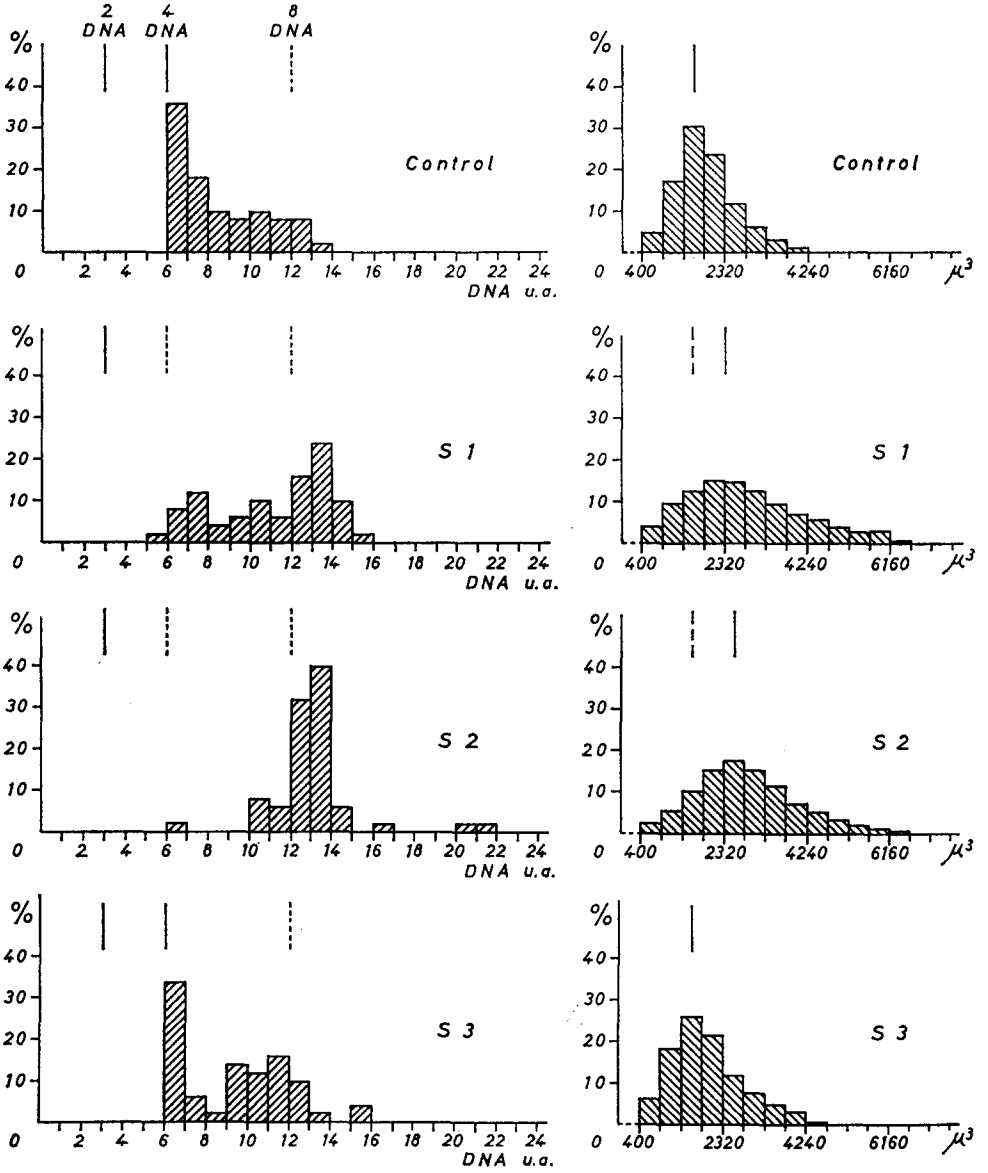


Fig. 8. *DNA contents and cell volumes in Ehrlich cells treated by sarcolysine and in controls. In each case, the DNA content and the cell volume have been measured in cells growing in the same animal. The control cells have been fixed* 5 *days after the inoculation. S* 1, *S* 2 *and S* 3*: Tumor cells treated by sarcolysine* (10 μg) *on the* 4*th day after the inoculation. S* 1*: cells fixed* 24 *hr after the injection of the drug. S* 2 *and S* 3*: cells fixed* 5 *days after the treatment. The reversibility of the block is visible in S* 3 *only (see the text). In abscissae, the cell volumes in cubic microns or the DNA contents in arbitrary units; in ordinates, the percentages of cells. The volumes of inflammatory cells and red blood cells (volumes under* 400 μ^3) *are not represented. At the top of the volume histograms, the full line indicates the modal value; in S* 1 *and S* 2 *(volume histograms), the modal value observed in controls is indicated by a dotted line. At the top of the DNA histograms, the* 2 *DNA value is the mean DNA content found in leucocytes situated on the same slides. The individual values obtained for these leucocytes are not represented here. The tetraploid* (4 *DNA) and octoploid* (8 *DNA) values have been either observed (full lines) or calculated (dotted lines) by doubling or quadrupling the diploid value. For the cell volumes, about* 25,000 *cells have been measured; for the DNA contents,* 50 *cells have been studied in each case.*

One, 3 or 7 days after the injection of sarcolysine, the mean number of cells per mouse is 69, 186 and 184 millions against 184, 400 and 600 millions respectively in the controls.

In the controls (Fig. 8), the post-mitotic DNA content is tetraploid as seen before. The modal volume is 1600 μ^3. Twenty-four hr after the treatment with sarcolysine (S 1, on the left) many cells are blocked in preprophase (8 DNA); the mitotic index is 2‰ against 12‰ in the controls. The modal volume has increased: 2320 μ^3 and giant cells have appeared (values above 4240 μ^3). In some mice, 5 days after the injection of sarcolysine, the number of blocked cells has increased (S 2 on the left) and the modal cell volume reaches 2560 μ^3 (S 2 on the right). The mitotic activity is then very low: 2‰. In other cases, 5 days after the treatment, the mitotic activity is high (44‰); some mitoses are abnormal. The DNA contents and cell volumes histograms become normal (S 3). The premitotic block is thus reversible as also shown above. However, the moment when the reversibility occurs varies from one animal to another.

It is interesting to note that when the DNA content is doubled (during the premitotic block) the cell volume tends also to double. The cell volume again becomes normal when cell multipication resumes. However, this relation between the DNA content and the cell volume is sometimes much less close. In some cases, after a treatment by sarcolysine, the modal cell volume is nearly 4 times or even 8 times higher than in controls. These giant cells are however blocked in preprophase (8 DNA). The mitotic activity observed in these populations is 15‰ or even 20‰. This relatively high index is due to a blockage in metaphase. Some of these metaphases become pyknotic but many others return into interphase. In this event, many micronuclei are formed because many chromosomes are dispersed in the cytoplasm. Some of these cells are able however to begin new DNA syntheses and reach the 16 DNA level. How can we explain the volume increase in these cells blocked in preprophase? It could be due to active protein synthesis or it could be related to some cellular degeneration. Measurements are in progress in order to try to find out the answer to this question. The second possibility seems to us probable because in some cases the ELT cells are destroyed by sarcolysine; the modal cell volume is then around 880 μ^3, as for ELD cells. The cytological and cytophotometric analyses of these smears have shown that ELT cells have disappeared and that mononucleated inflammatory cells have replaced them. The volume of the latter is larger however than that usually observed in cells of this type. This volume increase could be due to a greater activity of these cells, linked perhaps to the phagocytosis of degenerated tumor cells.

We have also analyzed the effects of sarcolysine by studying day after day small samples of tumor cells coming from the *same* mouse. These experiments have confirmed our results and have shown that cell multiplication resumes after a temporary blockage in preprophase.

The use of the electronic counter has been very precious for analyzing these tumor cell populations. However, it is important to note that the measurement of the number and volume of the treated cells is not sufficient to study and understand the effects of antimitotic agents. Though very often the cell volume seems to be closely related to the DNA content, sometimes this relation is missing and DNA measurements are then necessary to find out either at which stage of the cell cycle the cells are blocked or what is their degree of polyploidy. The combined use of these two methods is very useful to study such problems.

We have looked for chromosome alterations after a treatment by a relatively high dose of sarcolysine (5 or 10 μg). The aberrations are frequent and consist in chromatid or chromosome breaks, irregular chromatids, bridges between chromosomes and chromosome fusions with the formation of tri- or quadriradial figures (Fig. 9). The number of chromosomes is not modified or only slightly.

DISCUSSION

Three Ehrlich-ascites tumor cell lines propagated in mice have been studied. They contain an *abnormal number of chromosomes*: 45, 77 or 90. They also contain marker chromosomes. However, is there a relation between these anomalies of the karyotype and the fact that these cells are cancer cells? Aneuploidy is often secondary and due to the selection of more resistant lines [12]. An hypotetraploid or hyperdiploid cell can appear after a mitosis without cytodieresis [13] because the resultant polyploid cell can loose some chromosomes during abnormal mitoses, e.g. multipolar. It is to be noted that in our material the number of marker chromosomes depends on the number of genomes.

We have observed that each genome contains the same *DNA* amount. This agrees with classic notions. Ehrlich cells with 90 chromosomes contain twice as many DNA as Ehrlich cells with 45 chromosomes. During the prepara-

tion for mitosis and during the mitosis itself, DNA behave exactly in the same way as in actively multiplying normal cells. The DNA content is doubled before prophase; all the DNA of the nucleus are condensed in the chromosomes and are then equally distributed between the two daughter-cells. Polyploid cells with doubled chromosome number and DNA content exist in the three lines examined here. These giant cells can be formed either because cytodieresis does not occur at telophase or because the cell is blocked in preprophase and begins another cell cycle without dividing. Such a phenomenon is common in cells treated by alkylating agents [4, 26]. These polyploid cells are able to divide.

Within the limits of precision of our methods, we did not observe any absolutely specific characteristic of cancer cells in the three lines studied here. It is the reason why we think that cell multiplication is not fundamentaly different in normal and cancer cells, but *control mechanisms* could be altered in the latter. Some of the mitoses are abnormal in cancer cells but this fact is common in actively multiplying normal cells. These abnormal mitoses can give rise to aneuploidy, a situation frequently observed in cancer cells.

In the three lines studied here, the *cell volume* is related to the DNA content and the number of chromosomes. The volume is nearly twice as large ($\pm$ 1600 μ^3) in hypo- and hypertetraploid cells as compared to hyperdiploid cells ($\pm$ 800 μ^3). Moore *et al.* [27] have measured with the haematocrit the mean cell volume for ELD and ELT but they have not found any significant difference. According to Hauschka *et al.* [28], the mean cell volume for ELT (as measured with the haematocrit) and the DNA content (measured chemically) are as high as for ELD.

Sarcolysine, an alkylating agent, inhibits Ehrlich cells multiplication. Cytophotometric DNA measurements performed in individual cells have shown that these cells are blocked in preprophase (post-S or G2). This antimitotic agent has the same effect in normal fibroblasts cultivated *in vitro* [4, 29–31]. Other alkylating agents, such as myleran or mitomycine C, also block the cells in preprophase [4]. Probably nucleoprotein alterations prevent the cell from entering into prophase. The effects of alkylating agents are thus the same in normal and cancer cells. This confirms the conclusion drawn above according to which the tumor cell is not essentially different from the normal cell as far as the cell cycle is concerned.

In the cells *blocked in preprophase*, the DNA content is at the premitotic level; it has doubled. The volume of these cells tends also to double. This confirms the existence of a relation between the DNA content and the cell volume, a relation we have also observed when comparing ELT to ELD cells. The analysis of cell populations with the electronic counter is thus very interesting but care must be taken. In some cases, indeed, the cell volume may increase much more than the DNA content in cells blocked in preprophase. This cell volume increase is probably related to cell alteration. Several days after the inoculation, the volume of the hypotetraploid Ehrlich cell tends to increase in the peritoneal cavity of the mouse [22]. At this moment, the tumor growth is slower and the conditions for cell multiplication are not favourable anymore (increase of the cell population density, progressive weakness of the mouse).

The fact that the *block in preprophase* caused by sarcolysine is *reversible* under some conditions, is interesting. Indeed it explains how a strongly inhibited tumor is again able to grow very actively. This could be important to know when using alkylating agents in chemotherapy, especially when fractionated doses are given. Furthermore, we see that it is possible to block the majority of the tumor cells in preprophase and then to treat them with another antitumor agent to which they would be particularly sensitive at this stage of the cell cycle. One may ask whether the reversibility we have observed is due to the disappearance of the block or to the persistence of cells *resistant* to the alkylating agent. Both possibilities may exist. But we think that the Ehrlich cell blocked in preprophase is able, after some time, to enter into mitosis. The mitotic wave is indeed very important though some hours before the majority of the cells were blocked. Another argument is that the number of degenerating cells is low. It is possible that sub-lethal damages due to alkylation are repaired, as demonstrated in other materials [24, 25].

Our results agree with recent observations published by Wheeler *et al.* [32] who studied the effects of nitrogen mustards on H. Ep. No. 2 cells cultivated *in vitro*. The cells are blocked in preprophase but after some time this effect is reversible.

In our material, sarcolysine alters the chromosomes and disturbs the mitosis. Other alkylating agents have similar effects; Myleran for example [26]. These alterations are probably due to alkylation of nucleoproteins. Some cellular lesions which are not sufficient to block the cell in preprophase are visible however during the following mitoses: sticky chromosomes, lost or fused chromosomes, chromosome breaks.

Changes occurring during the growth of Ehrlich ascites cells in the mouse have recently been studied by Peel and Fletcher [33]. The rate of multiplication becomes progressively slower as the tumor ages and the modal cell size increases. Once the tumor mass is reduced by transplantation, the cell size diminishes and the cells are again able to divide quickly. These conclusions agree with the results of personal experiments now in progress concerning Ehrlich cells treated by Daunomycin; this will be the subject of a next publication [34].

REFERENCES

1. T. S. Hauschka and A. Levan, Cytologic and functional characterization of single cell clones isolated from the Krebs-2 and Ehrlich ascites tumors. *J. nat. Cancer Inst.* **21,** 77 (1958).
2. R. Bassleer, Etude cytologique et cytochimique de la tumeur d'ascite d'Ehrlich. Activité mitotique, caryotype, teneurs en protéines nucléaires totales et en acides désoxyribonucléiques. *C. R. Acad. Sci. (Paris)* **262,** 1032 (1966).
3. R. Bassleer, Etude cytologique et cytochimique de la tumeur d'ascite d'Ehrlich. Comportement des protéines nucléaires totales et des acides désoxyribonucléiques au cours de la préparation à la mitose et de la division cellulaire. *C. R. Acad. Sci. (Paris)* **262,** 1117 (1966).
4. R. Bassleer, Recherches sur les protéines nucléaires totales et les acides désoxyribonucléiques dans des fibroblastes cultivés *in vitro* et dans des cellules tumorales d'Ehrlich. Une étude cytochimique quantitative réalisée par micro-interférométrie et cytophotométrie. *Arch. Biol.* **79,** 181 (1968).
5. G. Lennes and R. Bassleer, Le test de linéarite de Wald-Bartlett et son application à l'étude des synthèses nucléaires prémitotiques. *Pathol. Biol.* **17,** 697 (1969).
6. B. M. Richards and H. G. Davies, The determination of mass and concentration by microscope interferometry. In *General Cytochemical Methods.* (Edited by J. F. Danielli), Vol. I, p. 55. Academic Press, New York (1958).
7. J. Seed, The synthesis of deoxyribonucleic acid and nuclear protein in normal and tumor strain cells. *Proc. roy. Soc. B* **156,** 41 (1962).
8. U. Leeman, F. Ruch and P. Strauli, Characterization of the nuclei of Ehrlich ascites carcinoma by means of quantitative cytochemistry. *Acta Cytol.* **12,** 381 (1968).
9. B. Lederer and W. Sandritter, A cytophotometric study of DNA and protein content during interphase growth of an Ehrlich ascites tumor. *Europ. J. Cancer* **3,** 21 (1967).
10. G. C. Mueller, The G1→S conversion: a target for cancer chemotherapy. *Cancer Res.* **29,** 2394 (1969).
11. R. Bassleer, Teneurs en protéines nucléaires, en acides désoxyribonucléiques et en matériel nucléolaire dans des cellules d'origines diverses. *C.R. Soc. Biol.* **160,** 705 (1966).
12. P. C. Koller, Chromosomes in neoplasia. In *Cellular Control Mechanisms and Cancer*, p. 175. Elsevier, New York (1964).
13. R. Berger, Chromosomes et tumeurs humaines. *Pathol. Biol.* **17,** 1133 (1969).
14. R. Bassleer, Effets de la sarcolysine sur la multiplication cellulaire dans la tumeur ascitique d'Ehrlich de la souris. *C.R. Acad. Sci. (Paris)* **268,** 1236 (1969).
15. R. Bassleer, Dosages cytophotométriques d'acides désoxyribonucléiques dans des cellules tumorales d'Ehrlich traitées par de la sarcolysine. *C.R. Acad. Sci. (Paris)* **268,** 1357 (1969).
16. R. Bassleer and Cl. Desaive, Effets de la sarcolysine sur des cellules tumorales d'Ehrlich chez la souris. Relation entre la teneur en acides désoxyribonucléiques et le volume cellulaire. *C.R. Acad. Sci. (Paris)* **270,** 1412 (1970).
17. R. Bassleer and Cl. Desaive, Relation entre le volume cellulaire et la teneur en acides désoxyribonucléiques dans deux souches de la tumeur ascitique d'Ehrlich. *C.R. Assoc. Anat.*, 55*e Reunion, Nancy* 131 (1970).
18. Cl. Desaive and R. Bassleer, Comparaison de deux souches de tumeur ascitique d'Ehrlich: etude du caryotype, de la teneur en acides desoxyribonucleiques et du volume cellulaire. *C.R. Acad. Sci. (Paris)* **270,** 436 (1970).
19. F. Bergel and J. A. Stock, Cyto-active amino-acid and peptide derivatives. Part I: Substituted phenylalanines. *J. chem. Soc.* **2,** 2409 (1954).

20. L. F. Larionov, A. S. Khokhlov, E. N. Shkodinskaya, O. S. Vasina, V. I. Troosheikina and M. A. Novikova, Studies on the antitumor activity of p-di (2-chloroethyl)aminophenylalanine (sarcolysin). *Lancet* **2,** 169 (1955).
21. N. Blokhin, L. F. Larionov, N. Perevodchikova, L. Chebotareva and N. Merkulova, Clinical experiences with sarcolysin in neoplastic diseases. *Ann. N.Y. Acad. Sci.* **68,** 1128 (1958).
22. Cl. Desaive, Etude comparée à l'aide d'un compteur electronique de la tumeur ascitique d'Ehrlich greffe chez la souris et le rat. *Bull. Cancer* **56,** 321 (1969).
23. F. Hartveit, The immediate cause of death in mice with Ehrlich's ascites carcinoma. *Acta path. microbiol. Scand.* **65,** 359 (1965).
24. J. J. Roberts, A. R. Crathorn and T. P. Brent, Repair of alkylated DNA in mammalian cells. *Nature* **218,** 970 (1968).
25. F. Mauro and M. M. Elkind, Comparison of repair of sublethal damage in cultured chinese Hamster cells exposed to sulfur mustard and X-rays. *Cancer Res.* **28,** 1156 (1968).
26. M. Chèvremont, J. Frederic and E. Baeckeland, Contribution à l'étude de l'action cellulaire du Myleran. *Bull. Acad. roy. Med. Belg.* **24,** 141 (1959).
27. J. Moore, J. Kieler and B. Biczowa, Comparative studies of a near-tetraploid and a near-diploid line of Ehrlich's ascites tumor propagated *in vivo* and *in vitro*. II. Cytology and transplantability. *Europ. J. Cancer* **4,** 81 (1968).
28. T. S. Hauschka, S. T. Grinnell, L. Revesz and S. Klein, Quantitative studies on the multiplication of neoplastic cells *in vivo*. IV. Influence of doubled chromosome number on growth rate and final population size. *J. nat. Cancer Inst.* **19,** 13 (1957).
29. J. Guimard and R. Bassleer, Effets cytologiques de la sarcolysine sur des fibroblastes normaux cultivés *in vitro*. *C.R. Soc. Biol.* **157,** 2337 (1963).
30. J. Guimard and R. Bassleer, Altérations cytologiques et modifications des synthèse d'acides désoxyribonucléiques dans des fibroblastes normaux cultivés en présence de sarcolysine. *Chemotherapia* **9,** 25 (1964).
31. R. Bassleer, Effets de la sarcolysine sur les protéines nucléaires et les acides désoxyribonucléiques de fibroblastes cultivés *in vitro*. *C.R. Soc. Biol.* **159,** 1242 (1965).
32. G. P. Wheeler, B. J. Bowdon, D. J. Adamson and M. H. Vail, Effects of certain nitrogen mustards upon the progression of cultured H. Ep. No. 2 cells through the cell cycle. *Cancer Res.* **30,** 100 (1970).
33. S. Peel and P. A. Fletcher, Changes occurring during the growth of Ehrlich ascites cells *in vivo*. *Europ. J. Cancer* **5,** 581 (1969).
34. Cl. Desaive and R. Bassleer, Mécanisme de l'action antimitotique de la daunomycine dans les cellules tumorales d'Ehrlich. *C.R. Acad. Sci. (Paris)* **272,** 1713 (1971).

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Reticulum Cell Sarcoma, an Oncologic Model for a System of Classifying the Malignant Lymphomas

K. MUSSHOFF, H. SCHMIDT-VOLLMER and D. MERTEN
Radiation Department Medical University Clinic, Freiburg im Breisgau, Germany

Abstract—*In three quarters of those patients with lymphoreticular tumors (reticulum cell sarcoma and lymphoreticular sarcoma) the disease begins in lymph nodes and in one quarter of the cases in extranodular organs. If patients are suffering from primary lymph node involvement, the disease tends to be widespread. In patients with primary organ involvement the disease remains limited to local regions for a longer time and shows a greater tendency towards a continuous rather than a discontinuous spread.*

Patients with primary lymph node and organ involvement have almost the same prognosis when receiving the same treatment: In stages I *and* II *the* 5 *year survival rates for primary lymph node involvement is* 50%, *for primary organ-involvement,* 59%. *The corresponding percentage rates of permanent cure are* 48% *and* 45%. *Under present therapy conditions, the prognosis of both forms in the advanced stages* III *and* IV *is very unfavourable.*

Comparative prognosis renders it possibly advisable to classify patients with primary lymph node and organ involvement together.

INTRODUCTION

UNIVERSAL agreement on a practical method of staging Hodgkin's disease and the lymphomas is rendered necessary for the exchange of information on the natural history of these diseases and response to treatment.

Unfortunately the four stage Rye classification for Hodgkin's disease, as originally defined [1, 2], was not suited to all clinical situations. This system was used in the erroneous opinion, that extranodal involvement in Hodgkin's disease was regarded as a manifestation of diffuse dissemination regardless of the extent of involvement (Stage IV). Subsequent to 1965, the concept that organ involvement is necessarily indicative of stage IV of dissemination has been disproved. Limited presentation of Hodgkin's disease with organ involvement is currently recognized as localized disease. When treated with intensive regional radiotherapy, limited organ involvement has been shown to have the same prognosis as a lymph node involvement of similar extent and therefore should be classified according to the same system [3–7].

Localized manifestations of organ involvement of Hodgkin's disease generally result from direct invasion from adjacent lymph node involvement. In contrast to Hodgkin's disease, approximately 25% of reticulum cell sarcoma and 10% of lymphosarcoma apparently originate in non-lymphatic tissue or organs.

A retrospective investigation was undertaken using reticulum cell sarcoma (RCS) and lympho-reticular sarcoma (LRS) as a model to determine whether a modified basis of the Rye system of staging Hodgkin's disease could be applied to extranodal presentation of the lymphomas, despite differences in kinds of origin and possible differences in kinds of spread.

As regards Hodgkin's disease localized to

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organs, the answers to three basic questions were required, i.e.

1. Are the patterns of spread for RCS and LRS present in organs the same as those of RCS and LRS of lymph node origin?

2. Does primary organ involvement without continuity and given the same treatment as primary lymph node involvement show the same prognosis?

3. What is the dose of radiation for the complete destruction of localized manifestations of RCS and LRS?

MATERIAL

One hundred and ninety-one primary untreated patients with RCS and 33 with LRS were treated in the University Medical and Ear-Nose-Throat clinics at Freiburg im Breisgau between 1948 and 1968. All patients were treated with radiotherapy. Some of the patients from the Ear-Nose-Throat clinic had preliminary surgery followed by radiotherapy and chemotherapy. Both groups, RCS and LRS, were examined together.

RESULTS

1. *Primary site and pattern of spread*

(*a*) In 77% of all patients with RCS and LRS the disease occurred primarily in the lymph nodes and lymphoid tissue, and in 23% in a non-lymphatic organ.

Table 1. *Reticulum cell sarcoma (RCS) and lymphoreticular sarcoma (LRS) Freiburg i. Br.*, 1948–1968, *patients not formerly treated* (n = 224)

		Primary site of disease Stages I through IV			
		in lymph nodes		in organs	
Collective	n	n	%	n	%
RCS	191	151	79	40	21
LRS	33	21	64	12	36
Total	224	172	77	52	23

(*b*) In both groups the disease started in the head and neck region in the great majority of the cases:

(i) in primary lymph node and extranodal lymphoid tissue involvement in the region of Waldeyer's tonsillar ring and neck lymph nodes, and

(ii) in primary extranodular organ and tissue involvement in the nasal- and epipharynx space, maxillary sinus and remaining regions of the head (Table 2).

(*c*) At the time of diagnosis one half of the patients with primary lymph node involvement

Table 2. *RCS and LRS* 1948–1968, *stages* I *and* II (n = 116)

	Distribution of primary disease					
	stage I		stage II		stage I and II	
Primary site	n	%	n	%	n	%
A primary LN involvement	39	100	42	100	81	100
Waldeyer's Ring	17	40	17	44	34	42
cervical	34	36	15	37	21	36
axillary	4	10	4	9	8	10
mediastinal	1	2,5	4	9	5	6
inguinal	2	5	2	5	4	5
others	1	2,5			1	1
B primary organ involvement					35	100
nasal- and epipharynx space					12	34
skull					6	16
skin and soft parts of face					5	14
maxillary sinus					3	9
parotid gland					2	6
other bones					2	6
lungs					2	6
larynx					1	3
stomach					1	3
soft tissue of extremities					1	3

were in stages I and II and the others in stages III and IV. On the other hand, two thirds of the patients with primary organ involvement belonged to stages I and II and only one third to stages III and IV.

Table 3. *RCS and LRS* 1948–1968 (n = 224)

Stage	Primary site of disease			
	in lymph nodes (n = 172)		in organs (n = 52)	
	n	%	n	%
I+II	81	47	35	67
III+IV	91	53	17	33

This distribution pattern suggests the conclusion that primary organ involvement remains localized for a longer time and that primary lymph node involvement tends to spread earlier.

(*d*) This different spreading tendency in relationship to the place of origin of the disease can also be confirmed by the evolution of the disease after termination of the first treatment: the patients with loco-regional involvement in stages I and II, whose disease has been arrested after the first treatment, are especially suitable for the investigation of this question. These patients, whose disease has been arrested, amounted to 69/81 = 85% in the group with primary lymph node involvement, and to 28/35 = 80% in the group with primary organ involvement.

The disease recurred in 33/69 = 48% of the patients with primary lymph node involvement and 16/28 = 57% of the patients with primary organ involvement.

We may distinguish between four different types of recurrence after a first disease-free interval:

1. Local recurrences.
2. Recurrences in neighbouring lymph nodes or neighbouring non-lymphatic organs.
3. Recurrences in distant lymph nodes.
4. Recurrences in distant non-lymphatic organs.

The first recurrences after a first disease-free interval were distributed as follows:

(i) Local and regional relapses were 1·5 times more frequent in cases with primary organ involvement than in cases with primary lymph node involvement.

(ii) On the other hand, recurrences in distant lymph nodes were 4 times more frequent in cases with primary lymph node involvement than in cases with primary organ involvement, in which they occurred relatively rarely.

(iii) In both groups first recurrences in distant organs were rare (Table 4).

(iv) If further recurrences occur in the later course of the disease, they show the same distribution tendency in relationship to the place of origin of the disease: if the disease began in a non-lymphatic organ, regional relapses were again predominant; if it began in lymph nodes, relapses in distant lymph nodes and especially in distant organs prevailed.

2. *Prognosis*

The 5-, 10-, and 15-year survival rates for patients with RCS and LRS limited to the organ of apparent origin were the same as for patients with a single lymph node involvement. In the loco-regional stage II the survival rates of the patients with primary organ involvement were more than twice as high as in the groups with primary lymph node involvement. In stage III the 5- to 15-year survival rates for patients with primary lymph node involvement amounted to

Table 4. *RCS and LRS* 1948–1968, *stages* I+II(n = 116)

Stage		Permanent local control	Subsequent new disease				
			total		1–2 local+ adjacent	3 in distant LN	4 in distant organ
	n	n	n	%	%	%	%
I+II	116	97	49	50	38	9	3
(*a*) begin in LN	81	69	33	48	33	12	3
(*b*) begin in organs	35	28	16	57	50	3·5	3·5

11% while it amounted to 0% for patients with primary-organ involvement. In stage IV with wide-spread organ involvement the 5-year survival rate was nil.

Taking a 10-year remission as an arbitrary definition of permanent cure [8], the cure rates and survival rates were parallel in patients in stage I with 67% for those with primary lymph node involvement and 44% for those with primary organ involvement. They are very much better in spreading stage II of primary organ involvement, the survival rate being 45% more than the 27% for those patients with primary lymph node involvement of equal extent.

Cure and survival rates of this nature were hardly attainable in stage III patients and not attainable in stage IV "per dissemination" patients, but were attainable in stage IV "per continuitatem" patients.

Table 5. *RCS and LRS* 1948–1968, *stages* I *through* IV (n = 224)

Stage	Primary site of disease							
	in lymph nodes				in organs			
	Survival rates % in							
		years				years		
	n	5	10	15	n	5	10	15
I	(39)	76	66	59	(13)	61	61	61
II	(42)	28	28	19	(22)	58	58	49
I+II	(81)	50	42	38	(35)	59	59	51
III	(34)	11	11	11	(7)	0	0	0
IV disseminated (p.d.)	(51)	0	0	0	(10)	0	0	0
IV localized (p.c.)	(6)	56	56					
Total	(172)	28	28	23	(52)	36	36	31

Comparative survival figures for primary lymph node versus primary organ involvement.

Table 6. *RCS and LRS* 1948–1968, *stage* I *through* IV (n = 224)

Stage	10 years disease free survival rates %	
	Primary site of disease in lymph nodes	Primary site of disease in organs
I	67	44
II	27	45
I+II	48	45
III	4	0
IV (p.d.)	0	0
IV (p.c.)	50	
Total	30	31

Percentage of patients surviving ten or more years in complete clinical remission following an initial treatment for disease originating in lymph nodes or disease originating in organs.

3. *Lethal focal doses*

We have tried to answer the third question by a retrospective investigation, similar to that for Hodgkin's disease, by comparing local radiation dose, time-length of radiation and appearance of a local recurrence according to Strandquist's method van der Werf-Messing [9] performed this in a similar way. In the loco-regional stages I and II, 8 local recurrences appeared in 116 patients following radiation therapy. By using this method the lethal focal dose amounted to 4500 rads in four-and-a-half weeks. The rate of local recurrences under these conditions is only about 1%. Taking 4000 rads in 4 weeks the rate of local recurrence is about 5% (Fig. 1).

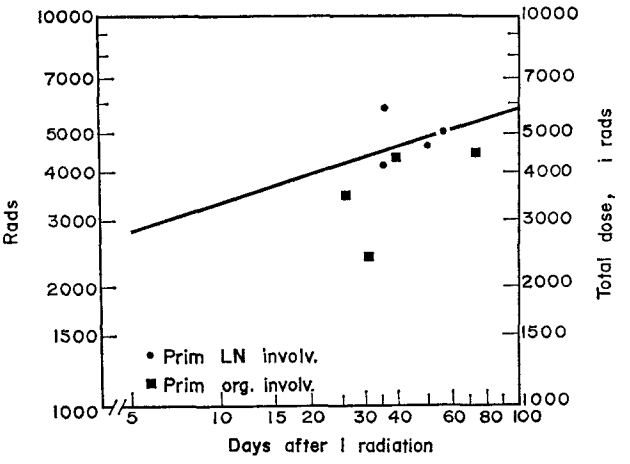


Fig. 1. *RCS and LRS, stages I and II* (n = 116). *Eight local relapses out of a total of* 116 *patients.*

DISCUSSION

1. Reticulum cell sarcoma and lymphoreticular sarcoma represent an oncologic model within

the group of malignant lymphomas, which differs in a characteristic manner from the most thoroughly investigated lymphoma model, the Hodgkin's disease model:

(*a*) In Hodgkin's disease primary extranodular organ involvement is extremely rare, and probably does not exist, whereas in RCS and LRS primary organ involvement accounts for approximately 20% of the cases regardless of the stage. This differentiation of nodal and extranodal sites of origin in RCS and LRS is based on differences in clinical behaviour: in patients with primary organ involvement the disease is localized for a longer time and recurrences appear predominantly in neighbouring regions. Patients with primary lymph node involvement suffer recurrences predominantly in distant lymph node regions and show a greater tendency towards dissemination.

(*b*) The first recurrence after disease-free interval is observed predominantly in regions of previous primary organ involvement and those adjacent to them whereas in primary lymph node involvement a large number of distant lymph node regions are attacked. Further recurrences during the development of the disease show the same tendency: if the disease begins primarily in organs, regional recurrences are again predominant; if it begins in lymph nodes then the recurrences occur in distant lymph nodes as well.

2. The lymphoreticular cell tumor is sensitive to radiation, whereby the lethal focal dose is somewhat larger than used in the treatment of Hodgkin's disease.

3. The following cure rates have been achieved in the loco-regional involvement stages of both disease groups by local radiation treatment: this percentage amounts to 44% for the involvement of an extranodular organ or tissue in stage I, and to 45% for additional regional involvement in stage II. Corresponding percentages of permanent cure in patients with primary lymph node involvement are 67% in stage I and 27% in stage II. In comparison, therapy results in advanced stages III of both groups are very unsatisfactory and practically nil. These results are a definite confirmation of the opinion first expressed by Dancot [10–13], after treating a large group of patients in Brussels, that lymphoreticular sarcomas in the stage of loco-regional involvement can be cured to a large extent by radiotherapy.

4. Although therapy results are not the same, they are similar and essentially comparable in both groups of patients with lymph node and extranodular organ involvement and this enables us to classify both forms of the disease together. Classification is determined not by the site of the primary involvement but solely by the extent of the disease. In relation to the Rye stage classification for Hodgkin's disease, our stage classification is then given in Table 7 as follows:

Table 7. Reticulum cell sarcoma, Hodgkin's disease, and lymphosarcoma staging classification (K. Musshoff, 1969 [14])

Stage	Involvement
I	Disease limited to (1) 1 lymphatic region, nodal or extranodal (Waldeyer's ring, spleen) or (2) 1 non-lymphatic region.
II	(1) Disease in 2 and more lymphatic regions on the same side of the diaphragm (2) including localized non-lymphatic organ involvement primary or incontinuity with lymph node involvement (p.c.-type).
III	(1) Disease in lymphatic regions on both sides of the diaphragm (2) including localized non-lymphatic organ involvement primary or incontinuity with lymph node involvement.
IV	Lymphatic and/or disseminated non-lymphatic organ involvement (p.d.-type).

In Hodgkin's disease each stage will be divided into A without and B with signs and symptoms of active disease.

The survival rates of the total number of patients with reticulum cell sarcoma and lymphoreticular sarcoma which have been classified (in this way) are grouped together in Table 8.

5. Following therapeutical conclusions may be derived from the results:

(*a*) In lymphoreticular tissue recurrence can be prevented by lethal focal doses of 4500 rads in 4½ weeks. As we have been able to demonstrate earlier, additional surgical treatment does not lead to better results [15].

(*b*) Good therapeutical results have already been obtained in the loco-regional involvement stages by local radiotherapy limited to the disease focus as carried out here. Considering the way in which the disease spreads after a disease-free interval, due to therapeutical treatment, it is probable that these can be vastly improved by prophylactic radiation of the surrounding area. B. van der Werf-Messing's

Table 8. *RCS and LRS* 1948–1968, *stages* I *through* IV(n = 224)

Stage		Survival rates after 1 to 20 years in %							
		n	1	2	3	5	10	15	20
I	Involvement of one lymph node region or one organ	52	77	75	75	72	72	57	48
II	Involvement of several lymph node regions on one side of the diaphragm including local limited organ involvement (formerly IV p.c.) primary or secondary site	68	75	55	45	38	35	32	32
III	Involvement of lymph nodes on both sides of the diaphragm including local limited organ involvement (formerly IV p.c.) primary or secondary site	43	55	24	21	13	13	13	13
IV	Lymph node- and disseminated (p.d.) organ involvement	61	28	8	1	0			
	Total	224	58	41	34	30	26	23	21

Overall results for RCS and LRS expressively staged regardless of clinical presentation.

research [9] has provided us with results which lead to the same conclusion. The varying ways in which the disease evolves according to primary lymph node or organ involvement, possibly render a different radiation prophylaxis desirable: radiation of the surrounding area in the case of organ involement and total-nodular radiation in the case of primary lymph node involvement. The correctness of these therapeutical conclusions can be checked by a prospective therapy series, as is now being carried out in the European Cancer Research Group.

The very unsatisfactory therapy results of disease involvement in stage III in primary organ as well as in primary lymph node involvement are possibly caused by the fact that this stage has, till now, not been subjected to systematic radiotherapy. This assumption is the more probable if it is remembered that the results of a therapeutic treatment of Hodgkin's disease in stage III were unfavourable before the introduction of systematic total nodular radiotherapy [16–18] and were improved enormously after its introduction by Kaplan [7] and others [19, 20].

6. The results which we found, regarding the lymphoreticular tumor disease and which have been published in this study, were also found in a small group of patients with lymphosarcoma [14]. These results point to the fact that the oncological model of lymphoreticular tumors (RCS and LRS) presented here is also basically applicable to the lymphosarcoma.

REFERENCES

1. J. Bernard, S. Chiappa, P. Denoix, H. S. Kaplan, A. Laugier, R. J. Lukes, G. Mathé, G. Nézeloff, M. V. Peters, A. Ratti and M. Tubiana, Proposition d'une classification des différentes formes cliniques de la maladie de Hodgkin, in *La Radiothérapie de la Maladie de Hodgkin*, Symposium international organisé à Paris le 15 février 1965. Supplément au *J. Radiol. Electrol.* **47,** 175 (1966).
2. S. A. Rosenberg, Report of the Committee on Staging of Hodgkin's disease. *Cancer Res.* **26,** 1310 (1966).
3. K. Musshoff, H. Renemann, L. Boutis and J. Afkham, The Differentiation of two different types of stage IV Hodgkin's disease. *Second International Congress of Lymphology*, Miami, U.S.A., March 15–20, 1968. *Progress in Lymphology* II, 272, Stuttgart (1970).
4. K. Musshoff, H. Renemann, L. Boutis and J. Afkham, Die extranodulare Lymphogranulomatose-Diagnose, Therapie und Prognose bei zwei unterschiedlichen Formen des Organbefalls. Ein Beitrag zur Stadieneinteilung des Morbus Hodgkin. *Fortsch. Röntgenstfahlen* **109,** 776 (1968).
5. M. V. Peters, A clinical classification of the malignant lymphomas related to the course of the disease. *Second International Congress of Lymphology*, Miami, U.S.A., March 15 (1968).
6. L. M. Fuller, J. F. Gamble and J. J. Butler, Current concepts of intensive regional radiotherapy in the treatment of localized and generalized Hodgkin's disease. XII. *International Congress of Radiology*, Oct. 6–11, 1969. *Book of Abstracts* **50,** 140.

7. H. S. KAPLAN, On the natural history, treatment, and prognosis of Hodgkin's disease. *The Harvey Lectures (Ed. Academic Press)*, Series **64,** 215 (1970).
8. K. MUSSHOFF and L. BOUTIS, Die Frage der Heilbarkeit der Lymphogranulomatose, beurteilt nach den Behandlungsergebnissen der Freiburger Medizinischen Klinik. *Verh. dtsch. Ges. inn. Med.* **73,** 322 (1967).
9. B. VAN DER WERF-MESSING, Reticulum cell sarcoma and lymphosarcoma, a retrospective study of potential survival in loco-regional disease. *Europ. J. Cancer* **4,** 549 (1968).
10. H. DANCOT, Etude du prognostic des lymphosarcomes et des réticulosarcomes. *J. belge Radiol.* **49,** 264 (1966).
11. H. DANCOT, Über die Heilungsaussichten bei Lymphosarkomen und Retikulosarkomen durch Bestrahlung (Ergebnisse einer Untersuchung bei 395 Fällen). *Deutscher Röntgenkongress* 1966, *Teil B Strahlenbehandlung und Strahlenbiologie. Sonderbänder zur Strahlentherapie* **64,** 283 (1967).
12. H. DANCOT, Etude comparée des résultats obtenus pour les lympho- et réticulosarcomes aéro-digestifs supérieurs et ganglionnaires. *Bull. Cancer* **55,** 439 (1968).
13. H. DANCOT, Lympho- et réticulosarcomes digestifs. Relations avec les autres lympho- et réticulosarcomes. *Acta gastro-ent. belg.* **32,** 141 (1969).
14. K. MUSSHOFF, Therapy and prognosis of two different forms of organ involvement in cases of malignant lymphoma (Hodgkin's disease, reticulum cell sarcoma, lymphosarcoma) as well as a report about stage division in these diseases. XII. *International Congress of Radiology*, Tokyo, October 1969. *Book of Abstracts* **50,** 138. *Klin. Wochenschr.* **48,** 673 (1970).
15. H. RENEMANN, K. MUSSHOFF and J. BECKER, Retikulumzellsarkom der oberen Atem- und Speisewege, einschließlich von Kopf und Hals. In *Gauwerky: Maligne Lymphome Deutscher Röntgenkongress*, Hamburg, 1968, München-Berlin-Wien 139 (1969).
16. M. V. PETERS, A study of survivals in Hodgkin's disease treated radiologically. *Amer. J. Roentgenol.* **63,** 299 (1950).
17. C. M. NICE and K. W. STENSTROM, Irradiation therapy in Hodgkin's disease. *Radiology* **62,** 641 (1954).
18. C. HILTON, Malignant lymphomas. Classification, prognosis and treatment. *Lancet* **283,** 1962. Ref. K. MUSSHOFF *et al.:* Die Behandlung der Lymphogranulomatose. In Heilmeyer-Hittmair, Hdb. der ges. *Haematologie* **2,** 421 (1969).
19. K. MUSSHOFF and L. BOUTIS, Die Behandlungsergebnisse der malignen Lymphogranulomatose (Morbus Hodgkin) in Abhängigkeit von individuellen und krankheitsspezifischen Faktoren und der Therapie. Freiburger Ergebnisse 1948–1967. *Klin. Wochenschr.* **47,** 93 (1969).
20. K. MUSSHOFF, P. KALKOFF, K. -H. STRICKSTROCK, A. BÖHMERT, W. MAYER and D. MERTEN, Zur Strahlenbehandlung der Hodgkin'schen Erkrankung. Die Ergebnisse der radikalen Megavolttherapie mit ausgedehnten Feldern im Vergleich mit der konventionellen Orthovolttherapie. 1. Mitteilung: Gesamtergebnisse Freiburger Krankengut 1948–1964 und 1964–1969. *Strahlentherapie* **141,** 381 (1971).

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Metabolic Controls in Precancerous Liver: Defective Control of Cholesterol Synthesis in Rats Fed N-2-Fluorenylacetamide

BRIAN J. HORTON and JOHN R. SABINE
Department of Animal Physiology, Waite Agricultural Research Institute, University of Adelaide, South Australia, Australia

Abstract—*We have measured synthesis of cholesterol from ^{14}C-acetate in liver slices from rats fed the hepatocarcinogen, N-2-fluorenylacetamide (FAA). Within 2–3 weeks of beginning FAA feeding (a) cholesterol synthesis was increased (b) feedback control of cholesterogenesis was partially or completely lost, (c) cholesterol synthesis was extremely variable within all groups fed FAA. Feedback control was regained within 16 weeks in the liver, but remained defective in the hyperplastic nodules present on some livers at that time.*

The study of such precancerous livers should prove useful in understanding both the mechanism of lost metabolic control shown by fully-developed hepatomas, and the relationships between loss of control and the neoplastic process.

INTRODUCTION

A MAJOR feature of all hepatomas studied is loss of metabolic control [1]. For example, Bottomley *et al.*, have shown loss of inducibility of the enzymes glucose-6-phosphate dehydrogenase [2], serine dehydratase [3], threonine dehydrase [3], tyrosine α-ketoglutarate transaminase [4] and tryptophan pyrrolase [5] in a variety of Morris hepatomas. But in most cases there are exceptions, in that each enzyme can be induced in some tumours but not in others, while most tumours retain at least some control of enzyme induction. On the other hand no hepatoma yet tested has been found to have normal control of cholesterogenesis [6]. Cholesterol synthesis is inhibited by cholesterol feeding [7] and by fasting [8] and is increased by feeding cholestyramine [9] in the normal liver, but not in the liver tumour, neither primary nor transplanted [10–14].

A similar loss of metabolic control has also been reported to occur in the livers of carcinogen-treated animals. For example, Poirier and Pitot [15–18] found that livers of rats fed FAA† or 3′MeDAB† resemble hepatomas in that some enzymes of amino acid and carbohydrate metabolism can not be induced. They found that in the first 5 weeks of FAA feeding 9 out of 10 enzymes tested showed loss of inducibility at some time, and that in rats fed 3′MeDAB 6 out of 10 enzymes showed faulty induction in the first 5 weeks, though control returned after 4 weeks on a normal diet [16].

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*Inhibition of cholesterol synthesis by cholesterol feeding is often referred to as "feedback" inhibition [6]. We use this term in a general sense only, and it is not intended to imply any particular mechanism of action.

†The following abbreviations have been used:
FAA: N-2-fluorenylacetamide (2-acetylaminofluorene);
3′MeDAB: 3′methyl-4-dimethylaminoazobenzene (N,N-dimethyl-*p*-(*m*-tolylazo)-aniline).

Furthermore, Siperstein has reported the loss of control of cholesterol synthesis in precancerous liver. He found that two strains of hepatoma-prone mice lacked feedback control of cholesterogenesis [6], as did both rainbow trout [19] and rats [20] within 2 weeks of treatment with aflatoxin.

Because of these findings it is likely that loss of metabolic control of cholesterol synthesis is important in the carcinogenic process, at least in the liver. The present studies were undertaken to examine control of cholesterol synthesis in the early stages of FAA carcinogenesis. A preliminary report of this work has already appeared [21].

MATERIAL AND METHODS

Male, hooded-Wistar rats were used and were 2–3 months old at the beginning of each experiment. All animals were housed in a windowless but well-ventilated room (21–26°C), with lighting strictly controlled; on at 6 a.m., off at 6 p.m. In all experiments they were killed between 9 a.m. and 10 a.m. They were fed a commercial laboratory ration (powdered Charlicks' M164 mouse cubes) *ad libitum*, supplemented where indicated with FAA (K & K Laboratories Inc., Plainview, N.Y.) and/or 5% cholesterol (American Cholesterol Products Inc., Edison, New Jersey). Water was freely available at all times. In our first experiment rats were fed FAA according to the schedule shown in Table 1. This was described by Epstein *et al.* [22] and was reported by him to produce large, hyperplastic, liver nodules in 50% of the rats at 16 weeks and hepatomas in 80% after 10 months. In all other experiments FAA was fed continuously.

Table 1. *Dietary regime for producing hyperplastic nodules on rat liver with FAA* [22]

Diet	Weeks on Diet
Basal + 0·05% FAA	3
Basal	1
Basal + 0·05% FAA	2
Basal	2
Basal + 0·05% FAA	3
Basal	5

Animals were killed by decapitation and their livers quickly removed, placed in ice-cold saline (0·9%) and then sliced with a McIllwain tissue chopper. The incubation of tissue slices with 1-^{14}C-acetate (4 μmoles) and the subsequent extraction and measurement of ^{14}C-cholesterol were carried out as described previously [12]. Cholesterol was measured as total digitonin-precipitable sterols. All incubations were carried out in duplicate, and duplicates were always in close agreement.

RESULTS

Control of cholesterol synthesis was tested at various times during carcinogen treatment by feeding 5% cholesterol for 3 days, then killing the animals and measuring cholesterol synthesis in liver slices. An important feature of our results is the wide variation in rates of cholesterol synthesis shown by all the FAA-treated animals, whereas there was little variation in the control rats. Thus in all Tables the percentage of incorporation of ^{14}C from 1-^{14}C-acetate into cholesterol is shown for each individual animal, and, where appropriate statistical evaluation has been carried out.

In the first experiment rats were fed FAA according to the schedule shown in Table 1 and control of cholesterol synthesis was measured at 3, 8, 11 and 16 weeks (Table 2). At all times the rats not receiving FAA showed normal control of cholesterogenesis. On the other hand, the rats receiving FAA had greatly enhanced levels of cholesterol synthesis, and feedback control appeared to be lost completely at 3 weeks and partially at 8 and 11 weeks. By 16 weeks hyperplastic nodules had formed on the livers of 2 of the 10 rats (Epstein *et al.* [22] found 50% with liver nodules) but all the livers had regained control, even those bearing nodules. The nodular tissue itself showed no control, but this last result must be interpreted cautiously, since only two rats developed nodules.

In all experiments no attempt was made to pair-feed the animals. Those rats fed FAA ate significantly less than control rats (Fig. 1). They lost weight initially, but regained weight quickly when FAA was removed from the diet (Fig. 2). This makes the increased levels of cholesterol synthesis observed in the FAA-fed animals even more significant, since in normal rats fasting lowers cholesterogenesis [8]. Cholesterol feeding caused no change in the quantity of food eaten, nor in weight gained.

Because in the previous experiment control had been lost by 3 weeks, rats were now tested after 1, 2, 3, 4 and 6 weeks of continuous FAA feeding (Table 3). Again cholesterol synthesis by FAA-fed rats was higher than by the controls, particularly for the first two weeks. Feedback

Table 2. The effect of FAA on feedback control of cholesterogenesis in rat liver

Tissue	Dietary supplement		Cholesterol synthesis			
	FAA	Cholesterol	Weeks on FAA feeding regime			
			3	8	11	16
Liver	–	–	0·51 0·42 (0·47)	1·53 0·86 (1·20)	0·47 0·19 (0·33)	0·12 0·32 (0·22)
		+	0·06 0·07 (0·07)	0·05 0·05 (0·05)	0·09 0·10 (0·10)	0·02 0·03 (0·03)
	+	–	0·59 1·62 (1·11)	1·52 1·76 (1·64)	0·52 0·34 (0·43)	0·96 0·68 0·87 0·77 0·82 0·93* (0·84 ± 0·04)
		+	0·98 0·57 (0·79)	0·20 0·40 (0·30)	0·21 0·19 (0·20)	0·07 0·04 0·18 0·11* (0·10 ± 0·03)
Hyperplastic Nodules		–				0·27
		+				1·14

Rats were given the basal diet ± FAA (according to the schedule shown in Table 1) ± cholesterol (5% for 3 days). Cholesterol synthesis is expressed as the percentage of recovery of ^{14}C in ^{14}C-labelled, digitonin-precipitable sterols, following incubation of liver slices with ^{14}C-acetate (4 μmoles). Each value is the average of duplicate assays on one rat liver, with the mean for each group in parentheses.

*These values are from the livers bearing hyperplastic nodules, but from which all nodular tissue was removed before testing.

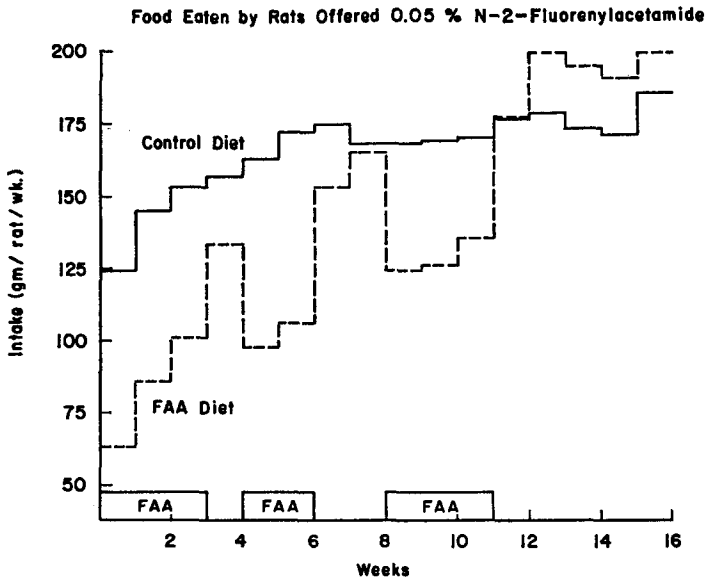


Fig. 1. Food eaten by rats offered chow alone or chow plus 0·05% N-2-fluorenylacetamide according to the schedule in Table 1.

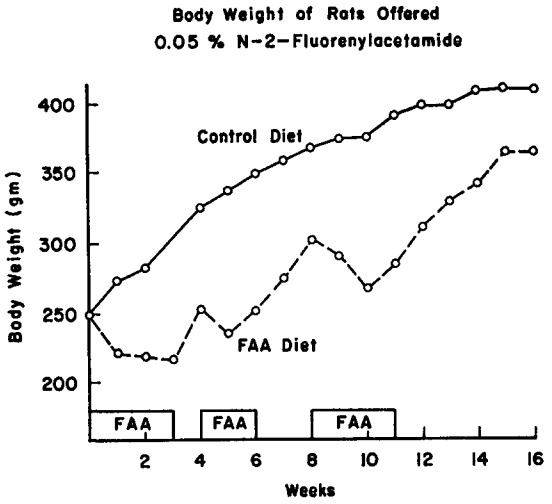


Fig. 2. Body weight of rats offered chow alone or chow plus 0·05% N-2-fluorenylacetamide according to the schedule in Table 1.

Previous work [12] has shown that fasting lowers cholesterol synthesis in the liver but not in the hepatoma. The results, shown in Table 4, indicate that the FAA-fed rats responded normally to fasting, but there appeared to be a marked loss of feedback control, with again wide variation in cholesterogenesis by rats fed FAA alone or FAA and cholesterol.

Finally, in an attempt to produce a consistent loss of control in all animals, we fed FAA at a higher level; 0·2% of the diet, as compared with 0·05% in the previous experiments. The results (Table 5) indicate that again the FAA-fed rats had high levels of cholesterol synthesis and had at least partially lost feedback control. The variation within each group was even greater than that obtained at a lower level of FAA feeding.

Table 3. Control of cholesterogenesis in rat liver during the early stages of FAA feeding

Dietary supplement		Cholesterol synthesis				
		Weeks on FAA diet				
FAA	Cholesterol	1	2	3	4	6
–	–	0·86	1·53	1·48	0·84	0·62
		0·41	0·86	0·78	0·82	1·44
		(0·64)	(1·20)	(1·13)	(0·83)	(1·03)
	+	0·08	0·06	0·39	0·07	0·46
		0·11	0·05	0·15	0·07	0·06
		(0·10)	(0·06)	(0·27)	(0·07)	(0·26)
+	–	2·05	3·36	0·47	1·67	0·57
		1·83	1·15	1·42	0·76	2·00
		(1·94)	(2·56)	(0·95)	(1·22)	(1·29)
	+	0·05	2·39	0·38	0·84	0·47
		0·12	1·43	0·15	0·16	0·60
		(0·09)	(1·91)	(0·27)	0·40	0·63
					0·87	0·12
					0·98	
					(0·65 ± 0·16)	(0·46 ± 0·12)

Rats were given the basal diet ± FAA (0·05%, fed continuously) ± cholesterol (5% for 3 days). Cholesterol synthesis is expressed as in Table 2, with the mean value for each group in parentheses.

control appeared to be normal at 1 week, completely absent at 2 weeks and only partially present at 3–6 weeks. At 4 and 6 weeks the substantial variations within each FAA-fed group were even more apparent. Some rats appeared to have normal control, others partial control and a few no control at all.

To examine further this defective control we tested animals fed the carcinogen for 4 weeks for both feedback control and fasting control.

DISCUSSION

Weis and Deitschy [23] suggested recently that cholesterol synthesis in the liver is under continuous, endogenous, feedback control, in that the cholesterol continuously absorbed from the intestine suppresses enzyme activity. According to this hypothesis cholesterol-feeding increases the amount of cholesterol absorbed, and thus increases enzyme inhibition. Our

Table 4. Dietary control of cholesterogenesis after 4 weeks FAA feeding

	Cholesterol synthesis		
	Diet (for 3 days before assays)		
FAA	Chow	+5% Cholesterol	Fast
−	0·72	0·04	0·10
	1·26	0·12	0·39
	0·45	0·08	0·12
	0·56	0·10	0·01
	1·01	0·07	
	1·35		
	(0·89 ± 0·15)	(0·08 ± 0·01)*	(0·16 ± 0·08)†
+	4·99	1·41	0·28
	3·70	0·72	0·36
	0·69	0·96	0·13
	2·45	2·28	0·23
	1·16	5·70	0·24
		1·05	0·05
		1·85	0·25
			0·31
	(2·60 ± 0·80)‡	(1·99 ± 0·65)§	(0·23 ± 0·04)‖

Rats were given the basal diet ± FAA (0·05% for 4 weeks) then fed chow or 5% cholesterol or fasted, as indicated. Cholesterol synthesis is expressed as in Table 2, with the mean values, with standard error, given in parentheses.

*Significantly different from chow-fed $P<0·001$.
†Significantly different from chow-fed $P<0·01$.
‡Not significantly different from chow-fed $P = 0·05$.
§ Not significantly different from FAA-chow.
‖ Significantly different from FAA-chow $P<0·02$.

Table 5. The effect of a high level of dietary FAA on feedback control of cholesterogenesis in rat liver

Dietary supplement		Cholesterol synthesis	
FAA	Cholesterol (5%, 3 days)	Weeks on FAA diet 2	4
−	−	0·31	0·86
		0·77	0·51
		(0·54)	(0·69)
	+	0·15	0·11
		0·17	0·03
		(0·16)	(0·07)
+	−	6·59	1·43
		0·76	1·38
		1·36	1·76
		(2·90 ± 1·85)	(1·52 ± 0·12)
	+	0·22	0·41
		1·31	1·30
		0·18	0·18
		2·10	1·20
		1·39	3·81
		(1·04 ± 0·37)*	(1·38 ± 0·65)*

Rats were given the basal diet ±0·2% FAA (fed continuously) ± cholesterol (5% for 3 days). Cholesterol synthesis is expressed as in Table 2, with the mean values, with standard error, given in parentheses.

*Not significantly different from the appropriate group not fed cholesterol.

results show that FAA feeding can markedly interfere with this control, in that animals given FAA showed both increased levels of cholesterogenesis, presumably due to loss of the endogenously-activated control, and also no reduction in cholesterol synthesis upon cholesterol feeding.*

It is interesting that the fasting control of cholesterol synthesis was retained at a time when other rats had lost feedback control (Table 4). This suggests that, although both controls act primarily through the same enzyme (β-hydroxy-β-methylglutaryl co-enzyme A reductase), they may act by different mechanisms. Of course, while the fasting animals were not receiving any food, they were also not receiving FAA.

A major feature of our results was the wide variability in rates of cholesterol synthesis shown by the FAA-fed rats, indicating marked differences in the degree of loss of control. The variation could indicate that some rats were markedly affected, some only partially and others not at all, or it could indicate that some rats lost control early in the feeding, then regained it quickly, while others which were normal early lost control later.

In the present experimental approach it is of course necessary to kill the animals in order to test for metabolic control, and hence it is not possible to tell whether any rat or all rats ever completely lose control, or more importantly, whether the rats that do lose control are those that develop tumours. These questions cannot be answered until a test for liver cholesterogenesis can be carried out frequently on the same animal, and we are at present developing such an assay [24].

The reason for partial loss of control is probably not that all cells partially lose control, but rather that there is a proportion of cells in which control is completely lost. It seems likely to us that most cells in the liver can lose control, but can also regain it. We suggest, however, that a few cells suffer an irreversible loss of control, and that these are the cells that develop into the tumour. We do not suggest that loss of control of cholesterol synthesis causes cancer, but we feel that it is an essential step in tumour development. Hence we believe that the study of precancerous livers should prove valuable in understanding the mechanism of the loss of metabolic control that is a characteristic of the fully-developed hepatoma.

*Since it has not been shown conclusively whether feedback control occurs at the level of new enzyme synthesis or enzyme inhibition, we are not attempting to specify the mechanism of action in this paper.

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REFERENCES

1. H. C. Pitot, Some biochemical aspects of malignancy. *Ann. Rev. Biochem.* **35,** 335 (1966).
2. R. H. Bottomley, H. C. Pitot, V. R. Potter and H. P. Morris, Metabolic adaptations in rat hepatomas. V. Reciprocal relationship between threonine dehydrase and glucose-6-phosphate dehydrogenase. *Cancer Res.* **23,** 400 (1963).
3. R. H. Bottomley, H. C. Pitot and H. P. Morris, Metabolic adaptations in rat hepatomas. IV. Regulation of threonine and serine dehydrase. *Cancer Res.* **23,** 392 (1963).
4. H. C. Pitot and H. P. Morris, Metabolic adaptations in rat hepatomas. II. Tryptophan pyrrolase and tyrosine α-ketoglutarate transaminase. *Cancer Res.* **21,** 1009 (1961).
5. Y. S. Cho, H. C. Pitot and H. P. Morris, Metabolic adaptations in rat hepatomas. VI. Substrate-hormone relationships in tryptophan pyrrolase induction. *Cancer Res.* **24,** 52 (1964).
6. M. D. Siperstein, Comparison of the feedback control of cholesterol metabolism in liver and hepatoma. In *Developmental and Metabolic Control Mechanisms and Neoplasia*, p. 427. Williams and Wilkins, Baltimore, (1965).
7. M. D. Siperstein and M. J. Guest, Studies on the site of the feedback control of cholesterol synthesis. *J. Clin. Invest.* **39,** 642 (1960).
8. G. M. Tomkins and I. L. Chaikoff, Cholesterol synthesis by liver. I. Influence of fasting and of diet. *J. biol. Chem.* **196,** 569 (1952).

9. J. W. Huff, J. L. Gilfillan and V. W. Hunt, Effect of cholestyramine, a bile acid binding polymer, on plasma cholesterol and fecal bile acid excretion in the rat. *Proc. Soc. exp. Biol. Med.* **114,** 352 (1963).
10. M. D. Denton and M. D. Siperstein, Control of fatty acid and cholesterol synthesis in mouse hepatoma. *Clin. Res.* **12,** 265 (1964).
11. J. R. Sabine, Control of cholesterol synthesis in hepatomas: the effect of bile salts. *Biochim. Biophys. Acta.* **176,** 600 (1969).
12. J. R. Sabine, S. Abraham and I. L. Chaikoff, Control of lipid metabolism in hepatomas: insensitivity of rate of fatty acid and cholesterol synthesis by mouse hepatoma BW 7756 to fasting and to feedback control. *Cancer Res.* **27,** 793 (1967).
13. M. D. Siperstein and V. M. Fagan, Deletion of the cholesterol-negative feedback system in liver tumours. *Cancer Res.* **24,** 1108 (1964).
14. M. D. Siperstein, V. M. Fagan and H. P. Morris, Further studies on the deletion of the cholesterol feedback system in hepatomas. *Cancer Res.* **26,** 7 (1966).
15. L. A. Poirier and H. C. Pitot, Dietary induction of some enzymes of amino acid metabolism during 2-acetylaminofluorene feeding. *Cancer Res.* **29,** 464 (1969).
16. L. A. Poirier, M. C. Poirier and H. C. Pitot, Dietary induction of some enzymes of carbohydrate metabolism during 2-acetylaminofluorene feeding. *Cancer Res.* **29,** 470 (1969).
17. L. A. Poirier and H. C. Pitot, Dietary induction of some enzymes of amino acid metabolism during azo dye feeding. *Cancer Res.* **29,** 475 (1969).
18. L. A. Poirier and H. C. Pitot, Metabolic adaptions during hepatocarcinogenesis: dietary induction of some enzymes of carbohydrate metabolism during 3′-methyl- and 2-methyl-N, N-dimethyl-4-aminoazobenzene feeding. *Cancer Res.* **30,** 1974 (1970).
19. M. D. Siperstein, Feedback control of cholesterol synthesis in normal, malignant and premalignant tissues. *Proc. 7th Can. Cancer Conf.* **7,** 152 (1967).
20. M. D. Siperstein, Deletion of the cholesterol negative feedback system in precancerous liver. *J. Clin. Invest.* **45,** 1073 (1966).
21. B. J. Horton and J. R. Sabine, Loss of control of cholesterol synthesis in precancerous liver. *Proc. Austral. Biochem. Soc.* **3,** 38 (1970).
22. S. Epstein, N. Ito, L. Merkow and E. Farber, Cellular analysis of liver carcinogenesis: the induction of large hyperplastic nodules in the liver with 2-fluorenylacetamide or ethionine and some aspects of their morphology and glycogen metabolism. *Cancer Res.* **27,** 1702 (1967).
23. H. J. Weis and J. M. Dietschy, Failure of bile acids to control hepatic cholesterogenesis: evidence for endogenous cholesterol feedback. *J. Clin. Invest.* **48,** 2398 (1969).
24. B. J. Horton, J. D. Horton and J. R. Sabine, Repeated esimation of liver cholesterol synthesis *in vivo*, using the inhibitor AY-9944, *Biochim. Biophys. Acta* (in press, 1971).

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Carcinoma of the Bladder Treated by Preoperative Irradiation Followed by Cystectomy

B. VAN DER WERF-MESSING
Rotterdamsch Radio-Therapeutisch Instituut, Rotterdam, The Netherlands

Abstract—*Eighty-eight patients with carcinoma of the bladder, not suitable for radium implant, under 70 years of age, and fit for major surgery, have been treated by cystectomy. The majority received preoperative irradiation (4000 rads in 20 fractions). In stage $T_I N_X M_O$ cystectomy, with its inherent mutilation, seemed unjustifiable as prognosis was not better than after a radical course of external irradiation.*

In stage $T_{II} N_X M_O$ prognosis after cystectomy, as first line of treatment, is significantly better than after a radical course of external irradiation.

The value of preoperative irradiation is not confirmed in stages T_I and T_{II}.

In stage $T_{III} N_X M_O$ preoperative irradiation reduces the T-stage in over two thirds of the cases. If T-reduction occurs, prognosis is improved. If no T-reduction occurs, the mutilating cystectomy seems unjustifiable in view of the poor prognosis.

INTRODUCTION

BETWEEN 1965 and 1970, 88 patients with bladder carcinoma were treated by cystectomy The majority of these cases received preoperative irradiation. The criteria for selection of patients for this type of treatment were as follows:

1. Proven bladder carcinomas of stage T_I, T_{II}, and T_{III} ($N_X M_O$), staging according to the international TNM classification system of the U.I.C.C. [1], which were considered unsuitable for radium-implantation due to a tumour diameter larger than 5 cm.
2. The patient should be fit for cystectomy.
3. The patient is under 70 years of age.

Patients who did not comply with the last two criteria were treated exclusively by radical external irradiation. Cystectomy was carried out as the primary pre-planned policy in the majority of cases. In some patients, cystectomy was resorted to as a secondary line of treatment due to a recurrence or a complication after a previous radium-implant or intracavitary cobalt bead application. A few cases with a T_{IV} growth, infiltrating into the prostate or fixed to the pelvic wall over a small area only, have been included in this treatment regime.

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MATERIAL AND METHODS

Eighty-four males and 4 female patients were submitted to cystectomy. The average age of the patients was 59·5 years (stand. dev.: on the average 6 months). The majority of the patients received preoperative irradiation: a midplane dose of 4000 rads was delivered to the whole small pelvis in 20 fractions over a 4-week overall time. Immediately following the last irradiation, cystectomy was performed.

One urologist preferred to perform the operation in 2 stages: ureteric deviation first, followed by external irradiation, then cystectomy.

Table 1 shows the number of patients and the type of treatment they received with respect to staging.

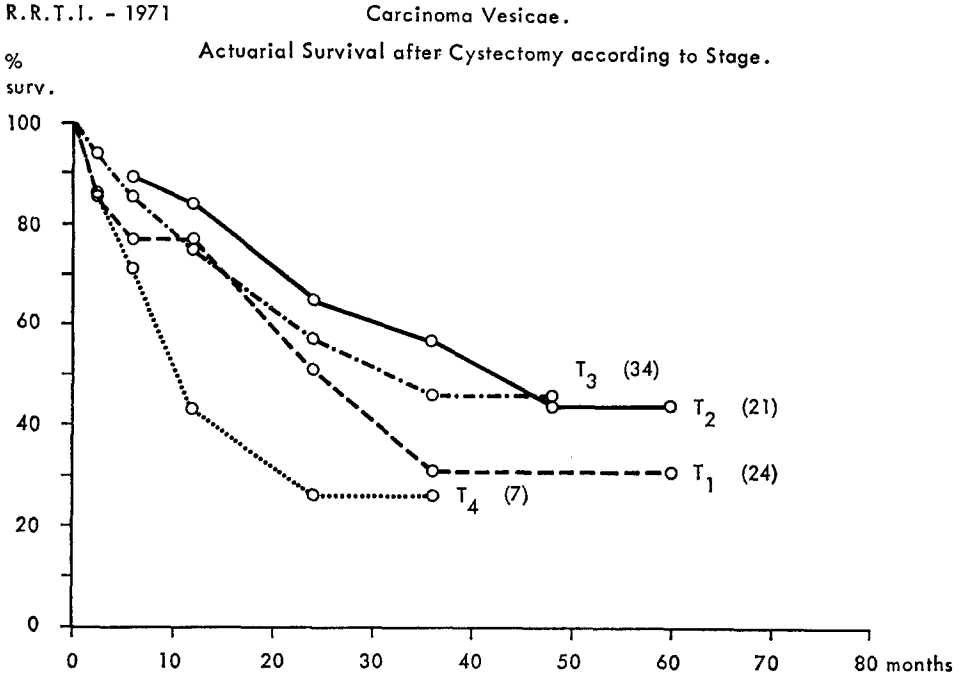
RESULTS

The actuarial survival by stage is demonstrated in Graph 1. In stage $T_I N_X M_O$ no difference in survival was demonstrable be-

Table 1. *R.R.T.I.* 1971. *Patients treated by cystectomy*

Stage	Preop. irradiation		No preop. irradiation	
	Prim. treatment	Sec. treatment	Prim. treatment	Sec. treatment
$T_1N_XM_O$ (24)	4	4	4	8 (Recurrence) 4 (Complications)
$T_2N_XM_O$ (21)	13	3	1	2 (Recurrence) 2 (Complications)
$T_3N_XM_O$ (34)	26	5	—	3 (Recurrence)
$T_4N_XM_O$ (7)	7	—	—	—
Ca *in situ* (2)	—	—	2	—
Total	50	12	7	19

Categorized according to stage, preoperative irradiation, primary treatment or secondary treatment after failure of previous radium therapy.



Graph 1

tween the group treated primarily and the group that underwent the operation as a secondary measure after failure of radiotherapy. There was no difference in survival either between the group that received preoperative irradiation and the group that did not.

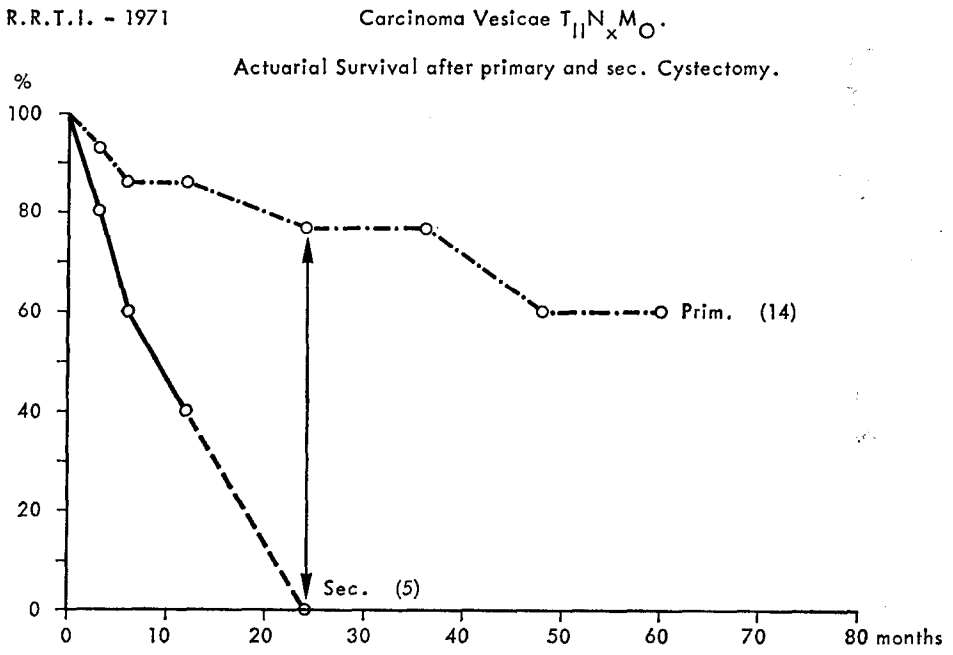
Stage $T_{II}N_XM_O$ showed no difference in survival with respect to whether or not a preoperative course of irradiation was given. A significantly better 2-year prognosis was noted in those cases which underwent surgery as their primary treatment policy as compared with the group which was operated on as a secondary measure (Graph 2).

In stage $T_{III}N_XM_O$ prognosis was similar for primarily and secondarily treated patients.

Operation mortality including deaths due to complications within 3 months postoperatively amounted to 14%. It was not demonstrably influenced by the preoperative irradiation (Table 2).

The causes of death in each stage are presented in Table 3.

In the majority of the cases the pretreatment T-stage could be compared with the P-stage, i.e. the microscopic depth of invasion as diagnosed by examination of the cystectomy specimen. Radiogenic T-stage reduction was noted in 69% of all cases (Table 4). In all



Graph 2

Table 2. *R.R.T.I.* 1971. *Operation mortality after cystectomy*—relation to stage and preop. irradiation*

Stage	Preop. irradiation	No preop. irradiation	No preop. irradiation (complications after previous treatment)	Total
$T_1N_XM_O$	2/8	3/12	2/4	7/24 29%
$T_2N_XM_O$	2/16	0/3	0/2	2/21 9·5%
$T_3N_XM_O$	3/31	0/3	—	3/34 9%
$T_4N_XM_O$	1/7	—	—	1/7 14%
Ca *in situ*	—	0/2	—	0/2 *r*
Total	8/62 12%	3/20 15%	2/6 33%	13/88 15%

*Included death due to complications within 3 months after operation.

Table 3. *R.R.T.I.* 1971. *Cause of death after cystectomy in each stage*

	$T_1N_XM_O$	$T_2N_XM_O$	$T_3N_XM_O$	$T_4N_XM_O$	Ca. *in situ*
Death due to operation (within 3 months)	**7**	2	3	1	—
Urosepsis more than 3 months after operation	1	2	1	—	—
Iliac+lumbar glands	1	1	**8**	—	—
General metastases	2	2	3	3	1
Recurrence in ureter region	—	1	—	—	—
Abdominal wall infiltration	—	1	—	—	—
Intercurrent	2	2	1	1	—

non-irradiated cases T-stage was in accordance with P-stage!

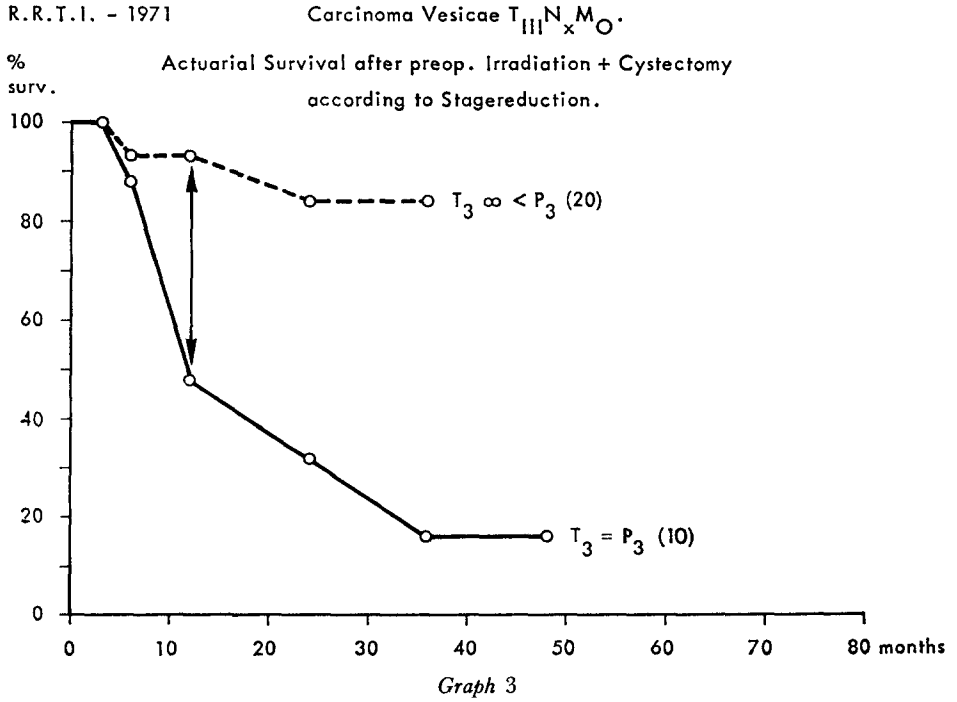
T-reduction appeared not to be related to the degree of differentiation of the growth (Table 5). The reduction in depth of growth of the primary (T-reduction) appeared to have no influence on prognosis in stages T_I and T_{II}, but in stage T_{III} this T-reduction resulted in a significantly increased 1-year survival rate (Graph 3). On examining the cystectomy specimen, in 12% of all cases cancer was found in the resection plane of a ureter or the urethra (Table 6). In the majority of those cases a secondary urethraectomy or nephro-ureterectomy was performed.

Table 4. R.R.T.I. 1971. "Stage reduction" by preoperative irradiation

Pretreatment clinical T-stage	No.	Postirradiation P-stage of cystectomy specimen	
T_I	8→	$<P_1$: 4 50%	(P_0 : 4)
T_{II}	16→	$<P_2$: 12 75%	(P_0 : 4)
T_{III}	30→	$<P_3$: 20 67%	(P_0 : 9)
T_{IV}	7→	$<P_4$: 6 85%	(P_0 : 1)
Total	61→	T-reduction : 42 69%	

Table 5. R.R.T.I. 1971. Reduction of depth of infiltration by preoperative irradiation in relation to histol. degree of differentiation

	High and medium degree of diff.		Low degree of diff.		Total
Stage reduction (T-stage higher than P-stage)	30	68%	12	70%	42
No stage reduction (T-stage = P-stage)	14	32%	5	30%	19
Total	44	100%	17	100%	61



Graph 3

Table 6. R.R.T.I. 1971. Ca. vesicae—cystectomy

	In urethra	In ureter	Total
$T_1N_XM_O$ (24)	2 (+1 also in ureter)	2	4/24 (17%)
$T_2N_XM_O$ (21)	—	3	3/21 (14%)
$T_3N_XM_O$ (34)	1	1	2/34 (6%)
$T_4N_XM_O$ (7)	2	—	2/7 (28%)
Ca. *in situ* (2)	—	—	0/2 (0%)
Total (88)	5 (+1)	6	11/88 (12·5%)

Cancer in resection plane of ureter and or urethra (by stage).

DISCUSSION

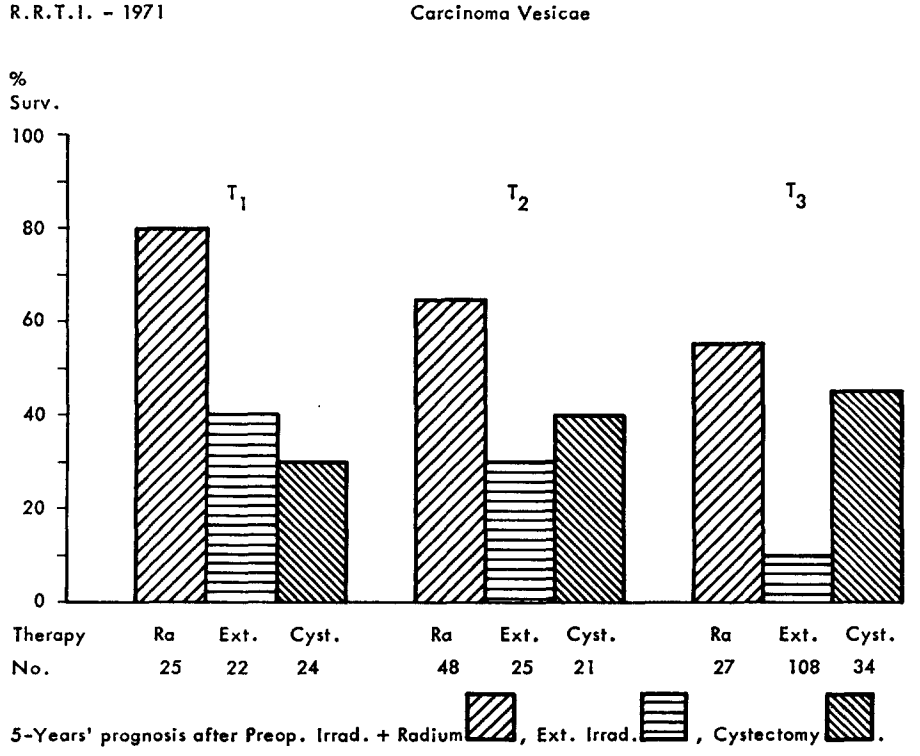
Graph 4 compares the 5-year survival rate between patients treated by external irradiation followed by a radium-implant, patients treated with a radical course of external irradiation [2], and those treated by cystectomy. As the patients who underwent cystectomy were not suitable for radium-implant because of the large diameter of their growth, only survival rates of patients treated with radical external irradiation and those treated by cystectomy can relevantly be compared in each stage. Even these two groups are not completely comparable, as those patients who were unfit or too old for cystectomy received a radical course of external irradiation.

In stage $T_IN_XM_O$ prognosis after cystectomy compares unfavourably with that after a full radical course of external irradiation (Graph 4). This is in part due to an unexplained high operation mortality. But even when excluding operation mortality, the 5-year survival rate is only about 40% (Graph 5). Hence it seems hardly justifiable to perform the mutilating operation in T_I category.

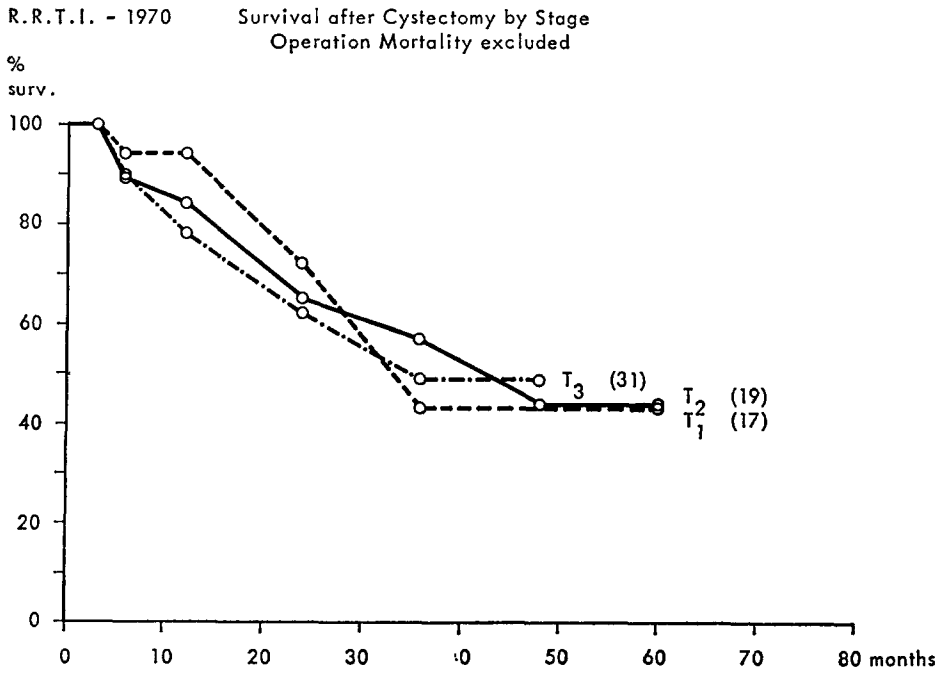
If failures after previous radiotherapy are excluded, in stage $T_{II}N_XM_O$ primary cystectomy is justified. It offers a significantly better prognosis than a radical course of external irradiation (Graph 2 and Graph 4).

In our small group of patients in stages $T_IN_XM_O$ and $T_{II}N_XM_O$, preoperative irradiation did not improve prognosis. This finding is in agreement with others [3].

In stage $T_{III}N_XM_O$, the group which shows T-reduction has a good prognosis which cannot be equalled by external irradiation (Graph 3 and Graph 4). In patients where no radiogenic stage reduction could be demonstrated, prognosis is as poor as after external irradiation. As the clinical T-stage assessment is most reliable for experienced clinicians and the only danger is understaging of a growth, especially in the case of its location on the anterior bladder wall, it may safely be postulated that the observed T-stage reduction is radiogenic and not the result of clinical error. It seems justifiable to re-examine the patient after having given



Graph 4



Graph 5

4000 rads and to evaluate by cystoscopy and bimanual palpation under general anesthesia whether a T-reduction has taken place. If so, cystectomy is apparently indicated. If not, a full radical course of irradiation might give the same result *quo ad vitam* without unnecessary mutilation.

As 50% of the deceased T_{III} patients died of metastases to the lower lumbar and iliac glands (Table 3) it is worth considering to include those glandular regions in the irradiation field. However, an increase in the risk of complications at the level of the ureteric anastomosis might occur after intensive irradiation of this vulnerable region. Unfortunately the degree of differentiation of the growth is no indicator of the chance of T-reduction. The number of T_{IV} cases is too small for conclusions but in this highly selected group the applied treatment modality was apparently adequate.

Of the two patients with carcinoma *in situ* one died of metastases, and one is "cured". The small number permits no conclusions.

In 12% of the cases cancer was found in the resection plane of a ureter or the urethra. This had serious therapeutical consequences. Therefore careful microscopical examination of these structures is necessary.

CONCLUSION

In carcinoma of the bladder stage $T_IN_XM_O$ cystectomy seemed too high a mutilating price for the obtained results. External irradiation might do as well. In previously untreated stage-$T_{II}N_XM_O$ patients cystectomy is justifiable as prognosis is better than after radical external irradiation. Preoperative external irradiation did not demonstrably improve the prognosis.

In stage $T_{III}N_XM_O$ precystectomy irradiation caused T-stage reduction in 69% of the growths. In case of stage reduction prognosis became significantly better than after radical external irradiation. In case of absence of such a T-reduction, prognosis was poor and cystectomy seems unjustifiable.

Acknowledgments—I am largely indebted to all Rotterdam urologists for their cooperation, to J. G. A. H. Kaalen for statistical analysis, to L. Ries for drawing the graphs and to Miss P. de Haan for the secretarial help.

REFERENCES

1. International Union against Cancer, TNM classification of malignant tumours. Geneva (1968).
2. B. van der Werf-Messing, Carcinoma of the bladder treated by suprapubic radium implants. *Europ. J. Cancer* **5,** 277 (1969).
3. W. F. Whitmore, H. Grabstald and A. R. Mackenzie, Preoperative irradiation with cystectomy in the management of bladder cancer. *Amer. J. Roentgenol.* **102,** 570 (1968).

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Structure and Activity in Chemical Carcinogenesis

Studies of Variously Substituted 7-Bromomethylbenz[*a*]anthracenes

A. DIPPLE and T. A. SLADE
Chester Beatty Research Institute, Institute of Cancer Research, Royal Cancer Hospital, Fulham Road, London, S.W.3.

Abstract—*Four new 7-bromomethylbenz*[a]*anthracene derivatives were prepared and their chemical reactivities studied. The abilities of these new agents and the previously studied 7-bromomethylbenz*[a]*anthracene and 7-bromomethyl-12-methylbenz*[a]*anthracene to initiate papilloma in mouse skin were compared. For five out of the six compounds a relationship was found between an increasing tendency to react through a first order process and an increasing ability to initiate papilloma in mouse skin.*

INTRODUCTION

WE HAVE previously studied both the chemistry and carcinogenic activities of 7-bromomethylbenz[*a*]anthracene and 7-bromomethyl-12-methylbenz[*a*]anthracene [1]. The latter compound was the more carcinogenic of the two and also reacted to a greater extent through a first order process in the model system used.

This indication of a direct relationship between biological activity and ability to react through a first order process was considered worthy of further investigation, particularly in view of similar indications from other laboratories [2–4]. Also, for certain types of mutation the mutagenic efficiency of alkylating agents has been related to their Swain-Scott substrate constants and this shows that a high mutagenic efficiency is related to "a relatively high reactivity towards centra with a low nucleophilic strength". [5]. This latter is one property which would be associated with reaction through a first order process and therefore, further studies could conceivably clarify, not only the mechanisms involved in carcinogenesis, but also the relationship, if any, between this process and that of mutagenesis.

Accepted 10 May 1971.

MATERIAL AND METHODS

1-Methylbenz[*a*]anthracene, 6-fluoro-7-methylbenz[*a*]anthracene, 4-chloro-7-methylbenz[*a*]anthracene and 4-bromo-7-methylbenz[*a*]anthracene were all generously provided by Prof. M. S. Newman, Ohio State University, Columbus, Ohio. 7-Bromomethylbenz[*a*]anthracene and 7-bromomethyl-12-methylbenz[*a*]anthracene were prepared as before [1]. All melting points are corrected. Analyses were by Strauss Microanalytical Laboratories, Oxford.

Syntheses

1-*Methyl-7-bromomethylbenz*[a]*anthracene* was obtained in 65% yield when 1-methylbenz[*a*]anthracene (0·2 g) mixed with the clear solution obtained by passing hydrogen bromide through a suspension of paraformaldehyde (0·07 g) in glacial acetic acid (2·5 ml), was kept at room temperature for 48 hr. After

precipitation with water, drying and recrystallization from benzene, the product was obtained as clusters of pale yellow needles, m.p. 193·5–195°C (Found: C, 72·1; H, 4·5; Br, 23·7. $C_{20}H_{15}Br$ requires C, 71·65; H, 4·5; Br, 23·85%). Reduction of this compound with stannous chloride/HCl yielded a product with the same m.p. as the known 1,7-dimethylbenz-[*a*]anthracene.

The other new 7-bromomethylbenz[*a*]anthracene derivatives were prepared by treating the appropriately substituted 7-methylbenz-[*a*]anthracene in carbon disulphide with a molar equivalent of bromine. The bromo-compounds precipitated out, and were collected, washed with dry ether and recrystallized.

6-*Fluoro-7-bromomethylbenz*[a]*anthracene* (yield 62%) was obtained as pale yellow needles, m.p. 195–197·5°C (decomp.) (from benzene) (Found: C, 67·6; H, 3·85. $C_{19}H_{12}BrF$ requires C, 67·25; H, 3·55%). 4-*Bromo-7-bromomethylbenz*[a]-*anthracene* (yield 57%) gave pale yellow silky needles from benzene-cyclohexane (1 : 1) m.p. 244–246°C (Found: C, 57·65; H, 3·4. $C_{19}H_{12}Br_2$ requires C, 57·2; H, 3·05%). 4-*Chloro-7-bromomethylbenz*[a]*anthracene* (yield 60%) gave pale yellow silky needles from benzene-cyclohexane (1 : 1) m.p. 233–235°C (Found: C, 64·6; H, 3·45; $C_{19}H_{12}BrCl$ requires C, 64·5; H, 3·4%).

Reactivity studies

The previously described colorimetric method using 4-(*p*-nitrobenzyl)pyridine [1] was followed exactly.

Papilloma induction experiments

The hair was shaved from the backs of 150 Swiss S female mice (age 8–10 weeks) and two days later the test compound (one micromole) dissolved in benzene was applied to the backs of a group of 25 of these animals. For 4-chloro-7-bromomethylbenz[a]anthracene this dose was administered in 0·15 ml benzene and for the 4-bromo- derivative in 0·1 ml benzene, whilst for the other four compounds 0·05 ml benzene was sufficient. Two weeks after administration of the initiators 10 μg of 12-myristoylphorbol-13-acetate (Hecker's croton oil fraction, A_1) in acetone (0·05 ml) was applied twice weekly to the back of each mouse until each mouse had received 25 such applications. For convenience, an occasional routine application was missed out so that the total 25 applications were administered over a period of 14 weeks.

RESULTS

The results of the animal experiments are presented in Fig. 1. It can be seen that the papilloma incidence was approaching a plateau towards the end of the experiment and that 7-bromomethyl-12-methylbenz[*a*]anthracene was by far the most active compound of the group. The average number of papilloma per papilloma-bearing mouse was 4·1 for this group. This factor was about 1·2 for 6-fluoro-7-bromomethylbenz[*a*]anthracene, the second most active compound, and for 1-methyl-7-bromomethylbenz[*a*]anthracene and 7-bromomethylbenz[*a*]anthracene, both of which gave similar papilloma incidences. The 4-chloro- and 4-bromo- derivatives were the least active agents and only one papilloma per papilloma-bearing mouse was observed.

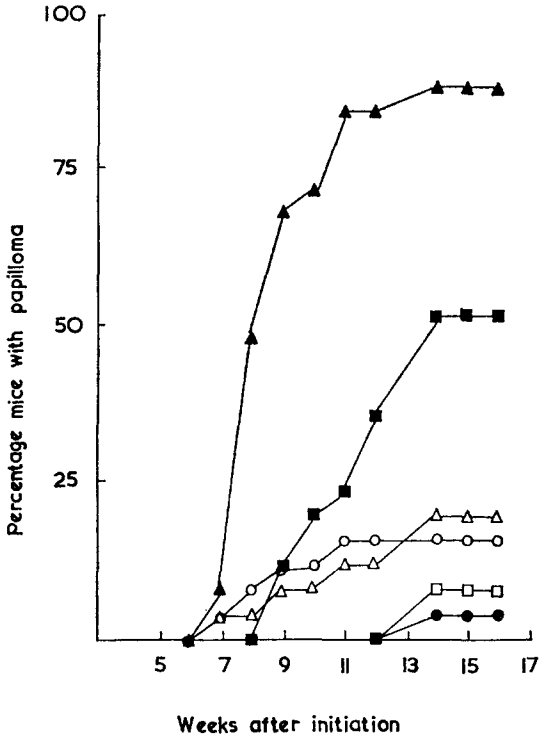


Fig. 1. *Papilloma incidence in Swiss S female mice promoted with* 12-*myristoylphorbol*-13-*acetate. One micromole of initiator was used:*

▲——▲, 7-*bromomethyl*-12-*methylbenz*[a]*anthracene;*
■——■, 6-*fluoro*-7-*bromomethylbenz*[a]*anthracene;*
△——△, 1-*methyl*-7-*bromomethylbenz*[a]*anthracene;*
○——○, 7-*bromomethylbenz*[a]*anthracene;*
□——□, 4-*bromo*-7-*bromomethylbenz*[a]*anthracene;*
●——●, 4-*chloro*-7-*bromomethylbenz*[a]*anthracene.*

The chemical reactivities of the four new 7-bromomethylbenz[*a*]anthracene derivatives were studied as before [1], by observing the colour produced with time on reaction with 4-(*p*-nitrobenzyl)pyridine. The pseudo first

order rate equation for the overall reaction, in terms of the measured quantities, is

$$\log E_\infty/(E_\infty - E_t) = k\,t/2{\cdot}303$$

where E_∞ and E_t are the absorbance at any given wavelength at infinite time (greater than five times the half life of the reaction), and at time, t, respectively. The experimental data for the new compounds is presented in Fig. 2 and it can be seen that k, the overall rate

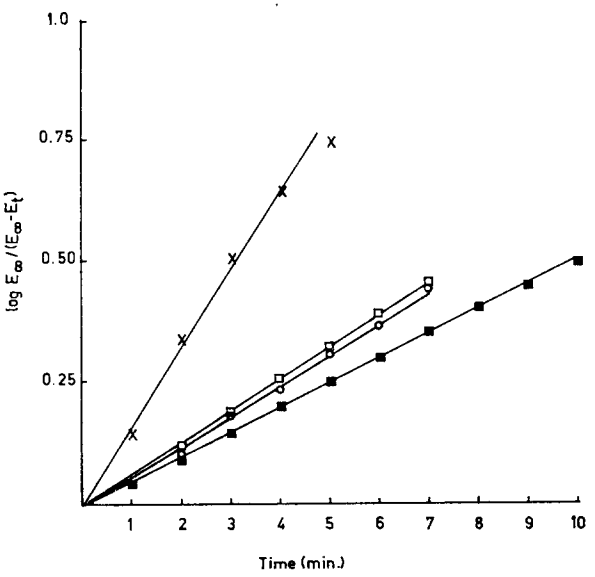


Fig. 2. Analysis of the data from the reactions of 1-*methyl-7-bromomethylbenz*[a]*anthracene* (×——×), 4-*bromo-7-bromomethylbenz*[a]*anthracene* (□——□), *4-chloro-7-bromomethylbenz*[a]*anthracene* (○——○), *and* 6-*fluoro-7-bromomethylbenz*[a]*anthracene* (■——■) *with* 4-(*p-nitrobenzyl*) *pyridine* [1].

constant, is easily obtained from the slopes of these lines. We have previously shown that two competitive reactions occur in this assay, a second order reaction with the pyridine derivative with a specific reaction rate, k_2' (this includes the pyridine concentration term) and a first order hydrolysis with a specific reaction rate, k_1 [1].

$$\text{Since, } k = k_1 + k_2'$$

$$\text{and } k_1/k_2' = (E_{\text{theor}} - E_\infty)/E_\infty$$

where E_{theor} is the absorbance which would have been obtained if all the arylmethyl bromide had reacted with the pyridine derivative, and is known to be 2·0 [1], the numerical values of k_1 and k_2' can be calculated.

These values are presented in Table 1 along with the previously published data for 7-bromomethylbenz[*a*]anthracene and 7-bromomethyl-12-methylbenz[*a*]anthracene for ease of comparison.

DISCUSSION

Comparison of the biological data in Fig. 1 and the chemical data in Table 1 suggests no clear general relationship between biological activity and chemical reactivity. However, if the 6-fluoro-7-bromomethylbenz[*a*]anthracene data are considered separately, the data for the other 5 compounds do provide a rough correlation between k_1, the first order rate constant and biological activity. Similarly, a reasonable correlation is seen between biological activity and the ratio k_1/k_2', whilst the correlation with k_2' alone would be somewhat less convincing. The 6-fluoro derivative must remain a clear exception but it is worth noting that this agent has a low k_2' value which could suggest some steric hindrance to a "backside" attack on the methylene carbon. Electrostatic repulsion between the neighbouring electronegative halogen atoms in this compound could result in such steric hindrance. It is possible that this effect could be more pronounced in reactions in an *in vivo* environment, thereby forcing this reagent to react more extensively through a first order process than is indicated by the data obtained from the model studies in organic solvents.

It is not surprising that absolutely clear cut correlations are not obtained from the type of experiments described here, since any measure of biological activity in animals must involve many unknown pharmacological factors such as solubility, membrane permeability, etc. We have tried to minimize these factors by studying the biological response at the site of admini-

Table 1. Comparison of chemical reactivities of various 7-*bromomethylbenz*[a]*anthracenes*

Compound	E_∞	$t_{\frac{1}{2}}$(min)	k(min^{-1})	k_1(min^{-1})	k_2'(min^{-1})	k_1/k_2'
7-Bromomethyl-12-methylbenz[*a*]anthracene	0·18	0·20	3·2	2·9	0·30	9·7
1-Methyl-7-bromomethylbenz[*a*]anthracene	0·37	1·8	0·38	0·31	0·070	4·4
7-Bromomethylbenz[*a*]anthracene	0·49	2·2	0·31	0·24	0·070	3·4
6-Fluoro-7-bromomethylbenz[*a*]anthracene	0·80	6·0	0·12	0·072	0·048	1·5
4-Chloro-7-bromomethylbenz[*a*]anthracene	0·97	4·8	0·14	0·074	0·066	1·1
4-Bromo-7-bromomethylbenz[a]anthracene	1·00	4·6	0·15	0·075	0·075	1·0

stration of the agent but they are, nevertheless, always present.

Within the context of such experimental limitations, some relationship between increased papilloma incidence and tendency to react through a first order process does emerge. This relationship is analagous to that found by Veleminsky *et al.* [5] for the mutagenicity of the alkyl methanesulphonates in plant systems. These authors have suggested that their relationship implies that the more mutagenic agents have a greater affinity for reaction with centres of low nucleophilicity in DNA. The description by Loveless [6] of alkylation at the O^6 position of deoxyguanosine by ethyl methanesulphonate, but not by methyl methanesulphonate, was clearly consistent with this view and has indicated a possible critical receptor site in DNA for these alkylating agents.

The benz[*a*]anthracene derivatives discussed here would be expected to react far more extensively through a first order process than ethyl methanesulphonate and could, therefore, be expected to be even more specific for DNA sites of low nucleophilicity. It is of some interest, therefore, that in aqueous solution 7-bromomethylbenz[*a*]anthracene attacks DNA on the amino group of guanine and adenine residues (and probably also on the amino group of cytosine residues), and does not attack the ring nitrogen atoms [7]. All the bromomethylbenz[*a*]anthracene derivatives studied are probably capable, therefore, of reaction with the amino groups in DNA, but since biological activity varies along the series of compounds it is to be expected that even amongst the amino groups, attack on those which are less nucleophilic may be more relevant to the observed papilloma incidences. It is also possible that other, even less, nucleophilic sites may be attacked and further studies aimed at the identification of the critical receptor sites in DNA for these agents are in progress.

Acknowledgements—We wish to thank Professor Melvin S. Newman for generously providing various benz[*a*]anthracene derivatives and Dr. J. D. Scribner for advice on the animal experimentation.

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REFERENCES

1. A. Dipple and T. A. Slade, Structure and activity in chemical carcinogenesis: reactivity and carcinogenicity of 7-bromomethylbenz[*a*]anthracene and 7-bromomethyl-12-methylbenz[*a*]anthracene. *Europ. J. Cancer* **6,** 417 (1970).
2. T. J. Bardos, N. Datta Gupta, P. Hebborn and D. J. Triggle, A study of comparative chemical and biological activities of alkylating agents. *J. Med. Chem.* **8,** 167 (1965).
3. R. F. Hudson, G. M. Timmis and R. D. Marshall, A physicochemical investigation into the biological action of myleran and related sulphonic acid esters. *Biochem. Pharmacol.* **1,** 48 (1958).
4. P. F. Swann and P. N. Magee, Induction of rat kidney tumours by ethyl methanesulphonate and nervous tissue tumours by methyl methanesulphonate and ethyl methane-sulphonate. *Nature (Lond.)* **223,** 947 (1969).
5. J. Veleminsky, S. Osterman-Golkar and L. Ehrenberg, Reaction rates and biological action of *N*-methyl-and *N*-ethyl-*N*-nitrosourea. *Mutation Res.* **10,** 169 (1970) and references cited therein.
6. A. Loveless, Possible relevance of 0–6 alkylation of deoxyguanosine to the mutagenicity and carcinogenicity of nitrosamines and nitrosamides. *Nature* **223,** 206 (1969).
7. A. Dipple, P. Brookes, D. S. Mackintosh and M. P. Rayman, unpublished data.

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Letter to the Editor

Preliminary Clinical Screening with 6-Aminochrysene in Lung Cancer

COOPERATIVE GROUP FOR CLINICAL TRIALS IN LUNG CANCER*
European Organization for Research on the Treatment of Cancer (EORTC)

THE PURPOSE of this trial was to evaluate the effect of 6-aminochrysene in lung cancer. It is a screening trial or a type-I trial according to the terminology of the EORTC [1]. This drug was found effective in a small number of patients with far-advanced breast cancer [2].

This study included 19 patients, 18 males and 1 female. The diagnosis was epidermoid carcinoma in 11 cases and anaplastic (oat cell) carcinoma in 8 cases. All patients had far-advanced cancer and were not suitable for surgery or radiation therapy, according to the criteria defined in a previous trial of the group [1]. Cases with a life expectancy of less than 6 weeks were not included.

6-Aminochrysene was given orally at a dose of 1 g for the first day, 1·5 g for the second day and 2 g daily thereafter. The treatment was discontinued in case of severe digestive toxicity (persistant vomiting not relieved by antiemetic drugs) and when no objective response was seen after a 6-week course treatment.

All patients in this study had lesions which were subject to evaluation. The lesions were measured with the aid of a slide caliper in the case of peripheral adenopathies and subcutaneous metastases. Measurements were made on radiographic plates in the case of lung tumours and hilar or mediastinal adenopathies.

Hemoglobin, white cell and platelet counts were carried out at least once a week during the course of the treatment. ECG was performed before and after the treatment.

RESULTS

Eleven patients with epidermoid carcinoma received 6-aminochrysene for 6 to 45 days (mean: 29 days) at a dose of 2 g per day (total dose: 12–90 g, mean: 59·7 g). No objective response was observed.

Nine patients with anaplastic carcinoma received 6-aminochrysene for 5 to 45 days (mean: 18 days) at a daily dose of 2 g (total dose: 10 to 90 g, mean: 34·8 g). No objective response was observed.

Hematologic tolerance was excellent in 19 cases. In 1 case there was a decrease of the red blood cells to 2,000,000 per mm^3, and a leukopenia of 4000 leukocytes per mm^3, after 15 days of treatment. No abnormalities of the ECG were detected. Seventeen patients had nausea and vomiting, severe enough for discontinuing the treatment in 11 cases.

CONCLUSION

This preliminary clinical screening of 6-aminochrysene in lung cancer was discontinued when no objective response had been observed among 20 consecutive cases. It was not considered valid to treat larger numbers of patients because the digestive toxicity was too severe.

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*Chairman: Y. Kenis; Secretary: P. Levy; Representative of the Council of the EORTC: D. Schwartz.
Participating members: Belgium, Institut Bordet (Brussels): Y. Kenis; France, Hôpital Xavier Arnozan (Bordeaux): P. Freout, P. Chomy; Hôpital Sabourin (Clermont-Ferrand): C. Molina, J. C. Cheminat; Hôpital Boucicaut (Paris): A. Meyer, M. Brunel, A. Tournant; Hôpital Foch (Paris): R. Israel-Asselain, J. Chebat; Institut Gustave-Roussy (Villejuif): G. Brulé.

Other 6-aminochrysene derivatives [3] are being studied for gastro-intestinal tolerance in the hope of finding a compound which could be better tolerated.

REFERENCES

1. Groupe Coopérateur d'Essais Thérapeutiques sur les Cancers Broncho-Pulmonaires, Résultats d'un essai d'une méthylhydrazine dans le traitement des cancers épidermoïdes et anaplastiques des bronches. *Europ. J. Cancer* **4,** 129 (1968).
2. Groupe Coopérateur des Cancers du Sein, Induction par le 6-aminochrysène de rémission des cancers du sein en phase avancée chez la femme. *Europ. J. Cancer* **3,** 75 (1967).
3. H. J. Tagnon, A. Coune, S. Garattini, R. Rosso, G. Lambelin, M. Gautier, and N. P. Buu-Hoï, The antitumoral activity of some derivatives of 6-aminochrysene. *Europ. J. Cancer* **6,** 81 (1970).

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Letter to the Editor

Measurement of the Antitumoral Immune Reactions Against a Strain-Specific Chemically-Induced Sarcoma in Syngeneic and F_1 Hybrid Mice*

D. OTH, M. DONNER and C. BURG
INSERM, Experimental Cancer and Radiobiology Research Unit, U 95–P.O. Box 18, 54–Vandoeuvre, France

MOST chemically-induced murine tumours possess tumour associated transplantation antigens (TATA). These can be detected *in vivo* by comparing the tumoral transplantability in genetically compatible hosts with different immune status. When the tumour grows better in immunodepressed animals than in untreated controls, it can be concluded that a first-set immune reaction against TATA exists [1, 2]. When the tumour grows less well in specifically immunized hosts, a second-set reaction against TATA is thus detected [3–6]. Comparisons of these differences can afford quantitative approaches on the intensity of the anti TATA immune reactions [7, 8]. Results obtained with first-set and second-set reactions do not necessarily correlate ([1] and Donner *et al.*, unpublished data).

In the present work, first-set and second-set reactions against the TATA of the dibenzanthracene-induced rhabdomyosarcoma TP10 were appraised in two sorts of genetically compatible recipients: in syngeneic highly inbred Swiss/B mice, to which TP10 is specific, and in (Swiss/B × C3H/He)F_1 hybrids. One hundred and twenty three Swiss/B and 170 hybrids were whole body irradiated (500 rads) one day before tumour graftings, to provide immunodepressed animals. Specifically immunized recipients were obtained according to Révész [6]: 135 Swiss/B and 115 hybrids. In addition, 34 hybrids were treated with splenic cells from healthy Swiss/B mice, in the same schedule as the antitumour immunizations. Finally, 196 Swiss/B and 197 hybrids were left untreated for controls. Transplantability of TP10 (graft passages 29*th*–36*th*) was assessed according to two criteria: the titration curves and the mean survival times of tumour-bearing recipients.

Titration curves were obtained by plotting the percentage of tumour takes in a group of mice grafted with a number of dispersed viable tumour cells, as a function of this number. Inocula from 5 cells to 100,000,000 cells were administered subcutaneously to groups comprising 8 to 12 mice. The data, rearranged according to Reed and Münch [9] are presented in Fig. 1. The upper part represents the tumour's behaviour in Swiss/B syngeneic mice. Only slight differences exist between untreated control and immunodepressed recipients on the one hand, and between controls and specifically immunized mice on the other hand. This means that both first-set and second-set reactions against the TATA have a very slight expression in this system. Contrastingly, if the same experiment is made using (Swiss/B × C3H)F_1 hybrids, a quite different pattern can be observed (lower part of Fig. 1): depression of the immune responses dramatically enhances the tumour transplantability, while specific immunization considerably diminishes it. Thus, it seems that very "strong" first-set as well as second-set immune reactions are established against the tumour, in this system.

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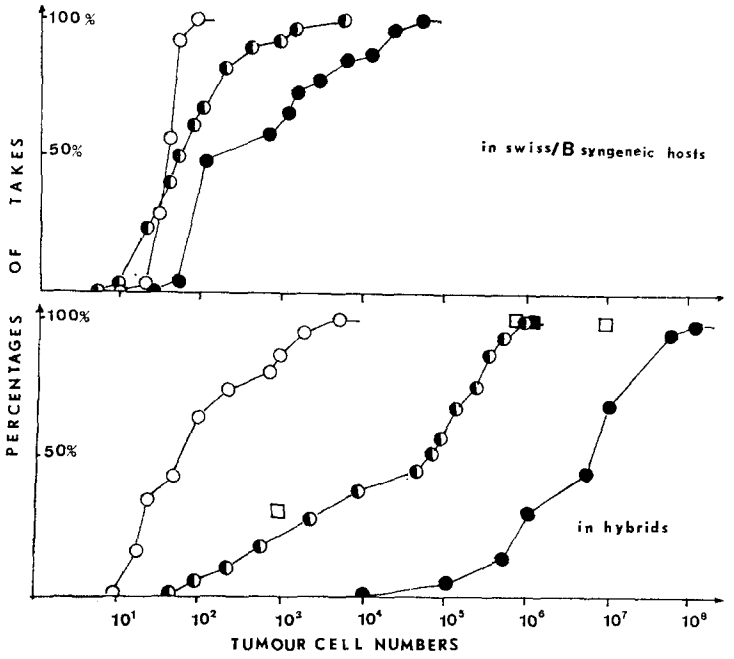


Fig. 1. Percentage of takes, as a function of tumour-cell numbers injected ○ irradiated mice; ◑ untreated mice; ● specifically immunized mice; □ hybrids pretreated with twice 0·5 10^6 splenic cells from healthy Swiss/B donors; ■ hybrids pretreated with twice 38 10^6 splenic cells from healthy Swiss/B donors.

Choosing the second criterion, i.e. the mean survival times of tumour-bearing mice which received comparable amounts of tumour cells, similar facts are observed. On the upper part of Fig. 2, it can be seen that immunodepression has a slight acceleration effect on deaths by the tumour, and that specific immunization has no effect in the Swiss/B. In contrast, tumour growth acceleration is very important in the irradiated hybrids, and a detectable augmentation of life span is observed in specifically immunized hybrids, even if they receive an important challenge of viable tumour cells.

The specificity of the reaction against TATA, and not against normal transplantation antigens (reaction supposed to occur from a residual heterozygosity in the Swiss/B, or from phenomena akin to hybrid resistance [10]) is suggested by the fact that treatment of the hybrids with normal parental cells do not modify the behaviour of further challenge with the tumour (lower part of Figs. 1 and 2).

From these results, it seems that the intensity of the immune reactions against TATA of the same tumour may considerably vary from one system to another. In the present case, the F_1 hybrids used in the experiment react more strongly than the syngeneic hosts. This could be the origin of the "hybrid effect" [11] observed in the case of this tumour [8] and in

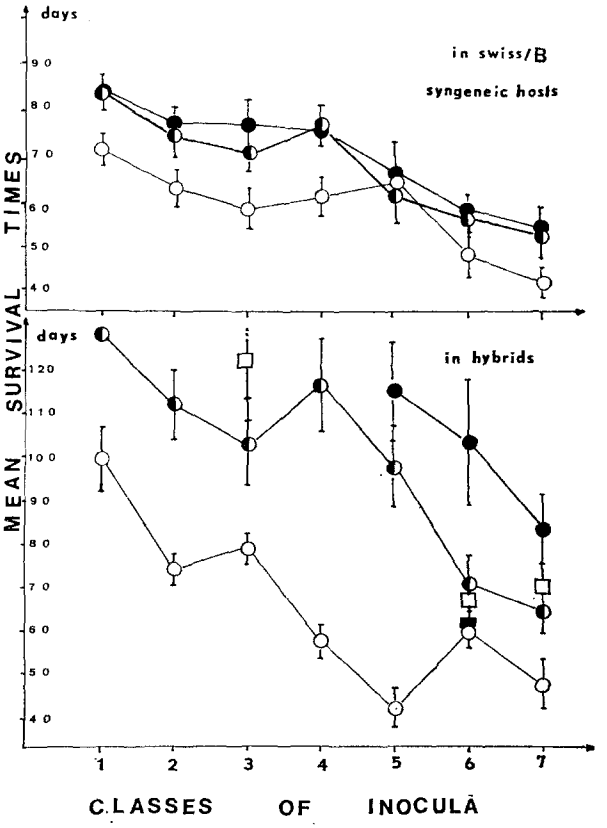


Fig. 2. Mean survival times ± standard error (days) of tumour-bearing mice, as a function of tumour inocula. Each value represents the mean of pooled data obtained from cell numbers having of the same $\log_{10}$ characteristic. Same symbols as in Fig. 1. The point in "class 1" of untreated hybrids represents one mouse.

the case of others which are less well accepted in some F_1 hybrids than in their strain of origin [8, 10, 12–15]. The fact that some F_1 hybrids may present such an increased reactivity to TATA of parent strain origin tumours [3, 5, 10, 12, 14] may have several explanations:

In some cases, this would be the result of Cudkowicz's "hybrid resistance", which is directed against "parental specific antigens" expressed on some non-neoplasic and neoplasic cells [10]. This action is operative only against marrow or marrow-derived cells, and in special genetic conditions [10]. In other cases, a neonatally-induced tolerance to the TATA, which is established in the native strain and not in the hybrids, can explain the differences of antitumoral reactivity. This was observed-with the "milk agent" —dependent mammary tumour of the C3H [15].

In the case of chemically-induced sarcomas, one must rise up other hypothesis.

A first possibility is the association of the present results with the "allogeneic inhibition" concept, according to which exposure of "target" cells with "killer" cells from an allogeneic (or F_1 hybrid) host can destroy them by means of a non-immunological mechanism [16].

In the present case, this action appears as negligible by itself (as shown when comparing the titration curves of immunodepressed F_1 and syngeneic animals), in comparison with the "hybrid effects" found when using normal and immuno-reinforced animals.

On the other hand, Huemer discussed several possibilities for explaining a better "recognition" or "processing" of the TATA in F_1 hybrids [13]. We also favour an explanation based on genetically dependent aptitudes for reacting to some antigens [17] to explain the differences observed between these both sorts of genetically compatible hosts. But the exact mechanism remains to be determined.

Acknowledgements—We wish to thank Mrs Beugnot-Llorens and Mr Liegey for technical assistance.

REFERENCES

1. R. T. Prehn, Influence of X irradiation and the milk agent on growth of transplanted mouse mammary tumors. *J. nat. Cancer Inst.* **43,** 1215 (1969).
2. W. Rosenau and H. D. Moon, Supression of the immune response to antigenic tumors in isogenic mice by whole body irradiation. *Cancer Res.* **27,** 1973 (1967).
3. A. Globerson and M. Feldman, Antigenic specificity of Benzo (*a*) pyrene-induced sarcomas. *J. nat. Cancer Inst.* **32,** 1229 (1964).
4. L. J. Old, E. A. Boyse, D. A. Clarke and E. A. Carswell, Antigenic properties of chemically induced tumors. *Ann N.Y. Acad. Sci.* **101,** 80 (1962).
5. G. Pasternak, Die unterschiedliche Reaktionsfähigkeit zweier mäuse inzuchtstämme gegen spezifische Antigene isolog transplantablen Carcinogen tumoren. *Acta Biol. Med. German.* **10,** 572 (1963).
6. L. Révész, Detection of antigenic differences in isologous host-tumor systems by pretreatment with heavily irradiated tumor cells. *Cancer Res.* **20,** 443 (1960).
7. R. F. Kallman, G. Silini and L. M. van Putten, Factors influencing the quantitative estimation of the *in vivo* survival of cells from solid tumors. *J. nat. Cancer Inst.* **39,** 539 (1967).
8. D. Oth and C. Burg, Strength of the tumor-specific antigens and of the "hybrid effect" of three chemically induced sarcomas of the same inbred strain origin. *Folia Biologica,* **16,** 374 (1970).
9. L. J. Reed and H. Münch, A simple method of estimating fifty per cent endpoints. *Amer. J. Hygiene* **27,** 493 (1938).
10. G. Cudkowicz, Hybrid resistance and parental-specific transplantation antigens, In *Int. Convoc. Immunol—Buffalo, N.Y.* 1968, p. 193, (Edited by N. R. Rose and F. Milgrom). Karger, Basel (1969).
11. G. D. Snell and L. C. Stevens, Histocompatibility genes of mice. III–H1 and H4, two histocompatibility loci in the first linkage group. *Immunology* **4,** 366 (1961).
12. B. H. Sanford, Evidence for immunological resistance to a parental line of tumor by F1 hybrid hosts. *Transplantation* **5,** 557 (1967).
13. R. P. Huemer, Further studies on inhibition and adaptation of a parental tumor in F1 hybrid mice. *Cancer Res.* **29,** 610 (1969).

14. B. H. SANFORD and S. F. SOO, Resistance to recent tumors by F1 hybrid hosts. *J. nat. Cancer Inst.* **46,** 95 (1971).
15. D. OTH, M. LECLERE, G. GRIGNON and C. BURG, Maternal factor in the "hybrid effect" given by a spontaneous mammary carcinoma of C3H origin. *Life Sci.* **7,** 599 (1968).
16. L. LINDHOLM, W. LAPP and E. MÖLLER, Allogeneic inhibition in thymectomized animals lacking homograft reactivity. *Nature* **228,** 1326 (1970).
17. H. O. MCDEVITT and M. L. TYAN, Genetic control of the antibody response in inbred mice. Transfer of response by spleen cells and linkage to the major histocompatibility (H2) locus. *J. exp. Med.* **128,** 1 (1968).

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Announcement

Conference on Biological and Clinical Basis of Tumor Radiosensitivity

This international Conference will be held on October 7, 8 and 9, 1971 at the Istituto Regina Elena, Rome.

Although many efforts are made to enhance radiosensitivity by means of oxygenation, adjuvant chemotherapy, fractionation patterns, etc., little attention has been given to the study of the phenomena of radiosensitivity.

The purpose of the Conference is to assemble and evaluate the known facts on radiosensitivity.

Some of the scheduled speakers are:

Dr. J. Robert Andrews, Dr. Roger J. Berry, Dr. Antonio Caputo, Dr. George Casarett, Dr. Mortimer Elkind, Dr. Gilbert Fletcher, Dr. Milton Friedman, Dr. Morton Kligerman, Dr. L. F. Lamerton, Dr. Carlo Nervi, Dr. Philip Rubin.

Direct inquiries to:

Prof. Carlo Nervi,
Istituto Regina Elena per lo Studio e la Cura dei Tumori,
Viale Regina Elena, 291,
00161 Rome, Italy.

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Chromosome Abnormalities in AKR/T1ALD Leukaemias

E. LEGRAND* and J. F. DUPLAN†

Fondation Bergonié, 33 Bordeaux, and Laboratoire Pasteur – Fondation Curie, 75 Paris, France

Abstract—*Chromosomal abnormalities have been observed in lymphosarcomas of AKR and AKR/T1ALD mice. AKR/T1ALD is a substrain of AKR in which a translocation occurred spontaneously, so that its chromosomal number is* 38, 36 *acrocentric chromosomes and* 2 *metacentrics, instead of* 40 *acrocentrics in AKR.*

Lymphosarcomas have been studied in animals unirradiated and exposed to sub-lethal fractionated irradiation or whole-body acute irradiation. The most frequent abnormality is an extra additional metacentric very often identical to the normal pair and probably originating from a non-disjunction. This trisomy is characteristic of leukaemic cells as it has never been found in normal mice. Its frequency is comparable with that of additional acrocentrics in AKR and quite independent of irradiation, as it is the same in control (25%) *and in irradiated T1ALD mice* (26·7%).

INTRODUCTION

CELLS OF murine leukaemias show less chromosome abnormalities than human leukaemic cells. Most metaphase plates correspond to diploid sets of 40 chromosomes. Such is the case with AKR strain leukaemias in which the most common abnormality is an extra acrocentric chromosome which is present in nearly 30% of the cells [1, 2]. In other respects, it is difficult to identify such specific chromosome alterations, owing to the fact that, in the mouse, all the chromosomes are acrocentric and have therefore to be classified according to their dimensions.

In 1966, a spontaneous translocation occurred in an AKR colony maintained by Leonard and Deknudt who isolated this substrain and designated it as the: T1ALD. This translocation results from the centromeric joining of pairs 4 and 13 and does not induce any phenotypic change, nor a change in the overall DNA content of the chromosomes [3]. However, the cells carrying this rearrangement at times tend to display some chromosomal instability, which renders them subject to additional alterations when exposed to physical mutagens such as radiations.

The present experiments were performed to study this possibility. For this purpose, the chromosomal abnormalities of leukaemic cells belonging to normal or X-irradiated AKR mice were analysed.

MATERIAL AND METHODS

Mice used in these experiments were males and females of the AKR and AKR/T1ALD strains. The former has been bred at the Institut du Radium for the last 18 yr and its cells have 40 acrocentric chromosomes (Fig. 1a). The latter, i.e. the AKR/T1ALD substrain, has also been maintained at the above Institute during the last 2 yr, and originates from breeders of the Nuclear Study Center of Mol, which were kindly given to us by Dr. Leonard. The cells of this line have 36 acrocentric and 2 metacentric chromosomes (Figs. 1a and 1b).

All the irradiated mice belonged to the T1ALD substrain. Some of them, 60–70 days old, were exposed to a whole body irradiation of 900 rads. The others, 30–37 days old when irradiated for the first time, receive 4 weekly exposures of 175 rads each. The conditions at which the X-ray machine was used were as

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*Attachée de recherches à l'INSERM.
†Directeur de recherches à l'INSERM.

follows: 225 kV peak, 11 mA, with an added filtration of 2mm Al and 0·3 Cu, HVL 1·1 mm Cu. The dose rate at 40 cm was slightly less than 90 rads/min. Within 4 hr after the acute exposure or after the 4th irradiation, the mice received an intravenous dose of 0·6–1 $\times 10^7$ nucleated bone marrow cells obtained from 50–60 days old AKR or T1ALD donors.

All mice exposed to 900 rads were restored whereas those exposed to 4 weekly irradiations were divided into 3 groups: one group was injected with AKR-bone marrow cells, another with T1ALD-bone marrow cells and the last one remained uninjected.

Control and treated animals received water and food *ad libitum*. They were sacrificed when showing obvious leukaemic symptoms. Ninety minutes before being killed, they were injected intra-peritoneally with 125 μg of colchicine. Thymus and femoral-bone marrow cells were suspended in a 2·2% solution of sodium citrate and processed according to the method described by Ford [4]. Metaphases plates were either photographed or directly analysed under the microscope. Fifty mitosis were scored per mouse for the thymus and the bone marrow and depending on the group, 10–23 animals were studied. Histological studies were carried out on the thymus, spleen, lymph nodes, liver and kidneys, and smears of bone marrow were examined.

RESULTS

As seen in Table 1, seven groups of animals have been studied: AKR and T1ALD controls, 900 rads irradiated T1ALD restored with AKR or T1ALD-bone marrow cells; T1ALD irradiated with 4 times 175 rads and unrestored or restored with T1ALD or AKR bone marrow cells. This last group appears twice in Table 1 with respect to the origin of the lymphosarcomas.

All leukaemias occurring in 900 rads irradiated mice developed from donor cells which had not been directly submitted to the action of X-radiation.

On the other hand, after fractionated irradiation (4 × 175 rads) and restoration, thymic lymphosarcomas were seen to develop from the host as well as from donor thymic cells. As a result, two kinds of lymphosarcomas could be distinguished in T1ALD mice restored with AKR-bone marrow cells: those which originated from the recipient (T1ALD) cells surviving irradiation and the other originating from donor (AKR) unirradiated cells. But such discrimination was impossible for the lymphosarcomas developed in T1ALD radiation induced chimaeras restored by T1ALD cells [5].

(1) *Abnormalities related to the number of chromosomes*

The following data are restricted to the abnormalities observed in the thymus, the reason for this choice being that the thymus is generally invaded by lymphosarcoma cells, while an unknown percentage of normal cells is always present in the bone marrow and makes a similar study difficult. Ninety-four per cent of the lymphosarcomas showed only one modal number in whatever group to which the mice belonged.

In 70% of the cases, this modal number was diploid (40 or 38 chromosomes for AKR or T1ALD respectively), hyperdiploid in 27%, and hypodiploid in 3%. The chromosomal number was 39 for T1ALD and 41 for AKR in 22% of the total hyperdiploid cell population.

In 6% of the leukaemias, no modal number could be fixed on account of the wide variations

Table 1. *Abnormalities related to the number of chromosomes*

Strain of Recipient	Strain of Donor	X-ray dose (rads)	Karyotype of thymic L.S.	Number of mice	Number of L.S.* according to the modal number 37	38	39	40	41	42	†
T1ALD	–	–		23		18	4				1
T1ALD	T1ALD	900		15		10	2				3
T1ALD	–	175 × 4	T1ALD	17	2	8	4	2			1
T1ALD	AKR	175 × 4		14		8	7				2
T1ALD	T1ALD	175 × 4		17		15	2				
AKR	–	–		10				5	3	1	1
T1ALD	AKR	900	AKR	12				7	4		1
T1ALD	AKR	175 × 4		11				8	3		

*L.S.: Lymphosarcomas.
†L.S.: with several modal numbers.

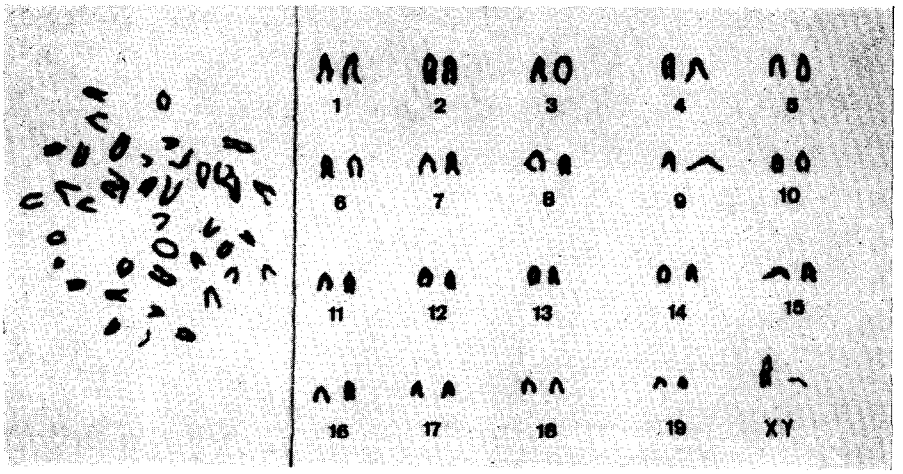


Fig. 1*a*. *Karyotype of the AKR strain.*

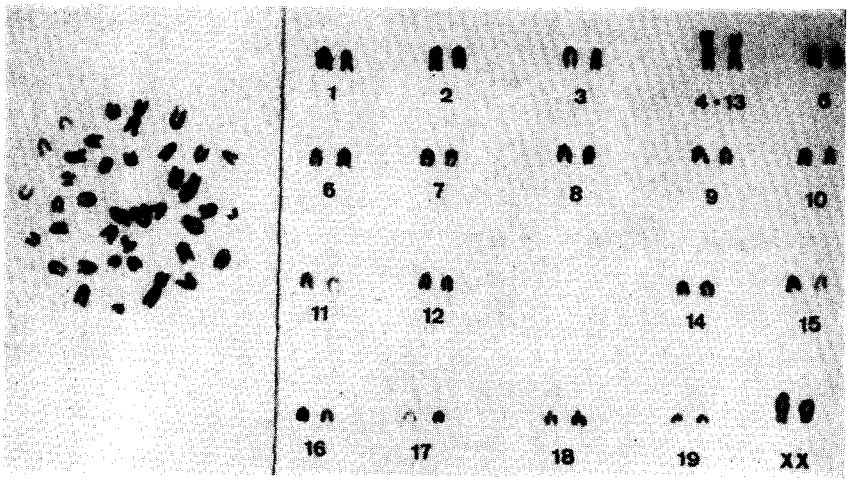


Fig. 1*b*. *Karyotype of the AKR/T1ALD substrain:* 38 *acrocentric and metacentric chromosomes resulting from the centromeric joining of* 4 *and* 13 *pairs.*

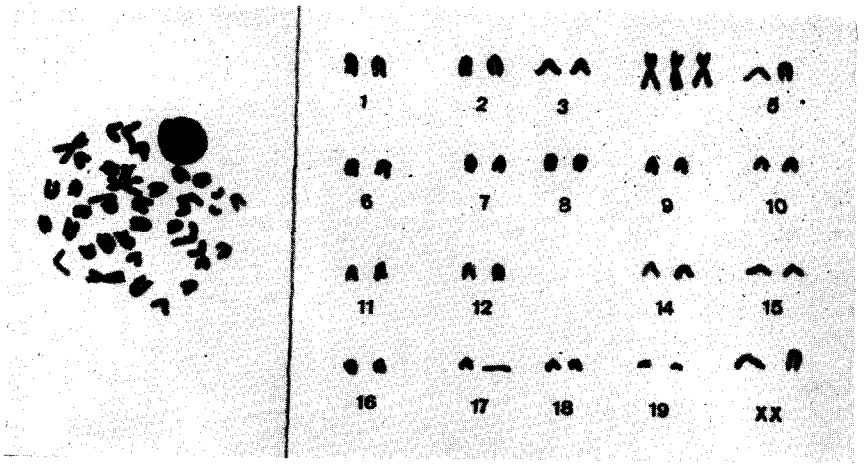


Fig. 2. *Karyotype of a leukaemic cell in AKR/T1ALD thymus: one additional metacentric chromosome.*

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in the number of chromosomes carried by different cells. In 2 lymphosarcomas developing from T1ALD recipient cells in radiation chimaeras exposed to fractionated irradiation and restored by AKR cells, several modal numbers were observed. In one case, there were two modal numbers for one leukaemia showing diploid and triploid cells, whereas in another case, three modal numbers were found namely diploid, triploid and tetraploid. The percentage of mitosis bearing the modal number of chromosomes is always included between 78 and 83 for AKR and between 82 and 89 for T1ALD. The chromosomal number generally varies between 36 and 43 for AKR and between 35 and 49 for T1ALD except for the two above cited leukaemias which had several modal numbers, from 38–78 in one case and from 35–77 in the other.

(2) *Abnormal or additional chromosomes*

In T1ALD leukaemias, hyperploidy with 39 chromosomes is very often related to the presence of an additional metacentric chromosome (Fig. 2). Its frequency has been determined in the various groups of leukaemias. Table 2 summarizes the results: to calculate the percentage of cells with additional metacentric or acrocentric chromosomes, all the cells which were analysed have been taken into account (Fig. 2).

Sixty-five per cent of *T1ALD leukaemic controls* showed a third metacentric chromosome which appeared in 25% of mitosis. No other abnormalities were observed except for an extra acrocentric chromosome in 3% of mitosis. An additional acrocentric was found in 40% of mitosis and two or more acrocentrics in 16·6% of *AKR leukaemic controls.*

In T1ALD radio-chimaeras exposed to 900 *rads and restored with T1ALD bone marrow,* 66% of the animals displayed an extra metacentric. The frequency of this abnormality was the same as in controls (24%) while 14% and 5% of mitosis had respectively one and two extra acrocentrics. In the corresponding *radiation chimaeras restored with AKR bone marrow,* all leukaemias were of donor type as shown by the absence of any metacentric. One extra acrocentric was found in 32% of the cells and two or three acrocentrics in 10% in the same group.

Among the mitotic T1ALD leukaemic cells present in animals exposed to *fractionated sublethal irradiation and unrestored,* 27% had a third metacentric, 24% an extra acrocentric and 3%, several acrocentrics. The additional metacentric was found in 41% of the animals. Moreover, various abnormalities were observed which were different for each animal, and therefore seemed specific for a given leukaemic clone.

When the leukaemic cells belonged to mice exposed to *fractionated irradiation and restored with T1ALD bone marrow,* the 3rd metacentric was present only in 6% of the cells. *If AKR bone marrow was injected instead of T1ALD,* and if the thymic lymphosarcoma retained the T1ALD karyotype, the extra metacentric was found in 21% of the mice and in 20% of the mitosis. In the same group, one extra acrocentric chromosome was found in 5·5% of the cells and 2 or 3 extra acrocentrics in 2·6%. Other abnormalities were present in 7·7% of the cells. In the animals of the same group which developed an AKR thymic lymphosarcoma, 31% of the cells had an extra acrocentric. This percentage is the same as in control AKR mice (36·5) or as in radiation chimaeras exposed to 900 rads and restored with AKR bone marrow (35·5). In this last group, all haemopoietic cells were of donor origin.

Table 2. Distribution of extra metacentric and acrocentric chromosomes among the different groups of mice

Strain of Recipient	Donor	X-ray dose (rads)	Karyotype of thymic L.S.	Number of mice	Mice with a 3rd metacentric (%)	Percentage of mitosis with extra chromosomes 3rd Metacentric	Acrocentric 1	Acrocentric 2 or more	Other abnormalities
T1ALD	—	—		23	65	25·0	3·2	0·4	—
T1ALD	T1ALD	900		15	66	24·0	14·0	4·8	—
T1ALD	—	175×4	T1ALD	17	41	26·7	24·6	2·9	23·2
T1ALD	AKR	175×4		14	21	20·4	5·5	2·6	7·7
T1ALD	T1ALD	175×4		17	23	6·0	7·3	1·6	
AKR	—	—		10	—	—	36·5	11·5	—
T1ALD	AKR	900	AKR	12	—	—	35·5	10·0	—
T1ALD	AKR	175×4		11	—	—	31·2	2·0	—

Table 3. Percentage of mitosis with extra acrocentric chromosomes in T1ALD lymphosarcomas according to the presence of a 3rd metacentric

X-ray dose (rads)	Origin of bone marrow	Percentage of extra acrocentrics in mitosis With 3rd metacentric	Without 3rd metacentric
–	–	4·8	2·7
900	T1ALD	20·7	13·3
175 × 4	–	44·8	17·7
175 × 4	AKR	1·4	6·2
175 × 4	T1ALD	6·0	9·5

A high number of control mice and of mice irradiated with 900 rads and restored with T1ALD bone marrow have an additional metacentric (65 and 66% respectively), while this same abnormality has a low frequency in the group exposed to fractionated irradiation and unrestored or restored with AKR or T1ALD bone marrow (41, 21 and 23% respectively). But it must be noted that the percentage of mitosis bearing the additional metacentric is 25 and 24 in the two first groups and 26·7, 20·4 and 6% in the last groups, respectively.

Table 3 summarizes an attempt to correlate the presence of extra acrocentric chromosomes with the trisomy of the metacentric in T1ALD. A few extra acrocentrics are observed in non-trisomic mitosis belonging to controls and 900 rads radiation chimaeras, and the percentage of additional acrocentric chromosomes is significantly higher in trisomic mitosis. The same finding is made in animals exposed to fractionated irradiation and unrestored, but in radiation chimaeras restored by AKR bone marrow and bearing leukaemias of recipient type, there are no additional chromosomes. In T1ALD restored by T1ALD bone marrow, trisomic mitosis were too scarce to provide significant information. But in animals exposed to fractionated irradiation, the findings are doubtful, due to a possible role played by irradiation in the duplication of acrocentric chromosomes.

In controls and 900 rads irradiated T1ALD restored with T1ALD bone marrow, the relative length and the centromeric indices of the third metacentric were identical with those of the normal metacentric pair, except in two controls where the length was shorter in 1 or 2% of the cells. On the contrary, in animals exposed to fractionated irradiation, the relative length had only once the control value and is shorter in 6 cases. The relative length and the centrometric indices of these smaller metacentrics were also variable, varying between 1/2 to 2/3 of the normal length. Sometimes, it was a real metacentric or a telocentric but, most frequently, a sub-metacentric.

Besides additional acrocentrics or metacentrics, other abnormalities were observed in animals exposed to fractionated irradiation. They were variable and seemed specific for a given leukaemic clone. In 4 mice, 100% of the mitosis showed deletions or rearrangements affecting one of the two metacentrics of the normal pair. Twice, a small submetacentric was found, the relative length of which was distinctly smaller than that of the normal pair. This abnormality was very likely the result of a deletion on the long arms of the third metacentric. On the other hand, in two different cases, we observed two metacentrics: one of them being 1·5 times and the other twice longer than the normal one. Such abnormalities were the result of complicated rearrangements, probably arising from translocation phenomena.

We also observed, in a lymphosarcoma, a long additional acrocentric, the length of which was identical to the metacentrics.

Other abnormalities were found in a few mitosis: small metacentrics or telocentrics were seen only in cells with a third metacentric. Secondary constrictions were also present. In every group of mice, the abnormalities observed in the thymus (3rd metacentric, additional acrocentric or others) were also found in the bone marrow when it was invaded by thymic leukaemic cells, but an accurate estimate of their frequency was difficult, owing to the presence there of normal cells of the haemopoietic series.

DISCUSSION

Our findings are in good agreement with the data of Kurita and Yosida [1] and with those of Wakonig and Stich [2]. Most of the mitosis they observed in leukaemic cells (spontaneous and transplanted leukaemias) had a diploid modal number and an apparently normal set, except in one third of the cases where an additional chromosome was found.

In T1ALD, hypo or hyperdiploid cells were more frequently observed when lymphosarcomas develop from regenerated thymus, that is from irradiated cells (12 out of 28) than when they occurred either spontaneously as in the controls or in the thymus repopulated by donor cells (6 out of 34). However, in all cases, the percentage of mitosis corresponding to the modal number and the spread of the chromosomal number were comparable. In 94% of leukaemias, most chromosomal sets were distributed according to one modal number, suggesting that most leukaemic mice die with a monoclonal sarcoma which is very likely the result of a selection. In the AKR mice hyperploidy is related to the presence of one or several extra acrocentric chromosomes. On account of their uncertain identification, it is impossible to decide to which pair they belong. On the other hand, in T1ALD, if we omit the unrestored mice which were exposed to sub-lethal irradiation, additional acrocentrics are seldom seen, but this is counter-balanced by a trisomy of the metacentrics. Moreover, it is striking that the frequency of additional metacentrics in T1ALD is quite comparable with that of extra acrocentrics in the AKR. It is also noteworthy that there is a low percentage of extra acrocentrics in T1ALD leukaemias, in which no cells with a 3rd metacentric are observed. This frequency is inferior to the percentage of such acrocentrics in AKR controls.

However, it is possible, that in the case of T1ALD lymphosarcomas with a diploid set of chromosomes, a few normal cells have been confused with leukaemic cells; such an error is impossible for cells with a 3rd metacentric as this abnormality is not observed in normal cells [6].

But, even if we omit leukaemias developed in sub-lethally irradiated mice, it seems that the trisomy of the metacentric marker goes with a higher percentage of additional acrocentrics than in mitosis in which not any trisomy is observed. Perhaps, it may be that the occurrence of trisomy in the metacentric pair induces a chromosomal unbalance involving other abnormalities, especially additional acrocentrics.

The rate of additional acrocentrics is significantly higher in radiation chimaeras exposed to fractionated irradiations and unrestored. This increase is probably the result of irradiation, and the same applies to chromosome rearrangements which are frequent in this same group but are absent in leukaemias of control or donor types. Besides, special attention must be paid to the high frequency of these rearrangements (translocations or deletions) selectively occurring on the metacentric pair. However, such translocations, when they concern acrocentrics may escape detection and are difficult to evaluate. They can be detected only in chromosomes of male germinal cells. Thus, as a result of homologous chromosomes pairing at meiosis, some rearrangements induced by radiation become visible, expecially translocations between non-homologous chromosomes. Leonard showed in experiments related to the radiosensitivity of germinal cells in various strains of mice, that, in T1ALD, the rate of chromosomal rearrangements induced by an irradiation of spermatogonies was not higher than in other strains, but metacentrics seemed frequently associated with radiation induced translocations, a fact which is in good agreement with our results [7, 8].

On the contrary, as pointed out in a previous paper, the trisomy of the metacentric is independent of irradiation: its frequency is comparable in controls, in radiation chimaeras exposed to acute irradiation where leukaemias are of donor type, and in mice which have received fractionated irradiation without restoration. However, in this last group we noted that the relative length of some of the additional metacentrics was distinctly smaller than the normal length of normal pairs, this may be the consequence of a deletion induced by irradiation.

The age at which leukaemias develop has no effect upon the frequency of the 3rd metacentric. This trisomy is characteristic of leukaemic cells, as it has never been found in any normal animal of the same age, but it appears only in 45% of all leukaemias and sometimes in a very small number of cells. This abnormality may appear at a very early stage of the leukaemic process [9] and its frequency does not increase when the disease progresses.

As the extra metacentrics, specially in controls, are very often identical with the normal pair, it can be assumed that they originate from a non-disjunction, although we have never observed mitosis with only one metacentric. However, such monosomic clones are probably at such a selective disadvantage that they are unlikely to be viable.

Similar findings have been reported by Ilbery and Barnes and by Wallis *et al.* [10, 11]. They relate a few cases of radioleukaemias in (C57BL × CBA T6 T6) hybrids, where one or several extra abnormal chromosomes, very identical to the T6 marker, were observed.

These observations relate abnormalities probably originating from a non-disjunction of the T6 marker like the one we observe in T1ALD, although in CBA, they are seen in radiation-induced leukaemias and in a very small number of cells. As in CBA, it is worth noting that the trisomy of the T1ALD marker applies to a chromosomal pair previously modified by a translocation.

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REFERENCES

1. Y. Kurita and T. H. Yosida, Chromosomal alterations and the development of tumors. VII. Karyological analysis of spontaneous and induced leukaemias in mice. *Gann* **52,** 257 (1961).
2. R. Wakonig-Vaartaja and H. F. Stich, Chromosomes in primary and transplanted leukaemias of AKR mice. *J. nat. Cancer Inst.* **25,** 295 (1960).
3. A. Leonard and G. H. Deknudt, A new marker for chromosomes studies in the mouse. *Nature* **214,** 504 (1967).
4. C. E. Ford, *Tissue Grafting and Radiation*, p. 197, (Edited by H. S. Micklem and J. F. Loutit). Academic Press, New York (1966).
5. J. F. Duplan and E. Legrand, Etude cytogénétique de la leucémogénèse lymphoïde chez les radiochimères isogéniques AKR–T1ALD. *C.R. Acad. Sci. (Paris)* **268,** 2990 (1969).
6. E. Legrand and J. F. Duplan, Presence of an additional metacentric chromosome in AKR/T1ALD leukaemic cells. *Nature* **225,** 737 (1970).
7. A. Leonard and G. H. Deknudt, Réarrangements chromosomiques induits par les rayons X chez les Souris de race AKR/T1ALD. *Experienta* **22,** 715 (1966).
8. A. Leonard and G. H. Deknudt, Quelques aspects des dommages provoqués aux chromosomes de la Souris par les rayons X. *Agricultura* **2,** 21 (1967).
9. E. Legrand and J. F. Duplan, Etude de la leucémogén`se AKR a l'aide d'un marqueur cytogénétique. *Int. J. Cancer* **7,** 443 (1971).
10. P. L. T. Ilbery and C. A. Barnes, Origin of neoplastic cells following prophylaxis of radiation-induced mouse leukaemia. *Int. J. Cancer* **5,** 124 (1970).
11. V. Wallis, A. J. S. Davies and P. C. Koller, Inhibition of radiation induced leukaemia by injection of haematopoietic tissue: a study of chimaerism. *Nature (Lond.)* **210,** 500 (1966).

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Studies on a Viral Nephroblastic Nephroblastoma of the Chicken:

an Electron Microscope Comparison of the Sequence of Development of the Virions in Different Organs

O. WEILER,* E. DELAIN† and F. LACOUR*

**Laboratoire d'Immunologie et de †Microscopie Electronique, Institut Gustave-Roussy, (94) Villejuif, France*

Abstract—*An ultrastructural study of a serially transmissible nephroblastoma DNV line showed the presence of virions similar, morphologically, to the avian leukosis group viruses. The particles were generally located in the basement membranes and the capillary endothelium.*

A chronological study indicated that the viral particles appeared and multiplied very early in the hematopoietic organs. The virions were later observed in the lung, the digestive tract, the ovary, the suprarenal gland and the heart. A very large number of viral particles were found in the kidney distal convoluted tubules 25 days after infection. Only the kidney actually underwent a malignant transformation; furthermore an unusual differentiation into cartilaginous or osteoid cells was sometimes observed and, strikingly enough, these elements were virogenic.

A TRANSMISSIBLE nephroblastoma line has been developed in chickens. The origin of this nephroblastoma was described elsewhere [1]. Briefly, a leukaemia appeared in a hamster after inoculation of Avian Myeloblastosis Virus (AMV) producing chicken cells, was successfully transmitted to hamster, and a transplantable tumour line "HP" was established [2]. Avian leukaemia gs antigen was found in these tumour cells, and AMV-like particles were observed by electron microscopy in early passage material [3]. Inoculation into the chickens of these hamster tumour cells HP associated with normal chicken embryo fibroblasts induced nephroblastomas. One of these chicken nephroblastomas was transmitted by cells and by cell-free extract to 1–6-day old chickens throughout 11 generations. The tumours listed in our laboratory DNV retained their histologic pattern and the ability to synthesize infectious virus. Kidney tumours occurred in about 70% of the animals, killing them as early as $1\frac{1}{2}$ to $3\frac{1}{2}$ months after inoculation. The virus produced transforms the nephrogenic cells *in vivo*; they express their inherent potentiality to differentiate into nephron elements, as well as cartilaginous, osteoid and hematopoietic cells [4]. A malignant transformation was observed in the kidney cells only. We never found any other neoplasia or leukaemia.

Electron microscopy demonstrated that virus production occurred not only in tumour cells, but also in hematopoietic, bone and epithelial cells. Moreover, virions were observed in hepatic, pancreatic, intestinal and ovarian tissues.

A chronological study was undertaken to show in which type of tissues the viral production begins and to consider the possibility of a secondary infection of the whole organism by viruses released from the transformed kidney cells.

Accepted 20 July 1971.

MATERIAL AND METHODS

The nephroblastoma-bearing chickens were sacrificed, the kidney tumour tissue excised, and a viral extract prepared according to the technique of Moloney [5]. The material was filtered on Seitz K00 and then on Millipore 0·45 μ in order to eliminate cell fragments. A quantity of 0·3 ml of virus suspension obtained from 300 mg tumour was injected intraperitoneally to 24-hour old brown Leghorn chickens (lymphomatosis free).

The mean survival time was 75 days, and tumours were observed in 100% of the cases. In a comparative series of birds infected with the same viral extract, 2 chickens were sacrificed daily from the 2nd to the 13th day following the infection, then every week until the 17th week. Fragments of liver, spleen, kidney, pancreas, lung, stomach, intestine, heart, gonads and bone marrow were immediately collected and processed for electron microscopic and histologic studies. In some instances, the suprarenal glands and the brain were also taken; so were the bones when osteopetrosis was clinically diagnosed.

No leukaemia cells were observed in the peripheral blood upon systematic examinations of blood samples (including one taken on the very day of the animal's death).

Electron microscopic techniques

Samples were taken immediately after sacrificing the birds, and fixed for 1 hr in 6% glutaraldehyde (v/v) in ice-cold Sörensen phosphate buffer at pH 7·2. They were then washed for 2 hr and postfixed for 1 hr in 1% osmium tetroxide in the same buffer. Alcoholic dehydration and Epon embedding were performed in the usual manner. Sections were cut with a "LKB" ultramicrotome and stained with 0·5% uranyl acetate in Michaëlis buffer at pH 5 for 20 min, then for 10 min with lead citrate as described by Reynolds [6].

RESULTS

After 5–6 days, virus particles appeared in the bone marrow and buds developed from the myeloblasts plasma membrane (Fig. 1) as in AMV BAI-A induced myeloblastosis. At this stage, virions could also be observed in the intracellular spaces of the spleen (Fig. 2), which kept its normal aspect. No virus budding could be detected in any other observed organs.

Thus, the initial viral production took place exclusively in the main hematopoietic organs. It remained constant, but moderate, for 3–5 weeks and becomes quite low afterwards.

After 10–12 days, the particles appeared in the viscera and were always found in a considerable amount in the lung, the liver, the pancreas, the ovary and the digestive tract.

In the lung, virus elaboration occurred by budding from the alveolar epithelium and the capillary endothelium plasma membrane. The lung ultrastructure was normal (Figs. 3 and 4).

As observed for AMV BAI-A in the liver, virions were produced in the canaliculi lumen by budding from microvilli. The ultrastructural integrity of the organ was maintained.

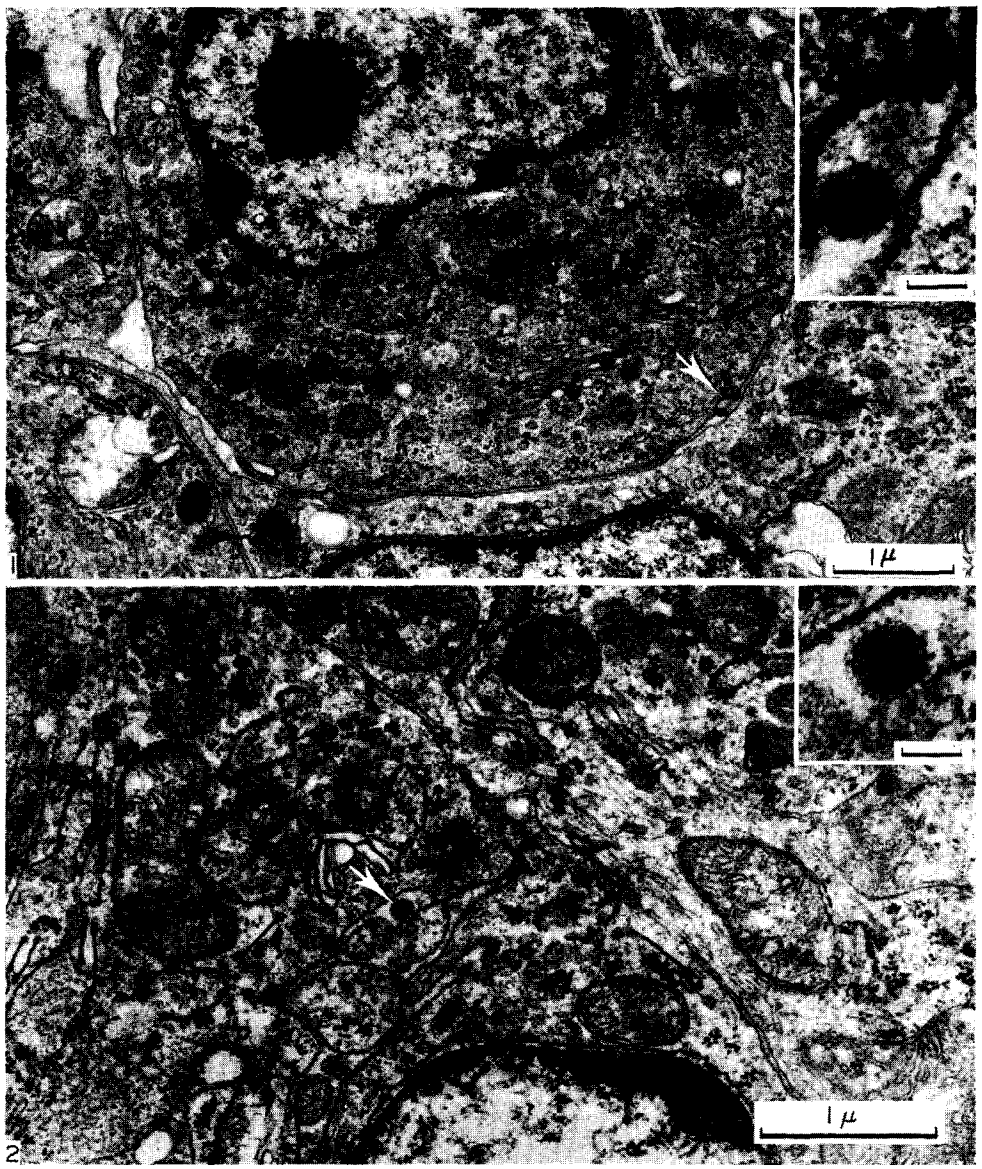
Many mature and immature virions were located between the pancreatic acinar cells. They accumulated within the secretion products in the acini lumen.

In the ovary, buds as well as mature and immature forms were found in large numbers near the interstitial stroma cells.

In the stomach and the intestine, the virions were in most cases of the mature type, but no preferential localization could be noted. However the particles seemed to be more numerous in the former organ.

In some organs, virions are observed only occasionally. In the heart, they were located near the muscle fibres; the occurrence of a production *in situ* was shown by the presence of buds (Figs. 5 and 6). In the suprarenal gland, the virions were very scarce and visible only in the pores of the capillary endothelium, in the basement membrane and in the blood. Mature virions were found in thigh bones with osteopetrosis, either trapped in bone lacunae, or free in the midst of the collagen fibrils located between the ossified area and the osteoblasts (Figs. 7 and 8). However, no evidence of virus elaboration by the osteoblasts could be obtained.

Virions were observed in the kidney tissue only 25 days after inoculation. Simultaneously, virus production decreases in the hematopoietic organs, in which the virus elaboration had begun soon after infection. At this stage, the macroscopical and the histological aspects of the kidney were still normal. The first virions were seen in the distal convoluted tubules. Buds were present in large numbers in the folds of the basal cell surface. Then the virions accumulated in the basement membrane and in the lumen of the tubule. A great number of particles were found between the cells lining the distal convoluted tubule (Fig. 9). At a later stage, virions were located in the same areas in the collecting tubules and in the Henle's loops. Only very seldom were particles observed in Bowman's capsular space. Virus production, which was slow at first, increased

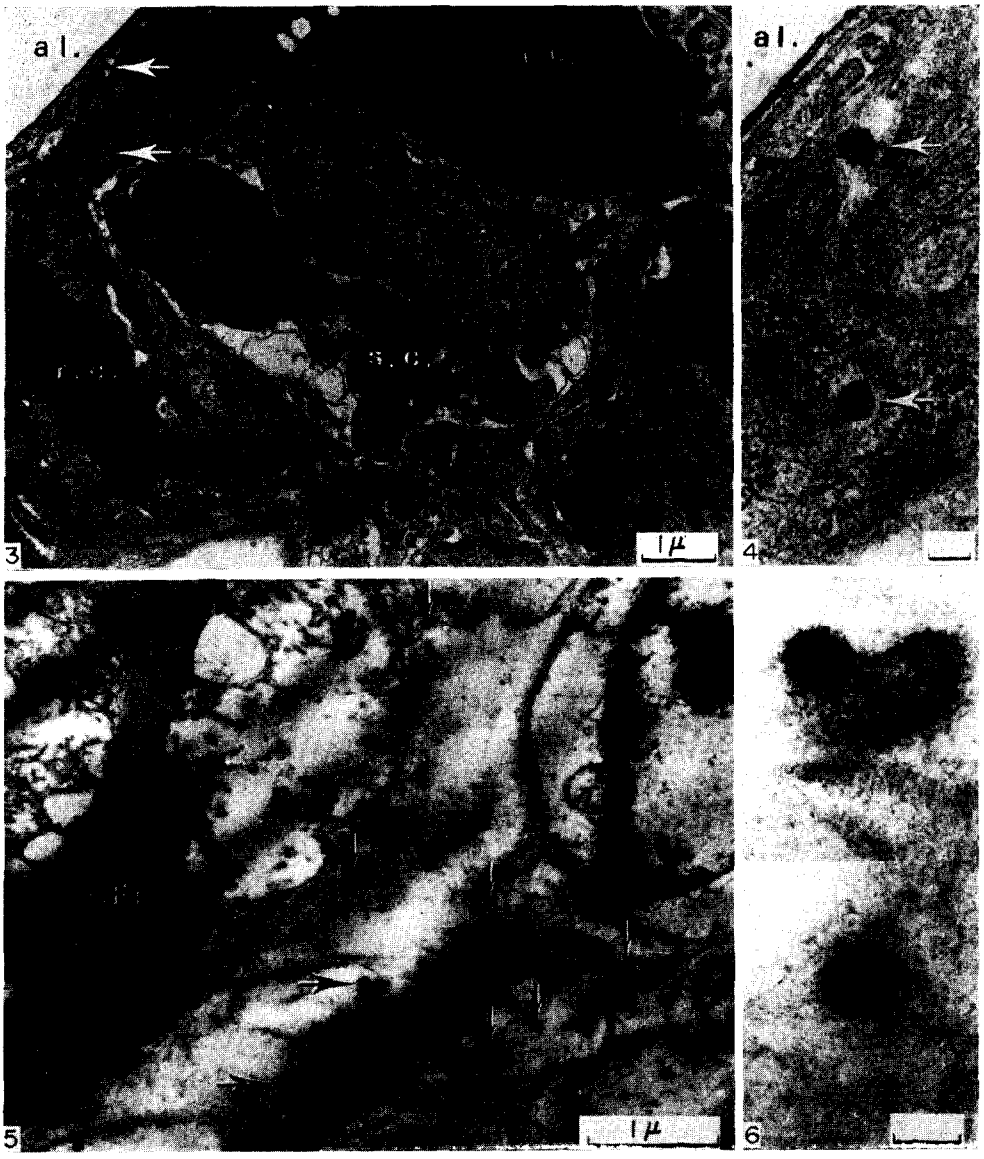


Unless otherwise stated, scales represent 0·1 μ.

Fig. 1. *Bone marrow* 18 *days after infection: budding from the plasma membrane of a myeloblast near a mature virion (white arrow). Note phagocyted mature virions (black arrows)* × 16,000. *Enlargement of the bud and of the mature virion* × 80,000.

Fig. 2. *Spleen* 25 *days after infection: characteristic mature form* × 31,000. *Enlargement of the virus particle* × 100,000.

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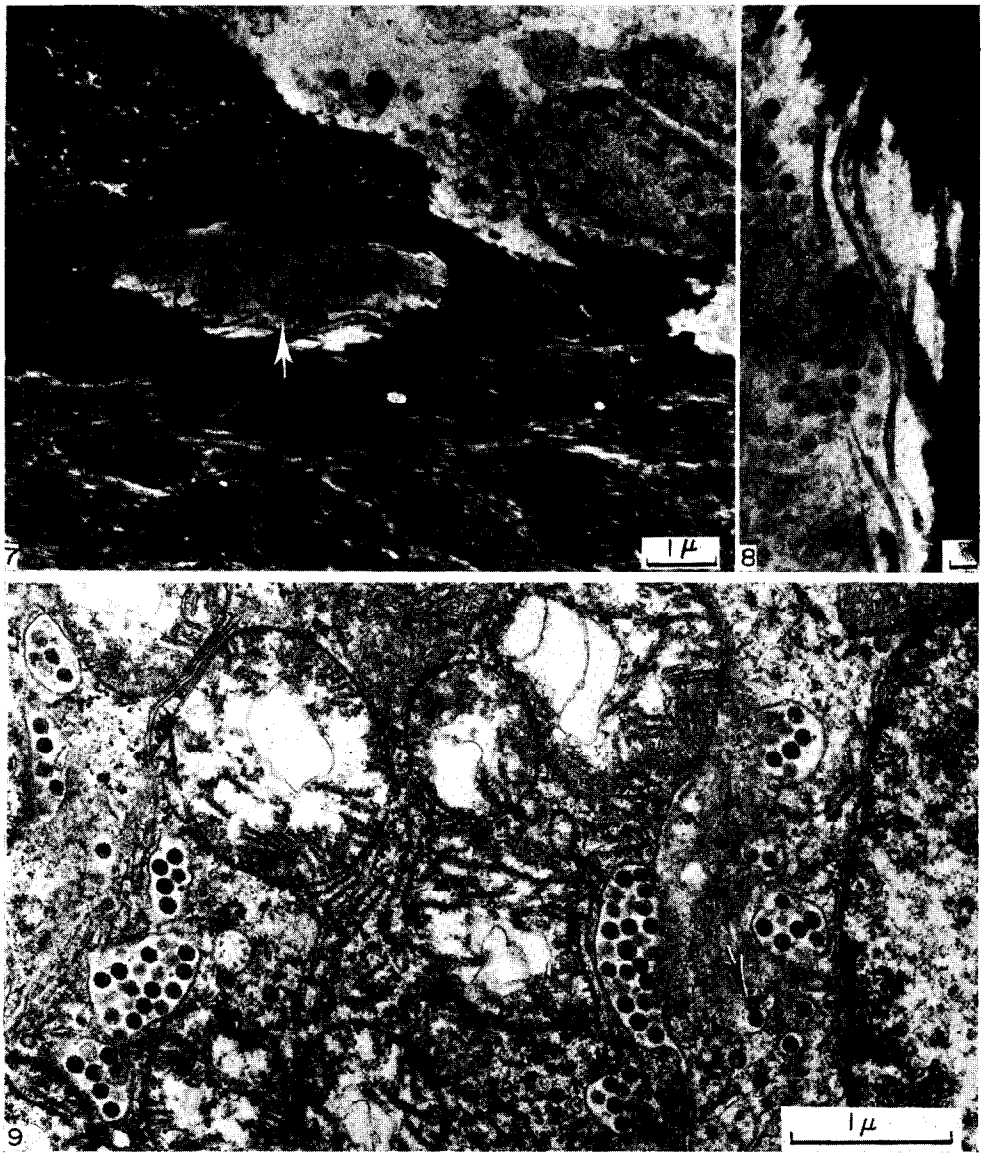
Unless otherwise stated, scales represent 0·1 μ.

Fig. 3. *Lung* 32 *days after infection: many virions* (*black arrows*) *are present both between the septal cells* (*s.c.*) *under the endothelium of the capillaries recognizable by the presence of red blood cells* (*r.c.*), *as in the basement membrane of the alveoli* (*al.*) × 9700.

Fig. 4. *Enlargement of two viruses located near the alevolus* (*white arrows of Fig.* 3).

Fig. 5. *Heart* 68 *days after infection. Note the presence of many extracellular virions* (*arrows*) *and one bud near the cardiac muscle fibres* (*Fi.*) × 18,000.

Fig. 6. *Detail of the bud and of a mature virion* × 40,000.

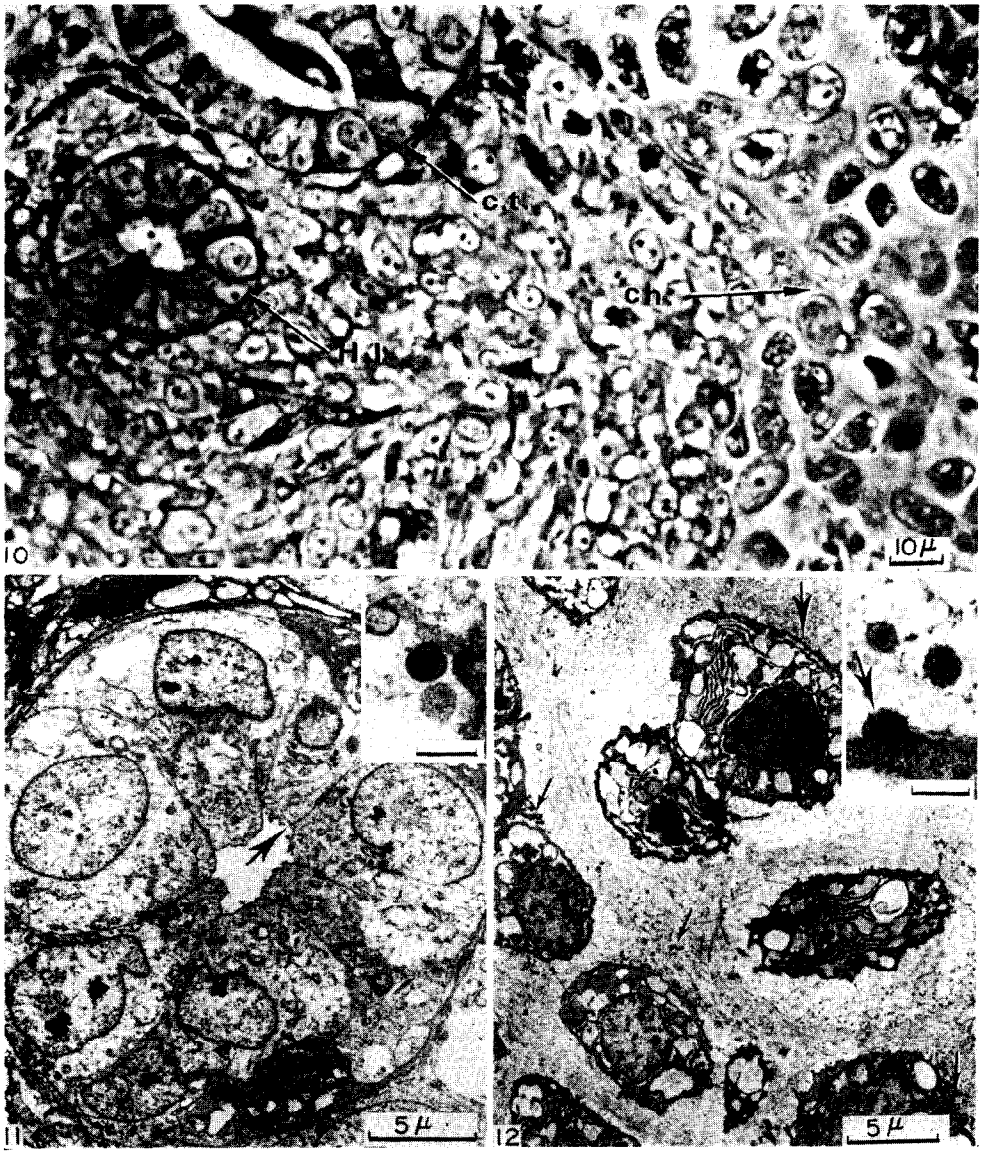


Unless otherwise stated, scales represent 0·1 μ.

Fig. 7. *Bone* 102 *days after infection; many virions are trapped in a lacuna (arrow)* × 10,300.

Fig. 8. *Detail of virions in the calcified zone* × 26,000.

Fig. 9. *Kidney* 38 *days after infection; many mature virions and one bud (arrow) are in the folds of the basement membrane of a distal convoluted tubule* × 25,700.



Unless otherwise stated, scales represent 0·1 μ.

Fig. 10. *Kidney 74 days after infection. Thick segment of Henle's loop (H.l.) and collecting tubule (c.t.) close to chondroid differentiated area (ch.) Toluidine blue stained 2 μ thick section. Phase contrast approximately* × 1000.

Fig. 11. *Same material as for Fig.* 10. *Intact structure of the Henle's loop segment: a few mature virions are located between the cells and in the tubule lumen (arrow)* × 3000. *Enlargement of the pointed virus in the lumen of the tubule* × 51,000.

Fig. 12. *Same material as for Fig.* 10. *Very large number of mature and immature virion (small arrows showing the greatest accumulations). A bud can be seen on a cell membrane (large arrow)* × 3000. *Enlargement of that bud (arrow) next to two mature virions* × 50,000.

Table 1. *Summary of observations after DNV inoculation*

	Number of observations	Positive observations*	Date of first positive case: days N°	Positive cases Total cases
Bone marrow	30	20	5	2/3
Spleen	30	19	6	2/3
Lung	24	16	10	2/3
Stomach	14	7	11	1/2
Intestine	11	6	10	1/2
Liver	27	15	12	1/2
Kidney	26	17	25	2/3

*Presence of AMV-type virions (buds, immature and mature).
Virus synthesis is as frequent in the hematopoietic organs as in the kidney.
Virus elaboration starts early in the bone marrow and the spleen.
Particles are then found in the lung and the digestive tract, and later in the kidney.

rapidly and remained steady in the kidney until the terminal stage when histological and ultrastructural modifications began to appear.

There was indeed an epitheliomatous transformation and differentiation of the nephrogenic cells with islets of chondroid tissue and hematopoietic cells. Besides this epitheliomatous proliferation of nephrogenic tissue, sarcomatous aspects of conjunctive tissue were sometimes observed. Figure 10 illustrates the proximity of cartilaginous cells and intact kidney tubules. Figure 11 shows the presence of a few virions in a thick segment of Henle's loop, in particular in its lumen (arrow); virus production also occurred in the chondrocytes (Fig. 12).

Virus and processes of virus synthesis were found in association with the cellular elements for all component tissues of the tumour.

DISCUSSION

It is known that AMV BAI strain A transforms *in vivo* specifically blood-forming cells and the nephrogenic tissue. Variations of the respective incidence of leukaemia and nephroblastoma in AMV infected chickens has been described [7].

In our laboratory, AMV BAI strain A injected into 1 to 5-day old brown Leghorn chickens of the J. Carr strain, induced in 94% myeloid leukaemia, and surviving chickens developed nephroblastomas within 4 to 10 months.

After heterotransmission of AMV to hamster the virus rescued from the transformed hamster cells HP, induced in chickens of the same strain only nephroblastomas. Nephroblastomas occurred as early as 6 weeks after inoculation and the incidence was up to 100% in some experiments.

We described in the present study the localization in different organs and the sequence of development of the virus particles from 2 days after inoculation of cell-free extracts of nephroblastoma DNV till the full development of the kidney tumours.

The morphology and elaboration of the virions described in this study are identical to those of the avian oncornavirus group. Virus elaboration occurred by plasma membrane budding; the virus then developed into an immature form with a hollow nucleoid. The mature form measured 1000–1200 Å and was composed of a dense nucleoid and an envelope with characteristic spikes.

The virus appeared at a very early stage in the hematopoietic organs, where it multiplied, as shown by the presence of buds. However, in contrast to AMV BAI-A, no proliferation or transformation was induced. It should be noted that the virus was injected to 24-hour old brown Leghorn chickens (lymphomatosis free), hence to a sensitive strain and at a particularly favourable age for myeloblastosis virus infection. Moreover, in our experiments the virus was inoculated at the dose (determined by C'F') customarily used for transmission of leukaemia [1].

Particles then developed in the lung, the digestive tract, the ovary, and less systematically in the suprarenal gland and the heart. The virus appeared in the kidney tissue only about 25 days after inoculation. Its localization in the different tissues corresponded partly to that of AMV BAI-A, as described by various authors [8–17]. It was often located between the basal cells and the basement membrane in most of the observed tissues.

However, a few characteristics were noted, including the special response of the kidney tissue to the virus, which was found almost

exclusively in the folds of the distal convoluted tubules basal cells membranes. In contrast to the observations of Beard *et al.* [8, 9] particles appeared only very seldom in Bowman's capsular space; moreover, no aberrant intracytoplasmic forms were noted.

The above study indicated that response to DNV is in fact a generalized infectious process and is not limited to the kidney. However, only the nephrogenic tissue undergoes a neoplastic transformation.

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REFERENCES

1. F. Lacour, E. Delain, R. Gérard-Marchant and O. Weiler, Dysembryome néphroblastique "lignée DNV" du poulet. Observations sur 10 générations de transmission expérimentale. *C.R. Acad. Sci. (Paris)* **271D,** 141 (1970).
2. F. Lacour, E. Delain, G. Camus and F. May-Levin, Hétérotransmission du virus de la myéloblastose aviaire (AMV) au hamster. *C.R. Acad. Sci. (Paris)* **268D,** 631 (1969).
3. E. Delain, O. Weiler et F. Lacour, Mise en évidence de particules virales de type AMV dans une tumeur transplantable du hamster: HP. *C.R. Acad. Sci. (Paris)* **268D,** 744 (1969).
4. F. Lacour, O. Weiler, R. Gerard-Marchant et E. Delain, Transformation et différenciation du tissu rénal par un virus oncogène à RNA. *Bull. Cancer* **57,** 335 (1970).
5. J. B. Moloney, Biological studies on the Rous Sarcoma virus. V. Preparation of improved standard lots of virus for use in quantitative investigations. *J. nat. Cancer Inst.* **16,** 877 (1956).
6. E. S. Reynolds, The use of lead citrate at high pH as an electron opaque stain in electron microscopy. *J. cell. Biol.* **17,** 208 (1963).
7. B. Thorell, Modification of the host reaction towards leukemia virus. *Proc. VII Int. Congress of Intern. Soc. Haematol.*, Rome, Sept. 7–13, p. 582 (1961).
8. H. Ishiguro, D. Beard, J. R. Sommer, U. Heine, G. de The and J. W. Beard, Multiplicity of cell response to the BAI strain A (myeloblastosis) avian tumor virus. I. Nephroblastoma (Wilm's tumor) gross and microscopic pathology. *J. nat. Cancer. Inst.* **29,** 1 (1962).
9. U. Heine, G. de The, H. Ishiguro, J. R. Sommer, D. Beard and J. W. Beard, Multiplicity of cell response to the BAI strain A (myeloblastosis) avian tumor virus. II. Nephroblastoma (Wilm's tumor): ultrastructure. *J. nat. Cancer Inst.* **29,** 41 (1962).
10. G. de The, H. Ishiguro, D. Beard and J. W. Beard, Multiplicity of cell response to the BAI strain A (myeloblastosis) avian tumor virus. VII. Elaboration of virus by non neoplastic hepatic cells. *J. nat. Cancer Inst.* **31,** 717 (1963).
11. R. F. Zeigel, Morphological evidence for the association of virus particles with the pancreatic acinar cells of the chick. *J. nat. Cancer Inst.* **26,** 1011 (1961).
12. U. Heine, G. de The, D. Beard and J. W. Beard, Multiplicity of cell response to the BAI strain A (myeloblastosis) avian tumor virus. V. Elaboration of virus by pancreas of chickens inoculated with the agent. *J. nat. Cancer Inst.* **30,** 817 (1963).
13. H. S. DiStefano and R. M. Dougherthy, Virus multiplication in the oviduct of hens infected with an avian leukosis virus. *Virology* **26,** 156 (1965).
14. R. M. Dougherthy and H. S. DiStefano, Sites of avian leukosis virus multiplication in congenitally infected chickens. *Cancer Res.* **27,** 322 (1967).
15. H. S. DiStefano and R. M. Dougherthy, Multiplication of avian leukosis in endocrine organs of congenitally infected chickens. *J. nat. Cancer Inst.* **42,** 147 (1969).
16. G. de The, Relations cellules–virus dans les leucémies et tumeurs aviaires. *Nouv. Rev. franc. Hémat.* **8,** 769 (1968).
17. D. Young, Osteopetrosis-virus growth in the kidney of infected chickens. *Europ. J. Cancer* **2,** 13 (1966).

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The Diethyl Esters of *Cis*- and *Trans*-Epoxysuccinic Acid, and other possible Carcinogens in Spirits

E. BOYLAND* and W. H. DOWN

Chester Beatty Research Institute, Institute of Cancer Research, Royal Cancer Hospital, Fulham Road, London, S.W.3 England

Abstract—*The diethyl esters of* cis- *and* trans-*epoxysuccinic acid were synthesised and examined. The compounds were stable for short periods at pH* 5·0, *as determined by gas–liquid chromatography. Both compounds were non-toxic to mice, possibly being detoxified in the liver by conjugation with glutathione. Both esters alkylate* γ-(4-*nitrobenzyl)pyridine, which may indicate that they are carcinogenic. Compounds with related structures possessed no alkylating properties. Gas–liquid chromatography has not yet indicated the presence of the epoxy esters in extracts of distilled drinks. The possibility that nitrosamines, lactones and some esters might be biologically active is discussed.*

INTRODUCTION

THE HIGHER incidence of cancer of the oesophagus in drinkers of some spirits rather than of undistilled alcoholic drinks suggests that carcinogens could be produced on distillation. *trans*-2,3-Epoxysuccinic acid is an intermediate in tartaric acid biosynthesis by *Aspergillus fumigatus* [1] and since tartaric acid occurs in the lees of wine, epoxysuccinic acid might also be present in the residues of fermentation. Since many simple epoxides are carcinogenic, epoxysuccinic acid, its esters or other derivatives might be present in alcoholic drinks and act as carcinogens. The only known carcinogenic epoxides are bis epoxides [2], but no esters of epoxy acids appear to have been tested for carcinogenic activity.

Epoxysuccinic acid could be formed when tartaric acid is heated (Fig. 1a) and esters of tartaric acid or epoxysuccinic acid could be produced on heating with ethanol during the distillation of the fermentation products (Fig. 1b). Diethyl succinate occurs in wine [3] so that diethyl epoxysuccinate could be present in wine and distilled spirits and thus be associated with the increased incidence of cancer of the oesophagus in drinkers of certain spirits [4]. The *cis*- and *trans*-isomers of diethyl epoxysuccinate have been prepared.

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*Present address: International Agency for Research on Cancer, Lyon, France and Department of Occupational Medicine, London School of Hygiene and Tropical Medicine, London, W.C.1, England.

EXPERIMENTAL

Materials

Maleic acid, fumaric acid, diethyl maleate, diethyl fumarate, diethyl succinate and triethylamine were obtained from British Drug Houses Ltd., Poole, Dorset, England. Diethyl tartrate and sodium tungstate were from Hopkin and Williams Ltd., England. Diethyl oxaloacetate was obtained from Sigma Chemical Co., Missouri, U.S.A., glutathione from Schwarz Bio-Research Inc., (Orangeburg, N.Y., U.S.A.), Ellman's reagent (5,5 dithiobis-2-nitrobenzoic acid) from Aldrich Chemical Co. Inc., Wisconsin, U.S.A., and γ-(4-nitrobenzyl)pyridine from Ralph N. Emmanuel, Wembley, England. All other reagents were of AnalaR grade.

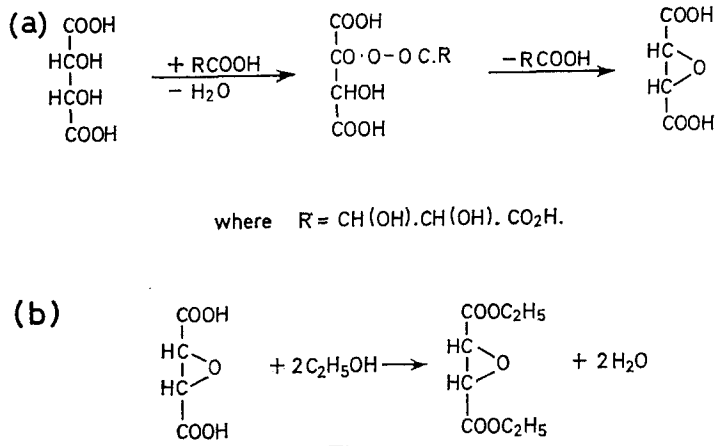


Fig. 1

Gas–liquid chromatography

Gas–liquid chromatography (glc) was carried out using a Perkin-Elmer Model 800 Gas Chromatograph, equipped with a flame-ionisation detector and a 0·25–2·5 mV Honeywell Electronik recorder. The chromatographic column was supplied by Perkin-Elmer (Beaconsfield, Bucks) and was stainless steel, 2 m in length, 3 mm o.d., packed with 80–100 mesh Chromosorb G. AW DMCS coated with 15% w/w Carbowax 20M. The operating conditions were:—hydrogen pressure 1 kg/cm^2; air pressure 3 kg/cm^2; nitrogen (carrier gas) flow rate 40 ml/min; injection block temperature 210°C; column temperature 175°C; detector temperature 200°C. Basic chart speed was 15 in./hr. The column was equilibrated for 24 hr at the operating conditions before use.

Gas–liquid chromatography enabled separation of the two isomers from related compounds to be carried out. Ethanolic solutions of the compounds were injected on to the chromatoraph using a 10 μl graduated syringe (Clark Hamilton Manufacturing AG. Switzerland). glc was used in an attempt to detect either diethyl *cis*- or *trans*-epoxysuccinate in extracts of distilled alcoholic drinks. Samples of 2–5 μl of extract were injected for each analysis.

Preparation of diethyl cis- *and* trans-*epoxysuccinate*

Cis- and *trans*-Epoxysuccinic acids were prepared by the epoxidation with hydrogen peroxide of maleic acid and fumaric acids respectively. The reaction was catalysed by sodium tungstate, and maintained in the pH range 4–5·5 [5]. The *cis*- and *trans*-epoxysuccinic acids were isolated as their barium salts and converted into their corresponding diethyl esters. Barium *cis*-epoxysuccinate was suspended in ether and treated with concentrated sulphuric acid, the temperature of the reaction mixture being maintained at 10–15°C. Ethanol was then added and the mixture refluxed overnight under anhydrous conditions [6]. The resulting pale green oil was dissolved in acetone and any insoluble free acid removed by filtration. The acetone was removed under reduced pressure and the oil fractionally distilled at reduced pressure to yield diethyl *cis*-epoxysuccinate as a colourless oil, b.p. 92–95°C at 0·005–0·01 mm (Found: C, 50·1; H, 6·5. Calc. for $C_8H_{12}O_5$: C, 51·1; H, 6·4%.) Diethyl *trans*-epoxysuccinate was prepared from barium *trans*-epoxysuccinate by an identical procedure as a colourless oil, b.p. 61–65°C at 0·005–0·01 mm (Found: C, 49·5; H, 6·5%). The infrared spectra of the two compounds were recorded using a Perkin-Elmer Model 257 Grating Infrared Recording Spectrophotometer. The spectra indicated esterification of the *cis*- and *trans*-epoxysuccinic acids by peak shifts from Wave Number 1670–1740 cm^{-1}. The appearance in the spectra of both compounds of a new peak at 790 cm^{-1} indicated the presence of epoxy compounds [7].

Determination of stability

The stabilities of the two epoxy esters were determined by keeping 30 μ moles of each ester at room temperature in 3·0 ml of 35% (v/v) aqueous ethanol at pH 4 and 5 (0·1 M-sodium acetate buffer) and pH 6 and 7 (0·1 M-sodium citrate buffer), or in aqueous solution at pH 5·0. Control samples were kept in absolute ethanol (in which the epoxy esters were stable). At intervals of 4, 8 and 18 days, aliquots (0·1 ml) were removed from each sample, made up to 1·0 ml with absolute ethanol and dried with anhydrous sodium sulphate. The solid was centrifuged off and 1 μl of supernatant used for glc analysis. Comparison of the glc analysis of the test solutions with that of the control, over a period of 18 days, gave an indication of the stability of each epoxy ester.

Acute toxicities in mice

Diethyl *cis*- and *trans*-epoxysuccinate were tested for toxicity by intraperitoneal injection into male C^- mice of an arachis oil suspension. The dose ranged from 5–625 mg/kg body weight.

Conjugation with glutathione

The two epoxy esters (1 mM in 5% aqueous ethanol) were each incubated with glutathione (1 mM) in the presence of 0·25 ml rat-liver supernatant (RLS) [8] in 0·1 M-sodium phosphate buffer, pH 7·4, at 37°C for 1 hr in evacuated Thunberg tubes. Aliquots (1·0 ml) of the reaction mixture were removed and added to 0·75 ml of 6% (w/v) sulphosalicylic acid. After centrifugation to remove protein, 0·5 ml supernatant was added to 4·0 ml Ellman's reagent (0·5 mM-5,5′-dithiobis-2-nitro-benzoic acid in 1·0 M-sodium phosphate buffer containing 1 mM-EDTA) [9]. The absorption at 412 nm was measured within 2 min against a blank of 0·5 ml water and 4·0 ml Ellman's reagent (using a 1 cm light path and a Unicam SP 500 Spectrophotometer). The E_{412} values were proportional to GSH concentration up to 0·25 μ moles of GSH in the final solution. The results were expressed as the percentage decrease in thiol groups of GSH in 1 hr, in the presence or absence of rat liver supernatant.

Alkylation of γ-(4-nitrobenzyl)pyridine

Solutions of 30 μ moles of each ester in 3·1 ml of 50% (v/v) aqueous ethanol were incubated at 60°C for a maximum of 22 hr in the presence of 0·6 M-sodium acetate buffer, pH 5·5 and 0·5 ml of 5% (w/v) γ-(4-nitrobenzyl)pyridine in methyl ethyl ketone [10]. Following incubation the reaction mixtures were made alkaline by the addition of 2·0 ml of 50% (v/v) triethylamine–acetone reagent. Absorption of the resulting purple solution was measured at 565 nm within 5 min, because of the instability of the purple colour. The incubation was continued for up to 22 hr to confirm the absence of any alkylating properties of compounds of similar structure.

The comparative rates of alkylation of γ-(4-nitrobenzyl)pyridine by diethyl *cis*- or *trans*-epoxysuccinate were determined by the incubation of either epoxy ester over 6 hr at 60°C. Reaction mixtures were identical to those previously described, and rates of alkylation determined by the increase in absorption at 565 nm. The alkylations of γ-(4-nitrobenzyl)-pyridine by some related diethyl esters (diethyl oxaloacetate, diethyl fumarate, diethyl maleate, diethyl succinate) were determined using this procedure.

Attempted dehydration of tartaric acid

Ten milligrams mesotartaric acid was refluxed in 10·0 ml absolute ethanol under anhydrous conditions for 72 hr. After removal of the ethanol under reduced pressure, the viscous residue was analysed by glc, using the conditions described. (+)-Tartaric acid and (−)-tartaric acid were refluxed with ethanol in a similar manner and analysed by glc.

RESULTS AND DISCUSSION

Determination of the stability of diethyl *cis*- and *trans*-epoxysuccinate in 35% aqueous ethanol indicated a maximum 10% loss of the epoxy ester at pH 4 and 5, rising to approximately 40% and 80% at pH 6 and 7, respectively. In aqueous solution a 10% loss occurred over an identical period. These results are shown in Table 1. The biological activity of these compounds in drinks consumed shortly after distillation would necessitate only short-term stability under the conditions encountered (35% aqueous ethanol, pH 5·5) in alcoholic drinks. From the stability determinations, both diethyl *cis*- and *trans*-epoxysuccinate could be present for at least 18 days following distillation.

Table 1. *The stability of diethyl* cis- *and* trans-*epoxysuccinate*

pH of medium (35% (v/v) ethanol)	Percentage loss of epoxy ester*		
	4 days	8 days	18 days
		cis	
4	2·1	2·4	1·9
5	3·3	6·4	10·2
6	10·4	18·7	35·6
7	20·7	31·2	70·6
5 (aqueous)	3·8	7·3	20·4
	trans		
4	2·2	2·6	2·4
5	4·1	7·3	10·2
6	9·3	19·7	60·3
7	25·7	49·1	85·6
5 (aqueous)	5·4	7·4	10·7

*Expressed as percentage loss with respect to control in absolute ethanol.

The intraperitoneal injection of either compound into mice to test for acute toxicity resulted in an initial weight loss, followed by a rapid recovery after the first day. No deaths resulted over a period of 12 days following the

injection of either ester. The non-toxic effect could be accounted for by a detoxication in the liver by conjugation with glutathione. A mechanism of detoxication was proposed [11] which involved conjugation of glutathione with epoxy-compounds, catalysed by glutathione-S-epoxidetransferase:

$$GSH + R'.CH\!-\!\underset{\backslash O /}{}\!-\!CH.R'' \longrightarrow GS.CH.R'.CH.R''.OH.$$

Investigation of the conjugation of diethyl *cis*- and *trans*-epoxysuccinate with glutathione indicated 95% conjugation over 1 hr in the presence of rat-liver supernatant, and 35% conjugation in the uncatalysed reaction. These results are shown in Table 2.

Table 2. *Conjugation of diethyl* cis- *and* trans-*epoxysuccinate with glutathione*

Compound	% decrease in SH of GSH in 1 hr	
	+RLS	−RLS
Diethyl *cis*-epoxysuccinate	95	39
Diethyl *trans*-epoxysuccinate	97	36

As many simple epoxides are carcinogenic by virtue of alkylating properties, the alkylation of γ-(4-nitrobenzyl)pyridine by diethyl *cis*- or *trans*-epoxysuccinic acid was investigated. Diethyl *cis*- and *trans*-epoxysuccinate both alkylate γ-(4-nitrobenzyl)pyridine, the *cis*-isomer at twice the rate of the *trans*-isomer (Fig. 2). Diethyl epoxysuccinate is isomeric with diethyl oxaloacetate, and the alkylating properties of this and other diesters were investigated. No alkylation of γ-(4-nitrobenzyl)-pyridine by diethyl oxaloacetate, diethyl maleate, diethyl fumarate or diethyl succinate occurred after 22 hr incubation. This indicates that the alkylating properties are due to a —CH(O)CH— epoxy group rather than to a —CH_2—C(=O)—OC_2H_5 ester group.

Gas–liquid chromatography was used to separate diethyl *cis*- and *trans*-epoxysuccinate from the related diethyl esters mentioned. The different retention times of the epoxy esters and diethyl oxaloacetate also indicates the stability of the epoxide ring at the high temperatures (210°C) which were employed. The retention of the related compounds (expressed as time from injection to elution) are listed in Table 3. Unesterified acids were not eluted under these conditions. glc was used to analyse extracts of alcoholic drinks. "African Gin" was analysed but contained no compound with a retention time equivalent to either diethyl *cis*- or *trans*-epoxysuccinate.

Traces of diethyl *trans*-epoxysuccinate were found in the residues when meso-tartaric acid was refluxed with absolute ethanol for 72 hr. glc analysis showed a major peak of retention time 6·9 min with a small shoulder at 7·3 min, which appeared to be diethyl *trans*-epoxysuccinate. This evidence, and the stability of the epoxy esters to heat and aqueous ethanol

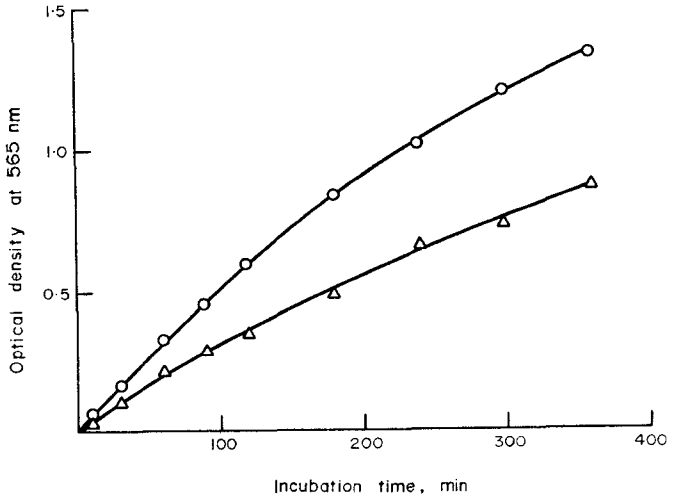


Fig. 2. *Alkylation of* -(4-*nitrobenzyl*)*pyridine by diethyl* cis- *and* trans-*epoxysuccinate. Incubation was at* 60°C. *Optical density values were taken within* 5 *min of addition of* 50% (*v*/*v*) *triethylamine–acetone reagent.*

Table 3. *Alkylation and gas–liquid chomatographic data of diethyl* cis- *and* trans-*epoxysuccinate*

Compound	Alkylation of -(4-nitrobenzyl)pyridine*	glc retention time (min)
Diethyl *cis*-epoxysuccinate	+	11·2
Diethyl *trans*-epoxysuccinate	+	7·3
cis-Epoxysuccinic acid	–	—
trans-Epoxysuccinic acid	–	—
Oxaloacetic acid	–	—
Diethyl oxaloacetate	–	12·6
Diethyl succinate	–	2·52
Diethyl maleate	–	4·1
Diethyl fumarate	–	3·0
Diethyl tartrate	–	19·1

*22 hr incubation.

implies that diethyl epoxysuccinate could be produced by dehydration of diethyl tartrate during the distillation of drinks.

Further attempts will be made to detect diethyl *cis*- or *trans*-epoxysuccinate in spirits, and to see if they are carcinogenic to animals.

Other theoretically possible carcinogens in spirits include:

(1) γ-Butyrolactone is present in some wines [3], is volatile in steam and on "gastric feeding" to rats produced carcinomata of the fore stomach. Other lactones that occur in plants or as fermentation products could also be present.

(2) Nitrosamines might be formed on distillation of fermentation products that contained nitrite or nitrate and secondary amines. Rebelein [12] and others have found nitrate in wines of different countries. Many wines contain 6 mg nitrate (expressed as N_2O_5) per l. McGlashan *et al.* [13] found nitrosamines in some African alcoholic spirits but this finding has not been confirmed.

(3) Methyl iodide, dimethyl sulphate and methyl methanesulphonate are esters and are carcinogenic [14] by virtue of their alkylating activity. Some of the esters present in alcoholic beverages might be alkylating agents although none of the esters tested in the present work with the exception of the epoxy compounds reacted with the γ-(4-nitrobenzyl)pyridine (Table 3).

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REFERENCES

1. R. H. Allen and W. B. Jakoby, Tartaric acid metabolism. IX Synthesis with tartrate epoxidase. *J. biol. Chem.* **224,** 2078 (1969).
2. B. L. Van Duren, L. Langseth, L. Orris, G. Teebor, N. Nelson and M. Kuschner, Carcinogenicity of epoxides, lactones and peroxy compounds. IV. Tumor response in epithelial and connective tissue in mice and rats. *J. nat. Cancer Inst.* **37,** 825 (1966).
3. J. C. Sapis and P. Ribéreau-Gayor, Etude dans les vins du tyrosol, du tryptophol de l'alcool phénylethylique et de la γ-butyrolactone produits secondaires de la fermentation alcoolique. *Ann. Technol. agric.* **18,** 207, 221 (1969).
4. A. J. Tuyns, Cancer of the oesophagus: further evidence of the relation to drinking habits in France. *Int. J. Cancer* **5,** 152 (1970).
5. G. B. Payne and P. H. Williams, Reactions of hydrogen peroxide. IV. Sodium tungstate catalysed epoxidation of α,β-unsaturated acids. *J. Org. Chem.* **24,** 54 (1959).
6. M. W. Miller, Derivatives of (-)-*trans*-2,3-epoxysuccinic acid and some of their biological effects. *J. Med. pharm. Chem.* **6,** 233 (1963)
7. L. J. Bellamy, *The Infra-red Spectra of Complex Molecules,* (2nd Ed.) p. 118. Methuen, London (1958).

8. J. Booth, E. Boyland and P. Sims, An enzyme from rat-liver catalysing conjugation with glutathione. *Biochem. J.* **79,** 516 (1961).
9. K. Harrap, The application of 5,5′-dithiobis-2-nitrobenzoic acid to the determination of the acid soluble disulphide content of blood. *Biochem. Pharmacol.* **16,** 725 (1967).
10. E. Boyland, M. D. Staunton and K. Williams, Experimental regional administration (perfusion and infusion) of chlorambucil [p-N,N-di (-chloroethyl)aminophenylbutyric acid]. *Brit. J. Cancer* **15,** 498 (1961).
11. E. Boyland and K. Williams, An enzyme catalysing the conjugation of epoxides with glutathione. *Biochem. J.* **94,** 190 (1965).
12. H. Rebelein, Beitrag zur Bestimmung und Beurteilung des Nitratgehaltes von Traubenmosten und Wein. *Deutsch. Lebensm. Tdsch.* **63,** 233 (1967).
13. N. D. McGlashan, C. L. Walters and A. E. M. McLean, Nitrosamines in African alcoholic spirits and oesophageal cancer. *Lancet* 1017 (1968).
14. H. Druckrey, H. Kruse, R. Preussmann, S. Ivankovic and Ch. Landschütz, Cancerogene alkylierende Substanzen. III. Alkyl-halogenide, -sulfate, sulphonate und ringgespannte Heterocyclen. *Z. Krebsforsch.* **74,** 241 (1970).

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Acute Effects of Hepatocarcinogens on Mouse-Liver Microsomal Antigens*

P. M. INGLETON
Department of Zoology, The University, Western Bank, Sheffield S10 2TN, England

Abstract—*The endoplasmic reticulum membranes of mouse liver have been shown to contain antigenic components which are affected by single injections of the hepatocarcinogen o-AAT. One of these antigens decreased in concentration after carcinogen treatment and another increased. Similar changes were not induced by the non-carcinogenic p-AAT, but o-AAT incorporated in the diet did affect these antigens during the early stages (2 weeks) of feeding.*

INTRODUCTION

CHANGES in the antigenic composition of a target tissue during chemical carcinogenesis have been postulated by Green [1] and by Molomut [2] as having particular relevance to cancer induction and progression. The disappearance of normal tissue antigens [3–10] and the appearance of novel antigens [11] in overtly malignant tissues are both well documented.

During the early stages of hepatocarcinogenesis disruption of the normal configuration of cell organelles, particularly the endoplasmic reticulum, follows application of a variety of carcinogens [12–17]. The potent liver carcinogen 3'methyl-4-dimethylaminoazobenzene has been detected in rat-liver microsomal protein during the uptake period [18] and firm binding of this dye to the ergastoplasm was reported by Fiala and Fiala [19]. Another hepatocarcinogen 2':3 dimethyl-4-aminoazobenzene (*o*-AAT) has also been located immunochemically in microsomes of rat and mouse liver during treatment with the dye [20–22].

In view of the obvious disruptive effects of hepatocarcinogens on the endoplasmic reticulum of rodent liver cells, and because of the presence of these substances in microsomes soon after administration, the present work was done to determine the acute effects of *o*-AAT on mouse-liver microsomal antigens, using the immunodiffusion technique of Ouchterlony [23].

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MATERIAL AND METHODS

The preparation of a sub-microsomal fraction of rat liver consisting essentially of endoplasmic reticulum membranes has been described by Vogt [24]. In the experiments reported here a similar fraction was prepared from livers of Strong A strain female mice, which display high incidence of liver tumour development after azo-dye administration [25]. The final pellet of membranal material, (M fraction) was either used immediately or lyophilized for storage at −20°C.

Production of antiserum

Antiserum was produced in rabbits by intramuscular injection of fresh M fraction emulsified in adjuvant (anti M). Normally 3 injections at 3 weekly intervals were given, each representing M fraction from c. 7·0 g wet weight liver. Serum was collected 10–14 days after the final injection. Similarly, antiserum was produced against M fraction from livers of mice that had received an intraperitoneal (i.p.) injection of 10 mg *o*-AAT 48 hr before sacrifice (anti DM).

Comparisons between the antigens of normal M fraction and carcinogen-treated M fraction were made by double diffusion in agar-gel [23]. Antigen solutions of standard protein concentration were prepared by adding c. 10 mg lyophilized M fraction to 1·0 ml Triton-phosphate (5% Triton × 100 in 0·1 M dipotassium hydrogen orthophosphate, pH 8·0).

RESULTS

(a) *Short-term effects of* o-*AAT*

Groups of Strong A female mice were given a single i.p. injection of 10 mg *o*-AAT in 0·5 ml olive oil and sacrificed at intervals up to 7 days.

The most marked changes in the antigenic composition of treated M fraction appeared 48 hr after injection of the dye. At this time a slow-diffusing component [Fig. 1, line (a)] present in normal M fraction had disappeared, subsequently showing a gradual recovery which was complete by the 7th day after the injection.

When anti DM was tested against 48 hr-treated M fraction a new precipitin line developed in the position nearest to the anti-serum well [Fig. 2, line (b)]. This was present in 3 day post-injection M fraction (DM_3) but not at 7 days.

(b) *Longer-term effects of* o-*AAT*

In this experiment, groups of Strong A female mice were maintained on diet 86 (MRC) which contained 0·1% *o*-AAT. The diet was fed continuously for 7 weeks but then many animals died; accordingly, 1 week of normal diet was alternated with 1 week of dye-diet, thus reducing the death rate. The results showed that M fraction from livers of mice maintained on dye-diet for 2 weeks had been affected in a similar way to that from 48 hr injected mice, in that there was a simplified pattern of precipitin lines, i.e. line (a) missing, when diffused against anti M serum, and line (b) present when diffused against anti DM serum (Figs. 3 and 4).

These gels also showed that at 17 weeks the antigenic composition of M fraction from treated mice more closely resembled normal M fraction. Thus there appears to be a period of initial damage followed by recovery.

(c) *Comparison of acute effects of* o-*AAT with effects of* p-*AAT*

4:5′dimethyl-2-aminoazobenzene (*p*-AAT) is not carcinogenic in rats and mice, in contrast to the *o*-AAT isomer [26].

A group of Strong A female mice was injected i.p. with 10 mg *p*-AAT in 0·5 ml olive oil, and a similar group received *o*-AAT as described previously, to see if the non-carcinogenic isomer had similar effects to *o*-AAT on M fraction. Both groups were sacrificed 48 hr later and M fractions prepared.

Figure 5 shows that the antigenic composition of *p*-AAT treated M fraction is similar to that of normal untreated M fraction. Furthermore the antigen present in DM which gave precipitin line (b) did not appear in *p*-AAT treated M fraction (Fig. 6).

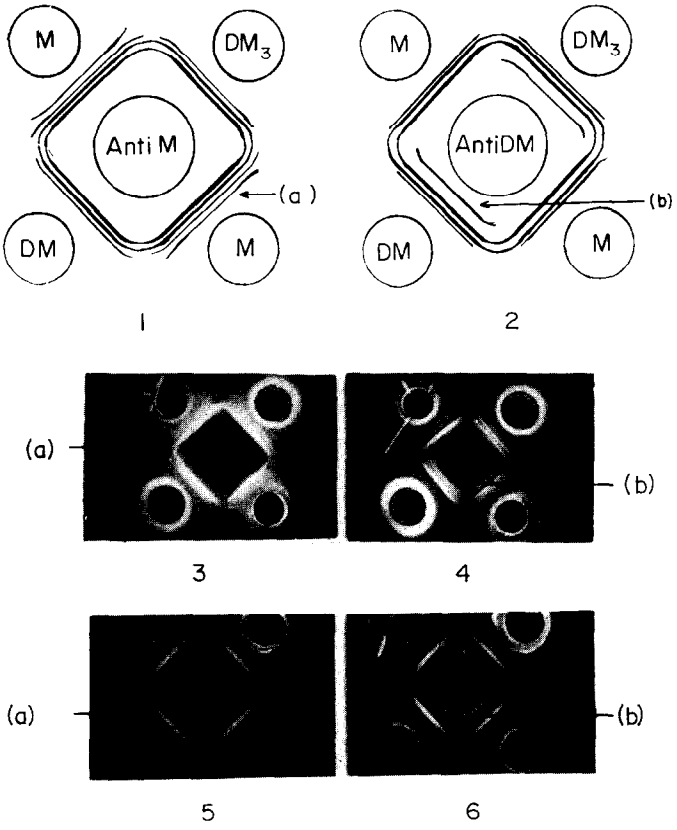
DISCUSSION

One of the acute effects of *o*-AAT is to alter the antigenic composition of liver endoplasmic reticulum membranes. The changes appear to be largely quantitative and repeated attempts to detect a qualitative change by absorption of antisera with either carcinogen treated M fraction or with normal M fraction never gave decisive results.

The lack of effect of the non-carcinogen *p*-AAT on these M fraction antigens lends support to the view that such changes, or gross changes in protein concentration, may be related to cancer induction. The results also suggest that initially the majority of liver cells are identically affected by the carcinogen, but that most of them are then able to recover. Ultimately, however, persistent attack by the carcinogen probably leads to permanent damage in some cells.

Friedrich-Freksa *et al.* [27] showed that rat liver cell sap contained proteins which cross-reacted immunologically with microsomal proteins, but none of these appeared to be the principal target for the binding of 3′MeDMAB or 2-acetylaminofluorene (2-AAF), although the content of one of them, which was liver specific, increased during carcinogen feeding. In contrast Kitagawa *et al.* [7] were able to show by immunoelectrophoresis of rat liver microsomes that 2–3 precipitin arcs fixed antibody to the 2-azofluorene hapten. They could not, however, detect any differences between normal rat liver microsomes and a similar fraction from rats injected with 2-AAF or 2-aminofluorene 72 hr previously, but rats fed 2-AAF for 16 weeks showed loss of some antigenic components and even more extensive changes were apparent in preparations from hepatomas.

M fraction is only a part of the total microsomal fraction which may account for the relatively few antigenic components detected in these immunodiffusion experiments. However, these results do indicate that hepatocarcinogens alter the antigenic properties of liver endoplasmic reticulum soon after administration. The inability of the non-carcinogenic *p*-AAT, and of an hepatomegalic agent ethyl-α-(*p*-chlorophenoxy)-isobutyrate [28] to induce similar changes, suggests that such alterations may be a significant in the carcinogenic process.



EXPLANATION OF PLATES

Figures 1, 3, 5. Centre well contains anti M serum.
Figures 2, 4, 6. Centre well contains anti DM serum.
Outside wells on all plates numbered from top left hand in clockwise direction (i), (ii), (iii), (iv).

Figs. 1 *and* 2. (i) *and* (iii) *Normal Strong A female mouse liver M fraction.* (ii) *M fraction from Strong A female mouse liver injected with* o-*AAT* 72 *hr previously.* (iv) *M fraction from Strong A female mouse liver injected with* o-*AAT* 48 *hr previously* (*DM fraction*).

Figs. 3 *and* 4. (i) *M fraction.* (ii) *and* (iv) *M fraction from Strong A female mouse liver maintained on dye-diet for* 17 *weeks.* (iii) *M fraction from Strong A female mouse liver maintained on dye-diet for* 2 *weeks.*

Figs. 5 *and* 6. (i) *M fraction.* (ii) *and* (iv) *M fraction from Strong A female mouse liver injected with* p-*AAT* 48 *hr previously.* (iii) *DM fraction.*

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REFERENCES

1. H. N. GREEN, An immunological concept of cancer: A preliminary report. *Brit. med. J.* (*ii*), 1374 (1954).
2. N. MOLOMUT, A hypothesis of progression of malignancy based on immune responses. *Neoplasma Bratisl.* **10,** 155 (1963).
3. R. C. NAIRN, H. G. RICHMOND and J. E. FOTHERGILL, Immunological differences between normal and malignant cells. *Brit. med. J.* (*ii*), 1335 (1960).
4. E. WEILER, Die Anderung der Serologischen Organspezifität bei Buttergelbtumor der Ratte im Vergleich zu normaler Leber. *Z. Naturforsch.* **78b,** 324 (1952).
5. E. WEILER, Die Anderung der serologischen Spezifität von Leberzellen der Ratte während der carcinogenese durch *p*-Dimethylaminoazobenzol. *Z. Naturforsch.* **11b,** 31 (1956).
6. E. WEILER, Loss of specific cell antigen in relation to carcinogenesis. *Ciba Found. Symp. on Carcinogenesis: Mechanisms of Action*, p. 165, (Edited by WOLSTENHOLME and O'CONNOR). Churchill, London (1959).
7. M. KITAGAWA, N. TANIGAKI, Y. YAGI, J. PLANINSEK and D. PRESSMAN, Carcinogen binding antigens in rat liver microsomes. *Cancer Res.* **26,** 752 (1966).
8. R. HIRAMOTO, J. BERNECKY, J. JURANDOWSKY and D. PRESSMAN, Immunohistochemical staining properties of the N-2-FAA rat hepatoma. *Cancer Res.* **21,** 1372 (1961).
9. V. I. KALNINS and H. F. STICH, Loss of liver cell antigens in azo-dye induced hepatomas of rats. *Nature (Lond.)* **200,** 189 (1963).
10. R. W. BALDWIN and C. R. BARKER, Antigen deletions in carcinogen-induced rat hepatomata. *Nature (Lond.)* **214,** 292 (1967).
11. U.I.C.C. Monograph Series No. 2, *Specific tumour antigens*, (Edited by R. J. C. HARRIS). Munksgaard/Copenhagen (1967).
12. K. R. PORTER and C. BRUNI, An election microscope study of the early effects of 3′-Me-DAB on rat liver cells. *Cancer Res.* **19,** 997 (1959).
13. J. M. PRICE, J. W. HARMAN, E. C. MILLER and J. A. MILLER, Progressive microscopic alterations in the livers of rats fed the hepatic carcinogens 3′-methyl-4-dimethylamino azobenzene. *Cancer Res.* **12,** 192 (1952).
14. R. G. GUSTAFSSEN and B. A. AFZELIUS, Comparative effects on rat liver cells after dimethylnitrosamine, 2-fluorenamine or prednisolone treatment studied by electron microscopy. *J. nat. Cancer Inst.* **30,** 1045 (1963).
15. P. EMMELOT and E. L. BENEDETTI, Changes in the fine structure of rat liver cells brought about by dimethylnitrosamine. *J. biophys. biochem. Cytol.* **7,** 393 (1960).
16. E. A. SMUCKLER, A. ISERI and E. P. BENDITT, An intracellular defect in protein synthesis induced by carbon tetrachloride. *J. exp. Med.* **116,** 55 (1962).
17. H. A. HARTMANN, Rat liver after N-hydroxy-2-acetylamino fluorene. *Arch. Path.* **79,** 126 (1965).
18. T. HULTIN, The distribution of protein-bound azo dye in subfractions of liver cytoplasm fractions. *Exp. Cell Res.* **10,** 697 (1956).
19. S. FIALA and A. E. FIALA, Intracellular localization of carcinogen and its relationship to the mechanism of carcinogenesis in rat liver. *Brit. J. Cancer.* **13,** 236 (1959).
20. T. A. KOROSTELEVA, About antigenic properties of tissue proteins during experimental liver malignization. Trudy Inst. of Oncology AMN USSR. (Leningrad) 95 (1957).
21. T. A. KOROSTELEVA, On haptenes produced in liver at the early stages of *o*-AAT action. *Vop. Onkol.* **11,** 67 (1965).
22. R. W. BALDWIN, Studies on rat liver cell antigens during the early stages of azo-dye carcinogenesis. *Brit. J. Cancer.* **16,** 749 (1962).
23. Ö. OUCHTERLONY, *In vitro* method of testing the toxin-producing capacity of diphtheria bacteria. *Acta path. microbiol. scand.* **25,** 186 (1948).
24. P. K. VOGT, The immunology of liver microsomes. III. Localization of the tissue-specific antigens in the structural components of endoplasmic reticulum. *Z. Naturforsch.* **15b,** 221 (1960).
25. J. P. GREENSTEIN, *Biochemistry of Cancer.* Academic Press, New York (1954).
26. H. G. CRABTREE, The relative carcinogenic activity of six isomeric aminotoluenes. *Brit. J. Cancer.* **3,** 387 (1949).
27. H. FRIEDRICH-FREKSA, E. LANKA, E. M. YOUNG and S. SOROF, Soluble microsomal antigens of normal and preneoplastic livers. *Cancer Res.* **28,** 491 (1968).
28. P. M. INGLETON, Aspects of microsomal antigenicity during chemical carcinogenesis. Ph.D. Thesis, University of Sheffield (1969).

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Aspects of the Biology and Radiation Response of Cloned C3H Mouse Mammary Carcinoma Cells *in vitro* and *in vivo**

NAOMI K. COHN† and KELLY H. CLIFTON‡
Radiobiology Laboratories, Departments of Radiology and Pathology, University of Wisconsin Medical School, Madison, Wisconsin 53706, *U.S.A.*

Abstract—*A combination of methods is described which has proven successful in establishing clonable cell lines from all primary or transplanted C3H/Wr or C3Hf/Wr mouse mammary carcinomas thus far tested. Both epithelioid and spindle cell types were observed in outgrowths from all tumors. All but two of the cell lines, both of the spindle type, gave rise to tumors after inoculation into histocompatible mice. Studies with two representative cloned epithelioid and one cloned spindle cell line showed that tumor growth in mice was more rapid and predictable when the cells had recently been grown in mice. Experiments designed to determine whether this mouse-passage affect resulted from pronounced antigenic differences between long-term cultured cells and the host mice were negative. Data from an experiment in which ^{125}I-5-iodo-2′-deoxyuridine-labelled cells were employed, indicated however that a greater percentage of the inoculated mouse passaged cells than of long-term cultured cells survived to contribute to tumor formation. When lethally-irradiated cells were included in the inocula, the 'effective number' of* in vitro *colony-forming units required for tumor formation* in vivo *was found to be about* 2 *and* 3 *for two epithelioid cell lines, respectively. Finally, the three cell lines studied had differing D_0 values when irradiated* in vitro. *The D_0 for irradiation* in vivo *of one epithelioid cell line was nearly identical to that of the transplanted tumor strain from which it was derived. Studies of the tumor- and radiobiology of these cell lines are continuing.*

INTRODUCTION

MAMMARY carcinoma of the mouse is one of the most widely used experimental models in cancer research. It is generally recognized however, that the growth rate and other biological characteristics vary among both primary and transplanted tumor strains [1, 2]. This report deals with the establishment of more uniform cell populations which can be cultured *in vitro* and which give rise to tumors *in vivo*, and with aspects of their biological and radiobiological characteristics.

Although reports involving the use of cultured cells derived from mouse mammary carcinomas have appeared [3–5], we are familiar with some of the difficulties in establishing such cell lines. A combination of procedures used successfully with all mammary carcinomas thus far tested in our laboratories is therefore described. With these methods, cell lines have been derived or re-established in culture from tumors more than fifty times. The general characteristics of nine-

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†Current address: Experimental Therapy Department, Burroughs Wellcome and Co., Research Triangle Park, North Carolina.
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teen cell lines, which originated from nine individual mammary carcinomas of aged multiparous C3H/Wr or C3Hf/Wr mice, and experiments *in vivo* and *in vitro*, with three representative cloned cell lines of differing morphology, form the basis of this report.

MATERIALS AND METHODS

Origin and transplantation of tumors

All tumors used were either primary or transplanted mammary carcinomas which originated in aged multiparous mice of the C3H/Wr* or C3Hf/Wr† strains. Transplant recipients were C3H or C3Hf mice or F1 hybrids of them with C57BL6/Wr mice.

Suspensions of solid tumors, when used directly for transplantation, were prepared with a Snell cytosieve as previously described [8]. Suspensions of cultured cells were prepared for transplantation by enzymatic digestion as outlined below. Preliminary experiments indicated that tumor growth from cultured cell inocula was stimulated by the inclusion of lethally-irradiated cells with the living cells [9], as has been reported for serially transplanted mammary tumors [8,10]. When lethally-irradiated cells were employed in transplantation or in immunization studies [11], they were obtained from transplanted C3H/Wr mammary tumor strain MTG-B [12], or were cells of the same type as the living cell inocula. All grafts were subcutaneous.

Cell culture methods

In contrast to methods which involve enzymatic treatment and/or immobilization of explants described in most standard texts [13–15], our best results in deriving cell lines have been obtained with minimal and gentle handling. A piece of asceptically excised tumor is washed thoroughly with Eagle's Minimum Essential Medium [16] containing 10% of horse serum (MEMS). A piece of grossly viable tumor tissue about 3 mm^3 is selected and minced in 5 ml MEMS in a 60-mm Petri dish. The mixture is suspended by gentle pipetting once or twice without forcible expulsion. The dish is incubated at 37°C in an atmosphere of 5% of CO_2–95% of air. After 12–24 hr, the surface of the dish and the adherent cells are gently rinsed 2–3 times with the medium containing unattached cells and tissue fragments. The latter is transferred to a test tube, and new MEMS is added to the dish which is again incubated.

The tissue fragments are allowed to settle in the test tube for a few minutes, and the medium is removed. The fragments are then transferred with 5 ml MEMS to a second Petri dish for incubation. The medium in both dishes is changed bi-weekly until mitoses are common in the attached cells, and daily thereafter.

When mitotic activity decreases, and the dishes contain many attached cells, the culture is subdivided using a modification of the method of Merchant *et al.* [14]. The medium is removed, bicarbonate-buffered glucose–saline, pH 8·0, containing 0·1% of trypsin and 0·025% of Viokase is added, and the dish is incubated for 3–5 min at 37°C. The enzyme solution is aspirated, and MEMS is added. If the cells have not rounded up, they are scraped off the dish with a rubber policeman and gently pipetted to achieve uniform dispersion. The cell concentration is adjusted to 2 to 5×10^5 cells per 5 ml for each of three or four 60-mm Petri dishes. Remaining cells are always saved until it is clear that the dilute inocula are growing. When the dishes were full, the cultures are again split using the same technique. The amount of inoculum can be readily adjusted according to the growth rate of the cell line, the plating efficiency and laboratory routine.

When cell lines are well established, clonal lines can be derived following dilute plating and colony isolation. We used stainless steel cylinders with sterile silicone stopcock grease to isolate a single colony from the remainder of the dish [17]. An isolated colony at least 1 mm in diameter was enzymatically dispersed, and transferred to a 35-mm Petri dish with 2 ml MEMS. The cloning procedure was repeated at least three times in an effort to maximize genetic homogeneity in those lines referred to as 'cloned', below.

In addition, differences in growth characteristics and susceptibility to enzymatic dispersion

*The original C3H/Wr breeding stock was obtained by H. Vermund from J. J. Bittner, University of Minnesota, in 1953, and the nucleus stock has since been maintained by brother and sister mating.

†The C3Hf/Wr mouse substrain was derived in 1965 by caesarian section of a C3H/Wr female with the cooperation of Dr. Charles Lattuada and Mr. Donald Barmore, A. R. Schmidt Co., Madison, Wisconsin. The pups were foster nursed in a bacteria-free isolator by a random bred albino mouse obtained from the stock from the Lobund Institute of South Bend, Indiana. The C3Hf/Wr breeding colony is maintained in a controlled access room and is stocked with isolator-born mice subsequently exposed to a combination of non-pathogenic bacteria [6]. The substrain is apparently free of the virulent form of the mammary tumor virus, although an antigen which reacts with rabbit anti-C3H/Wr mammary tumor virus has been found in low titres in their milk [7]. Mammary tumors are common in multiparous C3Hf/Wr mice at 14–16 months of age; i.e. 5–7 months later than in the parent C3H/Wr strain.

between cells of differing morphologic type can be used to obtain relatively uniform cell populations. Such uncloned cell lines are referred to as 'selected'.

Radiation dose-cell survival studies

Suspensions were prepared from cultures in the logarithmic phase of growth and were irradiated in MEMS in 60-mm plastic Petri dishes with gamma rays from a 2000-Ci ^{137}Cs source at room temperature in air. Aliquots of the irradiated suspensions were then distributed as rapidly as possible to 60-mm plastic Petri dishes and incubated in MEMS as above. Cell numbers in the irradiated suspensions were adjusted to yield approximately the same number of colony-forming units per dish. Depending on the growth rate of the particular cell line, the colonies which developed were fixed and stained 10–14 days later. Colonies containing more than 50 cells were then counted [18]. One 'colony-forming unit' (cfu) is defined as the cell(s) necessary to form one such colony.

During irradiation, the ^{137}Cs source was 25 or 11 cm above the surface upon which the dishes were placed. Thermoluminescent dosimetry performed with a thin layer of LiF crystals overlain with 5 ml of water in a 60-mm plastic Petri dish indicated exposure rates of 110 and 547 R per minute at the two distances, respectively. Assuming a rad to Roentgen ratio of 0·96 [19], the corresponding dose rates were 106 and 525 rads, respectively.

When large quantities of lethally-irradiated cells were required for transplantation studies, tumors were exposed to 11 krads ^{137}Cs gamma rays as previously described [20] and suspensions were prepared thereafter, or cell suspensions were irradiated with 11 krads ^{137}Cs gamma rays as above.

The survival of tumor cells irradiated *in vivo* was estimated in one experiment by the latency assay technique. Tumor-bearing mice were irradiated to the whole body in a plexiglass container, the floor of which was 35·9 cm from the 2000-Ci ^{137}Cs source. The dose rate estimated by LiF dosimetry was 53 rads per minute, assuming a rad per Roentgen ratio of 0·96.* Tumor suspensions were prepared and serially diluted, lethally-irradiated tumor suspensions were added, and five recipient mice were inoculated subcutaneously in both thighs with each cell concentration. Tumor latency was determined by daily palpation and cell survival was calculated according to methods previously described [8, 20].

Labelled DNA study

In one experiment, newly prepared cultures were exposed for 3 days to 0·002 μCi per ml of medium of 5-iodo-2′-deoxyuridine-^{125}I (^{125}IUdR) containing 2·5 Ci per m mole. At the end of the incorporation period, the cultures were rinsed three times and incubated 1–3 hr with MEMS containing 1·24 μM of unlabelled thymidine. In preliminary experiments such treatment did not alter the plating efficiency significantly, and resulted in 2–6 counts per minute per thousand cells. Mice were inoculated with such labelled cells and after tumors had grown, they were removed, rapidly weighed, and frozen on dry ice. The tumors were kept frozen during determination of total tumor radioactivity with a well-type crystal scintillation counter, and during storage until biochemical analysis. The specific activity of the DNA was determined after perchloric acid extraction as previously described [21].

RESULTS

General characteristics of cell lines

In general, cell lines were more readily established *in vitro* from tumors which had been transplanted and grew well *in vivo* than from primary tumors or tumors which were difficult to transplant. Lines were easily established within 10 days from the MTG-B and MTG-A mammary carcinomas which have a long history of transplantation in our laboratory [12]. In contrast, it required 6–8 weeks for explants of primary tumors to give rise to sufficient cells to subculture.

Spindle-shaped cells of fibroblastic appearance and polygonal epithelioid cells were observed in explants of the transplanted MTG-B and MTG-A tumor strains, and of the three primary C3H/Wr and four primary C3Hf/Wr mammary tumors investigated. Clonal or selected spindle cell lines were established from all nine; lines of the polygonal type were established from eight. Epithelioid cells which remained rounded in culture were observed in explants from four tumors, and were cloned from one. Other types of epithelioid cells which have been established as clonal lines include one which contains

*The radiation doses for the survival curve of MTG-B tumor cells irradiated *in vivo* with ^{137}Cs gamma rays included in Fig. 7 have been recalculated on the basis of this rads/R ratio from the initially-reported values based on ferrous–ferric dosimetry [20]. This recalculation does not alter the major conclusions drawn from these data.

many cytoplasmic vacuoles which give the cells a 'foamy' appearance, and one which gives rise in culture to hollow spherical stalked aggregates resembling mammary alveoli.

All spindle cell lines could be readily dispersed by gentle pipetting after enzyme exposure, whereas the polygonal cell lines were relatively resistant to such treatment. The rounded epithelioid cells and the 'alveolar' cells were intermediate in response.

On *routine* plating, 90–100% of the spindle cells gave rise to colonies, although lower plating efficiencies were obtained when experimental procedures required that they be held in suspension for 2–3 hr before plating. Spindle cell lines were subcultured weekly using 1/50th to 1/100th of a full dish for each new dish (a 'split-ratio' of 1 : 50 to 1 : 100). Polygonal epithelioid cell lines routinely plated with 25–40% efficiency, although efficiencies as high as 75% were observed. These were subcultured on a weekly basis with split-ratios of 1 : 20 to 1 : 50. Plating efficiencies and split-ratios of the rounded epithelioid cells were intermediate.

Eighteen of the 20 cloned or selected cell lines gave rise to tumors following injection into histocompatible mice. Cultured cell lines from transplanted tumors grew readily in adults, and the resulting tumors were composed primarily of the cell type injected as shown by histological examination of tumor sections and of colonies resulting from their re-explant into culture (Fig. 1). Cell lines from primary tumors grew poorly or not at all on initial trials in adult mice. All but two, however, gave rise to tumors when inoculated into newborn mice. Both cell lines which did not grow *in vivo* were of the spindle cell type from primary C3Hf/Wr tumors which were very slowly growing. An epithelioid line from one of these tumors did, however, grow *in vivo*, as did both spindle cell and epithelioid lines from two other tumors of C3Hf/Wr mice.

A polygonal epithelioid cell line (line CC1), a spindle cell line (line CC2) and a rounded epithelioid cell line (line CC3), all of which originated from a grafted MTG-B tumor and were thrice cloned, were chosen for the more detailed studies *in vivo* described in the following sections. The doubling times of these three cell lines during log phase growth *in vitro* were 18–20 hr, 14–15 hr and 17–18 hr, respectively.

Transplantation studies

Although explants of tumors which developed from inocula of cultured CC1, CC2, or CC3 cells gave rise on re-introduction to culture to cells of the same morphologic and growth characteristics as those inoculated, further tests indicated that a change in the cell populations was brought about by passage in mice. (Such recently mouse-passaged sub-lines are indicated by addition of 'M' after the cell line designation.) Latencies of tumors developing from long-term cultured cells were lengthy (23–42 days) and variable. In contrast, when such long-term cultured cells were grown for a single passage in mice, re-introduced to culture for a short period (up to 10 days in these experiments) and again inoculated in mice, latencies were reduced (12–26 days) (Fig. 2). This 'mouse-passage effect' was most marked in the polygonal epithelioid CC1 cell line.

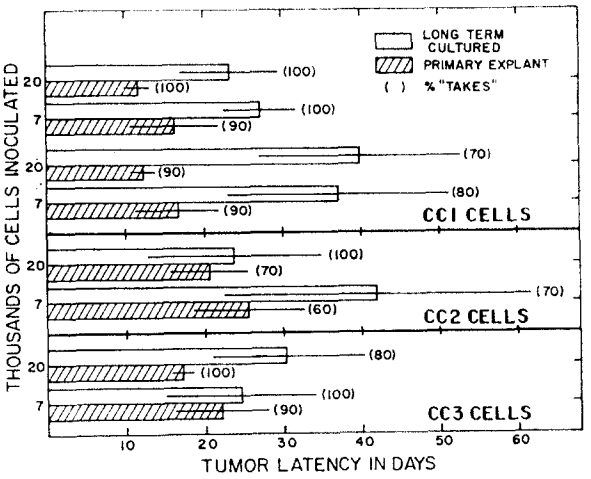


Fig. 2. Effect of recent passage in mice on latency and tumor incidence following inoculation of cultured cells. Differences between latencies of long-term cultured and recently mouse-passaged cells ('primary explants') were significant ($p < 0{\cdot}001$) *at all CC1 cell concentrations and at the higher CC3 cell concentration. Horizontal lines indicate standard deviations. Inocula did not contain lethally irradiated cells.*

Two types of experiment were performed to determine whether long-term cultured cells were antigenic to mice of the strain of origin, and whether the effect of a single mouse passage was to reduce such antigenicity. In the first series patterned after Révész [11] groups of mice were twice inoculated subcutaneously with $(1{\cdot}5–3) \times 10^5$ lethally irradiated cells of CC1, CC1-M, CC2 or CC3 types at 10–15 day intervals. The experiment with recently mouse-passaged CC1-M cells was performed in parallel to that with the parent long-term cultured CC1 line. Untreated mice served as controls. Five to 8 days after the second lethally irradiated cell injections, living cells of the same type, respectively, were injected in one hind leg. In no case did pre-treatment with lethally-irradiated cells affect tumor incidence or

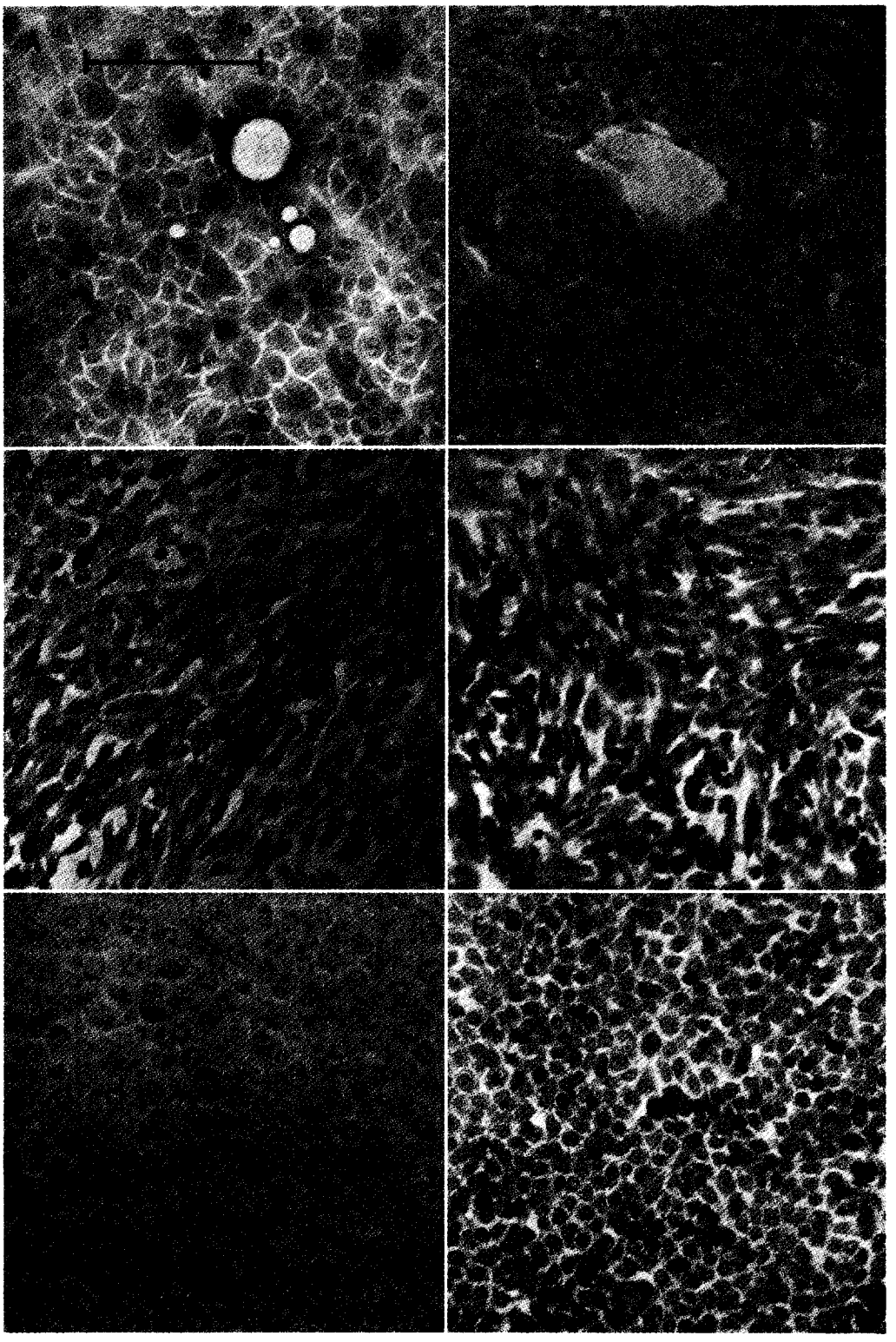


Fig. 1. *A and D; polygonal epithelioid CC*1 *cell line. B and E; spindle cell CC*2 *line. C and F; rounded epithelioid CC*3 *cell line. A to C are monolayer cultures of the three cloned cell lines stained with hematoxylin; D to F are sections of tumors which developed in mice after inoculation of the respective cultured cells, and are stained with hematoxylin and eosin. Bars (A and D) indicate* 50 μ. *The smaller apparent size of the cells* in vivo *is due, at least in part, to their three dimensional form in contrast to the virtually two dimensional form of the cultured cells.*

(to face p. 508)

latency at a statistically significant level (Table 1). However, the latencies of tumors from the higher concentration of CC1 cells tended to be longer than those from CC1-M cells.

In another experiment, half the recipient mice were treated with 600 rads ^{137}Cs gamma rays to the whole body before inoculation with CC1 or CC1-M cells. In only one case each were the differences in latency or tumor incidence between the irradiated immune-suppressed and control mice significant at the 5% level (Table 2).

Again, latencies of several groups of tumors from CC1-M cells were shorter than those from the parent CC1 cells. Thus, although these experiments do not rule out weak antigenicity, marked antigenic differences between the cultured cells and host mice which were histocompatible to the mouse strain from which they were derived were not demonstrated.

To gain information as to whether the mouse-passage effect was due in part to greater proportional survival of inoculated CC1-M as compared to CC1 cells, both sublines were exposed to ^{125}IUdR in culture for 2–3 doubling times. The resultant cells containing labelled DNA were inoculated into mice. The tumors which developed from them were assayed for total radioactivity and for the level of DNA labelling.

The final weights of tumors arising from CC1-M cells were about four times greater than those arising from CC1 cells in this experiment, and, expressed in terms of the percentage of

Table 1. *Effect of two pre-treatments with* $1{\cdot}5–3 \times 10^5$ *radiation-killed cells on tumor incidence and latency from cultured cell inocula*

Inoculum, cell type and number	Pre-treatment	Tumor incidence*	Tumor latency, days ± S. D.
2×10^5 CC1	Irradiated cells	18/18	16 ± 8†
2×10^5 CC1	None	16/17	14 ± 4†
2×10^4 CC1	Irradiated cells	11/18	21 ± 6
2×10^4 CC1	None	17/19	20 ± 4
2×10^5 CC1-M	Irradiated cells	17/17	10 ± 4
2×10^5 CC1-M	None	20/20	11 ± 5
2×10^4 CC1-M	Irradiated cells	12/18	19 ± 6
2×10^4 CC1-M	None	16/18	15 ± 4
2×10^5 CC2	Irradiated cells	20/20	7 ± 1
2×10^5 CC2	None	20/20	7 ± 1
2×10^4 CC2	Irradiated cells	18/19	9 ± 2
2×10^4 CC2	None	20/20	9 ± 3
1×10^5 CC3	Irradiated cells	18/18	9 ± 2
1×10^5 CC3	None	18/18	9 ± 3
1×10^4 CC3	Irradiated cells	14/17	11 ± 7
1×10^4 CC3	None	17/17	12 ± 4

*Number of tumors developing over number of sites inoculated.
†CC1 different from respective CC1-M at $p < 0{\cdot}05$.

Table 2. *Effect of* 600 *rads total-body irradiation before cell inoculation on tumor incidence and latency*

	CC1 Cells				CC1-M Cells			
	Incidence		Latency		Incidence		Latency	
Cell No.	Irradiated	Control	Irradiated	Control	Irradiated	Control	Irradiated	Control
20,000	10/10	12/12	19 ± 7	41 ± 21*	12/12	11/12	10 ± 2†	14 ± 7†
2000	10/10	10/11	25 ± 7	33 ± 22	12/12	11/12	18 ± 5‡	23 ± 15
400	0/2	4/12	—	62 ± 44	4/4	7/11	21 ± 0	35 ± 21
80	2/2	1/12	69 ± 45	56	2/2	6/8	40 ± 4	45 ± 16
16	3/12	0/12	79 ± 22	—	5/10	0/6*	81 ± 24	—

*Irradiated differ from control at $p < 0{\cdot}05$.
†CC1-M latency shorter than corresponding CC1 latency at $p < 0{\cdot}001$.
‡CC1-M latency shorter than corresponding CC1 latency at $p < 0{\cdot}025$.

counts inoculated, about three times as much radioactivity was retained in tumors from CC1-M as compared to CC1 cells (Table 3). If the data are further corrected for plating efficiency, i.e. adjusted for cfu as determined in culture on aliquots of the inocula, the difference is fivefold. The radioactivity per unit of DNA in the tumors from the two cell sublines was, however, very similar (Table 3).

Serial dilution assays of CC1-M cells and a mouse passaged CC3-M subline were performed to gain information both on the doubling time during tumor latency *in vivo* [8, 20] and to determine the number of cfu required to transfer one 'effective unit' [22, 23] for tumor formation (one tfu). The slopes of the plots of latency against log inoculated cfu number for doses of 6·8 to $6·8 \times 10^4$ cfu, i.e. those groups in which tumors grew at all inoculation sites, indicated volume doubling times of 0·9 days for CC1-M cells (Fig. 3) and about 1·0 day for CC3-M cells (*vide infra*). At lower cfu doses, where the tumor incidence was less than 100%, the effective numbers required on the average to successfully transplant one tfu were 1·7 CC1-M cfu, and 3·4 CC3-M cfu (Table 4). On the basis of hemocytometer counts, this corresponded to about 5 cells in both cases, disregarding cell agregation. At these inoculum concentrations, cell aggregates would account for a minority of the tumors observed (Table 4).

Table 3. *Effect of mouse passage on persistance of radioactivity in tumors* 6 *days after inoculation* of cells labelled in their DNA with* ^{125}*IUdR in culture*

Inoculum				Tumors		Tumor radioactivity			
Cells	Number of cells	Plating efficiency%	Total cpm	Number of tumors	Tumor weight mg ± S. D.	cpm/tumor ± S. D.	cpm, % inoculated	Corrected (%)†	cpm per mg DNA
CC1	2×10^5	76	831	19	3·1 ± 1·4‡	14 ± 5§	1·7	2·3	668¶
CC1-M	2×10^5	42	1432	26	21·1 ± 8·3	74 ± 50‖	5·2	12·3	683**

*All inocula contained 10% by volume lethally irradiated histocompatible mammary tumor cells.
†%of inoculated divided by the plating efficiency.
‡Different from CC1-M data at $p < 0·001$.
§Determined on samples each of which was comprised of 3–5 pooled tumors.
‖Determined on samples each comprised of 1–3 tumors.
¶All samples pooled for DNA extraction.
**Determined on 3 pooled samples. Range: 505–774 cpm/mg.

Table 4. *Colony-forming units (cfu) required for tumor growth from inocula containing cultured CC1-M or CC3-M cells**

Cell line	cfu number	Tumors/sites inoculated	tfu/cfu†	Mean tfu/cfu†	Effective number of cfu‡
CC1-M	5·1	9/10	0·45	0·61 (0·07–5·28)	1·7 (0·2–14·4)
	3·4	7/10	0·36		
	1·7	6/10	0·54		
	0·7	5/10	1·02		
	0·3	2/10	0·66		
CC3-M	10·1	7/9	0·15	0·29 (0·03–2·62)	3·4 (0·4–30·3)
	6·8	8/10	0·24		
	3·4	7/10	0·36		
	1·4	4/10	0·38		
	0·7	2/10	0·33		

*Suspensions contained 12–14% aggregates of 3 or more cells. *In vitro* plating efficiencies were 34 and 68% for CC1-M and CC3-M cells, respectively. All inocula contained 32% by volume supralethally irradiated tumor suspension of the same cell type as that in the living inocula.
†Individual and mean tumor forming units (tfu) per colony forming unit (cfu); i.e. the calculated fraction of the total cfu injected which were capable of giving rise to a tumor. These are similar to θ and $\hat{\theta}$ of Maruyama *et al.* [22, 23].
‡The 'effective number of cfu' is the number containing, on the average, one tfu. Numbers in parentheses are 95% confidence limits.

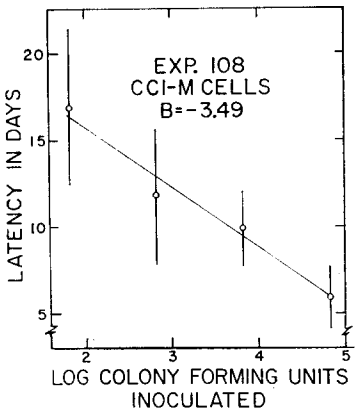


Fig. 3. Tumor latency plotted against the logarithm to the base 10 *of the number of cultured CC1-M colony forming units inoculated at the concentrations of inocula which yielded tumors in* 100% *of the transplantation sites. All grafts were subcutaneous in the thighs. Open circles are means of* 10–12 *observations; vertical bars are standard deviations; B is the slope of the regression indicated by the slanted line. All inocula contained* 32% *by volume lethally irradiated CC1-M tumor cells. See also Table* 4.

Radiosensitivity studies

The radiosensitivity of the three cell lines as determined by colony-forming capacity *in vitro* and expressed as D_0 values, differed markedly.

(The D_0 is defined by the equation: $S = n\,e^{-(D/D_0)}$, where S is the fraction of cells surviving, D is the radiation dose, n is the 'extrapolation number', i.e. the point on the y axis to which the exponential portion of the survival curve extrapolates at $D = 0$, and D_0 is the negative reciprocal of the slope of the exponential portion [18].

The two extreme D_0 values *in vitro* were for the epithelioid CC1 and CC3 cell lines (Fig. 4) while the CC2, spindle cells displayed intermediated sensitivity (Fig. 5). The D_0 values

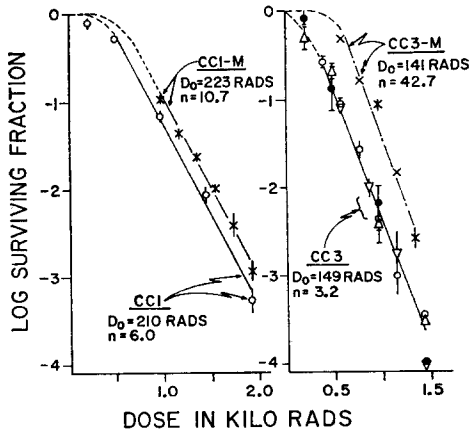


Fig. 4. Survival curves of cultured epithelioid CC1 and CC1-M cell lines (left) and CC3 and CC3-M cell lines (right) after irradiation in vitro *in air and estimated by colony-forming ability. Different CC3 symbols represent repeated experiments. Vertical bars are standard deviations where they exceeded the symbol dimensions. Logarithms are to the base* 10.

of mouse-passaged CC1-M and CC3-M cells were very similar to those of the parent cell lines, although, in both cases, the extrapolation number was greater (Fig. 4). Finally, the D_0 value of a second spindle cell line derived from the MTG-B tumor line 2 years later than the original CC2 cell line was virtually identical to that of the latter (Fig. 5), but displayed a slightly higher n value. As n values are subject to influence by many factors, such as aggregation of cells during handling, etc., whereas D_0 values are less sensitive, the significance of these differences is not clear at present.

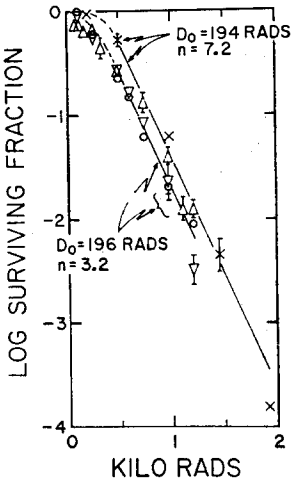


Fig. 5. Survival curves as estimated by colony-forming ability of the original cultured CC2 spindle cell line (open symbols), and a morphologically similar cell line derived from the same transplanted MTG-B tumor strain 2 years later (× marks) after irradiation in vitro *in air. Different open symbols represent repeated experiments; vertical bars are standard deviations where they exceeded the symbol dimensions. Logarithms are to the base* 10.

The sensitivity of CC3-M cells irradiated in tumors *in vivo* was estimated by the latency assay. As previously reported for the MTG-B tumor strain [8, 20] from which the CC3 cell line originated, the magnitude of the radiation dose had no sigificant effect on the slope of latency plotted against the logarithm of the volume of tumor suspension inoculated (Fig. 6). The tumor-volume doubling time during the latent period determined from the slope of these data [8, 20] was 1·0 days. The more variable data for latency vs. log cultured CC3-M cfu number discussed above is included for comparison. The independently calculated slope of the latter data was −3·33, also indicating a doubling time of about 1 day. The D_0 calculated from the data of the latency assay indicated that tumors composed of CC3-M cells irradiated in living mice was nearly identical to that for the MTG-B tumor line from which it was derived (Fig. 7).

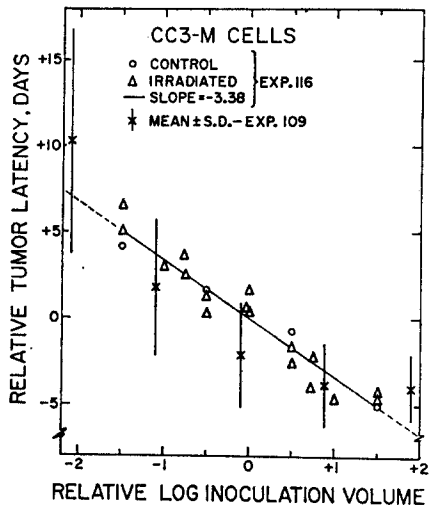


Fig. 6. Relative tumor latency plotted against the relative logarithm to the base 10 *of the volume of tumor suspension inoculated. Circles are mean latencies of tumors which developed from inocula prepared from unirradiated CC3-M tumors; triangles are mean latencies of tumors which developed from inocula from CC3-M tumors which had received* 710–3200 *rads* in vivo *(see also Fig.* 7*); line with broken ends indicates common slope.* × *marks with vertical lines are means and standard deviations of* 5–12 *tumor latencies after subcutaneous inoculation of* 13·6 *to* $1{\cdot}36 \times 10^5$ *colony-forming units of cultured CC3-M cells in the thighs of histocompatible mice, and are included for comparison (see also Table* 4*). All inocula contained* 32% *by volume lethally irradiated CC3-M tumor suspension.*

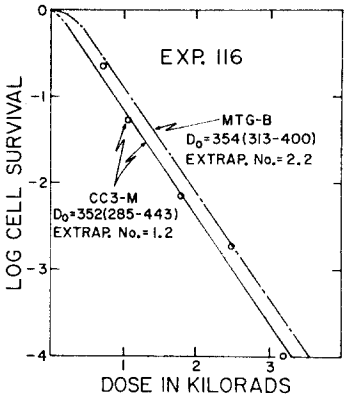


Fig. 7. Survival curve of CC3-M cells irradiated in vivo *as estimated by the latency assay* in vivo*. Survival curve, similarly determined, of mammary carcinoma MTG-B* [20]*, from which the CC3 cell line originated is included for comparison. Figures in parentheses are* 95% *confidence limits of the* D_0 *values. Logarithms are to the base* 10.

DISCUSSION

Cloned or selected cell lines with differing biological properties have been isolated from the two transplanted and seven primary mouse mammary adenocarcinomas tested, and the great majority of such cell lines gave rise to tumors when inoculated into either new-born or adult histocompatible mice. The epithelioid cell lines gave rise to typical carcinomas while the spindle cell lines gave rise to sarcoma-like tumors.

The origin of the spindle cells found in all cultures of primary tumor explants is not known. Such cells could be a neoplastic component of the original tumor cell population *in vivo*. The distinctive tumors to which selected or cloned lines of these cells give rise may not be more commonly observed in primary or long-term transplanted mammary carcinomas because their growth *in vivo* is distinctly slower than that of the epithelioid cell lines. In culture, in contrast, spindle cells appear to have a selective advantage over epithelioid cells.

Alternatively, these cells could represent the product of neoplastic transformation of normal supporting cells contained in the explant. If this is the case, the frequency of such transformation is much higher than that seen by Sanford *et al.* [24, 25]. In this connection it should be noted that the C3H/Wr and C3Hf/Wr mouse substrains have not been screened for viral antigens other than those of the mammary-tumor virus [7]. Finally, the spindle cells could arise by mutation and selection from the neoplastic epithelial elements after explantation. If this were true, it is unclear as to why they did not more commonly arise from selected or cloned epithelial cell lines during prolonged culture.

Although cells returned to culture from tumors were morphologically and behaviorally similar to the cultured cells inoculated, a mouse passage effect was noted on more detailed transplantation studies; i.e. tumors arose more rapidly and consistently from cultured cells which had recently been passed in mice than from long-term cultured cells. Experiments designed to determine whether long-term cultured cells bore antigens foreign to the mice of origin indicated that any such differences, if present, were weak.

Data from the experiment with CC1 and CC1-M cells containing ^{125}IUdR-labelled DNA suggest that the shorter latency of the latter was due in part to the fact that a greater percentage of inoculated CC1-M than CC1 cells survived transplantation. Although IUdR is known to be recycled from dying to living cells [10, 26, 27 and unpublished data], the recycling rate is much lower than that of thymidine. It is unlikely that re-utilization distorted the current results.

The small effective numbers of colony-forming units required for tumor formation calculated from the dilution studies with CC1-M and CC3-M cells approached that reported for lymphomas [22, 23]. Although the confidence limits are broad, the mean effective cfu

numbers for both cell lines were greater than one. More data will be required to determine whether this is due only to experimental variation, to host resistance of the type described for lymphoma cells by Maruyama *et al.* [22, 23], or to some other requirement for more than one viable unit to initiate a tumor. At the present time, expression of the data in terms of cfu rather than cell numbers seems preferable as it reflects at least one aspect of viability.

As noted above, passage of cultured cells in mice did not markedly affect their growth characteristics when they were re-introduced to culture. Furthermore, it did not markedly affect the radiation dose-cell survival characteristics of CC1-M or CC3-M cells *in vitro*. Finally, the D_0 of CC3-M cells *in vivo* was very similar to that of the MTG-B tumor from which it was derived. Previous studies indicated that the D_0 of MTG-B tumors in mice breathing air reflected the radiosensitivity of severely hypoxic cells which were calculated to comprise about 20% of the tumor cell population [20]. The reported D_0 of CC3-M cells *in vivo* also almost surely reflects the sensitivity of an hypoxic cell fraction. Although the results of the differing *in vivo* and *in vitro* assay methods must be compared with caution, the two D_0 values suggest an oxygen enhancement ratio of about 2·4.

Assuming the population doubling time of CC3-M cells during tumor latency was 1·0 day (Fig. 6) the latency to be expected from successful passage of a single tfu may be calculated to be about 20 days. The latencies of the 13 tumors which arose from inocula containing averages of 0·7–3·4 cfu (Table 4) ranged from 16–52 days, with a mode of 25 days. The calculated value thus falls within the lower end of the range of observed latencies for small inoculated cell numbers.

The number of tfu contained in a given volume of tumor suspension prepared with a cytosieve as in the latency assay can also be estimated. The most concentrated control inoculum in the *in vivo* radiosensitivity assay of CC3-M cells (Figs. 6 and 7) contained 5 μl. centrifugally packed material and $1{\cdot}3 \times 10^4$ calculated tfu. Assuming one tfu occupied 10^3 μ^3 (i.e. was equivalent to one cell), about 2·6% by volume of the centrifugally packed tumor suspension was comprised of tfu's. In other experiments (unpublished), we have used tumor inocula of similar concentration but prepared from MTG-B tumors which had been pre-labelled with ^{125}IUdR *in vivo*. In agreement with the above calculations, about 3% of the radioactivity in the inoculated cells was recovered in the tumors which developed from such pre-labelled inocula.

Cell lines of a variety of morphological types can thus be derived from primary or transplanted C3H mouse mammary adenocarcinomas and may be readily manipulated *in vivo* and *in vitro*. Studies with three representative lines have shown them to differ in radiosensitivity *in vitro*. All displayed more rapid and consistent growth in mice if they had been recently mouse-passaged. These and other cell lines similarly derived may prove useful as model systems in tumor and radiation biology.

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REFERENCES

1. K. H. Clifton and H. Vermund. Radio-orthophosphate uptake into DNA, autoradiography and histology during radiation-induced regression and regrowth of mouse mammary adenocarcinoma. *Radiat. Res.* **18,** 516 (1963).
2. M. L. Mendelsohn and L. A. Dethlefsen. Cell proliferation and volumetric growth of fast line, slow line and spontaneous C3H mammary tumors. (Abstract) *Proc. Amer. Ass. Cancer Res.* **9,** 47 (1968).
3. L. Dmochowski, C. E. Grey, P. L. Langford, W. C. Williams, J. A. Sykes, E. L. Young and P. J. Migliore, Viral factors in mammary tumorigenesis. In *Carcinogenesis: A Broad Critique.* Twentieth Annual Symposium on Fundamental Cancer Research, University of Texas M. D. Anderson Hospital and Tumor Institute, pp. 211–256. Williams and Wilkins, Baltimore (1967).
4. E. Y. Lasfargues and D. H. Moore, Cell transformation by the mammary tumor virus *in vitro*. In *Malignant Transformation by Viruses*, pp. 44–59 (Edited by W. M. Kirsten). Springer-Verlag, New York (1966).
5. J. A. Sykes, J. Whitescarver and L. Briggs, Observations on a cell line producing mammary tumor virus. *J. nat. Cancer Inst.* **41,** 1315 (1968).

6. R. W. Schaedler, R. Dubos and R. Cootello, Association of germfree mice with bacteria isolated from normal mice. *J. exp. Med.* **122,** 77 (1965).
7. J. Mitchen, Radiation studies on virus-like particles separated from C3H/HeJ and C3Hf/Wr strain female mice. M.Sc. Thesis, University of Wisconsin (1970).
8. K. H. Clifton and N. R. Draper, Survival curves of solid transplantable tumor cells irradiated *in vivo*: a method of determination and statistical evaluation; comparison of cell survival and ^{32}P-uptake into DNA. *Int. J. Radiat. Biol.* **7,** 515 (1963).
9. L. Révész, Specific effect of radiation-sterilized tissues on cellular multiplication. In *Sterilization and Preservation of Biological Tissues by Ionizing Radiation*, pp. 29–36. International Atomic Energy Agency, Vienna (1970).
10. M. B. Yatvin, D. T. Crouse and K. H. Clifton, Studies on the mechanism of the stimulatory effect of lethally irradiated cells on tumor inocula. *Proc. Soc. exp. Biol. (N.Y.)* **133,** 1123 (1970).
11. L. Révész, Detection of antigenic differences in isologous host-tumor systems by pretreatment with heavily irradiated tumor cells. *Cancer Res.* **20,** 443 (1960).
12. N. A. Hoffman, F. F. Gollin and K. H. Clifton, Acquired radioresistance of transplantable mammary adenocarcinoma in inbred mice after serial irradiation. *Radiology* **88,** 568 (1967).
13. G. Cameron, *Tissue Culture Techniques*. Academic Press, New York (1950).
14. D. J. Merchant, R. H. Kahn and W. H. Murphy, *Handbook of Cell and Organ Culture*. Burgess, Minneapolis (1964).
15. J. Paul, *Cell and Tissue Culture*. Williams and Wilkins, Baltimore (1965).
16. H. Eagle, Amino acid metabolism in mammalian cell cultures. *Science* **130,** 432 (1959).
17. T. T. Puck, P. I. Marcus and S. J. Cieciura. Clonal growth of mammalian cells *in vitro*. *J. exp. Med.* **103,** 273 (1956).
18. M. M. Elkind and G. F. Whitmore, *The Radiobiology of Cultured Mammalian Cells*. Gordon and Breach, New York (1967).
19. *Physical Factors of Irradiation*, Handbook 85, Table IA1. National Bureau of Standards, U.S. Department of Commerce, Washington (1964).
20. K. H. Clifton, R. C. Briggs and H. B. Stone, Quantitative radiosensitivity studies of solid carcinomas *in vivo*: Methodology and effect of anoxia. *J. nat. Cancer Inst.* **36,** 964 (1966).
21. K. H. Clifton and M. B. Yatvin, Cell population growth and cell loss in the MTG-B mouse mammary carcinoma. *Cancer Res.* **30,** 658 (1970).
22. Y. Maruyama and C. Ceman, Response of single mouse lymphoma cells to X-rays by effective fraction method *in vivo*. *Radiat. Res.* **41,** 552 (1970).
23. Y. Maruyama, C. Ceman and R. B. McHugh, Radiation dose-independent inactivation of isologous mouse lymphoma cells by non-specific resistance. *Cancer Res.* **30,** 2245 (1970).
24. K. K. Sanford and R. E. Hoemann, Neoplastic transformation of mouse and hamster cells *in vitro* with and without polyoma virus. *J. nat. Cancer Inst.* **39,** 691 (1967).
25. K. K. Sanford, W. K. Earle, E. Skelton, E. L. Schilling, E. M. Duchesne, G. D. Likely and M. M. Becker, Production of malignancy *in vitro*. XII. Further transformations of mouse fibroblasts to sarcomatous cells. *J. nat. Cancer Inst.* **11,** 351 (1951).
26. W. L. Hughes, S. L. Cummerford, D. Gitlin, R. C. Krueger, B. Schultze, V. Shah and P. Reilly, Deoxyribonucleic acid *in vivo*: cell proliferation and death as measured by incorporation and elimination of iododeoxyuridine. *Fed. Proc.* **23,** 640 (1964).
27. L. A. Dethlefsen, J. Cunningham and E. Powell, Reutilization of ^{131}I-5-iodo-2′-deoxyuridine as compared to ^{3}H-thymidine in the mouse duodenum and mammary tumor. *J. nat. Cancer Inst.* **44,** 827 (1970).
28. K. H. Clifton, N. K. Cohn and W. C. Summers, Clonal cell lines derived from C3H/Wr mouse mammary carcinoma MTG-B. (Abstract) *Fed. Proc.* **26,** 691 (1967).

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Evidence for the Inactivation and Repair of the Mammalian DNA Template after Alkylation by Mustard Gas and Half Mustard Gas

J. J. ROBERTS, T. P. BRENT* and A. R. CRATHORN
The Chester Beatty Research Institute, Institute of Cancer Research, Royal Cancer Hospital Fulham Road, London, S.W.3., England

Abstract—1. *Mustard gas or half mustard gas caused a rapid dose-dependent depression of DNA synthesis in HeLa cells.* 2. *Synchronous populations of HeLa cells treated prior to DNA synthesis in the G_1 phase of the cell cycle were not delayed in their progression into the S phase, but showed a dose-dependent inhibition of DNA synthesis when the cells entered the S phase.* 3. *Treatment during the S phase also resulted in a dose-dependent depression in the rate of DNA synthesis.* 4. *Cells in which the rate of DNA synthesis had been depressed exhibited a prolongation of the S phase and a corresponding mitotic delay.* 5. *Treatment early in G_1 produced less inhibition of DNA synthesis than treatment late in G_1 probably due to repair of DNA damage before its effects were manifest during the S phase.* 6. *The different effects on survival of treatment throughout the cell cycle were consistent with this possibility.* 7. *After cells passed through a mitotic block their subsequent progression was not impaired.* 8. *Treatment in G_2 did not delay the following mitosis but did induce inhibition of NDA synthesis during the next cell cycle and then mitotic delay.* 9. *The results accord with the view that the cytotoxic action of these agents was due to the direct inactivation of the DNA template which could, however, be repaired.*

INTRODUCTION

THE DIFUNCTIONAL alkylating agent sulphur mustard gas, di(2-chloroethyl)sulphide is markedly cytotoxic at very low doses where few biochemical alterations are detectable. Although it reacts with proteins and RNA, no effect on the overall synthesis of these macromolecules is observed even at concentrations which result in extensive cell death [1]. On the other hand, DNA replication is rapidly and dramatically suppressed at low concentrations of mustard gas which result in high survival. Since these low concentrations of mustard gas or higher concentrations of monofunctional alkylating agents do not inhibit the activity of some of the enzymes involved in DNA replication such as thymidine kinase, it is presumed that the inhibitory effect on gross DNA synthesis is due to the direct reaction of the agent with DNA. That such a reaction can occur has been established *in vitro* and both in bacteriophage [2] and mammalian cells [1]. The chloroethyl groups of mustard gas react predominantly with the N-7 position of guanine, resulting in a diguaninyl product (25%) and a monoguaninyl product (75%) [3, 1]. *In vitro* alkylation experiments with native and denatured DNA and reversible renaturation experiments with mustard-treated DNA [3, 4] indicate that some of the diguaninyl product is in the form of interstrand cross-links within the DNA molecule. Such covalent cross-links may be presumed to prevent the two strands of the DNA double helix from separating and hence

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*Present address: McGill University Cancer Research Unit, McIntyre Medical Sciences Building, 3655 Drummond Street, Montreal 2, Quebec, Canada.

replicating. Although the monofunctional alkylation product could not act in the same way, the larger number of alkylations occurring at equitoxic doses might interfere with the replicating enzymes.

The capacity for the repair of alterations in the structure of DNA has been described in some detail for ultra-violet irradiated bacteria [5, 6]. There is a variety of evidence that similar repair systems operate in mammalian cells [7]. The cellular response to an agent that damages its DNA is therefore complex, consisting of the cell's malfunction due to the damage, and at the same time modification or elimination of the damage.

In the present study the capacity of DNA to replicate in HeLa cells treated with mustard gas has been examined in some detail. The results, some of which have been reported elsewhere [8] extend our earlier studies [1] on DNA synthesis in asynchronous cultures, and provide further evidence appertaining to both the nature of the damage and of its repair. Other evidence for such repair of alkylation damage to DNA in mammalian cells has been discussed previously [9–11]. Some of the conclusions from this work regarding the progression of alkylated HeLa cells through the cell cycle confirm those of Levis *et al.* [12] who followed the effect of HN2 on strain AUP cultured cells and of Walker and Thatcher [13] who studied the effect of mustard gas on mouse L strain cells. These earlier findings have been amplified to show the effects of alkylation in the second and third cycles following treatment, and to compare the effects of a monofunctional analogue. The results have provided information on the time taken for recovery from DNA damage. Other studies reported elsewhere [14, 15] on the effects of alkylation on Chinese hamster V79-379A cells and of methylation in HeLa cells indicate that the pattern of response to DNA alkylation reported here is not universal.

MATERIAL AND METHODS

Cells and reagents

The conditions for growing HeLa cells in suspension or monolayer culture were as reported previously [10]. Synchronous cultures were obtained by selective detachment of mitotic cells from monolayer cultures as developed by Marcus and Robbins [16].

The preparation of mustard gas, and half mustard gas and their effects on the colony-forming ability have been described [10].

Measurement of DNA synthesis

DNA synthesis was measured by pulse-labelling 1 ml aliquots of cell suspension with [^{3}H]thymidine at 0·5 or 1 μCi/ml (12–20 Ci/mmole) for 10–20 min. The incorporation was stopped by dilution and cooling with 10 ml ice-cold saline followed immediately by filtering onto nitrocellulose disks, 0·2 μ pore size: 25 mm dia., [Millipore (U.K.) Ltd., London] or Whatman glass fibre paper disks GF/C (H. Reeve Angel Co. Ltd., London). The filter disks were washed once with cold saline (10 ml) and twice with ice-cold 5% trichloracetic acid (10 ml), before being placed in Bray's aqueous scintillation fluid for counting.

RESULTS

The effects of mustard gas and half mustard gas on cell survival and the corresponding levels of DNA reaction have been published [1, 10]. Table 1 indicates the effect of the concentrations of mustard gas and half mustard gas used during this investigation on HeLa cell survival.

Table 1. *The effects of mustard gas and half mustard gas on HeLa cell survival*

Agent	Concentration (μg/ml)	Survival (%)
Mustard gas	0·075	60
	0·15	10
	0·30	1
	0·6	negligible
Half mustard gas	0·75	70
	1·5	20
	3·0	1·5
	6·0	negligible

Effects on DNA synthesis in asynchronous cell cultures

It has been previously shown [1], that after treatment with mustard gas (c. 0·5 μg/ml), DNA synthesis in HeLa cells is inhibited preferentially as compared with RNA and protein synthesis. We have examined this inhibition in greater detail using pulse-labelling techniques and found that the response to mustard gas is complex. Figure 1 shows the rate of DNA synthesis at times after treatment with a range of concentrations. It can be seen that there is an initial rapid, dose-dependent inhibition which reaches a maximum at about 5 hr. This is followed by a period of constant or increased DNA synthesis for the next 5 hr, when it falls again over the next 10 hr. The curve is analogous to that seen for the fractionated dose-

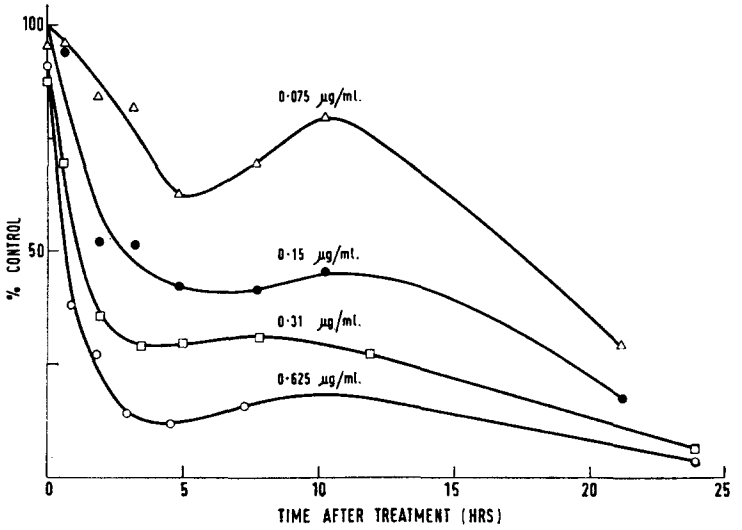


Fig. 1. The effect of mustard gas on DNA synthesis in HeLa cells. Replicate suspension cultures containing approximately 2×10^5 *cells/ml were treated with various concentrations of mustard gas in ether solution* (0·05%). *Duplicate 1 ml aliquots were then removed various times thereafter and the extent of DNA synthesis determined by incorporation of* [^3H] *thymidine into DNA as described; this was then plotted as a percentage of that in control (ether-only treated) cells against time. The ether-treated control cells showed the same amount of DNA synthesis as untreated cells.*
△—△ 0·075*µg/ml;* ●—● 0·15 *µg/ml;* □—□ 0·31 *µg/ml;* ○—○ 0·62 *µg/ml.*

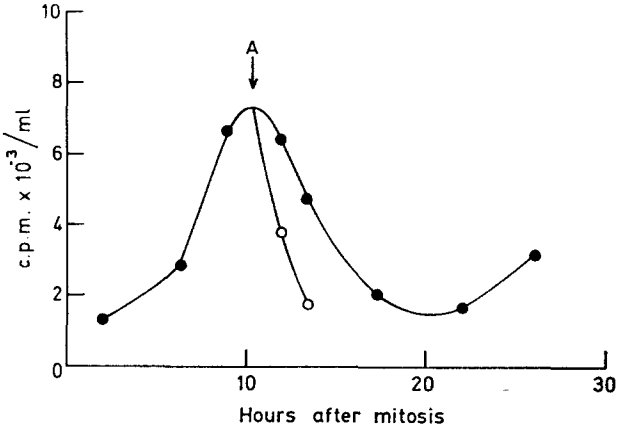


Fig. 2. The effect of mustard gas on DNA synthesis in synchronous HeLa cells. A suspension culture of synchronous HeLa cells (approx. 1×10^5*/ml) was treated with mustard gas* (0·2 *µg/ml) in ether* (0·05%) 10 *hr after harvesting (arrowed A). DNA synthesis was measured in treated and control (untreated) cells by the incorporation of* [^3H]*TdR into the DNA in a 1 ml sample of cells at various times both before and after treatment.* ●—●, *untreated control;* ○—○, 0·2 *µg/ml mustard gas.*

response in mammalian cells [17] suggesting that it is due to differential sensitivity of cells at different stages of the cell cycle, or to a synchronizing effect of the treatment.

Thus the initial fall in DNA synthesis might be due to a block in G_1 preventing cells from entering the S phase, and the subsequent rise due to a reversal of this block. On the other hand the initial fall could be due to an immediate inhibitory effect on cells already in S, those being at the beginning of S being maximally affected when they reach mid-S some 4–5 hr later. The subsequent rise in DNA synthesis might then indicate a decreasing subsequent effect on DNA synthesis the earlier in G_1 they were treated.

Effects on DNA synthesis in synchronous cell cultures
(a) *Early effects of treatment during S or* G_1. To test these possibilities experiments were performed with synchronous cultures. Figure 2 illustrates that DNA synthesis in cells treated in

mid-S is immediately and significantly inhibited. Since we know that these cells were in the S phase at the time of treatment this decrease in uptake of [^{3}H]thymidine relative to that into the same number of control cells represents a true depression in the rate of DNA synthesis. Figure 3 shows the results of an experiment where cells were treated early or late during

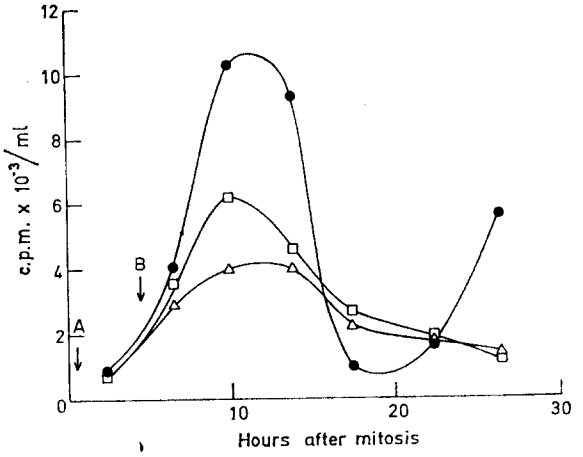


Fig. 3. The effect of treatment with mustard gas during different stages of the G_1 phase of the cell cycle on subsequent DNA synthesis in synchronous HeLa cells. Cells were treated as for Fig. 2 either 0·5 hr (arrowed A) or 4·5 hr (arrowed B) after harvest (mitosis), and DNA synthesis measured in control (untreated) and treated cells at various times thereafter as for Fig. 2. ●—●, control, untreated; □—□, mustard gas added at A; △—△, mustard gas added at B.

G_1. It is seen that in both cases cells enter S without any delay, but the extent of inhibition of DNA synthesis does vary according to the time of treatment; cells treated early in G_1 showing less subsequent depression of DNA synthesis than those treated in late G_1. This experiment also shows that the mustard-treated cells are continuing to synthesize DNA, albeit at a reduced rate, for more than 20 hr after mitosis. Furthermore, they became enlarged and failed to divide whereas by this time control cells had all divided and entered the S phase of the following cell cycle. The treated cells can therefore be said to have been blocked in the S phase. Again the decrease in uptake of [^{3}H]TdR relative to the control into cells in the S phase represents a depression in the rate of DNA synthesis in all cells and is not due to a proportion of cells synthesizing DNA at the normal rate and some at a very reduced rate. Had this been so, synchrony would not have been maintained, and some cells would have divided without delay. This interpretation was confirmed by autoradiographic studies of pulse-labelled cells during the S phase. All cells showed a decrease in the number of grains relative to those in control untreated cells.

(b) *Late effects of treatment during G_1.* When DNA synthesis was examined in asynchronous cultures for periods up to 48 hr after mustard gas treatment, it was noted that fluctuations continued to occur beyond the initial fall and rise (Fig. 4). The initial fluctuations can be explained on the basis of differential sensitivity during the G_1 and S periods. However, the later fall (10–20 hr) and subsequent rise (30–40 hr) may be due to other factors such as blocks and delay in progress of the cell cycle.

When this was examined in synchronous cultures it was indeed found that where cells have been treated in G_1 resulting in inhibition of DNA synthesis, there is a delay before the cells enter the S period of the following cycle

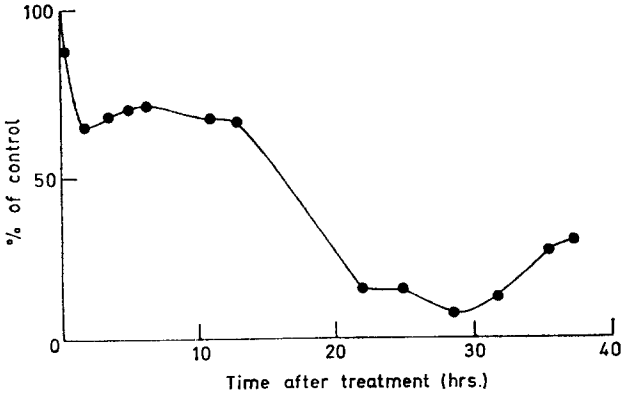


Fig. 4. The effect of mustard gas on DNA synthesis in asynchronous HeLa cells. The conditions for mustard gas (0·1 μg/ml) and measuring the effect on DNA synthesis were as in Fig. 1.

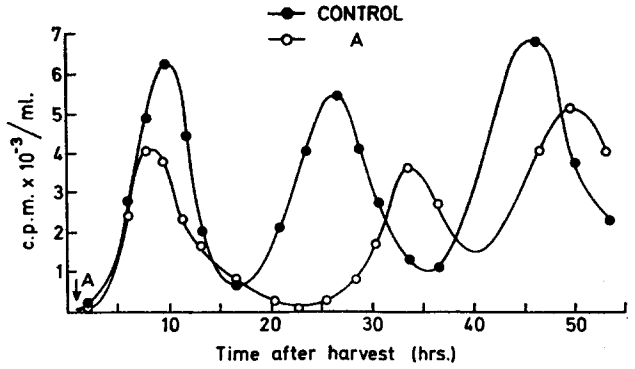


Fig. 5. The effect of treatment with mustard gas during the G_1 phase of the cell cycle (arrowed A) on subsequent DNA synthesis in a synchronous population of HeLa cells. The cells were treated with mustard gas [0·075 µg/ml in ether (0·05%)] 1 hr after harvesting mitotic cells (arrowed A) and extent of DNA synthesis in treated and untreated cells measured as in Fig. 2 at various times thereafter. ●—● *control;* ○—○ 0·075 *µg/ml mustard gas.*

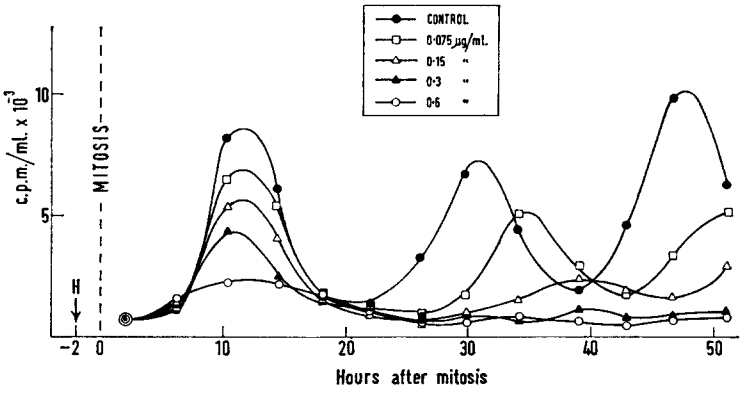


Fig. 6. The effect of treatment with mustard gas during the G_2 phase of the cell cycle (arrowed H) on subsequent DNA synthesis in synchronous HeLa cells. Replicate monolayer cultures were treated with various concentrations of mustard gas and mitotic cells harvested from each culture 2 hr later and placed in suspension culture. At intervals thereafter the amount of DNA synthesis was measured as in Fig. 2 in control and treated cells. ●—●, *control untreated;* □—□, 0·075 *µg/ml;* △—△, 0·15 *µg/ml;* ▲—▲, 0·30 *µg/ml;* ○—○, 0·60 *µg/ml.*

(Fig. 5). The treated cells become enlarged during this time suggesting that the delay is prior to mitosis and this was again confirmed by observations on cell numbers during this period. Cells treated early in S or the middle of S showed a similar response to those treated in G_1. It was also apparent that only the cell cycle which contains the initially inhibited S period is lengthened by the delay. When this block in S is eventually released subsequent cell cycle times are normal, since in treated cells the time between the second and third peaks of DNA synthesis after harvesting cells is the same as between the peaks of DNA synthesis in the control cultures.

(c) *Late effects of treatment during G_2.* At around 15 hr after harvesting mitotic cells the majority should be in the G_2 phase of the cell cycle. The inherent loss of synchrony by this time coupled with the short duration of G_2 (2–3 hr), result in poor resolution for this period. A significant proportion of such a supposed G_2 population may be in S, mitosis or G_1. Specific examination of G_2 cells was achieved by treating monolayer cultures 2 hr prior to selective detachment of mitotic cells. Provided there is no dose-dependent mitotic delay we can then examine the effects in the following cell cycle of such treatment. Figure 6 shows DNA synthesis in such synchronous cultures after a range of doses of mustard gas. The yields of mitotic cells from mustard-treated and untreated monolayers were the same, indicating that the treatments did not affect the entry into mitosis of the cells. It can be seen from Fig. 6 that there is also no delay of cells entering the subsequent S phase. There is then a marked dose-dependent depression of DNA synthesis in this S period, in the cycle following that in which treatment occurred.

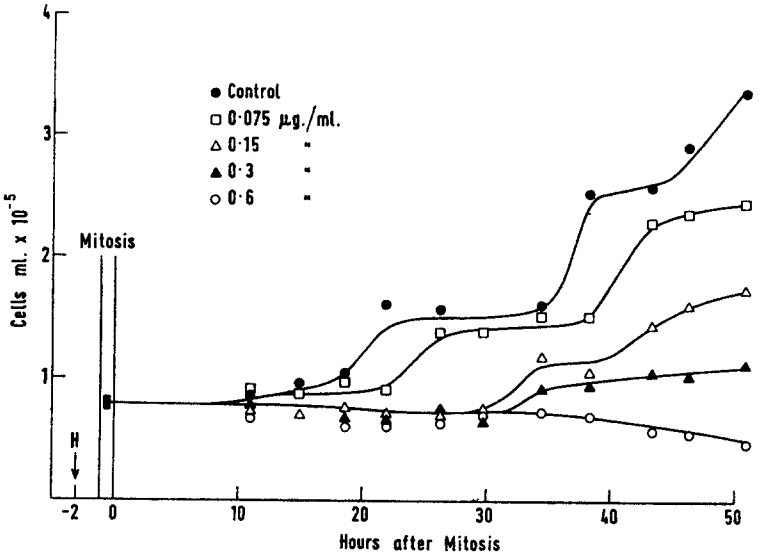


Fig. 7. The effect of treatment with mustard gas during the G_2 phase of the cell cycle (arrowed H) on subsequent cell division of synchronous HeLa cells. Haemocytometer counts were made on the various suspension cultures, obtained as described in Fig. 6 at intervals after the harvesting of mitotic cells. Initially all cultures had essentially the same cell counts. ●—●, control, untreated; □—□, 0·075 µg/ml; △—△, 0·15 µg/ml ▲—▲, 0·30 µg/ml; ○—○, 0·60 µg/ml.

Depression of DNA synthetic rate is accompanied by prolongation of this S phase leading to mitotic delay comparable to that seen following a similar depression arising from a treatment in the G_1 phase (Fig. 5). Again with the lowest concentration of mustard gas it is clear that following the delayed mitosis there is no delay of the succeeding S phase and no further mitotic delay. This lack of further mitotic delay is apparent in Fig. 7 which shows the changes in cell number of treated and control cultures over a period of 50 hr.

Survival following treatments during the cell cycle

Experiments to determine if the variation in sensitivity of DNA synthesis during the cell cycle was reflected in the viability of cells treated at different stages of the cycle are summarized in Fig. 8. It is seen that after a dose of 0·075 µg/ml of mustard gas survival is minimal at the G_1/S transition, and increases with time both backwards towards early G_1 and forwards through S towards G_2. This is essentially in agreement with the results of Walker and Helleiner [18] who found the G_2 period of mouse L cells to be the most resistant, and of Mauro and Elkind [19] for Chinese hamster cells (which have a very short G_1) but not with the results of Mauro and Madoc-Jones [20] who observed different effects on the survival of HeLa cells treated with mustard gas throughout the cell cycle than those reported here. We have no satisfactory explanation for this apparent discrepancy. The line of HeLa cells used by these investigators is obviously more resistant to the action of mustard gas than that used in this study. Furthermore there is a much greater variation in sensitivity during the cell cycle than we observed. Cell survival was determined by treating cells on plates prepared

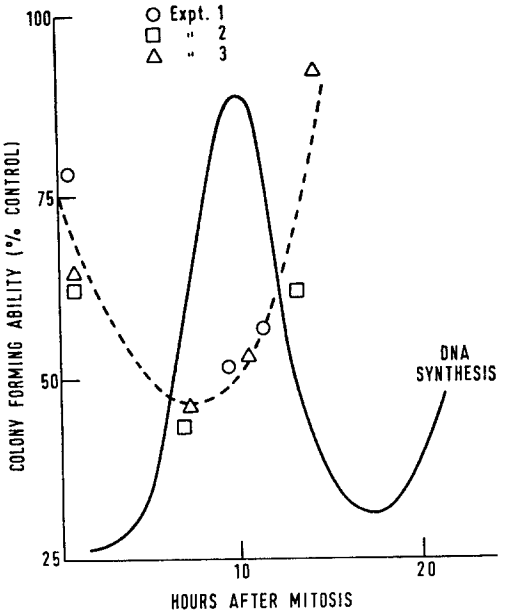


Fig. 8. The effect of treatment with mustard gas at various times during the cell cycle on subsequent cell survival. 30 min after treating synchronous populations of HeLa cells with mustard gas (0·075 µg/ml) at the times indicated the cells were diluted and plated to determine their colony-forming ability [10]. The solid line represents DNA synthesis in the control culture as shown in Fig. 5 in order to indicate the relative times of treatment throughout the cell cycle. The results of three separate experiments are indicated by the three symbols.

at the commencement of the experiment, whereas our cells were treated in suspension culture at various times after mitosis and then plated out. It does not, however, seem likely that this would be a significant factor since the response of synchronized cells to treatment with the monofunctional alkylating agent, N-methyl-N-nitrosourea, by the two regimens was found to be the same [unpublished result].

Effect of half mustard gas on DNA synthesis in synchronous cell cultures

Despite the difunctional nature of mustard gas it nevertheless reacts to form 75% monofunctional alkylation products in HeLa cells [1]. It is important to determine therefore whether the biological response is due to the mono- or bifunctional reaction product in DNA (or both). This problem has been studied by using the monofunctional analogue of mustard gas, 2-chloroethyl 2′-hydroxyethyl sulphide (half mustard gas). Ten times the dose of this agent is required to produce a toxic effect equal to that of mustard gas [10]. On examination of the DNA synthesis in asynchronous HeLa cells treated with half mustard, it was similarly found that ten times the dose is required for an equal degree of inhibition. Following treatment of synchronous cultures with half mustard gas 0·5 hr after mitosis it can be seen (Fig. 9) that there is again no delay of cells entering the S phase, when a clear dose-dependent depression in rate of DNA synthesis becomes apparent. The resulting variable delay in mitosis and delay in entering the succeeding DNA synthetic phase is proportional in duration to the extent of this initial inhibition of DNA synthesis. This is similar to the response to mustard gas, but it should be noted that the doses are ten times higher (cf Fig. 6). As with mustard gas, after the delay prior to the second cycle, the third cycle is of normal duration in every case, the time between the second and third peaks of DNA synthesis in all treated cultures being the same as that between the peaks of the control culture.

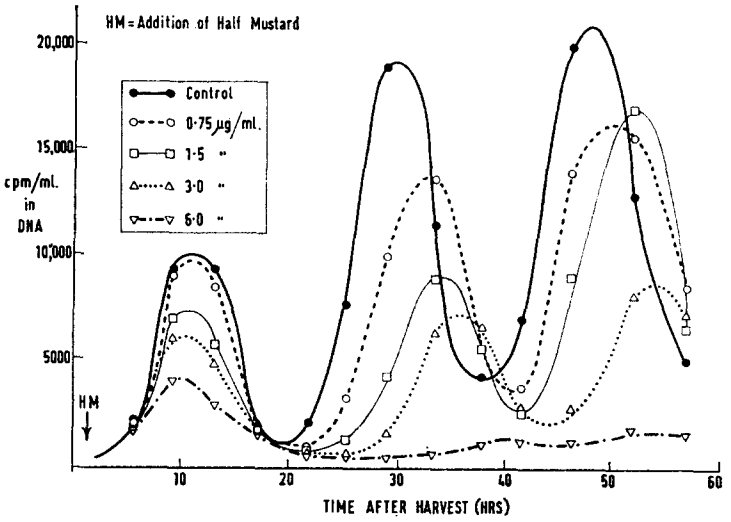


Fig. 9. The effect of treatment with half mustard gas during the G_1 phase of the cell cycle (arrowed HM) on subsequent DNA synthesis. Replicate cultures of synchronous HeLa cells were treated with various concentrations of half mustard gas 1 hr after harvesting mitotic cells. At intervals thereafter DNA synthesis was measured as in Fig. 2 in control and treated cells. ●—●, control, untreated; ○- - - ○, 0·75 µg/ml; □—□, 1·5 µg/ml; △···· △, 3·0 µg/ml; ▽-·-· ▽, 6·0 µg/ml.

DISCUSSION

It is possible to explain the response of mammalian cells to mustard gas on the basis of the agent's cross-linking action on the DNA double helix, and the subsequent removal of such cross-links by a repair mechanism. Figure 10 shows such a model where G_1-treated cells would be able to initiate DNA replication, but would become inhibited when the replication points reach a cross-link. Similarly S-treated cells would continue replication until a cross-link was encountered. G_2-treated cells however, since they have already replicated a double DNA complement, would be able to segregate this DNA and complete mitosis and G_1 before entering the subsequent S period when cross-links would be inhibitory. The excision of

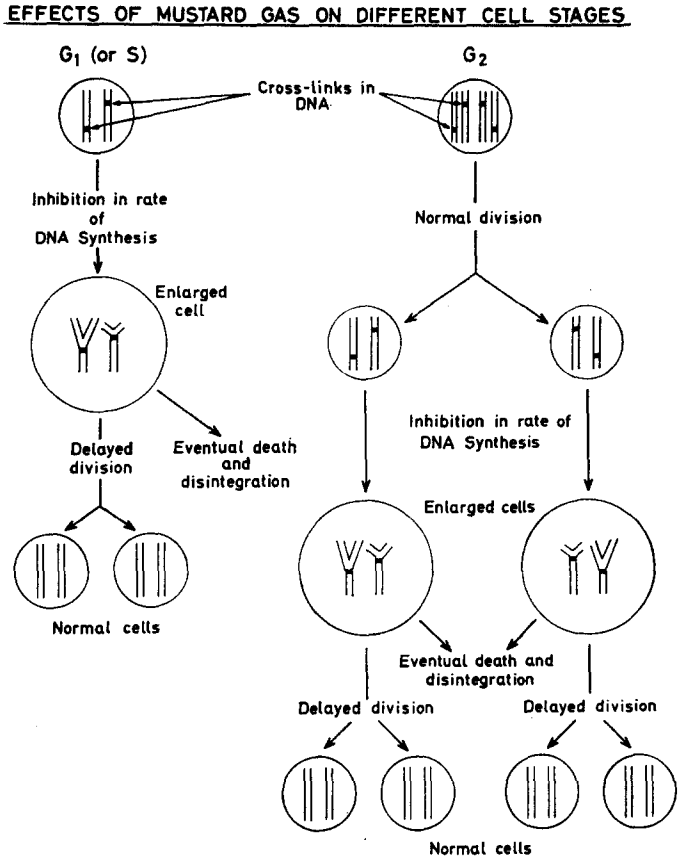


Fig. 10. Scheme showing the effect of treatment with mustard gas or half mustard gas during different phases of the cell cycle on subsequent DNA synthesis and cell division. Mustard gas-induced cross-links (or a larger number of half mustard gas type alkylations) are depicted by the interstrand bar and cause an inhibition in the rate of DNA synthesis and subsequent extension of the S phase. The resulting delay in cell division with continued RNA and protein synthesis leads to cell enlargement. Eventually following repair of DNA damage at concentrations which give high cell survival normal progeny may result. Alkylation of G_2 cells does not impair division but does produce a depression in the rate of DNA synthesis in the following cell cycle which then leads to the same sequence of events as described above for G_1-treated cells.

alkylations and the removal of cross-linkages from the DNA of mammalian cells has been previously described [1, 21]. If such a process was occurring throughout the cell cycle, then depending on the position in the cell cycle at the time of treatment, varying proportions of the cross-links would be removed prior to the next S period. Such a model would allow G_2 cells maximum repair and cells at the G_1/S transition minimum repair, which is indeed what is observed in terms of DNA synthetic capacity and cell survival.

Further, if the degree of inhibition of synthesis in the initial S period after treatment is a measure of the number of cross-links remaining at this time, then with a constant rate repair process the subsequent delay would be related to this initial degree of inhibition, as is in fact observed. According to the proposed model such a delay would be in the S period; however the low rate of DNA synthesis during the delay period and the deteriorating synchrony does not always make this obvious from our data. Investigations by Walker and Thatcher [13] indicate that mouse L cells are in fact blocked in the S phase of the cell cycle after mustard treatment, and that the premitotic amount of DNA is eventually synthesized. Whether the mitotic delay is only due to failure of cells to complete the synthesis of their full complement of DNA or to the failure of such cells to synthesize specific RNA or protein molecules required for division is not yet known. There is insufficient data on the precise DNA content of cells various times after treatment with a range of concentrations of mustard gas and of specific factors controlling cell division to answer this question unequivocally. It is always a possibility that a function other than DNA replication such as the centriole is damaged and requires a recovery process to operate before mitosis can occur, and such recovery could take

place in G_2. The fact that after the initial delay (i.e. block in S) the subsequent cell cycle durations are back to normal indicates that, in those cells which continue to proliferate, complete recovery of the replicative ability of DNA has been effected during 1–2 cell cycle periods.

Half mustard gas requires ten times the dose of mustard gas to elicit equal effects on survival and DNA synthesis. It is clear, therefore, that the monofunctional alkylation product which is also produced by reaction of mustard gas with DNA cannot be responsible for the latter's effects. On the other hand, a sufficient number of monofunctional alkylation reactions can lead to the same sequence of events as those produced by a mustard-induced cross-link in DNA. The dose-dependent delays, followed by cycles of normal duration after half mustard gas treatment are similar at equitoxic concentrations to those produced by mustard gas. This suggests a similar rate of recovery from the effects of monofunctional alkylation as from difunctional alkylation. Such a conclusion is possibly not readily reconciled with the findings of Reid and Walker [21], who reported that the half-life for removal of cross-links in L-cells is 2 hr while that for loss of total alkylations is 18 hr.

An alternative possibility is that at the higher levels of alkylation with half mustard gas, functions other than DNA replication are damaged and account for the delays observed. This is unlikely on target theory as previously discussed in detail [10] and because of the lack of effect of these concentrations of half mustard gas on RNA and protein synthesis. The results reported here therefore indicate that alkylation of the DNA molecule is the predominant cytotoxic causal event in mammalian cells following treatment with mustard gas and half mustard gas. Recovery from these agents occurs therefore, possibly by removal of the products of alkylation from DNA, although other repair mechanisms could be envisaged [15].

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REFERENCES

1. A. R. Crathorn and J. J. Roberts, Mechanism of the cytotoxic action of alkylating agents in mammalian cells and evidence for the removal of alkylated groups from deoxyribonucleic acid. *Nature* **211,** 150 (1966).
2. P. Brookes and P. D. Lawley, Effects of alkylating agents on T2 and T4 bacteriophage. *Biochem. J.* **89,** 138 (1963).
3. P. D. Lawley, Effects of some chemical mutagens and carcinogens on nucleic acid. *Progr. Nucleic Acid Res. and molec. Biol.* **5,** 89 (1966).
4. P. D. Lawley and P. Brookes, Interstrand cross-linking of DNA by difunctional alkylating agents. *J. molec. Biol.* **25,** 143 (1967).
5. R. B. Setlow, The photochemistry, photobiology and repair of polynucleotides. *Progr. Nucleic Acid Res. and molec. Biol.* **8,** 257 (1968).
6. P. Howard-Flanders, DNA repair. *Ann. Rev. Biochem.* **37,** 175 (1968).
7. J. E. Cleaver, Radiation repair mechanisms in mammalian cells. In Biological implications of the nuclear age. *A.E.C. Symposium Series* **16,** 283 (1969).
8. J. J. Roberts, T. P. Brent and A. R. Crathorn, The mechanism of action of the cytotoxic alkylating agents on mammalian cells. Biological Council *Symposium on the Interaction of Drugs and Subcellular Components in Animal Cells*, pp. 5–27, (Edited by P. N. Campbell, J. and A. Churchill). Biological Council, London (1968).
9. J. J. Roberts, A. R. Crathorn and T. P. Brent, Repair of alkylated DNA in mammalian cells. *Nature* **218,** 970 (1968).
10. J. J. Roberts, J. M. Pascoe, J. E. Plant, J. E. Sturrock and A. R. Crathorn, Quantitative aspects of the repair of alkylated DNA in cultured mammalian cells. I. The effect on HeLa and Chinese hamster cell survival of alkylation of cellular macromolecules. *Chem. Biol. Interactions* **3,** 29 (1971).
11. J. J. Roberts, J. M. Pascoe, B. A. Smith and A. R. Crathorn, Quantitative aspects of the repair of alkylated DNA in cultured mammalian cells. II. Non-semiconservative DNA synthesis ("repair synthesis") in HeLa and Chinese hamster cells following treatment with alkylating agents. *Chem. Biol. Interactions* **3,** 49 (1971).
12. A. G. Levis, G. A. Danieli and E. Piccini, Nucleic acid synthesis and the mitotic cycle in mammalian cells treated with nitrogen mustard in culture. *Nature* **207,** 608 (1965).

13. I. G. WALKER and C. J. THATCHER, Studies on the lethal effects of sulphur mustard on dividing mammalian cells, *Radiat. Res.* **34,** 110 (1968).
14. J. E. PLANT and J. J. ROBERTS, A novel mechanism for the inhibition of DNA synthesis following methylation: the effect of N-methyl-N-nitrosourea on HeLa cells. *Chem. Biol. Interactions* **3,** 337 (1971).
15. J. E. PLANT and J. J. ROBERTS, Extension of the pre-DNA synthetic phase of the cell cycle as a consequence of DNA alkylation in Chinese hamster cells: a possible mechanism of DNA repair. *Chem. Biol. Interactions*, **3,** 343 (1971).
16. P. I. MARCUS and E. ROBBINS, Mitotically synchronised mammalian cells: a simple method for obtaining large populations. *Science* **144,** 1153 (1964).
17. F. MAURO and M. M. ELKIND, Comparison of repair of sublethal damage in cultured Chinese hamster cells exposed to sulphur mustard and X-rays. *Cancer Res.* **28,** 1156 (1968).
18. I. G. WALKER and C. W. HELLEINER, The sensitivity of cultured mammalian cells in different stages of the division cycle to nitrogen and sulphur mustards. *Cancer Res.* **23,** 734 (1963).
19. F. MAURO and M. M. ELKIND, Differences in survival variations during the growth cycle of cultured Chinese hamster cells treated with sulphur mustard and X-rays. *Cancer Res.* **28,** 1150 (1968).
20. F. MAURO and H. MADOC-JONES, Age response of cultured mammalian cells to cytotoxic drugs. *Cancer Res.* **30,** 1397 (1970).
21. B. D. REID and I. G. WALKER, The response of mammalian cells to alkylating agents. II. On the mechanism of the removal of sulphur mustard-induced cross-links. *Biochim. biophys. Acta (Amst.)* **179,** 179 (1969).

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Serial Transplantation of Canine Lymphocytic Leukaemia

L. N. OWEN
*Radiobiological Institute TNO, Rijswijk, The Netherlands**

Abstract—*A canine lymphocytic leukaemia was successfully serially transplanted on three occasions by inoculation of canine foetuses* in utero. *Sixteen out of* 17 *dogs injected developed leukaemia. Four puppies injected at* 7 *days old also developed leukaemia. Peripheral w.b.c. counts reached up to* 280,000 *cells/mm*^{3} *and the main pathological lesions were similar to those occurring in lymphatic leukaemia in man.*

INTRODUCTION

A DESCRIPTION of the transplantation of canine lymphosarcoma [1] and canine osteosarcoma using intra-foetal injection methods into canine foetuses has been previously given [2]. Essentially similar methods using an injection volume of about 0·1 ml of canine leukaemic cells have been used in the experiments now to be described. Transplantation into whole-body X-irradiated and normal young dogs have also been made.

Lines of canine leukaemic cells in susceptible animals and in tissue culture would be of value in aetiological and chemotherapeutic studies. The study may provide the basis for an experimental test of whether or not canine lymphocytic leukaemia is transmissible via cell-free extracts. The haematology and pathology of the lymphatic leukaemia to be described is similar to that occurring in man.

Description of donor dog

The donor animal was a 2-year old Dalmatian male with a history of illness for one month. There was anorexia, diarrhoea, extreme emaciation and an enlarged liver. Haematological examination one week before euthanasia was as follows: haemoglobin 16·8 g, total w.b.c. count 29,000 cells/mm^3, lymphocytes 64%, neutrophils 34%, monocytes 1%, eosinophils 1%.

Post-mortem examination revealed a very enlarged whitish liver and enlarged mesenteric lymph nodes. Histologically there was infiltration of liver, abdominal lymph nodes, tonsils, bone marrow, spleen, pancreas, thyroid, kidney and lungs with cells of the lymphocyte series (Fig. 1).

METHODS

Horse anti-dog lymphocyte serum

This was prepared at the Radiobiological Institute and was a pool of 3 different batches with a final concentration of 14·7 mg gG/ml. Whole thickness skin transplantation was performed in unrelated adult dogs injected with the serum. A dose of 3 ml/kg of body weight was given 1–2 days before transplantation followed by a dose of 1·5 ml/kg of body weight three times a week for a further 18 days. Skin survival time averaged 27 days compared with a survival time in non-treated dogs of 10–12 days.

Passages

The donor animal was killed by an intravenous injection of sodium pentobarbitone and leukaemic puppies were killed by ether inhalation. A mixture of cells from mesenteric lymph nodes, spleen or bone marrow was added to white cells collected from the peripheral

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*Permanent address: Department of Animal Pathology, School of Veterinary Medicine, University of Cambridge.

whole blood. Cells were washed twice with Hank's BSS and injected intraperitoneally into foetuses at various stages of gestation and into normal or immunosuppressed newborn, young or adult animals.

RESULTS

First passage

Following the intraperitoneal injection of 4×10^7 donor cells into Beagle foetuses on the 45th day of gestation all puppies were born clinically normal but the five injected puppies all developed leukaemia and died or were killed at an advanced stage of the disease 34–59 days post-partum. The sixth non-injected puppy remained normal and was killed at 59 days. Nothing abnormal was diagnosed on post-mortem examination of this latter puppy.

White cell counts made just before death ranged from 43,800 cells/mm^3 to 280,000 cells/mm^3. In the dogs with very high counts over 90% of the circulating cells were lymphocytes with a diameter of 8–9 μ but a few larger lymphocytes or lymphoblasts of 10 μ occurred. Occasional lymphocytes of 7 μ or 11 μ were also present. Many bizarre cells were present and mitotic figures were seen in some smears.

The puppies showed a variable degree of anaemia and up to 7% normoblasts were seen in blood smears.

The main gross pathological findings were hepatomegaly and splenomegaly with some atrophy of the thymus. One puppy had also greatly enlarged mesenteric and peripheral lymph nodes and another had leukaemic infiltration into the anterior chamber of the eye.

The liver, spleen and thymus weights (in grams) of the leukaemic and control dog killed at 59 days were as follows:

	Liver	Spleen	Thymus
Leukaemic	470	57	10·5
Control	185	9	14·0

Histologically there was diffuse infiltration of lymphocytes in the bone marrow, spleen, liver, lungs, kidneys, intestine, thyroid, pancreas and prostate and atrophy of the thymus.

Second passage

Leukaemic cells from first-passage puppies were injected into dogs as follows:

(1) Beagle foetuses 47-day gestation—
2 foetuses 10^6 cells i.p.
1 foetus 5×10^6 cells i.p.
1 foetus 5×10^6 cells subcut.
3 foetuses 10^7 cells i.p.
2 foetuses not injected.

(2) Beagle foetuses 55 days gestation—5×10^7 cells i.p.

(3) Two Alsatian puppies 4 days old given 200 rads whole-body X-irradiation 24 hours previously—10^8 cells i.v.

(4) Two Alsatian puppies 4 days old given 3 ml/kg body weight ALS 24 hours previously—10^8 cells i.v.

(5) Two Alsatian puppies 11 days old given 3 ml/kg body weight ALS 24 hours previously—5×10^7 cells i.v.

(6) Two normal 11-day old Alsatian puppies—5×10^7 cells i.v.

(7) One adult Beagle dog given 200 rads whole-body X-irradiation 24 hours previously—8×10^8 cells i.v.

The foetuses injected at 55-day gestation were aborted 2 days later. Transplants were also not successful in any of the 8 normal or immuno-suppressed young puppies nor in the adult dog given whole-body X-irradiation.

The 7 puppies which were injected as 47-day old foetuses developed leukaemia and died or were killed in an advanced stage of the disease 11–24 days post-partum, while the 2 non-injected puppies remained normal.

The total w.b.c. counts of 2 leukaemic and the 2 normal puppies are shown in Fig. 2.

The haematological and pathological findings were similar to those described for the first transplantation.

Third passage

Cells from a second transplant Beagle puppy, killed when 11 days old, were injected as follows:

(1) Beagle foetuses 54-day gestation: 5 foetuses, cell numbers varying from 10^5 to 10^7 i.p. One foetus was not injected.

(2) Four normal Alsatian puppies 7 days old: These were injected i.p. or i.v. with 10^8–10^9 cells.

Four of the five Beagle puppies injected as foetuses developed leukaemia with similar pathological lesions to those previously described. Death or euthanasia because of advanced leukaemia occurred 18–25 days after birth. Total w.b.c. counts shortly before death ranged from 24,100 mm^3 to 130,400 mm^3, and the appearance of blood smears and pathological appearance of organs was similar to that which has been described for the other transplants.

All 4 of the 7-day old Alsatian puppies injected with leukaemic cells died 17–19 days later with leukaemia. Total w.b.c. count shortly before death ranged from 60,000 to 119,000.

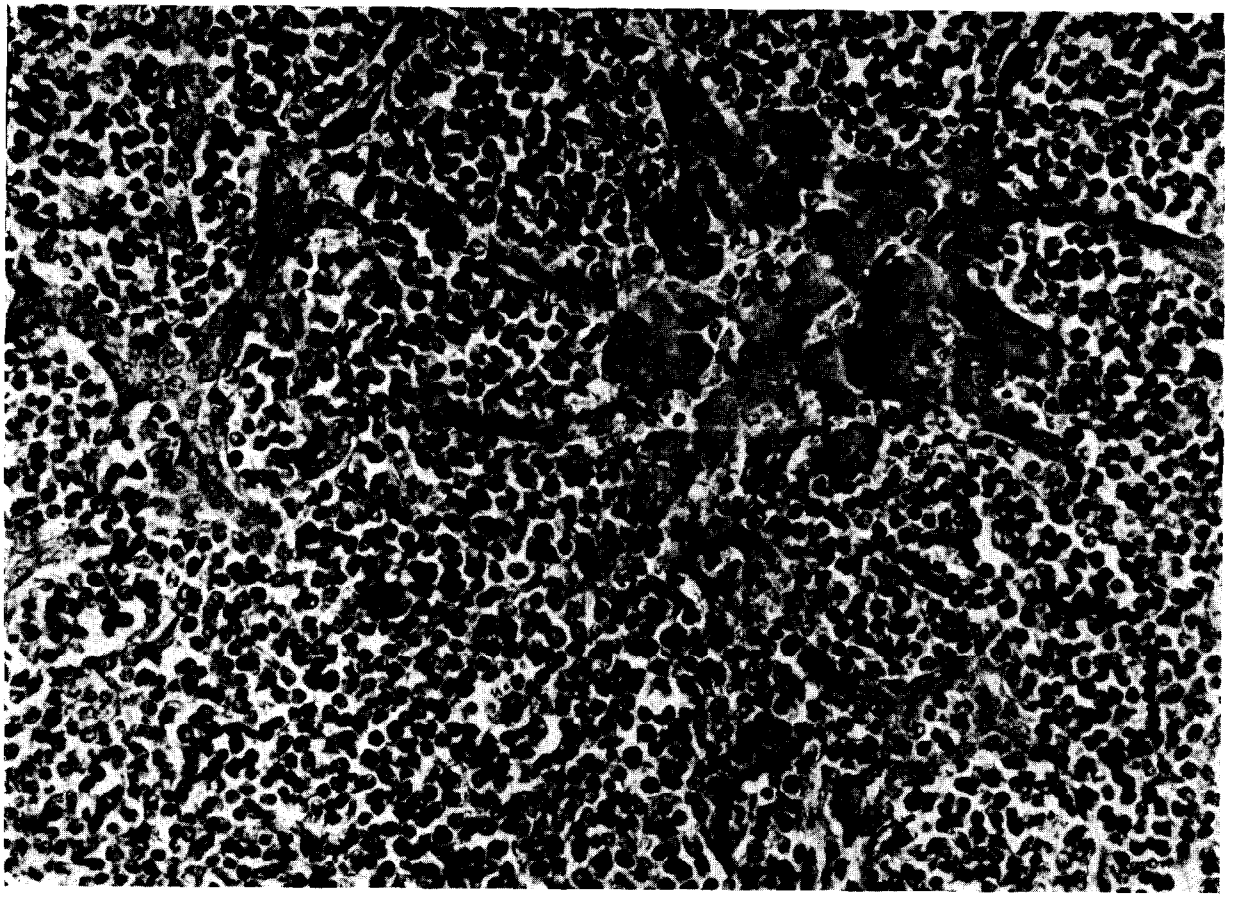


Fig. 1. *Liver of donor dog showing massive infiltration with lymphocytes.*

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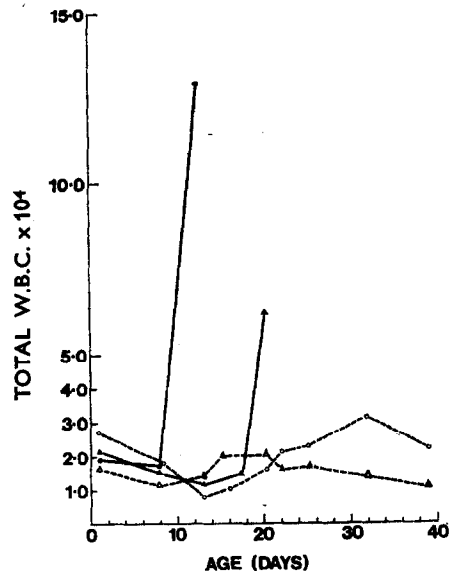
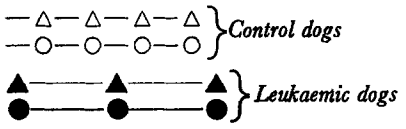


Fig. 2. Total w.b.c. counts of 2 control and 2 leukaemic (second transplant) puppies during the first few weeks of life. There was a rapid increase in the circulating lymphocytes of the leukaemic animals a short time before death.



Fourth passage

Cells from lymph nodes and spleen from a third transplant Alsatian, which was killed with advanced leukaemia 19 days after injection, were injected into two normal litter-mates aged 26 days, 8×10^8 cells being injected intravenously into each dog.

The 2 puppies developed, 2 months later, peripheral lymph node enlargement up to 2 cm in diameter. Histological examination of formalin-fixed specimens and impression smears from one of these nodes showed malignant cells but the blood picture remained normal. Regression of the nodes occurred after a few weeks and at 6 months of age the dogs are clinically and haematologically normal.

Tissue culture

Attempts to grow the donor cells and the various passages of the leukaemic cells in tissue culture in TC 199, Eagle's medium or McCoy's medium were unsuccessful.

DISCUSSION

While lymphosarcoma is of frequent occurrence in the dog, true lymphatic leukaemia with pathological features similar to those seen in man, and as described in the present Dalmatian dog, is uncommon. Cohen *et al.* [3] described one case of a leukaemic Beagle and were able to transplant cells in the buffy coat through one passage only in 2 of 11 animals inoculated.

In a previous experiment [4], we attempted transplantation of canine leukaemia from a 13-year old male Cairn with an enlarged liver and spleen and a total w.b.c. count of 650,000 cells/mm^3 (over 90% lymphocytes). Intraperitoneal injection of 4 normal new-born Collie puppies and 4 litter-mates, immunosuppressed with ALS, with up to 10^9 buffy coat cells was unsuccessful.

For success in transplantation at least in the initial stages it appears useful to inject the cells into foetuses at 6 or 7 weeks gestation. In the present experiments 16 out of 17 animals so injected developed leukaemia. It is likely that the one animal not developing leukaemia was the foetus injected with only 10^5 cells. In animals aged 4 days or older only 4 out of 15 injected developed leukaemia. The 4 puppies which became leukaemic were injected with large numbers of second passage cells when 7 days old. Littermates of these puppies, when injected with very large numbers of leukaemic cells at 26 days old, showed malignant lymph node enlargement 2 months later but by 6 months of age the lymph nodes had regressed and at no time were the animals leukaemic.

The appearance of a leukaemic blood picture in the transplanted dogs was of sudden onset with death (or severe illness necessitating euthanasia) occurring a few days later. The time between the injection of the foetuses and death from leukaemia was similar to that seen when canine lymphosarcoma was transplanted; animals dying with cellular transplanted lymphosarcoma [1].

Feline lymphosarcoma is of virus origin [5] but many attempts by various workers to isolate a virus from canine lymphosarcoma have failed. While a leukaemic blood picture sometimes occurs in lymphosarcoma in the dog—Jarrett *et al.* [6] found 13 such animals in 140 dogs with lymphosarcoma—the gross pathological picture differs from the canine lymphocytic leukaemia described in the present communication. It may be that they are different manifestations of the same disease but it seems particularly worth while, in view of the similarity to lymphatic leukaemia in man, to attempt cell free transmission of the leukaemia described in this article and such studies are in progress.

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REFERENCES

1. L. N. Owen and S. W. Nielsen, Transplantation of canine lymphosarcoma. *Europ. J. Cancer* **4**, 391 (1968).
2. L. N. Owen, Transplantation of canine osteosarcoma. *Europ. J. Cancer* **5**, 615 (1969).
3. H. Cohen, A. L. Chapman, J. W. Ebert, W. J. Boff and C. R. Gravelle, Cellular transmission of canine lymphoma and leukaemia in Beagles. *J. nat. Cancer Inst.* **45**, 1013 (1970).
4. L. N. Owen and D. E. Bostock, Unpublished data (1970).
5. W. F. H. Jarrett, E. M. Crawford, W. B. Martin and F. Davies, A virus-like particle associated with leukaemia (Lymphosarcoma). *Nature (Lond.)* **202**, 567 (1964).
6. W. F. H. Jarrett, G. W. Crighton and R. G. Dalton, Leukaemia and lymphosarcoma in animals and man. *Vet. Rec.* **79**, 693 (1966).

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Intracranial Tumors in Mice of Two Different Strains Maintained on Fat Enriched Diets*

J. SZEPSENWOL

Department of Anatomy, University of P. R. School of Medicine, San Juan, Puerto Rico

Abstract—*Two mice of BALB/c strain maintained on Purina chow supplemented with egg yolk developed, one at the age of* 619 *days and the other one at the age of* 429 *days, oligodendrogliomas originating from the cortex of the right hemisphere. The tumor of the first mouse invaded the interhemispheric fissure and extended to the brain stem and the posterior pole of the hemisphere. Two other mice of the T. M. strain, maintained on Purina chow supplemented with monoolein and cholesterol, and with lecithin and cholesterol, developed at the ages of* 804 *and* 700 *days respectively a fibrosarcoma associated with trigeminal nerve in the first case, and a brain-nerve cell tumor of the left hemisphere complicated by what appears to be a lymphoma in the second case. None of the control mice of the two strains developed intracranial tumors.*

INTRODUCTION

NEOPLASMS of the nervous system have been induced in mice and rats by direct application of carcinogenic hydrocarbons such as benzpyrene, dibenzanthracene, dimethylbenzanthracene and methylcholanthrene. They have also been obtained by administration, via the digestive tube, of 2-acetylaminofluorene [1]. More recently, various nitrosamine compounds, administered either intravenously of per os, were found to induce in rodents tumors of the central and peripheral nervous systems [2]. On the other hand, it was found that mice maintained on Purina chow supplemented with various lipids develop tumors of the cerebral cortex consisting entirely of differentiated nerve cells [3]. The present study is a report of 4 intracranial tumors, of a different nature, found in mice of two different strains maintained, as the above mentioned mice, on fat enriched diets. Two of these tumors were in mice of the BALB/c strain, maintained on Purina chow supplemented with raw egg yolk, and the other two in mice of the T. M. strain maintained on Purina chow supplemented with vegetable lipids.

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† The original BALB/c mice were supplied by H. B. Andervont. Nat. Cancer Inst. Bethesda, MD. to whom we are greatly indebted.

MATERIAL AND METHODS

The BALB/c mice† have been maintained on Purina chow supplemented with raw egg yolk (one egg yolk per 25–30 mice daily), while for the mice of the T. M. strain, like in previous experiments [3], the Purina chow was supplemented with small amounts of vegetable oil. In both cases, the mice were placed on the above diets at the age of 4 weeks, they were bred and their offspring maintained on the same diets. Mice of both strains maintained on Purina chow only, without supplements, were used as controls. The mice were killed when they were rapidly loosing weight. The skull was opened and the brain carefully examined. Those with tumors were fixed with Bouin's fluid, or with a 10% solution of formaldehyde, embedded in paraffin, sectioned serially and stained with hematoxylin and eosin. In some cases other staining techniques were used.

RESULTS

The two BALB/c mice with brain tumors (tumors 1 and 2) were both males; one was 619 and the other 429 days old. They were both on the Purina chow supplemented with egg yolk from the day they were born: they were offspring of mice maintained on the same diet. Macroscopically, the brain of the mouse 619 days old had a nodule projecting from the right hemisphere into the interhemispheric fissure and delimited laterally by a depressed area (Fig. 1). This nodule, as seen in sections, was actually not the tumor; it consisted of normal hemispheric tissue. It was separated from the rest of the cortex by a growing mass of cells extending from the depressed area of the hemisphere (Fig. 2) along the interhemispheric fissure to the brain stem and the inferior surface of the hemisphere (Fig. 3), and having all the characteristics of an oligodendroglioma. It was cellular and the cells had relatively large dark nuclei and clear cytoplasm. Many of them were in mitosis and some of the mitotic figures were abnormal (Fig. 4).

The second BALB/c mouse, 429 days old, presented a large depressed area on the lateral surface of the right hemisphere with a small whitish nodule emerging from its postero-superior end (Fig. 5). In microscopic sections this part of the hemisphere consisted, like tumor 1, of a mass of cells with large dark nuclei and clear cytoplasm (Fig. 6). Mitotic figures, some of which were abnormal, were abundant. This, as in the previous case, was without any doubt an oligodendroglioma.

Of the two intracranial tumors (tumors 3 and 4) in mice of the T. M. strain one was in a female 804 days old (maintained on Purina chow supplemented with monoolein and cholesterol) and the other one in a female 700 days old (maintained on Purina chow supplemented with lecithin and cholesterol). They were both offspring of mice maintained on the same diets. Tumor 3, in the mouse on the diet supplemented with monoolein and cholesterol, appeared as a large mass on the trajectory of the left trigeminal nerve (Fig. 7). It was continuous with the brain stem, with the lateral surface of the hemisphere, which was compressed and in part destroyed by it, and with the base of the skull. It consisted predominantly of interlacing bundles of fusiform cells. In some places the cells were uniform in appearance while in other places there were foci of pleomorphism, where among the fusiform cells, giant mononucleated cells of various sizes with large hyperchromatic nuclei were found. This tumor, with a large number of mitotic figures (Fig. 8), destroyed the adjacent brain tissue invaded and completely replaced the bone marrow of the neighboring part of the skull. It was either a fibrosarcoma or a malignant neurofibroma. The distinction between the two is often difficult. Its high malignancy and the absence of such microscopic features as nuclear palisading, short spindle cells in whorls, epithelioid cells with ample eosinophilic cytoplasm, etc. is in favor of the first possibility, namely that it was a fibrosarcoma.

Tumor 4, in a female of the T. M. strain maintained on Purina chow supplemented with lecithin and cholesterol, had macroscopically the appearance of a brain nerve cell tumor, at the most anterior end of the frontal lobe of the left hemisphere. Upon microscopic examination it was found that medially to the above tumor there was a mass of cells of various kinds: polymorphonuclear, plasma cells, and large, more primitive histiocyte-like cells. The latter formed masses or cords; many were in mitosis; and some were binucleated (Figs. 9 and 10). The differential diagnosis lies between an unusual inflamatory process and a lympho-proliferative phenomenon. The presence of polymorphs was in favor of the first hypothesis, while the large reticuloendothelial cells were suggestive of a lymph-proliferative process.

DISCUSSION

Mice maintained on a diet supplemented with egg yolk or with egg lipids develop a high incidence of lung tumors, lymphosarcoma and mammary cancer [4], while those fed vegetable fat have only a high incidence of lung neoplasms. The present results indicate that both, egg fat and vegetable oil, affect the nervous system. In a previous report it was shown that mice of the T. M. strain maintained on Purina chow supplemented with various vegetable lipids develop a relatively high incidence of nerve cell tumors of the frontal lobe [3]. The highest incidence of these tumors was found in the mice receiving lecithin and cholesterol. Contrary to a previous statement (that there are no mitotic figures in these tumors [3]), it was found recently, in tumors embedded in plastic and sectioned at one micron, that, among the completely differentiated nerve cells, there are many cells in mitosis (Fig. 11). This indicates that these are actively growing neoplasms and not masses of cells which, at an early stage, underwent proliferation, then secondarily differentiated and remained inactive.

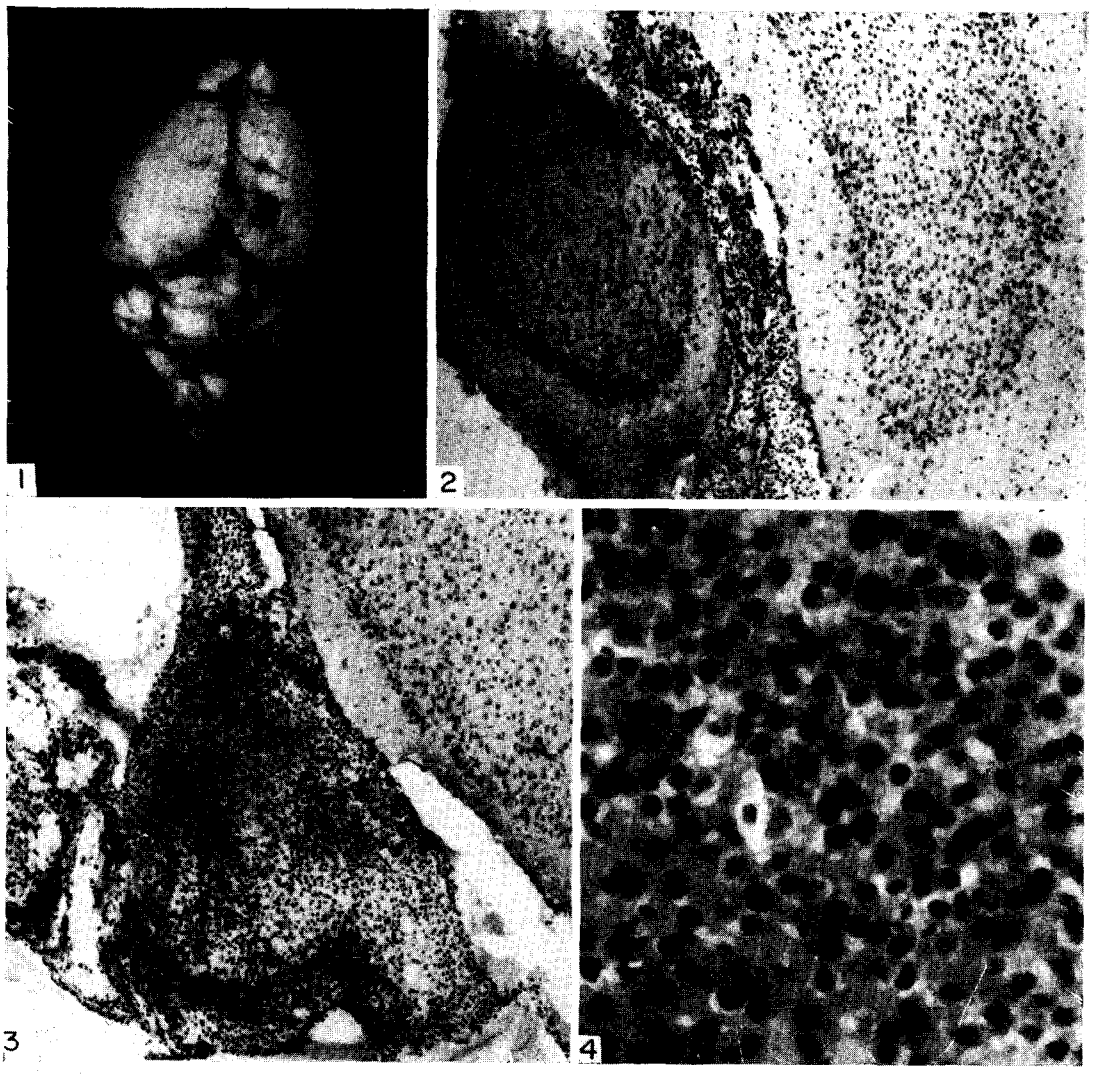


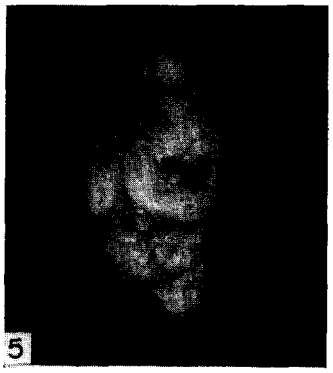
Fig. 1. *Brain of a BALB/c mouse, maintained on Purina chow supplemented with egg yolk.* 619 *days old.* × 3. *A nodule projecting from the right hemisphere into the interhemispheric fissure is delimited laterally by a depressed area which is the place of origin of the oligodendroglioma shown in Fig.* 2.

Fig. 2. *Horizontal section of the hemisphere of Fig.* 1, *passing through the nodule (to the left) the depressed area, (in the middle) and the remaining part of the cortex to the right. Hematoxylin and eosin,* × 100.

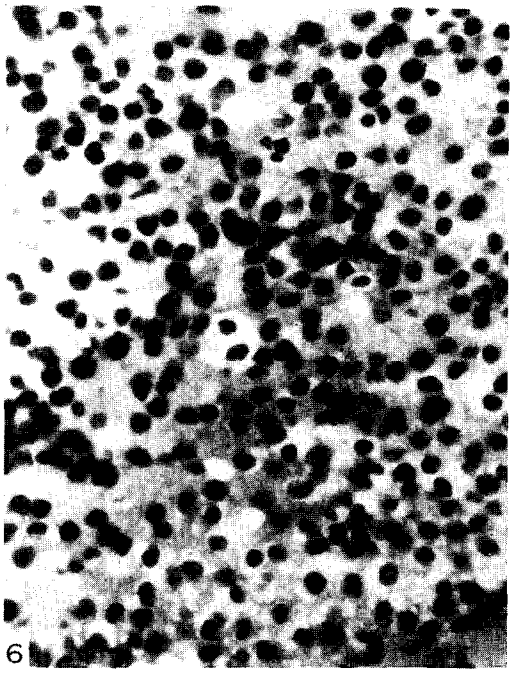
Fig. 3. *Section through the inferior and posterior end of the hemisphere (in the right upper corner) and of the tumor of Fig.* 1. *Hematoxylin and eosin,* × 100.

Fig. 4. *A higher magnification of the tumor of Fig.* 3. × 320. *The tumor consists of cells with large dark nuclei and clear cytoplasm. Many cells are in mitosis.*

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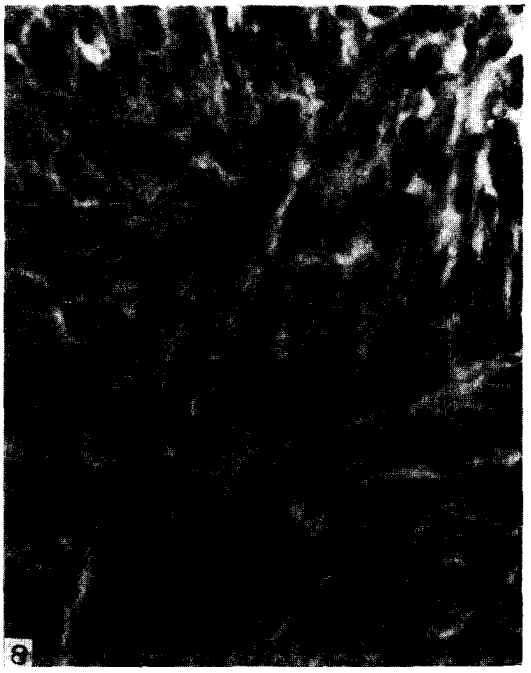


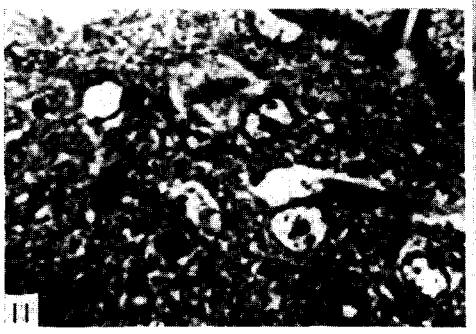
Fig. 5. *Brain of a BALB/c mouse,* 429 *days old, maintained on Purina chow supplemented with egg yolk.* × 1·5. *The depressed area on the right hemisphere and the whitish nodule at its postero-superior border correspond to the place of origin of the oligodendroglioma shown in the following figure.*

Fig. 6. *Section passing through the whitish nodule of Fig.* 5. *Hematoxylin and eosin* × 320. *The oligodendroglioma, like in Fig.* 4, *consists of cells with large dark nuclei and clear cytoplasm. Many cells are in mitosis and some of the mitotic figures are abnormal.*

Fig. 7. *Interior surface of the brain of a mouse of the T.M. strain maintained on Purina chow supplemented with monoolein and cholesterol.* × 2. *Left hemisphere is compressed (below) by a large tumor situated on the trajectory of trigeminal nerve. The tumor is continuous with the brain stem.*

Fig. 8. *Section of the tumor of Fig.* 7. *Hematoxylin and eosin.* × 320. *The tumor consists of bundles of fibroblasts running in all directions. A mitotic figure is seen slightly to the right of the middle.*





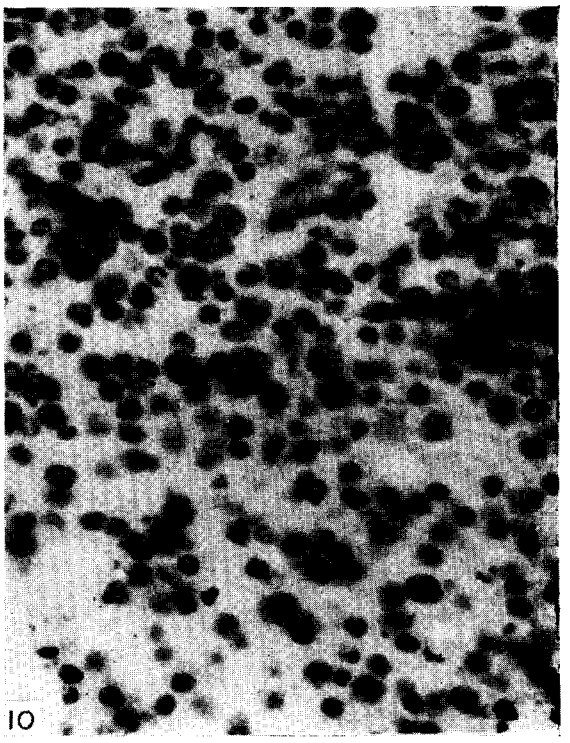


Fig. 9. *Section of the anterior end of the hemisphere of a mouse of the T. M. strain,* 700 *days old, maintained on Purina chow supplemented with lecithin and cholesterol. Hematoxylin and eosin.* × 100. *To the right of the brain-nerve cell tumor is a mass of cells which invaginates in part between the latter and the normal cortex, which is below.*

Fig. 10. *The mass of cells of Fig.* 9 *at higher magnification.* × 320. *The cells are polymorphonuclear and histiocytes. Many of the latter are in mitosis and some are binucleated.*

Fig. 11. *Section of a brain-nerve cell tumor of a mouse, of the T. M. strain,* 518 *days old, maintained on Purina chow supplemented with monoolein and cholesterol. Azur B and Methylene blue.* × 750. *It shows cross-sections of nerve fibers, a few differentiated nerve cells and a cell in mitosis, in metaphase.*

The present results demonstrate that the supporting elements of the nervous tissue may also undergo abnormal growth under the effect of a fat enriched diet. It is true that the number of tumors in this case was very small (only two of a total of 237 BALB/c mice on the diet supplemented with egg yolk developed oligodendrogliomas, one of 64 mice of the T. M. strain receiving monoolein and cholesterol developed an intracranial fibrosarcoma, and one of 88 mice on the diet supplemented with lecithin and cholesterol developed what was probably an intracranial lymphoma), but it is significant since none of the 197 BALB/c and of over 200 T. M. control mice developed such tumors. Moreover, if to the above 4 intracranial tumors are added the 37 brain nerve cell tumors, found in the mice on the diets supplemented with monoolein and cholesterol, and with lecithin and cholesterol [3], the incidence of the total number of intracranial neoplasms becomes highly significant.

As for the mechanism by which a fat enriched diet affects tumorigenesis in general and that of the nervous system in particular, it is still hard to understand. What seems to be striking is that cholesterol-enriched diets are particularly active. The incidence of brain-nerve cell tumors is higher in mice on a diet supplemented with glycerides and cholesterol than in those receiving glycerides alone. Cholesterol seems to affect also the pituitary gland. Of 117 mice of the T. M. strain maintained on Purina chow supplemented with refined corn oil and cholesterol, 15 developed pituitary tumors, while of 174 mice on a diet supplemented with corn oil alone only 3 developed such tumors (unpublished). It was previously reported that mice of the C57 Bl strain maintained on a diet supplemented with egg yolk (which is rich in cholesterol) had an incidence of 12% of pituitary tumors [6]. The question that could be raised is whether there is any relationship between the pituitary and the brain tumorigenesis. From the results so far obtained, it is still hard to draw any conclusion on the subject.

The mice with the intracranial tumors were offspring of mice maintained on the same diets; the question is consequently raised whether the lipids affected the animals already *in utero*, during the period of lactation, or during adult life. It is known that some chemical carcinogens have a transplacental effect. Druckrey and his co-workers found that following the injection of small, otherwise subthreshold doses of ethylnitroso-urea to pregnant rats practically all the offspring die of malignant tumors of the central or peripheral nervous system [7]. It has also been demonstrated that the aliphatic nitrosamines pass to the offspring of hamsters by way of the mother's milk [5]. The natural carcinogen cycasin and its Aglycon reach the offspring by way of the placenta and by the mother's milk [8]. According to Druckrey and his co-workers, during fetal development the sensitivity to the carcinogen is at least ten-fold higher than during the adult life of the animal, which could be attributed to the greater activity of the nucleic acids—the target of the primary carcinogenic action [7].

As for the fat enriched diets there is no indication that the lipids affect the nervous system of the offspring *in utero*. In no case did all the offspring of one litter die at an early age of tumors of the nervous system (brain nerve cell tumors and neoplasia of the supporting elements). Offspring of mice, on the fat enriched diets, foster nursed by control mice have, like the latter, a low incidence of tumors. There is some indication that the fat enriched diets affect the endocrine system during the period of lactation. In previous experiments it was found that a few of the mice, removed from the fat enriched diets immediately after weaning, developed pituitary tumors at the age of over 2 years. The problem is still being studied.

Acknowledgement—Some of the slides were examined by Dr. L. J. Rubinstein, Professor of Neuropathology at Stanford University School of Medicine, to whom I am greatly indebted.

REFERENCES

1. C. Hoch-Ligeti and D. S. Russell, Primary tumors of the brain and meninges in rats fed 2-acetylaminofluorene. *Acta Un. Int. Cancr.* **7,** 126 (1950).
2. G. Stoobrandt and J. M. Brucher, Etudes de tumeurs nerveuses obtenues par l'administration de méthylnitrosourée au rat. *Neuro-chirurgie* **14,** 515 (1968).
3. J. Szepsenwol, Brain nerve cell tumors in mice on diets supplemented with various lipids. *Path. Microb.* **34,** (1969).
4. J. Szepsenwol, Carcinogenic effect of ether extract of whole egg, alcohol extract of egg yolk and powdered egg free of the ether extractable part in mice. *Proc. Soc. exp. biol. Med.* **116,** 1136 (1964).

5. U. Mohr and J. Althoff, Carcinogenic activity of aliphatic nitrosamines via the mother's milk in the offspring of Syrian golden hamsters. *Proc. Soc. exp. biol. Med.* **136,** 1007 (1971).
6. J. Szepsenwol, Carcinogenic effect of egg yolk in mice of the C57 Bl strain. *Proc. Soc. exp. biol. Med.* **122,** 981 (1966).
7. H. Druckrey, R. Preussmann, S. Ivankovic and D. Schmahl, Organotrope carcinogene Wirkungen bei N-Nitroso-Verbindungen an BD-Ratten. *Z. Krebsforsch.* **69,** 103 (1967).
8. M. Spatz and G. L. Laqueur, Evidence for transplacental passage of the natural carcinogen cycasin and its aglycone. *Proc. Soc. exp. biol. Med.* **127,** 281 (1968).

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Triton WR 1339 (TWR), an Inhibitor of Cancer Dissemination and Metastases

G. FRANCHI, L. MORASCA, I. REYERS-DEGLI-INNOCENTI and S. GARATTINI
Istituto di Ricerche Farmacologiche "Mario Negri" Via Eritrea 62, 20157 *Milan, Italy, and European Organization for Research on Treatment of Cancer (E.O.R.T.C.)*

Abstract—*TRITON WR* 1339 (*TWR*), *a formaldehyde polymer of polyoxyethylene ether of octylphenol, decreases the formation of lung metastases in C* 57 *B*1/10 *J/Sel mice transplanted in the leg with Lewis lung carcinoma, it reduces the number of lymph node metastases in Swiss albino mice transplanted intratibially with Ehrlich carcinoma and it reduces the number of cancer cells in the lung of Swiss albino mice transplanted intracerebrally with Sarcoma* 180. *These effects are achieved without any effect on the primary tumor. Furthermore TWR does not affect the growth of other tumors of the mice transplanted in different sites.*

Two main effects of TWR may be involved in its antimetastatic action: an effect on the host because TWR enhances the reticular endothelial system activity in tumor-bearing animals for foreign particles; an effect on the cancer cells because TWR, at concentrations present in vivo, *affects the adhesiveness of cancer cells with an altered membrane, increases the volume, and decreases the movements of cancer cells. These effects may make in turn the cancer cells more susceptible to the defenses of the host.*

TWR is presented as the first example of antimetastatic agents differing from known antitumoral drugs also because it does not affect the primary tumor and the normal cells such as leukocytes.

INTRODUCTION

TRITON WR 1339 (TWR), a formaldehyde polymer of polyoxiethylene ether of octylphenol, is a non-ionic detergent provided with antitubercular activity in animals experimentally infected [1, 2]. Although it shows a considerable chemotherapeutical effect *in vivo* this compound has never been found effective *in vitro* on the growth of the Mycobacterium tuberculosis. Furthermore it is unlikely that the activity is due to the formation of a metabolic product because no tuberculostatic action was found in the blood of animals pretreated with TWR [3].

Since it is known that TWR may alter the superficial lipid layer of these bacilli [4] it was suggested that the micro-organisms may become more susceptible to phagocytosis by macrophages [5].

Alternatively the finding that TWR accumulates in the lysosomal fraction of the macrophages suggested that the antitubercular activity could be related to an increased lysis of bacilli for an activation of lysosomal enzymes [6, 7].

The capacity of TWR to bind to membranes suggested the possibility to affect the electrical charge of the malignant cancer cell membranes, which is considered more electro-negative than most of the normal cells [8].

This effect would have been perhaps important for changing the localization of cancer cell when they disseminate in the blood or in the lymph, or for making the cancer cells more susceptible to the phagocytic activity.

Since preliminary data indicated that TWR may decrease cancer dissemination and metastases [9] further studies were performed and are here summarized.

Accepted 17 May 1971.

MATERIAL AND METHODS

1. "In vivo" *experiments*

1.1. *Animals and tumors.* Female Swiss random-bred mice which had an average weight of 20 ± 2 g were used. Sarcoma 180 and Ehrlich carcinoma were maintained in ascitic form in this strain of mice for more than 300 passages; only ascitic fluids obtained from mice inoculated 7–10 days before were used in order to keep cell clumps and dead cells to a minimum.

Female and male C57/Bl/10J/Sel mice inbred at F : 104 which had an average weight of 18 ± 2 g were used. Lewis lung carcinoma (obtained from Prof. K. Karrer – Wien) was maintained in these mice by intramuscular implantation of 0·1 ml of tumor homogenate (1 : 10) in 0·2 M phosphate buffer pH 6·9. Spontaneous mammary carcinoma in $(C_3H \times O_{20})F_1$ hybrid mice (male castrated and treated with oestrone in drinking water 2 mg/l., and female "force bred") has been used. The tumor-bearing mice were supplied by Dr. O. Mühlbock, National Cancer Institute, Amsterdam.

The animals had free access to food (Diet ALAL 56) and water during the experiments. All the animals were kept at a room temperature of 23°C with a relative humidity of 60 % in cages of Makrolon ($20 \times 26 \times 13$ cm for 10 mice).

1.2. *Transplantation of tumors. Intracerebrally (i.c.).* 10^5 cells of Sarcoma 180 or Ehrlich carcinoma suspended in 10 μl of phosphate buffer were injected i.c. in the thalamic area on the medium line according to a technique previously described [10]. Histological serial examinations show proliferation of cancer cells at the site of implantation with an early infiltration of brain tissue : lymphocytes, which fill the perivascular lymphatic Virchow spaces during the first days after i.c. implantation, are replaced around the 4th day by cancer cells which then become detectable intraluminally (Sträuli and Hämmerli unpublished observations).

Intratibially (*i.t.*) 10^5 cells of Ehrlich carcinoma suspended in 10 μl of phosphate buffer were injected in the marrow cavity of the left tibia by means of a Hamilton microsyringe, according to a simple technique previously described [11]. Macroscopic metastases, mainly located in the lymphatic system were counted and weighed at the time of spontaneous death. Microscopic sections were prepared when doubt existed about the nature of the nodules.

Subcutaneously (s.c.) 5×10^6 cells of Ehrlich carcinoma or Sarcoma 180 were suspended in 0·2 ml of phosphate buffer and injected s.c. in the right flank.

Intraperitoneally (i.p.) 10^6 cells of Ehrlich carcinoma or Sarcoma 180 were suspended in 0·2 ml of phosphate buffer and injected i.p.

Intramuscularly (i.m.) 0·1 ml of Lewis lung carcinoma homogenate (1 : 5) in phosphate buffer pH 6·9 was injected in the muscle of the left posterior leg of mice. As previously described [12] the tumor growth results in metastatic nodules on the surface of the lung which are easily countable and measurable [13].

1.3. *Evaluation of cancer cell dissemination.* The presence of neoplastic cells in the blood and lungs of mice bearing i.c. tumors is evaluated by a bioassay procedure previously described [14]. Cancer cells in the lungs and blood may be detected on the 4th day and progressively increase in number until animal death (about the 14th day). In the experiments reported here, bioassay was performed 7 days after the i.c. implantation of tumor cells. At this time mice bearing i.c. tumors were killed by cutting the heart and the blood and lungs were collected aseptically. Blood clot and lung fragments (about 40 mg) were then transplanted subcutaneously (s.c.) into normal recipient mice. Every tissue of every donor was used for 2 s.c. transplantations. The number of tumor takes in recipient mice observed at the end of a 30-day period was shown to be a reliable parameter to evaluate different concentrations of cancer cells present in the transplanted tissue. An approximate quantitative estimation of the number of cancer cells was made possible according to a previously described procedure [15].

1.4. *Spectrophotometrical determination of Triton WR 1339 in plasma.* Triton WR 1339 shows an ultra-violet absorption peak at 278 nm as described by J. W. Cornforth [2]. A simple extraction of plasma with *n*-hexane added with 20% of *n*-propanol results in an organic phase concentration of Triton WR 1339 which is proportionally constant for the range of concentrations considered (1–20 mg/ml of plasma).

1.5. *Reticuloendothelial system (RES) activity test: rate of colloidal carbon clearance from blood.* The granulopectic activity of the RES has been evaluated by determining the rate of clearance of a standard dose of colloidal carbon, (160 mg/kg IV) (Pelikan 1141/a Gunther Wagner, Hannover, Germany) from the peripheral

blood according to the method of Biozzi *et al.* [16].

2. In vitro *experiments*

In vitro experiments have been carried out in medium NCTC 199 base Hank's plus 10% calf serum. Laboratory glassware was of borosilicate glass from Bellco Co. and the cleaning procedure was based on 30-min sonication in 7 × R detergent of wet glassware followed by multiple rinsing in tap and distilled water. After drying at 90°C the sterilization was performed at 160°C for 1 hr [17].

The following cell lines have been used:

EVE Normal human embryonal epithelium [18, 19]

KB Human carcinoma of pharynx [20]

BA 1112 Rhabdomyosarcoma of WAG/Rij rats [217]

Ch. L. Ching liver: line derived from normal human liver [22].

2.1. *Adhesion on glass.* KB cells grown in monolayer have been treated with 0·25% Difco trypsin dissolved in Ca++ and Mg++ free Hank's solution (CMFHS) for periods of time of 10 and 30 min. After treatment the cells suspensions were counted by an electronic cell counter (Coulter Counter mod. B) and diluted with growth medium, to give a final concentration of about 2×10^5 cells/ml. To half of this cell suspension TWR 1 mg/ml was added. Then 1 ml was distributed in test-tubes in a roller drum. Both cell suspensions were kept at 37°C and in 5% CO_2 in air during the whole procedure.

At different times random groups of test-tubes were drained of medium, gently washed with 8 ml of CMFHS and then treated again with trypsin to release the cells attached to the glass and to perform the counting.

The cells counted after this procedure represent the living fraction of the cells that were able to adhere to the glass at that time.

2.2. *Cell growth* [19]. Cell suspensions in growth medium containing 2×10^5 cells/ml have been seeded in test-tubes and incubated as for adhesion experiments.

After 48 hr of growth five tubes randomly chosen from the roller drum were evaluated for cell content by trypsinization (for 10 min) of the monolayer and electronic count of the cell population. The medium was then renewed in the other tubes with growth media containing graded doses of the drug. After 48 hr of incubation the cell content was evaluated as before.

The data are represented as per cent of growth of the control cultures during 48 hr of treatment by the relation

$$\% \text{ growth} = \frac{GI_T}{GI_C} 100.$$

The growth index (GI) of controls (GI_C) and treated cultures (GI_T) has been evaluated as

$$GI = \frac{N_0}{N_{48}} - 1,$$

where N is the mean number of cells counted at time 0 and 48 hr. It represents the number of doubling of the cell population during the 48 hr of growth and it assumes negative values when a total inhibition of growth is followed by a destruction of the initial population.

2.3. *Morphology and time lapse movie experiments.* KB cells have been implanted as small colonies, in Rose Chambers [23] under a sheet of perforated cellophane and when spreading was completed the time lapse observation was started, by a Kodak Special Camera driven at 1 frame per min. After 36 hr base line the treatment with TWR was started for different periods up to a maximum of 72 hr before changing the medium.

2.4. *Cell volume determination.* Sarcoma-180 cells were incubated in medium NCTC 199 + 10% calf serum in Spinner Flask with TWR.

The size distribution of the population was evaluated by the automatic plotter of a Coulter Counter mod. B.

The frequence distribution obtained has been transformed into cumulative percentage and plotted on probit paper.

Because of the skewiness of the distributions to the right, probably due to the poliploid part of the population, the threshold values on abscissae are expressed on a logarithmic scale.

RESULTS

Effect on transplanted and spontaneous tumors

In Table 1 are reported the results of the treatment with TWR of Sarcoma 180, Ehrlich carcinoma and mammary carcinoma in mice. Survival time or ponderal growth of animals transplanted with these tumors was not significantly changed by repeated treatments with Triton.

Effect on metastases

Lewis lung carcinoma transplanted in the right posterior leg of C57Bl/10J/Sel is not affected by the various TWR treatments in

Table 1. *Effect of Triton WR* 1339 *on various experimental tumors of mice*

	Sarcoma 180			Ehrlich carcinoma			Spontaneous mammary carcinoma in $(C_3H \times O_{20})F_1$ Mice
	s.c.	i.p.	i.c.	s.c.	i.p.	i.c.	
	g ± S.E.	days ± S.E.	days ± S.E.	g ± S.E.	days ± S.E.	days ± S.E.	g ± S.E.
Saline	0·81 ± 0·07	18·0 ± 0·5	13·4 ± 0·7	0·71 ± 0·06	20·8 ± 0·7	14·1 ± 0·7	5·540 ± 1·4
Triton WR 1339	0·80 ± 0·09	18·9 ± 1·8	12·9 ± 0·5	0·68 ± 0·08	19·9 ± 1·2	14·8 ± 0·3	3·945 ± 1·2

s.c. = subcutaneously; i.p. = intraperitoneally; i.c. = intracerebrally.

TWR was administered at the dose of 500 mg/kg intraperitoneally once a day during 6 following days beginning 5 days after subcutaneous tumor transplantation or 3 days after intraperitoneal tumor implantation or 1 day after intracerebral tumor implantation. In the case of s.c. tumors the day after the last treatment the mice were killed and tumor weights were evaluated. In the case of i.p. or i.c. tumors the survival time of the animals were recorded. The following schedule of treatment: days: 1, 2, 3, 4, 5, –, –, 8, –, 10, –, 12, –, –, 15, –, 17, –, 19, with a single daily injection of 500 mg/kg i.p. has been used in the case of the spontaneous mammary carcinoma. Three days after the last treatment the animals were sacrificed and tumor weight recorded.

No difference between control and treated animals are statistically significant (*t* Student's test).

its ponderal growth (Table 2). Daily treatment with TWR at the dose of 500 and 200 mg/kg decreases instead significantly the percentage of mice with metastasis as well as the number and size of metastases. The metastasis inhibitory effect is present also when the treatment (500 mg/kg) started 7 days after tumor transplantation but it is not evident when beginning the treatment on the 14th day after tumor transplantation. At the dose of 50 mg/kg TWR significantly reduces the number and the size of metastases without any effect on the percentage of mice showing metastases. The oral administration of TWR is uneffective, probably in relation to the fact that it is poorly absorbed by the gastro-intestinal tract.

Lymph nodal metastases from Ehrlich carcinoma transplanted i.t. are not affected in number and size by TWR at different doses and schedules of treatment but the number of mice which show metastases is significantly reduced. When the treatment starts late, i.e. 20 days after tumor implantation no difference has been observed between treated and untreated animals. It is remarkable that also in this experimental model the "primary" tumor growing in the leg is unaffected by the treatment with TWR (Table 3).

Table 2. *Effect of Triton WR* 1339 *on lung metastases in C57Bl/10 J/Sel mice transplanted intramuscularly with Lewis lung carcinoma*

Treatment	Dose mg/kg	Av. tumor* weight (g ± S.E.)	Mice with metastases† (%)	No. of metastases/ mouse with metastases	% Size distribution of metastases 1	2	3	4	5	6mm
Saline	—	5·9 ± 0·15	100 (74/74)	14·94 ± 0·9	69	24	5	0·9	0·1	0·4
Triton WR 1339	500*a*	5·9 ± 0·41	50 (13/26)‡	2·15 ± 0·3§	97	3	—	—	—	—
	500*b*	5·6 ± 0·29	82 (9/11)	6·81 ± 1·2§	91	9	—	—	—	—
	500*c*	6·2 ± 0·23	91 (20/22)	16·58 ± 2·4	77	18	4·5	0·5	—	—
	500*d*	5·8 ± 0·48	92 (11/12)	11·63 ± 1·2	98	2	—	—	—	—
	200*a*	4·8 ± 0·51	46 (6/13)‡	1·62 ± 0·7§	92	8	—	—	—	—
	50*a*	6·1 ± 0·22	80 (21/26)	3·20 ± 0·2§	96	2·5	1·5	—	—	—

*Tumor growing in the leg.

†In brackets number of animals with metastases/number of animals implanted. The animals were sacrificed 30 days after tumor transplantation and metastatic lung nodules were counted according to the technique of Hilda Wexler [17].

‡ χ^2 test, $P < 0{\cdot}01$.

§ *t* student test, $P < 0{\cdot}01$.

The i.p. treatment started (*a*) 3 (*b*) 7 or (*c*) 14 days after tumor transplantation, (*d*) oral administration beginning 3 days after tumor transplantation.

Table 3. *Effect of Triton WR* 1339 *on metastases in mice transplanted intratibially with Ehrlich carcinoma cells* (100,000 *cells/mouse*)

Schedule of Treatment	Treatment	Av. tumor wt. g ± S.E.	Survival time days ± S.E.	Mice with metastases* (%)	No. of metastases/ mouse with metastases
A	Saline	8·1 ± 0·8	47 ± 3	91 (31/34)	1·8 ± 0·13
	Triton WR 1339	7·0 ± 1·0‡	44 ± 3‡	62† (18/29)	2·1 ± 0·16‡
B	Saline	9·2 ± 0·5	47 ± 2	60 (23/38)	1·3 ± 0·13
	Triton WR 1339	10·5 ± 0·6‡	46 ± 2‡	37† (13/35)	1·8 ± 0·37‡
C	Saline	11·9 ± 0·7	50 ± 1	66 (14/21)	1·5 ± 0·22
	Triton WR 1339	11·7 ± 0·3‡	50 ± 2‡	57 (13/23)	1·6 ± 0·24‡

A: 500 mg/kg/i.p. daily beginning 3 days after tumor transplantation.
B: 1250 mg/kg/i.p. every 4 days beginning 3 days after tumor transplantation.
C: 1250 mg/kg/i.p. every 4 days beginning 20 days after tumor transplantation.

*In brackets number of animals with metastases/number of animals implanted.
†χ^2 test, $P < 0{\cdot}05$.
‡t student test, $P > 0{\cdot}05$.

Effect on cancer cells dissemination

The effect of TWR on cancer cells disseminated in blood or lung from Sarcoma 180 implanted i.c. has been determined. Different doses of the compound administered for 6 days by a single daily injection have been used.

Results reported in Table 4 show the percentage of takes of blood and lung obtained from i.c. tumor bearing mice and transplanted subcutaneously in normal recipient mice. The concentration of cancer cells in blood is not modified by the six day treatment with TWR at daily doses of 2000; 500; 200 and 50 mg/kg. On the contrary the number of cancer cells disseminated in lung is significantly reduced by the treatment with TWR at all doses of 50 mg/kg.

In separate experiments not reported here in detail it was excluded that in animals treated with TWR there may have been a shift of cancer cells in liver, kidney or spleen.

Effect on transpulmonary passage of cancer cells

The decrease of the number of cancer cells in lungs of mice treated with TWR can be interpreted as dependent by a reduction of the number of cancer cells reaching the lung (decreased leakage from the cerebral tumor) or by an increase of cancer cells filtered through the lung (decreased take in the pulmonary tissue). To approach the problem experiments were carried out for evaluating the number of cancer cells in the blood taken from the jugular vein, and in the arterial blood obtained from the left heart.

Table 4. *Effect of Triton WR* 1339 *on cancer-cell dissemination evaluated* 7 *days after intracerebral transplantation of Sarcoma* 180 *in Swiss albino female mice*

Treatment	Dose mg/kg/ip × 6 days	Blood		Lung	
		Takes* (%)	Cancer cells (ml)	Takes (%)	Cancer cells (g)
Saline	—	46 (43/92)	18×10^3	52 (48/92)	150×10^3
Triton WR 1339	2000	35‡ (7/16)	9×10^3	19† (3/16)	17×10^3
	500	40‡ (10/32)	11×10^3	22† (7/32)	22×10^3
	200	51‡ (10/16)	27×10^3	23† (4/16)	24×10^3
	50	56‡ (11/16)	40×10^3	58‡ (10/16)	220×10^3

The number of cancer cells disseminated in blood and lung from Sarcoma 180 transplanted intracerebrally in female Swiss albino mice was evaluated by a bioassy.
For technical details see references [18, 19].

*In brackets number of animals showing a tumor/number of animals implanted subcutaneously with blood or lung.
†χ^2 test, $P < 0{\cdot}05$.
‡$P > 0{\cdot}05$.

Results reported in Table 5 show that at the 7th and the 12th day after i.c. tumor transplantation it was not possible to observe any difference in the venous and arterial blood-cancer cell concentration between treated and control mice.

Effect on lung dissemination after intravenous injection of cancer cells

In this experiment we considered the possibility to evaluate cancer cells dissemination in lung using animals which have been treated with repeated doses of TWR in comparison to controls. Animals were treated with TWR 500 mg/kg i.p. daily during six following days till the day of the intravenous injection of $2{\cdot}5 \times 10^5$ Sarcoma 180 cells per mouse. Results reported in Table 6 show that the presence of cancer cells in lung evaluated within one hour after the cells injection is not significantly affected by the previous treatment of the animals with TWR.

Inhibitory activity on growth of normal and neoplastic cell lines

Chart 1 shows the inhibition of growth produced by graded doses of TWR on exponentially growing cell lines two of which are of normal and two of neoplastic origin. In particular the line BA 1112 is a rhabdomyosarcoma of the WAG/Rij rat that can be transplanted from the "*in vivo*" to the "*in vitro*" and vice versa without loss of viability. TWR was effective in inhibiting the growth at a concentration of 100 μg/ml but even at 1000 μg/ml there was no destruction of the cells. The different cell lines behave in a similar way and only the BA 1112 tumor appears to be somewhat less sensitive to the drug than the other three lines.

Effect on adhesion of KB cells on glass

The rate of adhesion of KB cells is modified by prolonged treatment of the cell population by trypsin. After 10 min of incubation with the enzyme the rate of adhesion is maximal and the population adheres to the glass at the same rate as untreated cells. When trypsin treatment is prolonged till 30 min the rate of adhesion is reduced. The acute treatment at time 0 of these cell suspensions with 1 mg/ml of TWR produces an increase of cellular adhesion and

Table 5. *Effect of Triton WR* 1339 *on transpulmonary passage of cancer cells disseminated from Sarcoma* 180 *transplanted intracerebrally in Swiss albino female mice*

Treatment	Days after transplantation	Jugular vein blood: Takes* (%)	Jugular vein blood: Cancer cells (ml)	Left heart blood: Takes* (%)	Left heart blood: Cancer cells (ml)
Saline	7	51 (15/29)†	27×10^3	0 (0/26)	< 100
Triton WR 1339 (500 mg/kg/i.p. × 6)	7	50 (13/26)	25×10^3	4 (1/23)	125
Saline	12	66 (8/12)	87×10^3	8 (1/12)	325
Triton WR 1339 (500 mg/kg/i.p. × 11)	12	69 (9/13)	112×10^3	7 (1/14)	275

The number of cancer cells disseminated in blood from Sarcoma 180 transplanted intracerebrally in female Swiss albino mice was evaluated by a bioassay. For technical details see references [18, 19].

*In brackets: number of animals showing a tumor/number of animals implanted subcutaneously with blood.
†No difference is statistically significant. χ^2 test, $P > 0{\cdot}05$ vs. saline.

Table 6. *Effect of Triton WR* 1339 *on the presence of cancer cells in lung of Swiss albino female mice injected intravenously with Sarcoma* 180 *cells* (250,000/0·1 *ml/mouse*)

Min after IV injection of cancer cells	Controls takes (%)	Treated takes (%)
1	75 (15/20)	45* (9/20)
15	65 (13/20)	72* (13/28)
60	50 (10/20)	30* (6/20)

*χ^2 test, $P > 0{\cdot}05$ in respect to controls.

Triton WR 1339 was administered at the dose of 500 mg/kg/i.p. daily during 6 following days till the day of cancer intravenous injection.

In brackets number of animals showing tumors/number of animals transplanted subcutaneously with lung.

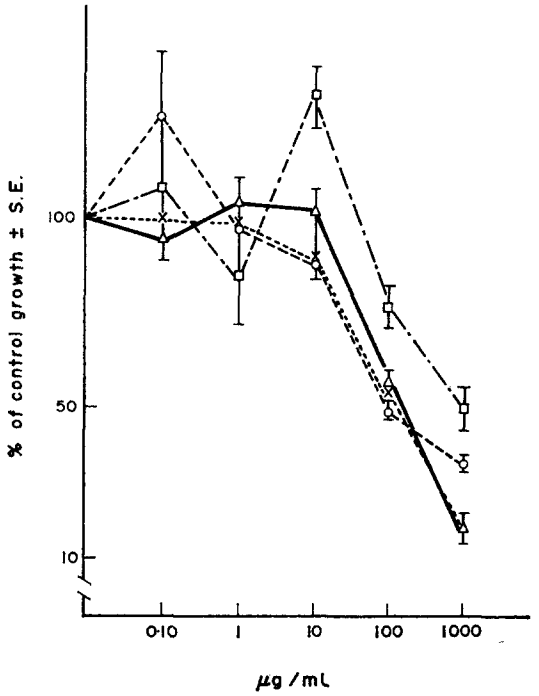


Chart 1. *Effect of various concentrations of Triton WR* 1339 *on the growth of* 4 *lines of tissue cultures* in vitro.
△——△ *EUE*
▲·····▲ *KB*
○- - -○ *CHANG LIVER*
□-·-·-·□ *BA* 1112

it inhibits cellular growth. The same experiment on the normal population shows only a reduction of the cellular growth (Chart 2).

These data suggest that the membrane of cells, modified in their adhesiveness by trypsin treatment, may be partially restored in their capability to adhere to the glass by the presence of TWR.

Effect on cell volume

In Chart 3 the cumulative frequency of the Sarcoma-180 cells against the threshold values of the Coulter Counter apparatus is plotted. The constant to transform these values in cell volumes is $K = 217\ \mu^3$. It is shown therefore that a population with a mean volume of $1630\ \mu^3$ is shifted after 4 hr incubation with TWR (1 mg/ml) to a mean volume of $2300\ \mu^3$ and after 8 hr to a mean volume of $868\ \mu^3$, that is in the range of the "cellular debris".

Effect on KB cells observed with time lapse movie

Chart 4 shows the analysis of a time lapse movie at 1 frame per minute during 120 hr. The percentage of cells in mitosis during a period of 5 hr in respect to the number of cells present is reported on the ordinates.

The sequence of events shows that 13 hr after TWR (1 mg/ml) the mitotic index falls to zero with a concomitant paralysis of cell movements. At this point the colony seems to be composed of fixed cells. Thirty hours after the treatment the cells were washed with fresh medium and 10 hr later the cell movement reappeared. The first outburst of mitoses is evident 20 hr after the change of the medium. Figures 1–6 demonstrate the morphological alterations appearing in the treated cell population. Cells are swelling with the formation of spikes in the membrane connecting neighbouring cells, after 12 hr of treatment. This phenomenon assumes a more pronounced aspect after 21 hr. Large cells are still remaining aggregated with fibroblastic-like ones. Large vacuoles of irregular volume and shape are present in the cytoplasma of cells.

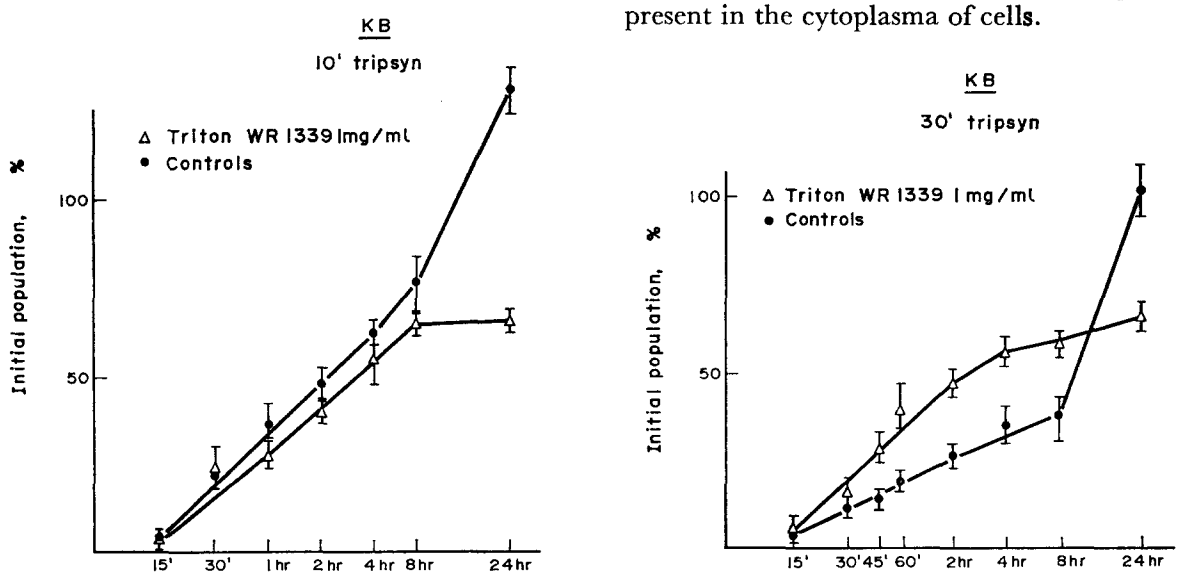


Chart 2. *Rate of adhesion on glass of KB cells in presence of Triton WR* 1339. *On the ordinates the percentage of cells attached to the glass wall; on the abscissae the time of incubation. KB cells were pretreated for* 10 *min* (*on the left*) *or for* 30 *min* (*on the right*) *with trypsin before the addition of TWR.*

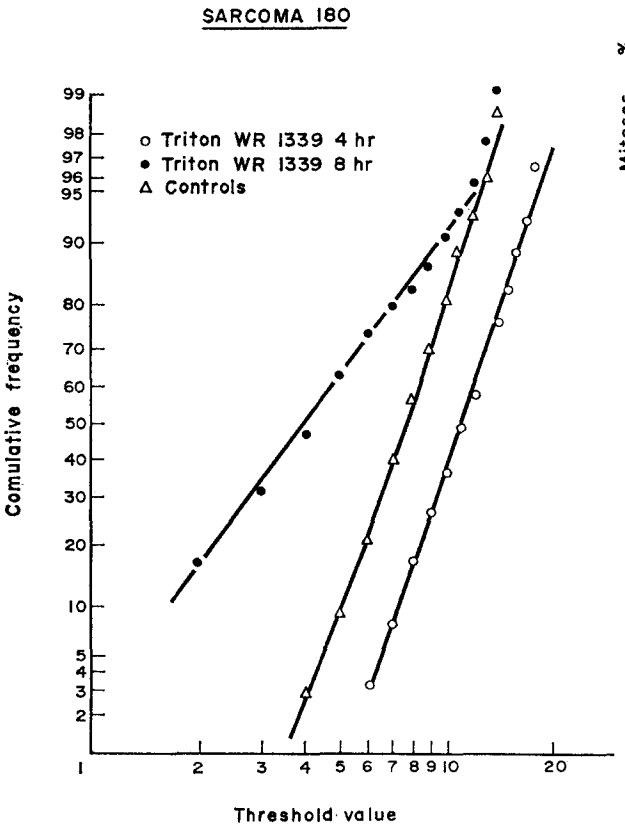


Chart 3. *Cumulative frequency of Sarcoma-*180 *cells (on the ordinates) is plotted against the threshold values of the Coulter Counter mod. B. The cell volume can be calculated by multiplying the threshold value for a constant* ($K = 217\mu^3$). *The mean volume of the untreated cells* (1630 μ^3) *is shifted to* 2300 μ^3 *and* 868 μ^3 *respectively after* 4 *or* 8 *hr of incubation with Triton WR* 1339 (1 *mg/ml*).

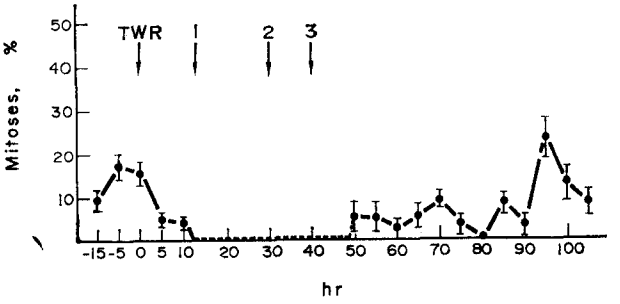


Chart. 4. *Sequence of events occurring after addition of Triton WR* 1339 (1 *mg/ml*) *to KB cells under time lapse movie.*

1. *Paralysis of cell movements*;
2. *washing*;
3. *reappearance of cell movements.*

Triton WR 1339 *plasma levels and related hyperlipemia*

The blood levels of Triton have been evaluated after a single intraperitoneal injection of 500 mg/kg i.p. The compound becomes detectable in blood 4 hr after the administration, its concentration increases with the time and it is still present in appreciable amount 24 hr later. A dose of 1250 mg/kg, administered by a single intravenous injection, is cleared from the blood very slowly reaching unmeasurable levels only after more than 72 hr. Repeated intraperitoneal injections of the compound result in high blood levels which remain constant for at least 24 hr. The triglycerides and cholesterol blood concentrations seem to be well related with the presence of Triton in blood (see Table 7).

Table 7. *Plasma levels of Triton WR* 1339 *in Swiss female mice: relationship between drug concentration and lypemia*

Dose (mg/kg)	Time after treatment (hr)	Plasma Triton WR 1339 (mg/ml)	Plasma cholesterol (mg%)	Plasma triglycerides (mg%
500 i.p.	0	< 1·0	200	140
	2	< 1·0	176	87
	4	2·0 ± 0·3	200	244
	8	5·3 ± 0·7	255	1080
	24	2·7 ± 0·2	194	420
	48	< 1·0	120	138
1250 i.v.	0	10·3 ± 1·1	94	490
	24	15·0 ± 0·3	178	2000
	48	11·0 ± 0·5	544	2000
	72	4·0 ± 0·3	212	1080
	96	< 1·0	77	440
500* i.p.	0	12·7 ± 0·4	272	1650
	8	15·7 ± 1·1	341	2300
	24	10·4 ± 1·8	730	4000

*Single daily injection during 6 following days.

The cholesterol and tryglyceride determination has been performed by using a pool of plasma from three mice. The lipids were extracted according to the method of Carlson [38]. Cholesterol and triglicerides were determined according to the methods respectively of W. R. Bloor [39] and E. van Handel–D. B. Zilversmit [40].

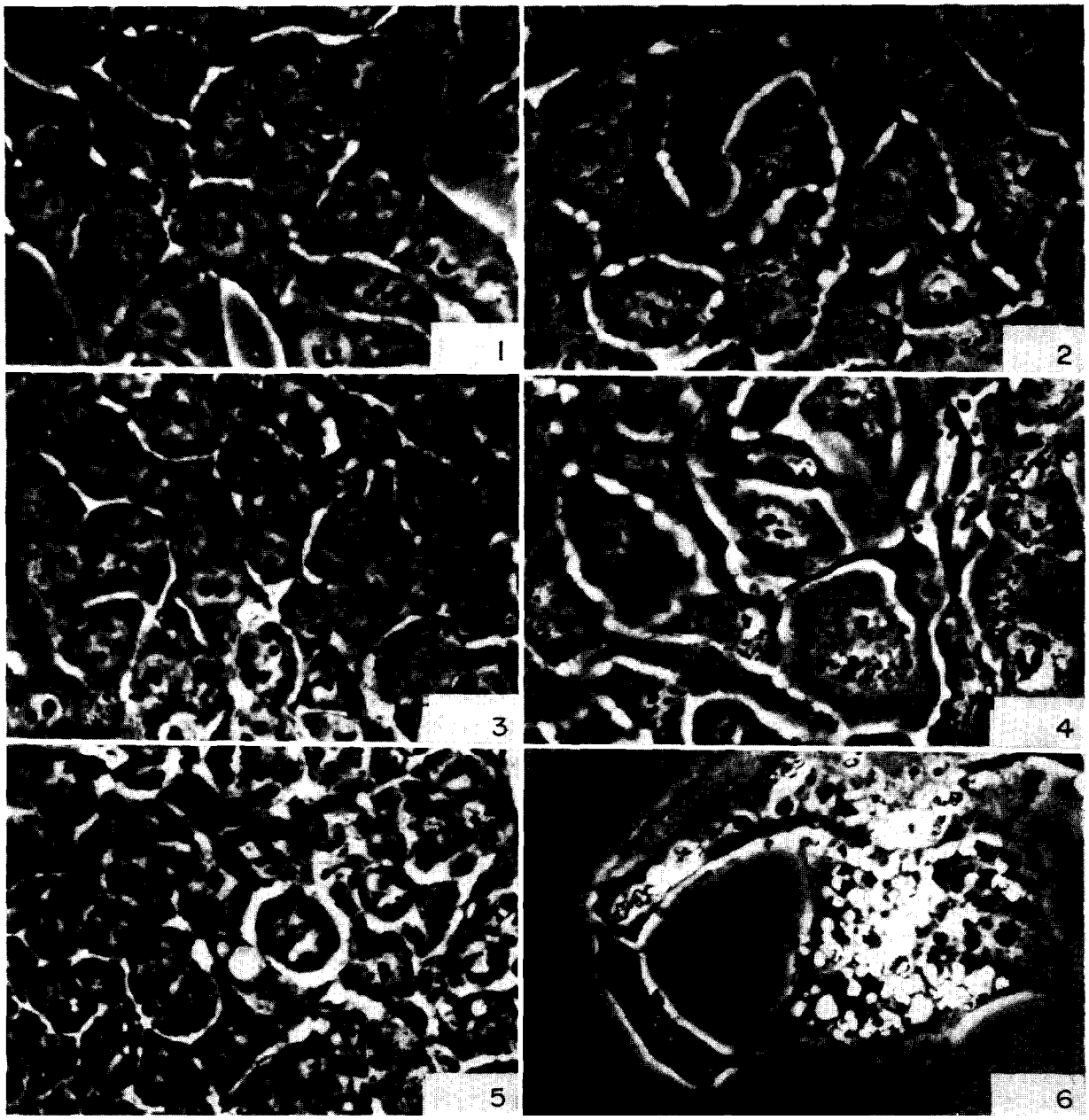


Fig. 1. 600× *phase contrast. KB cells* 12 *hr culture. Typical population of a young culture of epithelial cells still expanding on a large surface.*

Fig. 2. 600× *phase contrast. Sister culture* 12 *hr. Treatment with* 1 *mg/ml TWR. Cells are swollen, with well marked borders and formation of spikes connecting neighbouring cells.*

Fig. 3. 600× *phase contrast. KB cells* 21 *hr culture. Cells are more packed together with the aspect of cubic epithelium.*

Fig. 4. 600× *phase contrast. Sister culture* 21 *hr. Treatment with* 1 *mg/ml TWR. Cells are extremely swollen with marked borders and spikes connecting neighbouring cells.*

Fig. 5. 600× *phase contrast. KB cells* 37 *hr of culture. Epithelium is made of closely packed cells. One mitosis is evident in the center of the field.*

Fig. 6. 600× *phase contrast. Sister culture* 37 *hr. Treatment with* 1 *mg/ml TWR. A giant cell on the right is filled with vacuoles. On the lower left one fibroblastic like cell is connected with a second giant cell on the upper side of the picture.*

Effect on the Reticulum endothelial system (RES)

In order to establish if TWR could modify the activity of the RES the clearance from the blood of a standard preparation of colloidal carbon was determined in animals which had been transplanted intracerebrally with Sarcoma 180 seven days before the experiment. TWR was administered by a daily intraperitoneal injection of 500 mg/kg during 6 following days beginning the same day of the i.c. tumor transplantation till 24 hr before the evaluation of the rate of carbon clearance. Results reported in Table 8 show that TWR is able to stimulate the RES activity of the intracerebral tumor-bearing mice, which show without any treatment a phagocytic index significantly lower than normal animals.

TWR, at doses effective on metastases, was unable to modify the weight of the Ehrlich carcinoma and the Sarcoma 180 transplanted subcutaneously, the growth of a spontaneous mammary carcinoma of the mice [24] or the survival time of animals transplanted intraperitoneally or intracerebrally [14] with Ehrlich carcinoma or Sarcoma 180.

The explanation for an inhibitory effect on metastases not accompanied by a similar effect on the primary tumor may range in one or more of the numerous processes going from the leakage of the cancer cells from the primary tumor to the take, survival and proliferation in a normal tissue. Several experiments have been performed in an attempt to reveal the site of action of TWR. By using the intracerebral

Table 8. *Effect of Triton WR* 1339 *on the activity of the reticuloendothelial system evaluated by the clearance of colloidal carbon from blood*

No. of mice	Tumor	Treatment 500 mg/kg i.p. × 6 days	Liver+spleen weight (g/20 g)	Phagocytic index (K)*
7	—	—	1·299 ± 0·026	0·041 ± 0·002
8	Sarcoma 180 i.c.	—	1·172 ± 0·020	0·030 ± 0·001
7	Sarcoma 180 i.c.	Triton WR 1339	1·274 ± 0·064	0·145 ± 0·01†

*$K = \log C_1 - \log C_2 / T_2 - T_1 \cdot C_1$ and C_2 were the concentration of carbon in the blood at times T_1 (3 min) and T_2 (15 min) respectively.
†Significantly different from the mean of tumor bearing or control mice with a $P < 0{\cdot}01$.

DISCUSSION

The results obtained in this investigation consistently demonstrate that Triton WR 1339 (TWR) is an inhibitor of metastases in two experimental models, the Lewis lung carcinoma transplanted intramuscularly in the leg of C57Bl/10 J/Sel mice [12] and the Ehrlich carcinoma transplanted intratibially in the Swiss Albino mice [11].

In the first experimental condition where the metastases occur in the lung, TWR decreases the percentage of mice showing metastases as well as the number of metastases for mouse. In the second model, where the metastases are present in the lymph nodes, TWR reduces only the percentage of mice with metastases.

In both systems the effect of TWR is obtained with doses not exhibiting signs of toxicity for the host, only if the treatment was starting at a relatively short time (up to 7 days) after transplantation of the tumor.

It is remarkable that this effect on metastases was obtained without any reduction of the weight of the primary tumor. Furthermore transplantation of Sarcoma 180, a model which allows a quantitative determination of the cancer dissemination in blood and lung [14, 15], it was possible to establish that TWR reduces the number of cancer cells in blood. This effect was not a result of a shift of the cancer cells to other organs such as liver, kidney or spleen.

Furthermore TWR did not change the number of cancer cells flowing from the primary tumor into the jugular vein neither did it affect the capacity of the lung to trap the cancer cells because the number of cancer cells in the left heart was the same in both control and TWR treated mice. The fact that the take of cancer cells in lung is not influenced by TWR is shown also by the fact that animals pretreated with TWR and injected intravenously with cancer cells show in their lungs a number of cancer cells not significantly different from the untreated animals. There is however one main limitation in the conclusions which can be drawn from the results of these experiments. The data obtained show in fact a large variability and therefore a small decrease in the number of cancer cells present in blood,

although not detectable with the available methods, may in due time justify the decrease of cancer cells in the lung and therefore the presence of metastases.

Similar considerations could be put forward for the model of the intratibial transplantation of tumors where the dissemination of cancer cells is occurring through the lymphatic vessels [11, 25]. With the above mentioned limitations it may be tentatively concluded that TWR does not grossly affect the process of disseminations (various steps bringing the cancer cells from the primary tumor to the normal tissue) but it may rather affect some processes which result in a higher vulnerability of the cancer cells immediately after the lodgement. Previous experiments have in fact showed that the lung possesses a considerable capacity to destroy cancer cells because the block of cancer cell flow in the blood results in an immediate fall of the number of cancer cells present in the lung [26]. However, when the cells are lodged and when they start proliferating, TWR is without effect as shown by the experiments where TWR was given at late time after the implantation of the primary tumor. If TWR makes the cancer cells more susceptible to the defence of the organism this may occur for an effect of TWR on the host defence or for a direct effect on the cancer cells.

As far as the first possibility is concerned our experiments indicate that TWR increases the RES activity in tumor-bearing animals for foreign particles; however the significance of this finding for the effect of TWR on cancer metastases is still to be established. An increase of the RES could be in fact important for an activation of the immunological reactions which are unfavorable for the survival of cancer cells. It should also be noted that the antitumoral activity of formaldehyde-inactivated corynebacterium parvum was ascribed to an enhancement of the RES activity [27–29].

The hyperlipemic effect of TWR [30, 31, 32], which is long lasting also in mice, should not be responsible for the antimetastatic activity of TWR because, if any, Cliffton *et al.* [33] reported that an increase of blood lipids facilitates the formation of metastases.

As to the second possibility according to which TWR may act directly on the *c* cancer cells, several experiments seem to support this view. TWR changes "*in vitro*" the rate of adhesiveness of cancer cells with an altered membrane; it increases the volume and it blocks the ameboid movements of cancer cells. These effects, which are obtained with the concentrations of TWR measured in the blood of animals treated with antimetastatic doses of TWR, may be explained by a binding of TWR to the membrane. This binding, which results in a decrease of the cellular metabolic activity [34], may also explain the inhibition exerted by TWR on the growth of four cell lines "*in vitro*". The fact that cancer and normal cells are sensitive to the action of TWR is at variance with the data obtained by Miller [35, 36] who postulated a specific effect of TWR for malignant cells.

Since TWR remains for many hours in the blood stream it may be suggested that this agent may affect cancer cells when they are transported in the blood in such a way that changes in cellular adhesiveness, volume or movements make the cells more sensitive to the defences of the normal tissues, one of which may be the RES which is concomitantly enhanced by TWR as previously reported.

Although this hypothesis is supported by a number of experimental data it should be stressed that the mechanism of action of TWR remains a problem open to many alternative explanations.

In any case it should be underlined that TWR may be considered the first example of chemical agents showing an effect on the formation of metastases without influencing the growth of the primary tumor. This action is different from the known antitumoral drugs which, by blocking the cell proliferation, affect both primary and metastatic tumors [37]. TWR is different from antitumoral drugs also as far as its effect on normal proliferating cells is devoided of cytotoxicity for circulating leucocytes [unpublished results]. For this reason it is believed that TWR or other agents provided with similar activity may prove useful in the treatment of cancer in combination with surgery, irradiation or conventional chemotherapy. The fact that the effect of TWR was detected with the experimental methods here employed, but that it would have been missed with the conventional screening procedures, indicate that models for investigating cancer dissemination and metastases may be useful for revealing new types of antitumoral drugs.

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REFERENCES

1. J. W. Cornforth, P. D'Arcy Hart, G. A. Nichols and R. J. A. Stock, Antituberculous effects of certain surface-active polyoxyethylene ethers. *Brit. J. Pharmacol.* **10,** 73 (1955).
2. J. W. Cornforth, P. D'Arcy Hart, R. J. W. Rees and J. A. Stock, Antituberculous effect of certain surface-active polyoxyethylene ethers in mice. *Nature (Lond.)* **168,** 150 (1951).
3. R. J. W. Rees, Antituberculous activity of certain nonionic detergents. *Proc. roy. Soc. Med.* **46,** 581 (1953).
4. J. E. Lovelock and R. J. W. Rees, Possible site and mode of action of certain lipotropic macromolecules in tuberculosis. *Nature (Lond.)* **175,** 161 (1955).
5. G. B. Mackaness, Artificial cellular immunity against tubercle bacilli. *Amer. Rev. Tuberc.* **69,** 690 (1954).
6. P. D'Arcy Hart and A. H. Gardon, Suggested role of lysosomal lipid in the contrasting effects of "Triton WR 1339" and Dextran on tuberculous infection. *Nature (Lond.)* **222,** 672 (1969).
7. F. Leighton, B. Poole, H. Beaufay, P. Bandhuin, J. W. Coffey, S. Fowler and C. de Duve, The large-scale separation of peroxisomes, mitochondria and lysosomes from the livers of rats injected with Triton WR–1339. Improved isolation procedures automated analysis, biochemical and morphological properties of fractions. *J. Cell Biol.* **37,** 482 (1968).
8. A. D. Bangham, J. C. Glover, S. Hollingshead and B. A. Pethica, The surface properties of some neoplastic cells. *Biochem. J.* **84,** 513 (1962).
9. R. Rosso, M. G. Donelli, G. Franchi and S. Garattini, Effect of Triton WR 1339 on cancer dissemination and metastases. *Europ. J. Cancer* **5,** 77 (1969).
10. R. Rosso, V. Palma and S. Garattini, A model of a cerebral tumor for studies in cancer chemotherapy. *Experimentia (Basel)* **22,** 62 (1966).
11. G. Franchi, I. Reyers-Degli Innocenti, R. Rosso and S. Garattini, Lymph-node metastases after intratibial transplantation of tumors. *Int. J. Cancer* **3,** 765 (1968).
12. A. S. Ketcham, H. Wexler and J. P. Minton, Experimental study of metastases. *J. Amer. med. Ass.* **198,** 157 (1966).
13. H. Wexler, Accurate identification of experimental pulmonary metastases. *J. nat. Cancer Inst.* **36,** 641 (1966).
14. R. Rosso, M. G. Donelli and S. Garattini, Studies on cancer dissemination. *Cancer Res.* **27,** 1225 (1967).
15. M. G. Donelli, R. Rosso and S. Garattini, Quantitative studies on cancer dissemination. *Cancer Res.* **29,** 414 (1969).
16. G. Biozzi, B. Benacerraf and B. N. Halpern, The granulopectic activity of the reticuloendothelial system. II. The kinetics of the granulopectic activity of the reticuloendothelial system in relation to the dose of carbon injected, relationship between the weight of the organs and their activity. *Brit. J. exp. Path.* **34,** 441 (1953).
17. S. Nordling, K. Penttinen and E. Saxén, The effects of different methods of washing drying and sterilizing glass surfaces on cell attachment and growth behavior. *Exp. Cell. Res.* **37,** 161 (1965).
18. M. Terni and G. B. Lo Monaco, Coltura continua di cellule derivate da embrione umano. *Sperimentale* **108,** 77 (1958).
19. F. Nuzzo, Variazioni nel numero modale di cromosomi durante la fase iniziale di subcultura del ceppo EUE. *Atti. Ass. Genet. It.* **10,** 327 (1965).
20. H. Eagle, Utilization of dipeptides by mammalian cells in tissue culture. *Proc. Soc. exp. Biol. (N.Y.)* **89,** 96 (1955).
21. H. S. Reinhold, Quantitative evaluation of the radiosensitivity of cells of a transplantable rhabdomyosarcoma in the rat. *Europ. J. Cancer* **2,** 33 (1966).
22. H. Eagle, Propagation on a fluid medium of human epidermoid carcinoma strain. *Proc. Soc. exp. Biol. (N.Y.)* **89,** 362 (1955).
23. G. G. Rose, Biologic crystals and particles produced in tissue culture. *Cancer Res.* **23,** 279 (1963).
24. J. S. Gelzer and P. Loustalot, Biological and chemotherapeutic studies on primary mammary tumors in C_3H and C_3HO mice. *Int. J. Cancer* **2,** 179 (1967).

25. G. Franchi, I. Reyers-Degli Innocenti, S. Garattini, O. Alfieri, G. Cademartiri and G. Ottaviani, Lymphatic dissemination of cancer cells in mice transplanted intratibially with Ehrlich carcinoma. *Lymphology* In press (1971).
26. G. Franchi and A. W. van Bekkum, Effect of local X-irradiation on the dissemination of Ehrlich carcinoma transplanted intracerebrally. *Europ. J. Cancer* **6,** 173 (1970).
27. J. C. Fisher, W. R. Grace and J. A. Mannick, The effect of nonspecific immune stimulation with corynebacterium parvum on patterns of tumor growth. *Cancer (Philad.)* **26,** 1379 (1970).
28. A. Lamensans, C. Stiffel, M. F. Mollier, M. Laurent, D. Mouton and G. Biozzi, Effet protecteur de corynebacterium parvum contre la leucémie greffée AKR. Relations avec l'activité catalasique hépatique et la fonction phagocytaire du système réticulo-endothélial. *Rev. franç. Etud. clin. biol.* **18,** 773 (1968).
29. M. F. A. Woodruff and J. L. Boak, Inhibitory effect of injection of corynebacterium parvum on the growth of tumour transplants in isogenic hosts. *Brit. J. Cancer* **20,** 345 (1966).
30. R. L. Hirsh and A. Kellner, The pathogenesis of hyperlipemia induced by means of surface-active agents. I. Increased total body cholesterol in mice given Triton WR 1339 parenterally. *J. exp. Med.* **104,** 1 (1956).
31. R. L. Hirsh and A. Kellner, The pathogenesis of hyperlipemia induced by means of surface-active agents. II. Failure of exchange of cholesterol between the plasma and the liver in rabbits given Triton WR 1339. *J. exp. Med.* **104,** 15 (1956).
32. M. Friedman, S. O. Byers, K. Omoto, W. Kayoshi and B. Tsai, Mechanism underlying hypercholesteremia induced by Triton WR 1339. *Amer. J. Physiol.* **191,** 551 (1957).
33. E. E. Cliffton, D. Agostino and K. Minde, The effect of hyperlipemia on pulmonary metastases of Walker 256 carcinosarcoma in the rat. *Cancer Res.* **21,** 1062 (1961).
34. M. Zimmerman and T. M. Devlin, The effect of Triton WR 1339 on the metabolism of KB cells in culture. *Fed. Proc.* **26,** 513 (1967).
35. G. W. Miller and B. W. Janicki, Effect of Triton WR 1339 on malignant and normal cell cultures. *Cancer Chemother. Rep.* **39,** 1 (1964).
36. G. W. Miller and B. W. Janicki, Further studies on selective cytotoxicity of Triton WR 1339. *Cancer Chemother. Rep.* **52,** 243 (1968).
37. K. Karrer, S. R. Humphreys and A. Goldin, An experimental model for studying factors which influence metastasis of malignant tumors. *Int. J. Cancer* **2,** 213 (1967).
38. L. A. Carlson, Determination of serum triglycerides. *J. Atheroscler. Res.* **3,** 334 (1963).
39. W. R. Bloor, The determination of cholesterol in blood, *J. biol. Chem.* **24,** 227 (1916).
40. E. van Handel, D. B. Zilversmit and B. Kirk, Micromethod for the direct determination of serum triglycerides. *J. Lab. clin. Med.* **50,** 152 (1957).

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Growth Fraction, Resistance, Schedule–Doubling Time Relationship Sequential versus Simultaneous Combinations, as evaluated by a Mathematical Model of Response to Chemotherapy*

MICHEL DUCHATELLIER† and LUCIEN ISRAEL‡
†*Laboratoire de Physique des Structures et Systèmes Biologiques (Prof. Barraud)*, 12, *rue Cuvier, Paris*, V^e^, *France*
‡*Consultation de Médecine et Groupe d'Etudes et Recherches Cancérologiques Centre Hospitalier Universitaire Lariboisière*, 2, *rue Ambroise-Paré, Paris* X^e^, *France*

Abstract—*It has been shown previously* [6] *that the growth of tumours with a constant doubling time may be described by an equation linking the growth fraction and cellular losses. It is shown in this paper that analysis of chemotherapeutic response of measurable tumours may lead to the quantitative evaluation of the growth fraction and of the resistant population to the drug employed. The mathematical model describing the response also allows decisions to be made about the connection between schedule and doubling time, and about the superiority of a simultaneous combination over a sequential one. The parameters employed in such a model may be measured and used by the therapist in clinical cases in order to optimize the treatment.*

WE WERE able to show in previous clinical and theoretical papers: (1) that the doubling time of human solid tumors is constant during the clinical, observable period, as stated by Collins and many other investigators [1–3]; (2) that it is possible to deduce from this fact a growth equation which links the growth fraction, the cellular losses, the observed doubling time and the duration of the cell cycle [4]; (3) that it is possible on growth curves of treated measurable human tumors to evaluate the initial percentage of resistant cells against the drugs employed for the treatment. This evaluation can be performed only when all the sensitive cells have been destroyed by the treatment [4].

These important parameters have been also evaluated by means of other mathematical models, such as the ones developed by Steel for growth fraction [5, 6], by Bagshawe for cellular losses [7] and Priore for response to chemotherapy and resistance [8].

We have tried in this paper to develop a single model including many parameters important to the therapi. t and to use it in an attempt to help make decisions about schedules and combinations.

1. DESCRIPTION OF A MODEL INCLUDING THE GROWTH FRACTION, THE PERCENTAGE OF RESISTANCE TO A GIVEN TREATMENT AND THE DOUBLING TIME

In all the following equations, c stands for the growth fraction ($0<c<1$) and r for the fraction n'/n of the resistant cells against a given drug over the total number of cells at time t.

(1) Response to one drug effective against all the phases of the cell cycle. At time t_0, there

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are n_0 cells, some sensitive and some resistant:

$$n_0 = n_0.r+n_0.(1-r). \quad (1)$$

In both groups, some cells are proliferating and some are not, which leads to:

$$n_0 = n_0.r.c+n_0.c.(1-r)+n_0.r.(1-c) \\ +n_0(1-r)\ (1-c). \quad (2)$$

One shot of the drug can then kill at maximum:

$$n_0.c.(1-r) \text{ cells}, \quad (3)$$

so that after one shot, the number of living cells is at maximum:

$$n'_0 = n_0.[r+(1-r).(1-c)] \quad (4)$$

and after the elimination of the destroyed cells, the growth of the tumor is described by the equation:

$$n = n'_0.2^{t/DT}, \quad (5)$$

where t is the time at which there are n cells and DT the doubling time.

If then a second shot of the same drug is given at time t_1, and assuming (see Discussion) that c remains constant, the number of remaining cells is at maximum:

$$n'_1 = n_0[r+(1-r)\ (1-c)^2].2^{t_1/DT} \quad (6)$$

and the growth equation becomes:

$$n = n_0[r+(1-r).(1-c)^2].2^{t/DT}, \quad (7)$$

where the origin of t is t_0, when the first shot was given.

It is also possible to write the same equation not in relation to the number of cells, but in relation to the diameter of the tumor, a value the clinician can measure. It gives:

$$d = d_0[r+(1-r).(1-c)^2]^{1/3}.2^{t/3DT}. \quad (8)$$

In the schematic example of Fig. 1, a measurable tumor is treated by means of a shot every 15 days, and then measured every 15 days, 10 days after each shot. Values for d, d_0 and d_1 are read directly on the curve.

It can be seen that these values are linked by the following equations:

$$A = \frac{d}{d_0.2^{t_0/3DT}} = [r+(1-r).(1-c)]^{1/3} \quad (9)$$

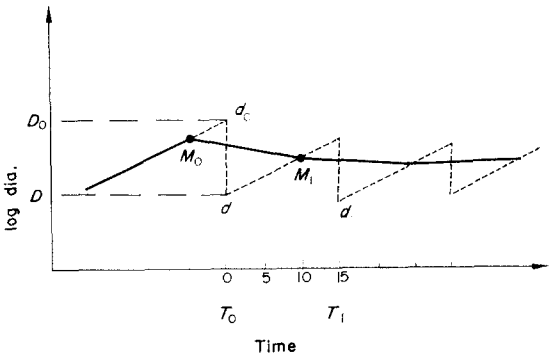


Fig. 1. Knowing the spontaneous growth curve and the clinical measurement it is possible to obtain by extrapolation the values of d_0, d *and* d_1 *and then by means of the equations* (14) *and* (15), *the values for* r *and* c.

$$B = \frac{d_1}{d_0.2^{t_1/3DT}} = [r+(1-r).(1-c)^2]^{1/3}. \quad (10)$$

Then, knowing the values for A and B by direct measurement of d, d_0 and d_1, it becomes possible to calculate the values for c and r by means of the following equations:

$$A' = A^3 = r+(1-r).(1-c) \quad (11)$$

$$B' = B^3 = r+(1-r).(1-c)^2 \quad (12)$$

$$A'-B' = (1-r).(1-c).c \quad (13)$$

$$c = 1-\frac{A'-B'}{1-A'} \quad (14)$$

$$r = 1-\frac{A'-B'}{c(1-c)}. \quad (15)$$

It is not then necessary, as in our previous model, to wait for the entire destruction of sensitive cells in order to evaluate r, as it appears that this evaluation can be deduced from the response to the second shot of chemotherapy.

(2) The above model can be easily modified if the drug employed is effective only against a given phase of the cell cycle. One has only to change c for $c' = c.p$, where p is the fraction of the cell cycle duration occupied by the corresponding phase.

On the other hand, if the drug is able to kill only a fraction, q, of the sensitive cells, according for example to the duration of an effective blood level, c' must be replaced by $c'q$. It is already clear that in fact only $c.q$ or $c'.q$ are read on the curves and represent the apparent growth fraction which is important to the clinician in order to optimize the treatment.

2. SEQUENTIAL VERSUS SIMULTANEOUS COMBINATIONS

For many practical reasons it is difficult to compare in clinical cases of solid tumors the effectiveness of sequential and simultaneous combinations. The above model provides a means to theoretically approach this problem.

The calculations have been made in examples sharing the following conditions: doubling time: 100 days; cell cycle: 2 days; $s = 8$ hr; growth fraction: 0·75; q: 0·8; r : 0·05 against each of the 3 drugs to be employed; time elapsed between 2 shots : 15 days; pretreatment diameter of the tumor : 2 cm.

(a) If the 3 drugs are equally effective against all the phases of the cell cycle it is not necessary to give the detail of the calculations. The term r in equation (8) has only to be changed for r^3. As it may be seen in Fig. 2, the total resistance,

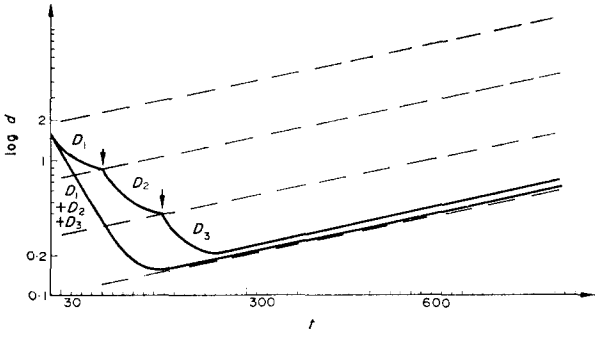


Fig. 2. The three drugs employed are effective against all the phases of the cell cycle. The higher curve represents the diameter of a tumor treated sequentially with the 3 drugs. The lower curve represents the changes induced in the same tumor by a simultaneous combination of the same tumor by a simultaneous combination of the same three drugs. The maximum decrease is more important and reached 90 days sooner with the simultaneous combinations.

when the drugs are used simultaneously, is reached after the 13th shot (195 days). If drugs are used in a sequential way, the total resistance is reached for each of them after 6 shots, so that the total resistance for the combination is reached after 18 shots (270 days). Besides the final response is poorer, as far as volume is concerned, in this last case.

(b) If we choose 3 different drugs (Fig. 3), one effective against all the phases, one effective against the S phase, and one effective against the M phase (with $m = 1/48°$ of the cell cycle), and the coefficients of resistance being respectively r_1, r_2 and r_3, the simultaneous combination give the following results. The number of sensitive cells before the first shot is:

$$n_s = n_0.c.s.(1-r_1.r_2)+n_0.c.m(1-r_1.r_3) + n_0.c.(1-s-m).(1-r_1). \quad (16)$$

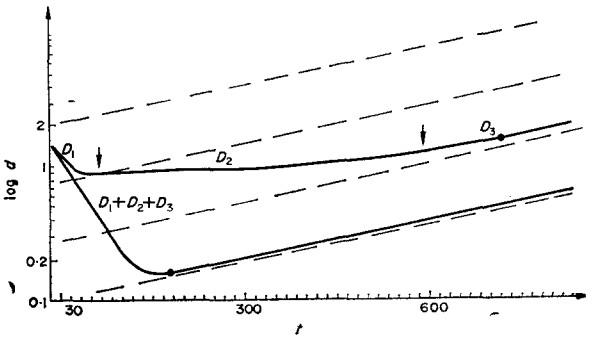


Fig. 3. Drug 1 is effective against all phases of the cell cycle; drug 2 against S phase; drug 3 against M phase. The higher curve represents the tumor changes induced by a sequential combination of the 3 drugs and the lower curve the tumor changes induced by a simultaneous combination. In this situation very similar to actually available drugs, the difference is highly significant.

If the fraction q of the above sensitive cells are killed by the first shot, there remain in the tumor $n_s(1-q)$ sensitive cells and the resistant cells.

The sensitive cells amount to:

$$n_s(1-q) = n_0.c.(1-q)\,[s.(1-r_1.r_2) + m(1-r_1.r_3)+(1-s-m).(1-r_1)] \quad (17)$$

and the resistant cells amount to:

$$n_r = n_0.r_1.r_2.r_3+n_0.(1-c).(1-r_1.r_2.r_3)+n_0.c.s.r_1.r_2+n_0.c.m.r_1.r_3+n_0.c.(1-s-m).r_1-n_0.c.r_1.r_2.r_3. \quad (18)$$

The same calculations can be made for each shot, and the results appear on Fig. 3. The total resistance is reached after 14 shots, a time at which the whole tumor has reached the diameter of the "resistant" tumor. This can be shown also by the following calculations.

The resistance against the combination of the 3 drugs is:

$$r = 0{\cdot}05^3 = 125\times10^{-6} \quad (19)$$

and at time t_0 the number of resistant cells is:

$$r.n_0 = 125\times10^{-6}.n_0. \quad (20)$$

The diameter of the "resistant tumor", d, is:

$$d/d_0 = (n/n_0)^{1/3} = r^{1/3} \quad (21)$$

$$d = d_0.(r)^{1/3} \quad (22)$$

$$d = 2.(125\times10^{-6})^{1/3} = 0{\cdot}1 \text{ cm}, \quad (23)$$

which is the value obtained by extrapolating back the resistant portion of the growth curve.

As for the sequential combination, it can be seen graphically on Fig. 3 that it is not effective enough to kill all the theoretically sensitive cells, this being due to the fact that the drug D_2 effective during the S phase can kill only in the kinetic model choosen here, a small fraction of the sensitive cells, not to speak of the drug D_3, effective only during the M phase.

3. SCHEDULE–DOUBLING TIME RELATIONSHIP

If we consider the case of one drug, the diameter of the resistant population of cells is the following:

$$d = d_0[r+(1-r).(1-c)^x]^{1/3}.2^{t/3DT} \qquad (24)$$

the resistance being reached, when the term $(1-r).(1-c)^x$ is very small, i.e. for example $= r/10$.

x then represents the minimum number of shots to be given in order to kill all the sensitive cells and to obtain an entirely resistant tumor. It then becomes possible to evaluate the maximum delay between two shots with respect to one condition: that the total number of resistant cells be at most equal to the population of tumor cells when the treatment was initiated.

This condition is expressed by the following relation:

$$d_0.[r+(1-r).(1-c)^x]^{1/3}.2^{t/3DT}<d_0,$$

which gives:

$$t < \frac{DT.\ln\,(1/r)}{\ln 2}.$$

It follows that the maximum delay between 2 consecutive shots is:

$$t/x = \frac{(1/x).DT.\ln\,(1/r)}{\ln 2}.$$

If we take $r = 0{\cdot}05$, $c = 0{\cdot}75$ and $q = 0{\cdot}8$, the calculations show that an entirely resistant tumor of the same diameter as the initial one is reached after 4·2 doublings, providing that the delay between 2 shots is: 0·7 doubling, with a drug effective against all the phases of the cell cycle; 0·08 doublings with a drug effective against the S phase and 0·02 doubling with a drug effective against the M phase. The same result is reached with a shot every doubling if the 3 drugs are given simultaneously.

4. DISCUSSION

(a) The very fact of setting up and discussing models of tumor growth and of response to chemotherapy implies that the real phenomena are unknown and that one has to make various hypotheses and assumptions. The main assumption in the above study is that, despite variability in local conditions, the growth fraction remains constant, that is chemotherapy does not induce major changes in the respective percentages of dividing and not dividing cells. In fact, it is known that this is not exactly true. After cell killing has occurred, there is more space left within the tumor mass, and more metabolites available, and changes in vascularisation, so that an acceleration of the growth rate takes place during a few days, due to an increase in growth fraction as well as a decrease in the duration of the cell cycle [9]. But, we are making the assumption that, in the long run, the growth fraction has to remain constant within certain limits, because of the fact that the doubling time of a measurable tumour resumes its previous value, as soon as the treatment is interrupted [3, 9, 10]. So, a certain kind of regulating mechanism seems to exist, which keeps grossly constant the growth fraction during some periods of time. Our model is based upon such a possibility, which remains to be proved.

(b) Secondly, we have to assume that the killed cells are eliminated from the tumor in a few days. If the calculations of Iversen [11] are true, that is if the duration of this disappearance is about 2 days, then measuring the diameter 10 days after the shot, as in our model, may be a safe procedure.

We never registered in the observed clinical curves a lengthening in the doubling time, which would be implied by the remaining of dead cells in the tumor [7].

(c) As stated by van Peperzeel [9], the growth rate is probably faster during the few hours following a shot. But, as seen in Fig. 4, the model choosen represents, probably in a correct way, the reality due to the long delay between the shot and the measurement.

(d) Anyhow, the parameters c and r must be considered as apparent parameters. They can describe some events registered by the clinician but their connection with the real parameters is unknown.

(e) It would be probably safer, as stated by Priore [8] to take into account the duration of the effective blood level of drugs. But, on one hand, one could question the parallelism between the tumor level and the blood level, on the other hand, what is important for the model described is the absolute number of the destroyed cells due to the drugs chosen by the

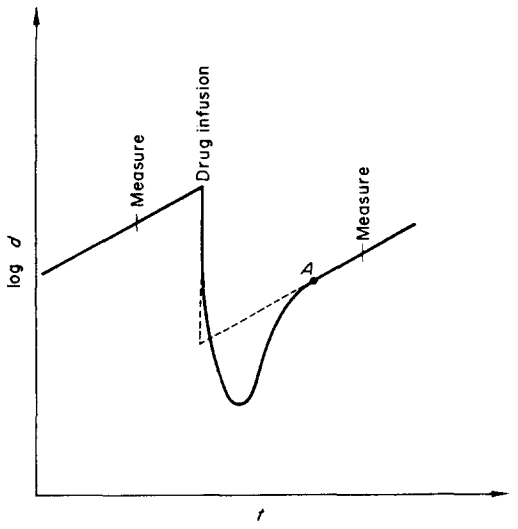


Fig. 4. The interrupted line represents the mathematical model and the plain line the actual events. From point A the mathematical model describes the real curve.

therapist, and not the relation between the dose given and the percentage of cell killing.

(f) According to the above considerations, it must be stated that the present model has to be tested on real clinical curves before to be taken as a valid one. Such tests are possible, since r can be independently calculated by a graphic method, as shown before [4].

(g) Anyhow, it is of some interest to find, when applying this model to real conditions, that simultaneous combinations are more effective than sequential ones and that there is a connection between the doubling time and the best theoretical schedule.

(h) It can be clearly seen that such a model can be employed in order to calculate the theoretical requirements for total eradication. This is being done and the results will be published in another paper.

To conclude, it is possible, in measurable tumors with known doubling times to evaluate from the response to cytostatic agents both the resistant population and the growth fraction, or, more precisely, the "apparent" parameters that are important to the therapist. The proposed mathematical model also allows some progress to be made about the question of schedule–doubling time relationship and about the choice between simultaneous or sequential combinations. Whether these combinations may apply to unmeasurable cases remains to be shown.

REFERENCES

1. V. P. Collins, R. K. Loeffler and H. Tivey, Observations on growth rates of human tumors. *Amer. J. Roentgenol.* **76,** 988 (1956).
2. M. Schwartz, A biomathematical approach to clinical tumor growth. *Cancer* **14,** 1272 (1961).
3. L. Israel and P. Chahinian, Le temps de doublement des cancers bronchiques. *Rev. franç. Etud. clin. biol.* **14,** 703 (1969).
4. L. Israel, M. Duchatellier and P. Chahinian, Les tumeurs mesurables bronchopulmonaires comme moyen d'évaluation de la chimiorésistance et du coefficient de prolifération cellulaire. Conséquences thérapeutiques. *Presse méd.* **25,** 78 (1970).
5. G. Steel, Cell loss as a factor in the growth rate of human tumor. *Europ. J. Cancer* **3,** 381 (1967).
6. G. Steel, K. Adams and J. Barret, Analysis of the cell population kinetics of transplanted tumours of widely differing growth rate. *Brit. J. Cancer* **20,** 784 (1966).
7. K. D. Bagshawe, Tumour growth and antimitotic action: the role of spontaneous cell losses. *Brit. J. Cancer* **22,** 698 (1968).
8. R. Priore, Using a mathematical model in the evaluation of human tumor response to chemotherapy. *J. nat. Cancer Inst.* **37,** 635 (1966).
9. N. A. van Peperzeel, *Patterns of Tumour Growth after Irradiation*, Vol. 1, (Edited by Drukherig van Denderen). Groningen (1970).
10. P. Chahinian and L. Israel, Survival gain and volume gain. Mathematical tools in evaluating treatments. *Europ. J. Cancer* **5,** 625 (1969).
11. O. H. Iversen and R. Bjerknes, *Kinetics of epidermal reaction to carcinogens in Norwegian Monographs on Medical Science.* Scandinavian University Books (1963).

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Growth of Sarcoma 180 in Splenectomized Mice Bearing Diffusion Chambers Containing Spleen or Tumor Cells*

LIA RUMI,† CHRISTIANE DOSNE PASQUALINI‡ and S. L. RABASA§
Department of Experimental Leukemia, Instituto de Investigaciones Hematologicas, Academia Nacional de Medicina, Las Heras 3092, *Buenos Aires, Argentina*

Abstract—*Sarcoma* 180 (*S*-180) *is* 100% *lethal in BALB mice and leads to spontaneous regressions in AKR, A and inbred Rockland mice. Splenectomy significantly increases the regression rate of S*-180 *in these strains when performed either* 7 *days before or after the tumor challenge. That this effect is specific is suggested by the fact that partial hepatectomy has no effect and that the growth of* 3 *other sarcomas, either syngeneic or allogeneic, remains unaffected by splenectomy. In BALB mice, diffusion chambers with* 0·22 *μ filters, enclosing viable syngeneic spleen cells or S*-180 *cells, introduced intraperitoneally at the time of splenectomy and* 7 *days before S*-180 *challenge, are both capable of reversing the effect of splenectomy. This is not obtained in control groups including either diffusion chambers enclosing cells from a different sarcoma or empty chambers. Hypothetically these data would indicate that S*-180 *cells trapped within the diffusion chambers would permit the elaboration of humoral antibodies in the absence of the spleen. Upon tumor challenge,* 7 *days later, the presence of these antibodies would alter the temporal relationship between the humoral and cellular immune responses, favoring progressive tumor growth.*

INTRODUCTION

SARCOMA 180 (S-180) is a non-specific murine tumor; it has proved to be 100% lethal in BALB mice, in our laboratory, while in Rockland, AKR, A and C57Bl mice, varying degrees of spontaneous regression have been observed, leading to the development of long-lasting immunity to the tumor. The intrasplenic route for S-180 transplantation favors the development of large tumors and metastases, not observed after subcutaneous implantation. Once the animals have rejected S-180, reimplantation, even in the spleen, does not lead to tumor development; this secondary immunity is specific for S-180 [1]. On the other hand, removal of the spleen has been shown to increase the incidence of tumor regression [2–4]. The data described in the present report confirm this effect of splenectomy in different strains of mice, determining its specificity, and indicate that spleen cells within diffusion chambers, impermeable to cells, were capable of restoring the normal response; unexpectedly, this was also obtained with S-180 cells within the diffusion chambers.

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†Fellow of LALCEC (Liga Argentina para la Lucha contra el Cancer).

‡Member of the Research Career, CNICT.

§From Instituto de Investigaciones Medicas, Rosario, Argentina.

MATERIAL AND METHODS

S-180 was supplied to us in 1957 in the Rockland non-inbred strain. Since then, our colony of Rockland mice has been inbred into 5 sublines, by sib matings, for 24 generations. The solid form of S-180 has been maintained by serial transplantation both in subline 3 of

Rockland mice (Rockland/3) and in the BALB inbred strain. The inbred AKR and A mice were transplanted with S-180 from the serial cell line maintained in the BALB strain. In all cases, pieces of the tumor were uniformly cut and transplanted subcutaneously by trocar into the axillary region of the animals. The tumor growth was estimated by measuring 2 of its diameters with a caliper, every 5 days. Four other serially-transplanted tumors which originated in our colony were used as controls; these included 3 fibrosarcomas induced in the BALB strain, S11, S12 and S15 [5] and a spontaneous fibrosarcoma which developed in a Rockland/3 mouse.

The animals used in these experiments were from 2 to 4 months old, of both sexes, and belonged to the following inbred strains: BALB, AKR, A; Rockland/3, Rockland/5 and their F1 hybrids.

Splenectomy was performed through a left-flank incision under anesthesia. It was carried out 7 days *before* the tumor implantation except for one group of Rockland/3 mice in which the spleen was removed 7 days *after* the tumor challenge. Partial hepatectomy was carried out by removal of 75% of the liver in a control group of Rockland/3 mice.

The diffusion chambers consisted of a plastic (lucite) ring covered at each end with a cellulose Millipore filter of 0·22 μ pore [6]. Suspensions of 2×10^5 viable syngeneic spleen or tumor cells in tissue-culture medium 199 were incorporated into the chambers. Both tumor and spleen-cell homogenates contained approximately 90% of viable cells, as determined by the Trypan-Blue exclusion method. The diffusion chambers were introduced into the peritoneal cavity of BALB mice at the time of the removal of the spleen and 7 days prior to the tumor challenge. These chambers proved to be impermeable to cells since none of the animals bearing chambers containing tumor cells ever developed tumors. Furthermore, in other experiments in which syngeneic leukemic cells were introduced into these diffusion chambers, no animal ever developed leukemia.

RESULTS

Murine S-180 is a malignant tumor which grows rapidly in all strains of mice. As it can be seen in Table 1, S-180 was 100% lethal in the BALB strain, while in the AKR, A and Rockland/3 strains a spontaneous complete and permanent reabsorption of the tumor was observed in a number of animals. Splenectomy 7 days before subcutaneous tumor challenge led to a significant increase in tumor regression in all 4 strains, ranging from 28 to 100% of survival. A group of 18 Rockland/3 mice splenectomized 7 days *after* the tumor challenge (when the tumor had already started to grow) presented a regression rate of 55% similar to the 56% of survival in those splenectomized 7 days *before* the tumor challenge. Hepatectomy, by surgical removal of 75% of the liver in another group of 35 Rockland/3 mice, did not increase the incidence of survival above that of the controls, only 2 mice surviving. This demonstrates that the surgical removal of most of the liver does not have an effect comparable to that of splenectomy.

Table 1. *Effect of splenectomy on the growth of S-180 and of other tumors in different strains of mice*

Tumor	Strain	Splenectomy* no./ total	%	Controls no./ total	(%)
S-180	BALB	13/47†	28§	0/23	0
	AKR	15/40	38§	1/41	2
	A	13/13	100§	9/14	64
	Rockland/3	85/153	56§	28/138	20
		19/34	56§	4/28	14
		10/18‡	55§	3/18	17
S11	BALB	0/16	0	0/16	0
S12		6/21	29	5/20	25
S3	Rockland/3	1/52	2	2/55	4
	Rockland/5	9/26	35	4/11	36
	F1/3,/5	0/17	0	2/18	11

*Animals splenectomized 7 days *before* the tumor challenge.

†Number of survivors/total number of tumor-bearing mice.

‡Animals splenectomized 7 days *after* the tumor challenge.

§Significantly different from the controls, $P < 0{\cdot}001$, as estimated by X_2.

The data summarized in Table 1 also include the comparative results obtained with 3 fibrosarcomas transplanted subcutaneously 7 days after splenectomy; these included 2 syngeneic tumors transplanted in the BALB strain (S11 and S12) and 1 syngeneic for Rockland/3 mice (S3) also transplanted as allogeneic graft in Rockland/5 and in their F1 hybrids. In no case was there any difference between these experimental groups and their respective non-splenectomized controls, thus showing that in our experimental model only S-180 could evoke a stimulating effect of splenectomy on tumor regression.

The data recorded in Table 2 summarize the response to S-180 transplant of splenectomized BALB mice bearing diffusion chambers containing either syngeneic spleen or tumor cells.

Table 2. *Regression of S-*180 *in splenectomized BALB mice bearing diffusion chambers containing spleen or tumor cells*

Group	Tumor challenge	Diffusion* chambers	Survival			Tumor size‡ cm² ± S.E.
			no./total	%	days†	
	Splenectomy§					
1	S-180	—	11/23¶	50‖	66‖	3·50 ± 0·47‖
2	S-180	Spleen cells	0/12	0	44	4·52 ± 0·66
3	S-180	S-180 cells	0/18	0	40	4·55 ± 0·77
4	S-180	S15 cells	10/14	71‖	55‖	3·50 ± 0·15‖
5	S-180	Empty	13/16	81‖	71‖	3·05 ± 0·86‖
	Non-splenectomized controls					
6	S-180	—	0/24	0	39	4·87 ± 0·54
7	S-180	Empty	0/18	0	34	5·35 ± 1·20
8	S-180	Spleen cells	0/6	0	34	4·45 ± 0·39
9	S-180	S-180 cells	0/24	0	35	4·27 ± 0·33

*Containing 2×10^5 cells within 0·22 μ millipore filters, introduced intraperitoneally at the time of splenectomy.
†Average day of death from tumor (all survivors excluded).
‡30 days after subcutaneous implantation.
§7 days before tumor challenge.
¶Number of survivors/total number of tumor-bearing mice.
‖Significantly different from the controls, $P < 0{\cdot}01$, estimated by X_2 and t statistical tests.

It can be seen that there was a 50% of permanent tumor regression in splenectomized mice (Group 1) with an average of 66 days of survival for those dying of tumor. The presence of 2×10^5 syngeneic spleen cells in the diffusion chamber reversed this result, all the animals dying in an average of 44 days (Group 2). The same result was obtained with diffusion chambers containing a suspension of 2×10^5 S-180 cells, the 18 mice dying in an average of 40 days (Group 3). These 2 latter groups behaved in the same way as the non-splenectomized controls challenged with S-180 (Group 6) in which all 24 animals died in an average of 39 days. When the same number of cells from a different tumor, S15, syngeneic for the BALB strain were enclosed in the diffusion chambers introduced i.p. into splenectomized mice (Group 4) 71% of the animals survived, the others dying in an average of 54 days; these results are comparable to those obtained with empty chambers (Group 5) where 81% of the animals survived, the others dying in an average of 71 days; there was no significant difference between these 2 groups and the splenectomized mice not bearing diffusion chambers (Group 1). On the other hand, Groups 1, 4 and 5, gave results significantly different from those obtained in all other groups challenged with S-180, including non-splenectomized controls with or without empty diffusion chambers (Groups 6 and 7). Moreover, neither spleen cells nor tumor cells within diffusion chambers had any effect on tumor growth in non-splenectomized controls (Groups 8 and 9). Thus, the results presented in Table 2 show that the resistance of splenectomized mice to the growth of S-180 can be reversed by the implantation of diffusion chambers containing S-180 or syngeneic spleen cells, but not by empty chambers or chambers containing S15 cells.

Both the spleen and tumor cells within the diffusion chambers remained viable and increased in number up to the 30th day. Chambers were removed from the peritoneal cavity 1–10 months after their implantation and their cellular content, in the form of a white clot, was homogenized and injected into BALB mice; these animals all died within 30 days as a result of vigorous tumor growth.

As far as the tumor size is concerned, the measurements by caliper in cm² obtained every 5 days did not show any marked difference between groups until the 30th day; for this reason only this value is included in Table 2. It must be pointed out that the tumors grew to the same size in all groups up to the 20th day; only at that time did they begin to regress in Groups 1, 4 and 5, which on the 30th day presented significantly smaller tumors than their respective controls, even though the animals bearing reabsorbed tumors were not taken into account.

These experiments ended when the animals had either died of tumor or survived after complete tumor reabsorption. However, it is interesting to note that among the survivors, which were presumably immune to S-180, out of 35 splenectomized BALB mice, 20 or 57% developed leukemia as compared to 11% in splenectomized controls, both with an average latency of 18 months. The incidence of leukemia

was also significantly increased in S-180 presumably immune Rockland/3 survivors, up to 35% (34/95) in intact and 50% (18/37) in splenectomized mice as compared to 6% in the controls. These results confirm previous experiments [7, 8] describing the development of *Leukemia 180* in S-180 immune Rockland mice, extending them to BALB mice. The development of leukemia 180 does not appear to be spleen-dependent; in BALB mice it was possible to obtain it only in splenectomized animals because S-180 is 100% lethal in non-splenectomized animals.

DISCUSSION

The investigation described herein is concerned mainly with the effect of the removal of the spleen on the growth of S-180. In the BALB strain in which this tumor is 100% lethal, splenectomy led to tumor reabsorption and survival of up to 50% of the animals. In other strains, such as AKR, A and Rockland (inbred), in which there is a background of spontaneous regression, a significant increase in survival also followed splenectomy. Surgery by itself was not responsible for this effect since partial hepatectomy did not alter tumor growth.

Removal of the spleen had the same effect when carried out 7 days *before* or 7 days *after* the tumor challenge, in spite of the fact that in the latter case the animals already had tumors of 1 cm^2. It must be pointed out that up to the 20th day after the tumor challenge, it was impossible to determine which animal would reabsorb the tumor, the size of which was comparable in all groups. On the basis of previously published information[3, 9], 20 days after the S-180 implantation the animals presumably have significant levels of humoral anti-S-180 antibodies. The presence of antibody against S-180 has been demonstrated in the serum of mice with spontaneously-regressing tumors [10] while thymectomy shortly after birth prevented S-180 regression [11].

Only S-180 transplants and not syngeneic or allogeneic passages of 3 fibrosarcomas were affected by splenectomy. This would be in accordance with the observations of Möller [12] that non-specific tumor sublines are particularly susceptible to regression and of Bradner and Pindell [13] who suggested that immunogenetic differences between tumor and host may be largely responsible for tumor regression.

The increased resistance of splenectomized mice to S-180 challenge was first observed by Old *et al.* [3] and later confirmed by Pasqualini *et al.* [4] and Ferrer and Mihich [2]. As an explanation, it was suggested that this resistance may be the result of a reduction in humoral antibodies which in the intact host facilitate the growth of S-180 through a process of self-enhancement [2, 14]. Moreover, Ferrer [12] showed that, in the induction of tumor enhancement with S-180 antisera, the presence of the spleen was essential; however, he also demonstrated that when a small dose of antiserum was injected the incidence of enhancement was lower but could be obtained in the absence of the spleen [9].

The use of intraperitoneal diffusion chambers impermeable to cells led to interesting results: viable syngeneic spleen cells within these chambers were capable of inhibiting the effect of splenectomy and furthermore viable S-180 cells (and not cells of a different tumor nor empty chambers) had the same effect as spleen cells. These results are difficult to explain. It could be speculated that the presence of syngeneic spleen cells would be capable of restoring enough splenic function to ensure the humoral antibody response needed for sustained tumor growth. However, it is more difficult to imagine by what mechanism S-180 cells within diffusion chambers are capable of a similar restoring effect in an animal without a spleen. This would lead to the suggestion that if humoral antibodies are responsible for tumor enhancement, the spleen would not be the only site of their production. It could therefore be speculated that S-180 cells trapped within diffusion chambers introduced intraperitoneally 7 days before the tumor challenge, would elicit the production of circulating antibodies. At the time of tumor implantation the animals would already possess enhancing antibodies. These antibodies being available before the development of an effective cellular immune response would ensure progressive tumor growth.

REFERENCES

1. C. D. Pasqualini, E. A. D. Holmberg, S. L. Rabasa and A. Pavlovsky, Inmunidad al Sarcoma 180. *Rev. Soc. Argent. Biol.* **36,** 403 (1960).
2. J. F. Ferrer and E. Mihich, Effect of splenectomy on the regression of transplantable tumors. *Cancer Res.* **28,** 1116 (1968).
3. L. J. Old, D. A. Clarke, B. Benacerraf and E. Stockert, Effect of prior splenectomy on the growth of S180 in normal and Bacillus Calmette-Guérin infected mice. *Experientia* **18,** 335 (1962).

4. C. D. PASQUALINI, E. A. D. HOLMBERG, A. PAVLOVSKY and S. L. RABASA, Aumento de inmunidad al sarcoma 180 por la esplenectomia. VI Congreso de la Asoc. Latinoamericana de Ciencias Fisiologicas, p. 79. Chile (1964).
5. C. D. PASQUALINI, F. SAAL and S. L. RABASA, Leucemia murina. *Medicina (B. Aires)* **28,** Suppl.1, 116 (1968).
6. E. E. CAPALBO, J. F. ALBRIGHT and W. E. BENNETT, Evaluation of the diffusion chamber culture technique for the study of the morphological and functional characteristics of lymphoid cells during antibody production. *J. Immunol.* **92,** 243 (1964).
7. C. D. PASQUALINI, E. A. D. HOLMBERG, S. L. RABASA and A. PAVLOVSKY, Immunity to Sarcoma 180 and the induction of leukemia. *Cancer Res.* **21,** 387 (1961).
8. C. D. PASQUALINI, E. A. D. HOLMBERG, S. L. RABASA and A. PAVLOVSKY, Leukemia induced by Sarcoma 180 and the development of reciprocal immunity. *Cancer Res.* **23,** 974 (1963).
9. J. F. FERRER, Role of the spleen in passive immunological enhancement. *Transplantation* **6,** 167 (1968).
10. B. SHOHAT and H. JOSHUA, Host defence mechanisms in the spontaneous regression of S180. *Europ. J. Cancer* **5,** 69 (1969).
11. J. F. FERRER and E. MIHICH, Dependence of the regression of Sarcoma 180 in Vitamin B_6 deficient mice upon the immunological competence of the host. *Cancer Res.* **27,** 456 (1967).
12. E. MÖLLER, Interaction between tumor and host during progressive neoplastic growth in histoincompatible recipients. *J. nat. Cancer Inst.* **35,** 1053 (1965).
13. W. T. BRADNER and M. H. PINDELL, Strain specificity of stimulated regression of Sarcoma 180. *Cancer Res.* **25,** 859 (1965).
14. J. F. FERRER, Enhancement of the growth of Sarcoma 180 in splenectomized and sham-operated AKR mice. *Transplantation* **6,** 160 (1968).

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Tumour Growth after Intravenous, Intraperitoneal and Subcutaneous Injection of Syngeneic Monodispersed Tumour-Cell Suspension

B. BOERYD, P. M. LUNDIN and K. NORRBY
Department I of Pathology, University of Göteborg, Sweden

Abstract—*The transplantability of monodispersed tumour cells was tested in two syngeneic murine tumour-host systems. A much higher number of tumour cells was required for progressive tumour growth after intravenous transplantation than after intraperitoneal or subcutaneous transplantation. Probably this may be accounted for by extensive and fairly rapid disintegration of the transplanted cells in the circulation. The cause of this is unknown, but such factors as micro-environmental requirements, immunological reactions and mechanisms related to allogeneic inhibition must be considered.*

THE NUMBERS of transplanted tumour cells necessary for progressive growth vary between tissues and between tumour-host systems [1]. A general experience is that large numbers of cells are needed for successful intravenous transplantation, and experimentally it has been shown that the number of metastases is related to the number of intravenously injected tumour cells [2]. Southam [3] demonstrated that a tumour cell dose given subcutaneously at a single site has a higher frequency of takes than an equal cell dose distributed among several implant sites. He suggested that the number of cells deposited locally may be a more important factor in development of metastases than the total number of disseminated cells. Successful tumour transplantation from a single cell has been made only with particular tumours, viz. some leukaemias and ascites tumours [4–6].

Experimentally the subcutaneous tissue and the peritoneum are often used for tumour transplantation. However, the spread of metastases to the peritoneum and via blood-borne tumour cells is also clinically important. The aim of this study was to compare the minimal number of monodispersed syngeneic tumour cells required for progressive growth after transplantation intravenously, subcutaneously and intraperitoneally.

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MATERIAL AND METHODS

The syngeneic tumours were the MC-induced sarcoma MCG1–SS in CBA mice [7, 8] and the spontaneous Melanoma B16 in C57BL/6J mice (Jax Mice Laboratory, Bar Harbor, Maine, U.S.A.). Cell suspensions were prepared from subcutaneously growing transplants using trypsin and DNAse [9, 10]. These suspensions were resuspended in medium 199 containing 10% syngeneic serum to prevent primary aggregation [10]. A cellular viability index between 0·85 and 0·90 was estimated by the trypan blue dye exclusion test. All suspensions were kept in ice water until just before use in order to retain high cellular viability and to reduce cell losses before injection [11]. The

suspensions were diluted to contain 10, 10^2, 10^3, 10^4 and 10^5 cells per dose of 0·05 ml. In parallel experiments using samples from the same suspension 3 groups of animals (4 or 5 in each group) received injections in a tail vein, intraperitoneally and subcutaneously in one groin. No pretreatment was given to the animals which were of similar ages (10–12 weeks) and of both sexes.

Animals with subcutaneous transplants were killed when the tumour reached a diameter of about 2 cm. Animals given intravenous and intraperitoneal transplants were autopsied and in all animals the presence of tumours was ascertained. Mice without tumour takes were observed for 5 months. While the possibility of dormant cells cannot be ruled out in the animals without takes, the period of observation was roughly as long as 5–10 times that of the mean survival time after the largest intravenous cell dose.

RESULTS

The number of takes and the survival time are given in Figs. 1 and 2. For progressive growth after intravenous injection 10^2–10^4 times as many cells were needed than after

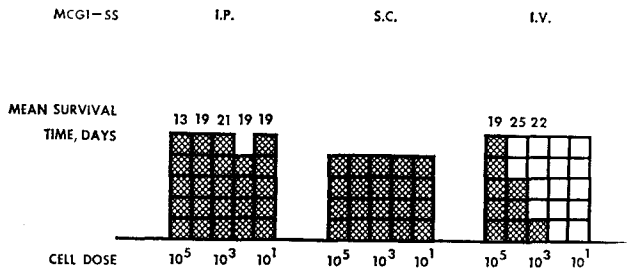


Fig. 1

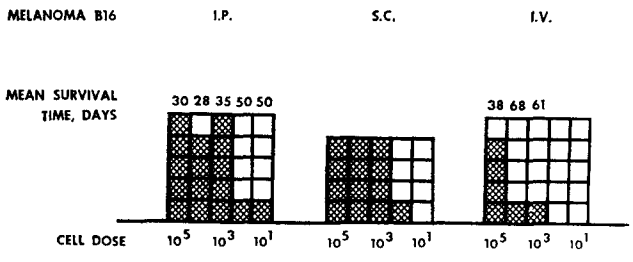


Fig. 2

injection by the other routes. The cell number needed for 100% takes in the subcutaneous tissue and the peritoneal cavity, respectively, was similar in each system, although approximately a hundred times more melanoma cells than MCG1–SS were necessary.

DISCUSSION

The present investigation shows that the subcutaneous tissue and the peritoneal cavity are more suitable milieux for the tumour cells to grow than is the intravascular space.

The number of cells required for successful intraperitoneal and subcutaneous transplantation of the MCG1 sarcoma was very low and this also indicates that the tumour cell suspension had a high viability. The reason why many more tumour cells are needed for growth after intravenous injection may depend on the fact that cells in the vascular bed may have difficulties to acquire a suitable micromilieu [12]. If close proximity of tumour cells in the vascular systems is necessary for proliferation this requirement must be more difficult to achieve in the systemic circulation than in the subcutaneous tissue. The similar minimal cell doses for successful transplantation subcutaneously and intraperitoneally is remarkable since the cells probably are more scattered in the peritoneal cavity than in the subcutaneous tissue.

Earlier investigations with enzymatically produced cell suspensions of the MCG1 sarcoma showed that a large percentage of intravenously injected tumour cells were trapped in the lungs but most of these cells were destroyed within the next few hours [13]. Similar results were obtained also with syngeneic ascites tumour cells (MCG1–AA) not exposed to trypsin. However, the same types of tumour cells injected subcutaneously survived at the injection site for much longer time and in a high percentage [14]. Thus the high cell dose required for progressive tumour growth after intravenous transplantation in the present study is consistent with a substantial cell loss.

The role of immunoligical factors in the defence against blood borne metastases is not elucidated. It can be assumed that these factors are different if the tumour cells are injected intravascularly, as in the present study, or if they are spontaneously released from a tumour which may have had time to sensitize the host. Tumour cells in the circulation may be more susceptible to the cytotoxic and phagocytic activities of bordering cells of the RES. A mechanism comparable to the allogeneic inhibition with some sort of a surveillance function eliminating abnormal cell variants may be important [15].

REFERENCES

1. W. H. Cole, G. O. McDonald, S. S. Robert and H. W. Southwick, *Dissemination of Cancer. Prevention and Therapy.* Appleton-Century-Crofts, New York (1961).
2. E. R. Fisher and B. Fisher, Experimental studies of factors influencing hepatic metastases. I. The effect of number of tumor cells injected and time of growth. *Cancer* **12,** 926 (1959).
3. C. M. Southam, Factors influencing the growth of tumour autotransplants. In *The Proliferation and Spread of Neoplastic Cells.* Williams and Wilkins, Baltimore (1968).
4. J. Furth and M. C. Kahn, The transmission of leukemia of mice with a single cell. *Amer. J. Cancer* **31,** 276 (1937).
5. K. Ishibashi, Studies on the number of cells necessary for the transplantation of Yoshida sarcoma. *Gann* **41,** 1 (1950).
6. G. Klein and E. Klein, Conversion of solid neoplasms into ascites tumours. *Amer. N.Y. Acad. Sci.* **63,** 640 (1956).
7. J. Mellgren, E. Bartholdson, B. Boeryd and K. Norrby, A spontaneously metastasizing 20-methylcholanthrene induced rhabdomyosarcoma and its transformation to ascites form in the CBA mouse. *Acta path. microbiol. scand.* **76,** 61 (1966).
8. B. Boeryd, P. M. Lundin, K. Norrby and U. Schelin, Ultrastructure of a mouse sarcoma in its solid, enzymatically dissociated and ascitic forms. *Int. J. Cancer* **3,** 283 (1968).
9. B. Boeryd, O. Eriksson, F. Knutson, P. M. Lundin and K. Norrby, On the viability of tumour cells in artificially produced suspensions. *Acta path. microbiol. scand.* **65,** 514 (1965).
10. K. Norrby, F. Knutson and P. M. Lundin, On the single cell state in enzymatically produced tumour cell suspensions. *Exp. Cell Res.* **44,** 421 (1966).
11. F. Knutson, P. M. Lundin and K. Norrby, Syngeneic serum and ascitic fluid in enzymatically produced tumour cell suspensions. I. Preservation of viability and single cell state during short-term storage. *Path. Europ.* **6,** 34 (1971).
12. L. Weiss, *The Cell Periphery, Metastasis and Other Contact Phenomena.* North-Holland, Amsterdam (1967).
13. B. Boeryd and P. M. Lundin, The fate of intravenously transfused, tritium-labelled sarcoma cells in an isologous system. *Acta path. microbiol. scand.* **67,** 303 (1966).
14. P. M. Lundin, B. Boeryd and F. Knutson, Distribution and disappearance of intravenously and subcutaneously injected tritium-labelled sarcoma cells in a syngeneic system. To be published.
15. K. E. Hellström and I. Hellström, Cellular immunity against tumour antigens. *Adv. Cancer Res.* **12,** 167 (1969).

Europ. J. Cancer Vol. 7, pp. 561–563. Pergamon Press 1971. Printed in Great Britain

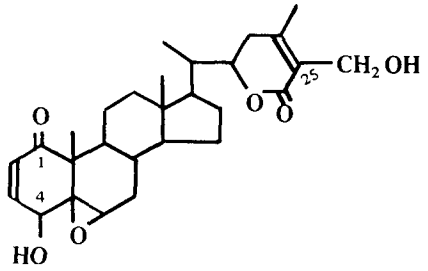
The Effect of Withaferin A on Plant Cells

B. SHOHAT and H. JOSHUA
The Clinical Laboratory, Beilinson Medical Centre, Petah Tikva, Israel

Abstract—*Withaferin A in a concentration of* 0·01–0·5% *in water inhibited the growth of Allium cepa roots. Furthermore, withaferin A induced c-tumor formation and lateral root formation on treated roots. Microscopical examination of withaferin A-treated root tip cells showed a clear arrest at metaphase as soon as* 2 *hr after treatment.*

INTRODUCTION

WITHAFERIN A $C_{28}H_{38}O_6$, a steroidal lactone obtained from Withania somnifera Dun, has been shown to have carcinostatic properties [1, 2]. More recently it was demonstrated that



withaferin A produced a mitotic arrest in the metaphase of dividing Ehrlich ascites carcinoma cells as well as in Hela cells [3–5]. In order to clarify further the effects of withaferin A on cell division the actions of this chemical on the structure and behaviour of root tip cells of Allium cepa was investigated.

MATERIAL AND METHODS

Small bulbs of Allium cepa were grown in tap water at 20°C in the dark. When roots were 1 cm long they were divided into several series of 2–4 bulbs each, placed into jars containing the following solution (1) tap water, (2) withaferin A in concentration of 0·01 to 0·5% in water, (3) colchicine 0·01% in water, (4) colchicine and withaferin A in concentration as above in water. These series of experiments were repeated three times. The solutions were changed every two days. Daily measurements of the Allium cepa roots were performed during a period of 14 days. At 2, 24 and 48 hr intervals several root tips from each experiment were carefully removed, fixed in Carnoy solution and stained with aceto-carmine. About 1000 cells were counted for each treatment. The mitotic index expresses the number of mitoses per 1000 cells counted.

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RESULTS

Treatment with withaferin A in a concentration of 0·01% highly inhibited the growth of Allium cepa roots 2 days after treatment (Fig. 1). The inhibition of root tip growth obtained by the highest concentration of

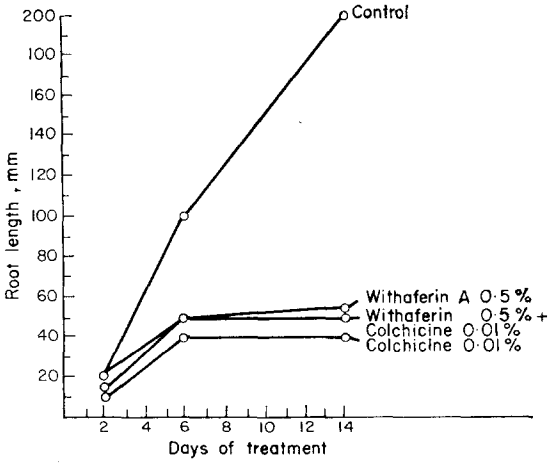


Fig. 2. Graph of the growth in length of roots treated by withaferin A 0·5%, *withaferin A and colchicine, and colchicine alone, as compared with control.*

withaferin A tested (0·5%) as compared to withaferin and colchicine, colchicine alone and control, is shown in Fig. 2. All points shown on reaction curves are the means of not less than three root tips per point.

Microscopical examination of aceto-carmine stained smears preparations of Allium cepa roots (Fig. 3) revealed numerous mitoses at metaphase with scattered chromosomes 2 hr after withaferin A treatment. Structural abnormalities such as aberrant chromosomes at anaphase as well as enlargement of cells and micronuclei were seen at the end of 48 hr of treatment. The mitotic index (Fig. 4) was elevated already 2 hr after withaferin A treatment, and a high percentage of cells in metaphase was still present after 24 hr. The mitotic index started to drop at the end of 48 hr, probably due to escape of mitotic inhibition, as suggested by the reappearance of anaphase figures at this time.

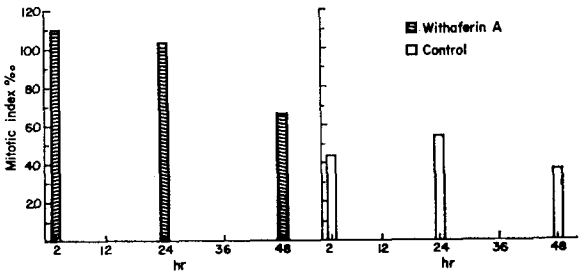


Fig. 4. Mitotic indices of Allium cepa roots exposed for 48 hr to 0·01% withaferin A as compared to control.

Twelve days after exposure to withaferin A set at the highest concentration used (0·5%), numerous lateral roots appeared (Fig. 5). Three days later, enlargements of root tips were seen (Fig. 6), similar to the so called c-tumours obtained after colchicine treatment and attributed to undiscriminated multidirectional growth of the cells.

Allium cepa roots treated with colchicine alone showed c-tumor formation at the end of the second day but no side roots were ever formed. Addition of withaferin A 0·5% simultaneously with colchicine 0·01% greatly inhibited c-tumor formation in number and in time of appearance (Table 1 and Fig. 7).

DISCUSSION

The findings of the present study clearly indicate that withaferin A acts as a mitotic inhibitor on plant cells even at the low concentration of 0·01%. The mitotic arrest at metaphase and the appearance of c-tumors closely resemble the effect of colchicine [6]. However, the lagging chromosomes at anaphase suggested that even after a prolonged arrest at metaphase, some of the cells could continue to anaphase and telophase. The striking formation of lateral roots in all experiments with withaferin A could be compared to the effects of naphthalene acetic acid as described by Levan and Lotfy [7]. These authors have shown that phytohormones such as naphthalene acetic acid and indole acetic acid initiate the secondary root meristem formation along the pericycle, and induce transversely oriented mitoses. Another point of similarity between these compounds and withaferin A resides in the antagonistic effects to colchicine with respect to c-tumor formation. Although witha-

Table 1. Effect of withaferin A on growth of Allium cepa roots

Time (days)	Length of roots (cm)	No. of c-tumors on each bulb	Appearance of lateral roots
Control			
2 days	2	0	0
6 days	10	0	0
14 days	20	0	0
Colchicine 0·01%			
2 days	1	11	0
6 days	4	30	0
14 days	4	30	Few lateral roots
Withaferin A 0·5%			
2 days	2	0	0
6 days	5	1	0
14 days	5·5	10	Many lateral roots
Colchicine 0·01% and withaferin A 0·5%			
2 days	1·5	2	0
6 days	5	3	0
14 days	5	10	Lateral roots on c-tumors



Fig. 1. *Inhibition of growth of Allium cepa root after* 48 *hr of treatment with* 0·01% *withaferin A* (*right root*) *as compared to control* (*left root*).

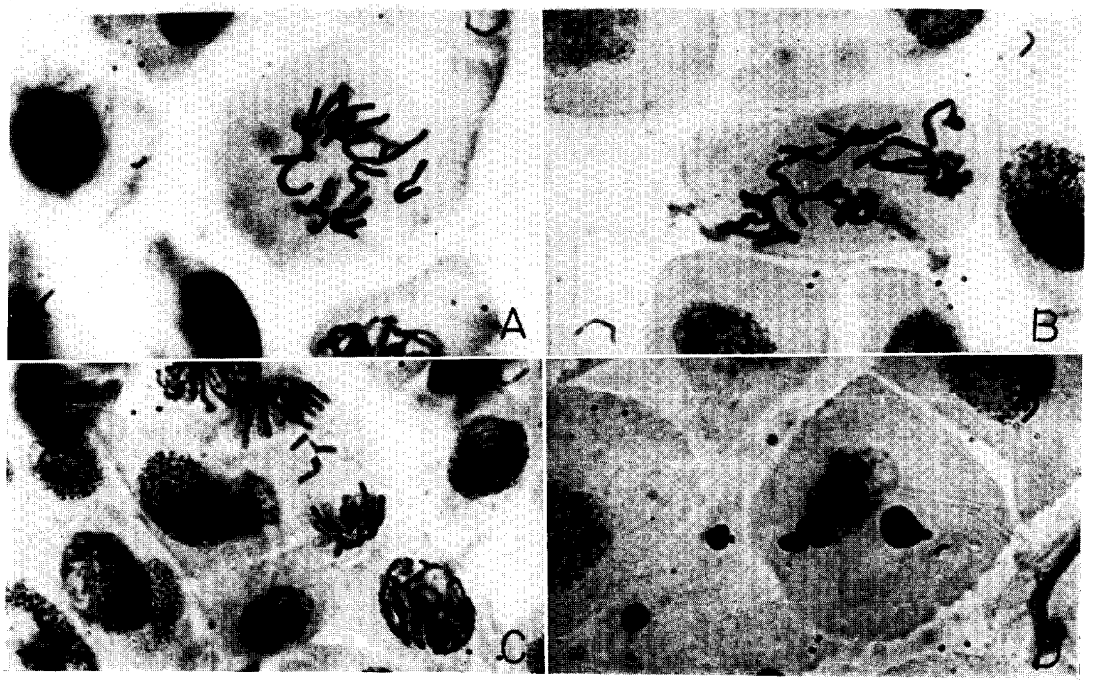


Fig. 3. *Aceto-carmine stained smear preparations of root tip exposed to* 0·01% *withaferin A for* 48 hr × 1000.

A. B. *Withaferin A treated cells arrested at metaphase* 2 *hr after treatment.*

C. *Lagging chromosomes at anaphase* 48 *hr after withaferin A treatment,*

D. *Micronuclei* 48 *hr after treatment.*



Fig. 5. *Bulb of Allium cepa exposed to* 0·5% *withaferin A for* 12 *days. Lateral root formation.*

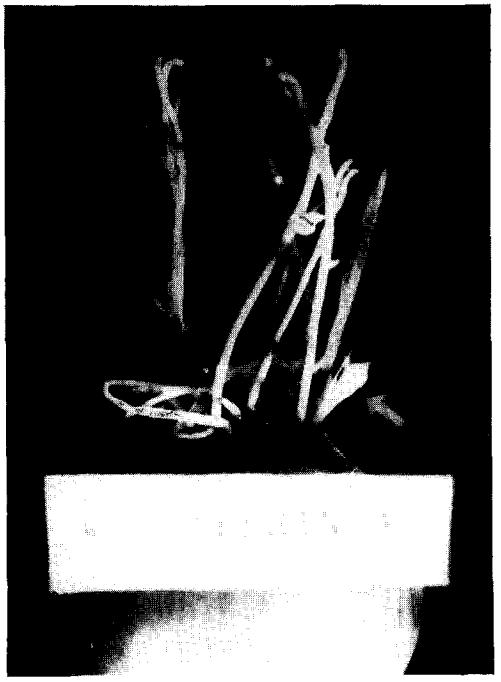


Fig. 6. *Bulb of Allium cepa exposed to* 0·5% *withaferin A for* 15 *days. c-Tumor formation.*

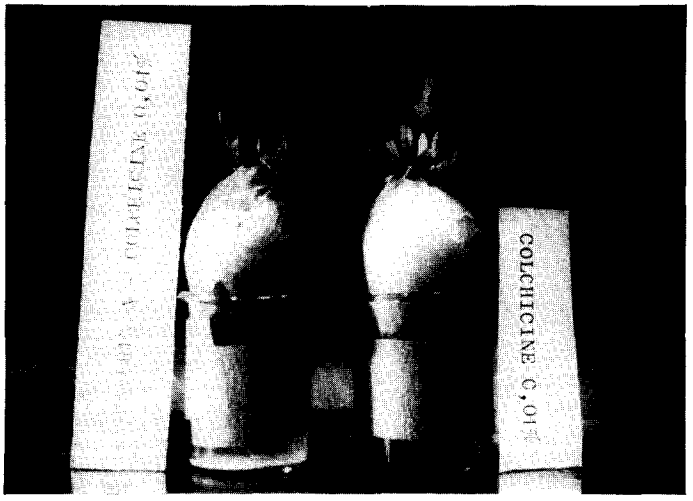


Fig. 7. *Bulbs of Allium cepa exposed to* 0·01% *colchicine and to* 0·01% *colchicine and* 0·4% *withaferin A for* 48 *hr. c-Tumors are already formed on the colchicine treated bulbs while no c-tumors appeared in the withaferin A and colchicine treated bulbs.*

ferin A *per se* causes c-tumor formation, the combined treatment results in a delay of c-tumor formation. These findings may indicate that the c-tumors of withaferin A and colchicine are induced by different mechanisms. The lagging chromosomes at anaphase and formation of micronuclei induced by withaferin A may be due to the arrest in metaphase "*per se*" (colchicine like effect) and/or to a direct deleterious effect on the chromosomes similar to the changes induced by nitrogen mustard, TEM, azaserine, actinomycin C and actinomycin D on meristem of Allium cepa, as described by Truhaut and Deysson [8].

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REFERENCES

1. B. Shohat, S. Gitter, A. Abraham and D. Lavie, Anti-tumor activity of withaferin A. *Cancer Chemother. Rep.* **51,** 271 (1967).
2. S. M. Kupchan, R. W. Doskotch, P. Bollinger, A. T. McPhail, G. A. Sim and J. A. S. Renauld, The isolation and structural elucidation of a novel steroidal tumor inhibitor from Acnistus arborescens. *J. Amer. chem. Soc.* **87,** 5825 (1965).
3. B. Shohat, S. Gitter and D. Lavie, Effect of withaferin A on Ehrlich ascites tumor cells—cytological observations. *Int. J. Cancer* **5,** 244 (1970).
4. B. Shohat, S. Gitter and D. Lavie, *Proceedings of Second International Symposium on Animal and Plant Toxins*, Israel (Feb., 1970).
5. J. Palyi, E. Tyihak and V. Palyi, Cytological effects of compounds isolated from Withania somnifera Dun. *Herba Hungarica* **8,** 73 (1969).
6. A. Levan, The effect of colchicine on the root mitosis in Allium. *Hereditas* **24,** 471 (1938).
7. A. Levan and T. Lotfy, Naphthalene acetic acid in the Allium test. *Hereditas* **35,** 337 (1949).
8. R. Truhaut and G. Deysson, Sur l'intérêt de l'utilisation de la cellule végétale comme réactif d'étude des composés dits radiomimétiques employés en chimiothérapie anticancéreuse. *Bull. Cancer* **44,** 22 (1957).

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Impairment of Drug Metabolism in Tumor-Bearing Animals*

R. ROSSO, M. G. DONELLI, G. FRANCHI and S. GARATTINI
Istituto di Ricerche Farmacologiche "Mario Negri", Via Eritrea 62, 20157 *Milan, Italy*

Abstract—*Rats and mice transplanted with different solid or ascites tumors are more sensitive than controls to the paralyzing effect of zoxazolamine and to the hypnotic action of pentobarbital.*

This increased effectiveness of pentobarbital can be partly explained by the fact that tumor-bearing animals show, in relation to controls, higher serum and brain concentrations of these two drugs. Other experiments indicate that zoxazolamine is less metabolized "in vitro" *when incubated with liver homogenates obtained from tumor-bearing animals relative to controls. Also other substrates of liver microsomal enzymes, such as aniline,* p*-nitro-anisol and amidopyrine, are metabolized to a lesser extent, per unit of liver proteins, in tumor-bearing vs. control animals.*

In the case of pentobarbital, it is demonstrated that the impairment of metabolism is not the only factor responsible for the increased effect of pentobarbital in tumor-bearing animals because the onset of the narcosis is present at lower concentrations of brain pentobarbital as compared to controls.

The surgical removal of the tumor abolishes the impairment of drug metabolism. Preliminary experiments seem to indicate that the serum of tumor-bearing animals contains principle(s) able to inhibit the activity of the microsomal liver enzymes.

INTRODUCTION

DRUG METABOLISM is modified by many physiological factors (species, strain, sex, age, time of the day etc.) [1] and it may be altered also by the presence of a pathological process. It is for instance well known that liver disturbances [2, 3] or an excess of thyroxine in the body [4] reduce the activity of the liver–reticulum endoplasmic system containing the enzymes able to metabolize foreign compounds [5–7]. It was recently observed that tumor-bearing animals respond to several drugs differently from normal animals [8, 9]. Kato [8] and workers from this laboratory [10–12] demonstrated that tumor-bearing animals show higher blood levels of drugs such as zoxazolamine, pentobarbital, amphetamine, as compared to normal animals. Furthermore the liver microsomal enzymes obtained from tumor-bearing animals metabolize several drugs at a lower rate than liver microsomal enzymes prepared from normal rats [10, 13, 14].

This paper intends to summarize and enlarge these observations and to analyze several factors which may be responsible for explaining the reduced drug metabolism resulting from the growth of a tumor.

MATERIAL AND METHODS

Male and female random-bred Sprague–Dawley rats (ALAL, Milan), female inbred WAG/Rij rats (kindly obtained from Dr. D. van Bekkum, TNO, Rijswijk, The Netherlands), weighing 150 ± 5 g, and male and female random-bred Albino Swiss mice (ALAL, Milan), weighing 20 ± 1 g were used. Animals had free access to food (diet ALAL 56, of ALAL, Milan) and water during the experiments. All animals were kept at a room temperature of

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22°C with a relative humidity of 60% in cages of Makrolon (20 × 36 × 13 cm for 10 mice, 47 × 25 × 15 cm for 6 rats). Walker 256 carcinosarcoma, Flexner-Jobling carcinoma, T-8 Guérin uterine epithelioma, and Sarcoma 45, maintained in solid form for more than 50 serial passages, were transplanted subcutaneously in the right flank of Sprague–Dawley rats; rhabdomyosarcoma, a syngeneic slowly-growing tumor [15], kindly supplied by the Radiobiological Institute TNO, Rijswijk, The Netherlands, was transplanted similarly in inbred WAG/Rij rats. Ascites tumors (Walker, Yoshida AH-130 or AH-602), maintained in ascites form for more than 50 serial passages, were implanted intraperitoneally in Sprague–Dawley rats by injecting 1 ml of ascites per rat. Sarcoma 180 and Ehrlich carcinoma, maintained in ascites form for more than 300 serial passages, were implanted subcutaneously or intraperitoneally by injecting 5 or 1 million cells per mouse, respectively.

1. "In vivo" *drug metabolism*

Blood and tissue levels of zoxazolamine and pentobarbital were determined respectively according to the method of Juchau *et al.* [16] and Brodie *et al.* [17].

2. *Pharmacological endpoints*

The sleeping time induced by pentobarbital was determined by following the period of loss of righting reflex. The duration of paralysis induced by zoxazolamine was evaluated by means of a rotarod test (9-cm diameter bar rotating at 2 rev/min).

3. "In vitro" *drug metabolism*

3.1. *Preparation of liver fractions.* The livers were removed immediately after sacrifice and homogenized with 4 volumes of ice-cold 1·15% KCl solution in a Potter-type Teflonhomogenizer. The homogenates were centrifuged at 9000 × **g** for 20 min. The 9000 × **g**-supernatant fractions were either used or centrifuged at 105,000 × **g** for 1 hr and the soluble fractions discarded. Microsomal pellets were resuspended in ice-cold 1·15% KCl solution.

3.2. *Enzyme assay.* Three milliliters of 9000 × **g** supernatant, equivalent to 600 mg of liver, were mixed with 2 ml of a solution containing NADP (1·5 μm), glucose-6-phosphate (50 μm), magnesium chloride (25 μm), nicotinamide (100 μm), potassium phosphate buffer (280 μm) (pH 7·4), substrate. The amounts of the substrates added were as follows: 3 μm of para-nitro-anisol, 5 μm of aniline, 5 μm of amidopyrine, 3 μm of zoxazolamine. The mixture was incubated for 30 min at 37°C.

In some experiments, the incubation mixture contained 2·5 ml of microsomes, equivalent to 1 g of liver, 1·5 μm of NADP, 50 μm of glucose-6-phosphate, 50 μm of nicotinamide, 25 μm of magnesium chloride, 0·5 units of glucose-6-phosphate dehydrogenase, 280 μm potassium buffer phosphate (pH 7·4) and 0·45 ml of 1·15% KCl.

The final incubation volume was always 5 ml with a concentration of microsomal proteins of about 3 mg/ml which is the optimal concentration for testing the enzymatic activity of liver microsomes as described by Fouts [18].

O-demethylation of para-nitro-anisol, para-hydroxylation of aniline, N-demethylation of amidopyrine, were determined by measuring the formation of para-nitro-phenol, para-amino-phenol and 4-amino-antipyrine respectively, according to the method described by Gilbert and Goldberg [19]. Hydroxylation of zoxazolamine was determined by measuring the disappearance of the substrate according to the method described by Juchau *et al.* [16]. Protein determinations were performed according to Lowry [20].

RESULTS

1. *Zoxazolamine: pharmacological effect and* "in vivo" *metabolism*

The duration of zoxazolamine paralysis in tumor-bearing rats was increased, independently from the type of the tumor implanted, to a highly significant degree ($P<0·01$) (Table 1).

The increase ranged from a minimum of 29% in the case of Sarcoma 45 transplanted in male Sprague–Dawley rats to a maximum of 285% in the case of rhabdomyosarcoma, a syngeneic tumor transplanted in the inbred WAG/Rij rats.

The measurements of the levels of zoxazolamine in plasma, liver and brain, indicate, as reported in Table 2, that the tumor-bearing animals always show a higher level of zoxazolamine. The increased brain level of zoxazolamine is consistent with the above-mentioned increased pharmacological activity of zoxazolamine in tumor-bearing animals.

2. *Pentobarbital: pharmacological effect and* "in vivo" *metabolism*

The hypnotic activity of pentobarbital was evaluated in rats bearing different types of solid tumors. Table 3 summarizes these findings. In all cases there was a significant increase in the sleeping time when pentobarbital was given to tumor-bearing animals relative

Table 1. *Zoxazolamine paralysis* (50 *mg/kg i.v.*) *in normal and tumor-bearing rats*

Group	Body wt.* (g ± S.E.)	Tumor wt. (g ± S.E.)	Duration of paralysis (min ± S.E.)	% of increase	*P*
Normal	240 ± 6 (30)	—	161 ± 4	—	
Walker†	188 ± 12 (13)	23 ± 2·7	328 ± 13§	103	<0·01
Flexner-Jobling†	207 ± 9 (9)	23 ± 2·5	300 ± 8	86	<0·01
Sarcoma 45†	210 ± 10 (6)	11 ± 1·3	209 ± 10	29	<0·01
T_8 Guérin†	162 ± 9 (6)	32 ± 4·8	515 ± 32	219	<0·01
Normal	186 ± 3 (9)	—	36 ± 5	—	
Rhabdomyosarcoma‡	152 ± 3 (10)	19 ± 1·2	139 ± 17	285	<0·01

*Body weight was calculated without tumor weight. Number of rats used, between brackets.
†Tumors implanted s.c. 16 days before the experiment in male Sprague–Dawley rats.
‡Tumors implanted s.c. 48 days before the experiment in female WAG/Rij inbred rats.
§4 animals died during the paralysis.

Table 2. "In vivo" *metabolism of zoxazolamine in normal and tumor-bearing rats*

	Controls	Walker	Controls	Rhabdomyosarcoma
Body wt. (g ± S.E.)	236 ± 13	182 ± 15	186 ± 3	162 ± 3
Tumor wt. (g ± S.E.)	—	17·8 ± 3·2	—	18·9 ± 1·0
Zoxazolamine in brain (μg/g ± S.E.)	3·8 ± 0·3	12·5 ± 0·8*	9·6 ± 1·3	25·0 ± 3·6*
Zoxazolamine in liver (μg/g ± S.E.)	7·3 ± 1·0	13·7 ± 1·8*	25·2 ± 3·5	49·2 ± 5·1*
Zoxazolamine in plasma (μg/g ± S.E.)	3·3 ± 0·7	6·5 ± 1·0*	3·5 ± 0·2	6·7 ± 0·1*

The concentration of zoxazolamine in the brain, liver and plasma was determined 6 hr later in the case of Walker tumor-bearing rats (male Sprague–Dawley rats) and 4 hr later in the case of rhabdomyosarcoma-bearing rats (female inbred WAG/Rij rats). Zoxazolamine was given at the dose of 50 mg/kg i.v.
Each point is the average of 7 determinations.
*$P < 0·01$.

Table 3. *Effect of sodium pentobarbital* (25 *mg/kg/i.p.*) *in normal and tumor-bearing rats*

Body-weight change relative to controls (%)	Tumor implanted subcutaneously	Days after tumor implantation	Tumor wt. (g ± S.E.)	Increase of sleeping time relative to controls* (%)	*P*
+3	Walker	4	n.d.	92	<0·01
0	Flexner-Jobling	4	n.d.	141	<0·01
+1	T_8 Guérin	4	n.d.	109	<0·01
−4	Walker	8	0·4 ± 0·1	65	<0·01
−2	Flexner-Jobling	8	0·6 ± 0·1	60	<0·05
−3	T_8 Guérin	8	0·4 ± 0·1	57	<0·05
−18	Walker	12	5·9 ± 1·0	68	<0·01
−28	Flexner-Jobling	12	4·4 ± 1·2	115	<0·01
−15	T_8 Guérin	12	4·0 ± 0·3	132	<0·01
−39	Walker	16	18·3 ± 2·1	107	<0·01
−31	Flexner-Jobling	16	22·5 ± 2·7	250	<0·01
−22	T_8 Guérin	16	22·0 ± 3·3	220	<0·01
−19	Sarcoma 45	16	12·9 ± 1·9	83	<0·01
−19	Walker†	16	20·2 ± 1·4	118	<0·01
−11	Rhabdomyosarcoma‡	48	18·9 ± 0·8	48	<0·01

Body weight was considered without tumor weight.
Tumors were transplanted subcutaneously in male Sprague–Dawley rats (157 ± 4 g); n.d. = not detectable.
*Sleeping times in control animals were the following: 31 ± 4 min (130 male Sprague–Dawley rats); 56 ± 2 min (30 female Sprague–Dawley rats); 60 ± 3 min (15 female WAG/Rij rats).
†Tumor transplanted in female Sprague–Dawley rats (135 ± 8 g).
‡Tumor transplanted in female WAG/Rij inbred rats (110 ± 3 g).

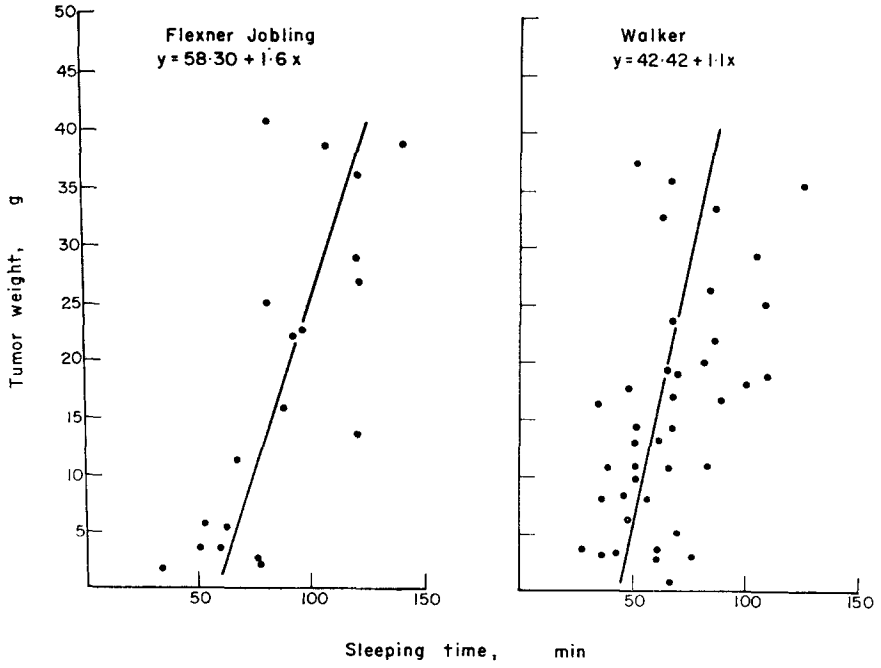


Fig. 1. Relation between tumor weight (ordinates) and sleeping time (abscissae) in individual rats implanted subcutaneously with Flexner-Jobling carcinoma or Walker carcinosarcoma.

to controls. Table 3 indicates also that for all the tumors examined there was a progressive increased duration of action of pentobarbital in relation to the time after implantation of the tumor. It is interesting to note that even when the tumor was not appreciable (4 days after implantation) there was already an increased effect of pentobarbital relative to controls.

In the case of Walker carcinoma, it is also evident that the increase of sleeping time after pentobarbital occurred in a similar manner when the tumor was implanted either in female or male rats.

Figure 1 establishes also that there is a linear correlation between the mass of the tumor and the duration of sleeping time. In addition, different types of rat ascites tumors, namely Walker, Yoshida AH-130 and AH-602, were able to induce a prolonged hypnosis by pentobarbital (see Table 4).

Tables 5 and 6 indicate that the sleeping time by pentobarbital in mice bearing either Sarcoma 180 or Ehrlich carcinoma, in solid or ascitic form, is higher than in their respective controls. In both sets of data it is also apparent that female mice are considerably more affected by the presence of the tumor in regard to the response to pentobarbital. It is remarkable that the sleeping time may be increased by the presence of the tumor up to a factor of ten times.

An explanation for the increased effect of pentobarbital in tumor-bearing animals may be given by considering the levels of plasma and brain pentobarbital. The data summarized in Table 7 show that tumor-bearing animals have always significantly higher levels of pentobarbital in plasma or brain than the control rats at comparable times after injection.

Figure 2 shows also that the initial distribution of pentobarbital after an intravenous administration is not different in normal or Walker tumor-bearing rats. However, while the levels of pentobarbital rapidly declined in plasma or brain of normal rats, they did not change from 20 to 60 min after pentobarbital administration in tumor-bearing rats.

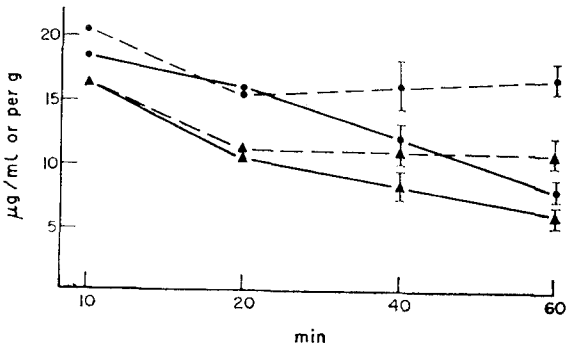


Fig. 2. Rate of disappearance of pentobarbital (25 mg/kg i.v.) in normal (——) and Walker tumor- (– – –) bearing rats. (▲) plasma (●) brain. Each point (±S.E.) is the average of 6 determinations.
On the ordinates, concentration of pentobarbital in plasma or in brain; on the abscissae, minutes after pentobarbital administration.

Table 4. Effect of sodium pentobarbital (25 mg/kg i.v.) in normal and ascites tumor-bearing rats

Body-weight change relative to controls (%)	Tumor implanted intraperitoneally	Days after tumor implantation	Ascites volume (ml ± S.E.)	Increase of sleeping time relative to controls* (%)	*P*
−4	Walker	7	n.d.	0	>0·05
−3	Yoshida AH–602	7	n.d.	10	>0·05
−20	Yoshida AH–130	7	13 ± 1	0	>0·05
−27	Walker	14	47 ± 3	62	<0·05
−24	Yoshida AH–602	14	49 ± 3	55	>0·05
−31	Yoshida AH–130	14	70 ± 2	110	<0·02
−28	Walker	21	62 ± 8	78	<0·05
−27	Yoshida AH–602	21	74 ± 10	102	<0·01
−39	Yoshida AH–130	21	79 ± 11	97	<0·01

Body weight was considered without the weight of the ascites. Tumors were transplanted intraperitoneally in male Sprague–Dawley rats (160 ± 5 g).
*Sleeping times in normal animals was 33 ± 3 min (30 rats); n.d. = not detectable.

Table 5. Effect of sodium pentobarbital (40 mg/kg i.v.) in normal and tumor-bearing mice

Sex	Body-weight change relative to controls (%)	Tumor	Days after tumor implantation	Tumor wt. (g ± S.E.)	Increase of sleeping time relative to controls (%)	*P*
Male	0	Sarcoma 180	7	0·33 ± 0·05	5	>0·05
	+3	Ehrlich	7	0·32 ± 0·02	24	>0·05
Female	0	Sarcoma 180	7	0·79 ± 0·10	23	>0·05
	+6	Ehrlich	7	0·73 ± 0·09	28	>0·05
Male	−3	Sarcoma 180	14	0·92 ± 0·12	43	<0·01
	+2	Ehrlich	14	0·98 ± 0·09	57	<0·01
Female	−5	Sarcoma 180	14	1·64 ± 0·33	115	=0·01
	+7	Ehrlich	14	1·09 ± 0·18	105	<0·05
Male	+5	Sarcoma 180	21	1·69 ± 0·15	36	>0·05
	+9	Ehrlich	21	2·09 ± 0·18	96	<0·01
Female	+12	Sarcoma 180	21	3·81 ± 0·53	342	<0·01
	+10	Ehrlich	21	1·70 ± 0·24	268	<0·01

Body weight was considered without tumor weight.
Tumors were obtained by transplanting subcutaneously 5 million cells in 0·2 ml/mouse in Swiss mice.
Sleeping time in normal animals was the following: 33 ± 4 min (30 male mice), 21 ± 2 min (30 female mice).

Table 6. Effect of sodium pentobarbital (40 mg/kg i.v.) in normal and ascites tumor-bearing mice

Sex	Body-weight change relative to controls (%)	Tumor	Days after tumor implantation	Ascites volume (ml ± S.E.)	Increase of sleeping time relative to controls (%)	*P*
Male	−3	Sarcoma 180	7	1·4 ± 0·2	126	<0·01
	+2	Ehrlich	7	1·7 ± 0·2	190	<0·01
Female	+4	Sarcoma 180	7	1·6 ± 0·2	185	<0·01
	+6	Ehrlich	7	1·1 ± 0·2	300	<0·01
Male	−9	Sarcoma 180	11	6·3 ± 0·6	290	<0·01
	−7	Ehrlich	11	7·7 ± 1·1	290	<0·01
Female	+2	Sarcoma 180	11	6·7 ± 1·0	500	<0·01
	−5	Ehrlich	11	7·6 ± 1·0	504	<0·01
Male	−11	Sarcoma 180	14	17·4 ± 1·1	306	<0·01
	−9	Ehrlich	14	19·0 ± 0·6	290	<0·01
Female	−9	Sarcoma 180	14	12·7 ± 1·5	995	<0·01
	−7	Ehrlich	14	13·6 ± 1·0	660	<0·01

Body weight was considered without the weight of the ascites tumor.
Tumors were obtained by transplanting intraperitoneally 1 million cells in 0·2 ml/mouse in Swiss mice.
Sleeping time in normal animals was the following: 31 ± 4 min (30 male mice), 23 ± 2 min (30 female mice).

Table 7. Levels of pentobarbital in brain and plasma of rats and mice transplanted with various tumors

Tumor implanted subcutaneously	Sex	Days after tumor implantation	Dose (mg/kg)	Route	Time of sacrifice (min)*	Tumor wt. (g±S.E.)	Levels of pentobarbital in plasma (μg/ml±S.E.)		Levels of pentobarbital in brain (μg/ml±S.E.)	
							Control	Tumor	Control	Tumor
Walker	Male	16	25	i.v.	60	17·8±2·0	6·0±0·5	11·1±1·2†	8·0±0·8	16·7±1·2†
	Male	4	25	i.p.	60	n.d.	10·2±0·4	15·3±0·7†	7·1±0·5	13·8±1·0†
	Male	16	25	i.p.	60	18·3±1·9	10·0±0·6	17·0±0·5†	6·9±0·8	17·0±2·1†
	Female	16	25	i.p.	120	20·4±2·0	—	—	9·4±0·5	14·0±1·0†
Flexner-Jobling	Male	16	25	i.p.	60	21·0±1·5	10·0±0·6	13·0±0·6†	6·9±0·8	11·0±0·6†
Rhabdomyosarcoma	Female	48	25	i.p.	120	17·9±2·1	—	—	20·9±0·7	26·8±1·1†
Sarcoma 180	Female	21	40	i.v.	60	2·9±0·3	11·2±1·3	23·1±1·2†	8·0±2·0	21·2±1·3†

* Time after sodium pentobarbital administration.
† $P < 0{\cdot}01$ (10 animals for each determination).
n.d. = not detectable.

3. *Quantitative correlations between sleeping time and brain pentobarbital*

In order to establish whether the changes of pentobarbital activity in tumor-bearing rats were related only to the increased level of brain pentobarbital or also to a change in sensitivity at the central nervous system level, an experiment was carried out by injecting control or Walker tumor-bearing rats with equiactive doses of pentobarbital (respectively 25 and 15 mg/kg i.p.).

Table 8 summarizes the results obtained. It is evident that Walker tumor-bearing rats require a shorter time than control animals for the beginning of the sleep although the level of the serum and brain pentobarbital is lower than in the controls. However, at the end of the narcosis, or 90 min after pentobarbital administration, the level of serum and brain pentobarbital are similar in the two experimental groups.

As previously reported [21] a correlation is present in individual normal animals between the duration of narcosis and the level of brain pentobarbital. However, such correlation does not exist in the Walker group suggesting that brain pentobarbital concentration is not the only factor responsible for the duration of narcosis (see Fig. 3).

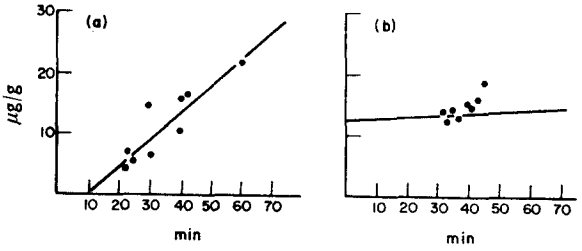


Fig. 3. Relation between level of brain pentobarbarbital (ordinates) and sleeping time (abscissae) in controls (a) *and Walker tumor-bearing* (b) *rats treated respectively with* 25 *and* 15 *mg/kg i.p. of pentobarbital. Rats have been implanted with Walker carcinosarcoma* 16 *days before the experiment. Determinations have been made* 90 *min after pentobarbarbital administration. In the groups* a *and* b *the regression lines and the* r *are respectively* $y = -4{\cdot}33 + 0{\cdot}44x$ and $y = 12{\cdot}27 + 0{\cdot}01x$, 0·857 ($P < 0{\cdot}01$) and 0·024.

4. "In vitro" *metabolism*

A possible explanation for the higher levels of serum and tissue pentobarbital and zoxazolamine observed in tumor-bearing animals may be related also to an impairment of the liver enzymes involved in the metabolism of foreign compounds. The enzymatic microsomal activity of the liver present in normal or tumor-bearing animals was therefore measured by using two liver fractions, namely the 9000 and the 105,000 × **g**. The enzymatic activities measured were hydroxylation of zoxazolamine and aniline, N-demethylation of amidopyrine and O-demethylation of p-nitro-anisol.

The results reported in Table 9 show that in all instances the liver of tumor-bearing animals was less active in metabolizing the various substrates. The effect was statistically significant both considering the disappearance of the substrate or the production of the metabolite per g of liver or per 100 mg of liver protein.

5. *Inhibition and induction*

In order to obtain more information about the reason for the decreased drug metabolism in tumor-bearing animals, it was of interest to investigate if a known inducer of liver microsomal enzymes such as phenobarbital, or a known inhibitor of drug metabolism such as SKF 525A, were able to change, in the same

Table 8. Levels of serum and brain pentobarbital at different times after pentobarbital administration

	Controls	Walker
Beginning of narcosis (min ± S.E.)	7 ± 1	4 ± 1*
Serum pentobarbital (μg/ml ± S.E.)	21·6 ± 0·2	12·1 ± 1·0*
Brain pentobarbital (μg/g ± S.E.)	23·9 ± 1·3	15·9 ± 1·3*
End of narcosis (min ± S.E.)	47 ± 7	53 ± 6
Serum pentobarbital (μg/ml ± S.E.)	14·6 ± 1·2	13·0 ± 1·4
Brain pentobarbital (μg/g ± S.E.)	23·2 ± 1·7	23·1 ± 1·6
Ninety minutes after pentobarbital administration†		
Serum pentobarbital (μg/ml ± S.E.)	11·6 ± 1·4	9·7 ± 1·3
Brain pentobarbital (μg/g ± S.E.)	11·3 ± 1·9	14·5 ± 0·6

Each figure is the average of 8 determinations.

Pentobarbital was given intraperitoneally at the dose of 25 mg/kg to controls and of 15 mg/kg to Walker tumor-bearing rats (tumor implantation was performed 16 day before the experiment).

*$P < 0{\cdot}01$.

†In this experiment controls slept 35 ± 4 min and Walker tumor-bearing rats 41 ± 3 min.

Table 9. Liver microsomal activity "in vitro" *of controls and tumor-bearing animals*

Substrate	Liver fraction	Group	Tumor wt. (g ± S.E.)	mμm substrate metabolized or metabolite produced* /g of liver	mμm substrate metabolized or metabolite produced* /100 mg of protein
Zoxazolamine	9000 × g	Normal	—	803 ± 113	392 ± 41
		Walker	15·5 ± 3·2	160 ± 29†	83 ± 17†
Aniline	9000 × g	Normal	—	245 ± 26	184 ± 19
		Walker	17·3 ± 2·0	89 ± 9†	65 ± 7†
		Normal	—	1110 ± 44	758 ± 30
		Sarcoma 180	2·6 ± 0·3	740 ± 53†	574 ± 41†
Amidopyrine	9000 × g	Normal	—	276 ± 42	207 ± 31
		Walker	20·2 ± 2·8	64 ± 14†	47 ± 11†
		Normal	—	416 ± 26	283 ± 17
		Sarcoma 180	2·6 ± 0·3	149 ± 17†	114 ± 11†
P-NO_2-anisol	9000 × g	Normal	—	173 ± 14	114 ± 9
		Walker	19·8 ± 2·1	82 ± 11†	55 ± 7†
		Flexner-Jobling	23·4 ± 4·7	86 ± 12†	64 ± 9†
		T_8 Guérin	17·0 ± 3·0	78 ± 10†	56 ± 7†
		Sarcoma 45	21·7 ± 2·9	69 ± 10†	56 ± 8†
		Normal	—	743 ± 16	507 ± 11
		Sarcoma 180	2·6 ± 0·3	517 ± 49†	401 ± 38†
	105,000 × g	Normal	—	107 ± 10	621 ± 58
		Walker	19·8 ± 2·1	74 ± 9†	368 ± 47†
		Flexner-Jobling	23·4 ± 4·7	53 ± 7†	308 ± 45†
		Normal	—	418 ± 18	2926 ± 125
		Sarcoma 180	2·6 ± 0·3	265 ± 21†	2190 ± 143†

*Incubation was carried out for 60 min in the case of zoxazolamine and for 30 min for the other substrates.
†$P < 0{\cdot}01$.

In control animals the liver protein content of rats and mice was respectively 151·6 ± 0·4 and 135·0 ± 2·8 mg/g liver for 9000 × g fraction, 16·2 ± 0·3 and 13·2 ± 2 mg/g liver for 105,000 × g fraction. The values of tumor-bearing animals were not significantly different.

way, the metabolism and the pharmacological activity in normal and in tumor-bearing animals. Table 10 indicates that both in normal and Walker carcinoma-bearing rats SKF 525A prolongs the sleeping time induced by pentobarbital and increases the brain level of this barbiturate. However, the increase of brain pentobarbital in tumor-bearing animals was relatively smaller than in control animals. Phenobarbital decreases the sleeping time induced by pentobarbital and it considerably lowers the level of brain pentobarbital in a similar manner both in controls and Walker carcinoma-bearing animals.

Another example of the inducing effect of phenobarbital is given in Table 11, where the metabolism of zoxazolamine in 9000 × **g** liver homogenates is summarized. It is evident that phenobarbital increases the metabolism of zoxazolamine in both normal and tumor-bearing rats. However, the absolute level of zoxazolamine metabolism in tumor-bearing animals was still only 50% of the level observed in control animals after the induction.

6. *Effect of food intake on drug metabolism*

Since it is known that tumor-bearing animals show a considerable decrease of food intake, it seemed necessary to establish that the restriction of the diet was not the major factor responsible for the observed change in drug metabolism. Results reported in Table 12 do not support this possibility. First of all, a 48-hr fasting does not affect in normal animals the sleeping time following pentobarbital injection or the levels of plasma or brain pentobarbital.

In other experiments, groups of normal rats and mice were pair-fed respectively with Walker carcinoma-bearing rats or mice implanted with Sarcoma 180. In both cases the pair-fed animals were less sensitive to the hypnotic effect of pentobarbital than the tumor-bearing animals. Furthermore, tumor-bearing animals always exhibited a higher level of plasma or brain pentobarbital than the respectively pair-fed controls. In additional experiments, not reported here in detail, glucose was injected subcutaneously at the dose of 1 g per kg in tumor-bearing animals, 15 min before injecting pentobarbital. The sleeping time and the levels of pentobarbital were not significantly different relative to untreated Walker carcinosarcoma-bearing rats.

7. *Effect of tumor removal*

Walker tumor-bearing rats were submitted to surgery twelve days after subcutaneous transplantation of the tumor. A group of these animals, before surgery, was injected with pentobarbital for the determination of sleeping time and pentobarbital levels, while another group was submitted to the same treatment 13 days after tumor removal.

Table 13 indicates that the excision of the tumor resulted in a decreased sleeping time and brain pentobarbital concentration. Data not reported here in details indicate that the liver homogenate (9000 × **g**) of animals in which the tumor was removed shows a higher activity in metabolizing *p*-nitro-anisol, aniline and amidopyrine in comparison to the liver of tumor-bearing animals.

8. *Are substances released from tumor responsible for the impairment of drug metabolism?*

In order to answer this question, normal animals were treated twice a day for two days or once a day for four days, with 0·5 ml of serum obtained from controls or Walker tumor-bearing rats. Results presented in Table 14 show that the liver 9000 × **g** fraction of rats treated with the serum obtained from Walker tumor-bearing animals was significantly less active in metabolizing *p*-nitro-anisol or aniline than livers of animals injected with saline or serum obtained from control rats.

Preliminary experiments demonstrated that this "inhibitor" present in serum could not be removed by dialysis.

DISCUSSION

The growth of a transplantable tumor induces a number of effects in the host animals. This paper summarizes several observations indicating that tumor-bearing animals (TBA) respond differently than controls to zoxazolamine and pentobarbital. In fact the paralyzing activity of zoxazolamine and the hypnotic activity of pentobarbital are increased in rats and in mice implanted with a variety of experimental tumors including Walker carcinosarcoma (solid and ascitic forms), Flexner-Jobling carcinoma, Sarcoma 45, uterine epithelioma T_8 Guérin, rhabdomyosarcoma, Yoshida AH-130 and 602, Sarcoma 180 and Ehrlich carcinoma (solid and ascitic forms). This effect is therefore present in different animal species (rats and mice) in both sexes, for solid and ascitic tumors, for allogeneic and syngeneic tumors (rhabdomyosarcoma in WAG/Rij rats) and for rapid and slowly-growing tumors.

However, quantitative differences are present. For instance female mice are more sensitive

Table 10. *Effect of phenobarbital and SKF* 525*A on pentobarbital activity and brain level in control and Walker tumor-bearing rats*

Group	Tumor wt. (g ± S.E.)	Treatment	Body wt. (g ± S.E.)	Liver wt. g/100g b.w. ± S.E.	Sleeping time (min ± S.E.)	Change %	Brain pentobarbital after (μg/g ± S.E.) 60 min	90 min
Control	—	A	262·2 ± 9·7	4·71 ± 0·15	33 ± 1 (75)	—	10·6 ± 0·9	4·9 ± 0·8
	—	B+A	221·0 ± 1·2	4·34 ± 0·20	15 ± 1 (33)	−55	0·1 ± 0·1	—
	—	C+A			280 ± 30 (100)	+748	—	18·7 ± 0·5
Walker	16·9 ± 1·0	A	170·7 ± 9·7	6·62 ± 0·52	67 ± 4 (100)	—	22·4 ± 2·3	17·0 ± 3·0
	13·7 ± 3·1	B+A	151·4 ± 9·4	7·54 ± 0·34	29 ± 6 (60)	−57	0·7 ± 0·1	—
	18·9 ± 3·0	C+A			390 ± 25 (100)	+482	—	20·8 ± 1·3

A sodium pentobarbital, 25 mg/kg i.p.
B sodium phenobarbital, 30 mg/kg i.p., twice a day (5 treatments) till 48 hr before pentobarbital.
C SKF 525A, 100 mg/kg i.p., 1 hr before pentobarbital.
Tumors were transplanted subcutaneously in male Sprague–Dawley rats 16 days before the experiments.
Between brackets, the percentage of sleeping rats (12 animals per group).

Table 11. *Effect of phenobarbital on the* in vitro *metabolism of zoxazolamine in normal and Walker tumor-bearing rats*

Group	Phenobarbital*	Body wt. (g ± .S.E)	Tumor wt. g/100g b.w. ± S.E.	Liver wt. (%) (g ± S.E.)	mμm Zoxazolamine disappeared g liver/hr	mμm Zoxazolamine disappeared 100 mg protein/hr
Normal	−	249·1 ± 13·0	—	4·50 ± 0·17	869 ± 208	435 ± 59
Normal	+	268·1 ± 10·0	—	4·58 ± 0·19	4934 ± 297	2232 ± 297
Walker	−	171·6 ± 17·2	17·4 ± 2·1	6·23 ± 0·31	262 ± 17	136 ± 35
Walker	+	198·0 ± 5·4	19·2 ± 3·0	6·75 ± 0·28	2402 ± 190	1220 ± 59

*Sodium phenobarbital, 30 mg/kg i.p., twice a day (5 treatments) till 48 hr before pentobarbital.
Walker carcinosarcoma was transplanted subcutaneously in male Sprague–Dawley rats 16 days before the experiment.
Zoxazolamine was added to a 9000 × **g** liver supernatant at the concentration of 3 μm. The 9000 × **g** liver protein content was in normal rats 146·5 ± 2·8 mg/g of liver and in Walker tumor-bearing rats 140·5 ± 3·3 mg/g of liver.

Table 12. *Influence of food intake on pentobarbital activity and tissue levels*

Group	Body-weight change relative to controls (= 100)	Treatment	Tumor wt. (g ± S.E.)	Increase of sleeping time relative to controls(%)	Pentobarbital* Plasma (μg/ml ± S.E.)	Brain (μg/g ± S.E.)
1	100	Controls fed *ad libitum*	—	—	10·1 ± 0·4	6·7 ± 0·9
2	94	Controls 48-hr fasted	—	5	10·2 ± 1·4	7·1 ± 0·5
3	68	Walker fed *ad libitum*	13·8 ± 1·9	190†	14·5 ± 0·5†	10·4 ± 0·5†
4	83	Controls pair fed as group 3	—	30	10·8 ± 0·5	7·4 ± 0·6
5	100	Controls fed *ad libitum*	—	—	11·2 ± 1·3	8·0 ± 1·4
6	107	Sarcoma 180 fed *ad libitum*	3·0 ± 0·4	203†	23·1 ± 1·2†	21·2 ± 1·3†
7	81	Controls pair fed as group 6	—	16	14·8 ± 1·7	12·8 ± 1·8

Walker 256 carcinosarcoma and Sarcoma 180 were respectively transplanted subcutaneously in male Sprague–Dawley rats and in female Swiss mice 16 and 21 days before the experiment.
Sodium pentobarbital was given at the dose of 25 mg/kg i.v. in rats (groups 1–4) and of 40 mg/kg i.v. in mice (groups 5–7). Weight of the animals is calculated without tumor.
*Determinations of serum and brain pentobarbital were performed 60 min after pentobarbital administration.
Each figure is the average of 10 determinations.
†$P < 0{\cdot}01$.

Table 13. *Effect of surgical ablation of tumor on* "in vivo" *metabolism of pentobarbital* (25 *mg/kg i.p.*)

Body wt. (g ± S.E.)	Group	Tumor wt. (g ± S.E.)	Days after operation	Increase of sleeping time relative to controls (%)	Brain pentobarbital (μg/g ± S.E.)
219 ± 8	Controls	—	—	—	6·7 ± 0·4
175 ± 4	Walker	6·3 ± 0·9	—	150*	11·6 ± 1·6*
302 ± 12	Controls	—	—	—	5·9 ± 0·3
305 ± 8	Sham-operated controls	—	13	22	5·8 ± 0·4
225 ± 11	Walker amputated	—	13	60†	8·5 ± 0·3†

Tumors were transplanted subcutaneously 12 days before surgery (anaesthesia with ether).
Determination of pentobarbital in the brain was performed 90 min after pentobarbital administration.
*$P < 0·01$ relative to controls.
†$P < 0·05$ relative to Walker group without operation.

Table 14. *Effect of serum administration on rat liver* (9000 × **g**) *metabolism*

Group	Schedule of treatment	mμm metabolite produced p-NO_2-phenol /g of liver	p-NO_2-phenol /100 mg of protein	p-NH_2-phenol /g of liver	p-NH_2-phenol /100 mg of protein
Saline	twice/day × 2 days	402 ± 31	289 ± 23	276 ± 32	199 ± 23
Control serum	twice/day × 2 days	358 ± 32	249 ± 24	288 ± 27	199 ± 18
Walker serum	twice/day × 2 days	233 ± 18*	174 ± 13*	193 ± 17*	142 ± 15*
Saline	once/day × 4 days	307 ± 17	195 ± 12	251 ± 27	161 ± 19
Control serum	once/day × 4 days	305 ± 29	185 ± 18	232 ± 28	141 ± 17
Walker serum	once/day × 4 days	188 ± 17°	115 ± 11°	130 ± 12*	79 ± 7*

*$P < 0·01$ relative to both saline and normal serum.
Ten male Sprague–Dawley rats for each group were treated intravenously with 0·5 ml/rat of saline or serum obtained either from control or from rats bearing a subcutaneous Walker carcinosarcoma transplanted 16 days before.
The animals were sacrificed 20 hr after the last serum administration; incubation with p-nitro-anisol and aniline were carried out for 30 min.
The 9000 × **g** liver protein content was, in control rats, 157·8 ± 3·6 mg/g liver; in serum treated rats the values were not significantly different.

than males to pentobarbital when they are implanted either with Sarcoma 180 or Ehrlich carcinoma. Female mice bearing an ascitic form of Sarcoma 180 may sleep up to 10 times more than controls when they are injected intravenously with pentobarbital. Although an increased effect of pentobarbital is already present a few days after tumor transplantation when the tumor (Walker, Flexner-Jobling or T_8 Guérin) is not yet visible, there is a clear positive correlation between the mass of the tumor and the effect (sleeping time) of pentobarbital. The effect observed does not depend on a change in the absorption of the drugs from the peritoneal cavity because it is present also when zoxazolamine or pentobarbital are given intravenously. Furthermore the fact that TBA show a lower weight and a decreased food intake relative to controls is not a major factor in explaining the increased activity of pentobarbital in TBA. Indeed this effect is already present when there is no appreciable body-weight decrease of TBA, and control animals do not change their response to pentobarbital when they are submitted to a considerable restriction of food intake. Biochemical determinations revealed that the increased effect of zoxazolamine or pentobarbital in TBA may be related to the higher concentrations of these drugs in serum and brain of rats and mice of both sexes, bearing different types of tumors, at different times after pentobarbital administration, either intraperitoneally or intravenously. Although the concentrations of brain pentobarbital in TBA were consistent with the duration of narcosis, an attempt was made to establish if the changes in sleeping time were entirely related to the levels of

brain pentobarbital. Suitable experiments revealed that when the sleeping time was similar in TBA and control animals (this situation was obtained by decreasing by 40% the dose of pentobarbital in Walker carcinosarcoma-bearing rats) the levels of brain pentobarbital, were also comparable at the end of narcosis. However, it was also shown that with equiactive doses of pentobarbital the TBA started to sleep at an earlier time and with lower brain pentobarbital level than controls. This suggests that the TBA may have also an increased sensitivity to pentobarbital. This suggestion is reinforced by the fact that in TBA the correlation, existing between sleeping time and brain pentobarbital in control individual rats, is disrupted. Previous work [21] has shown that this may be taken as an indication that other factors besides brain pentobarbital concentration may be important in determining the final duration of the narcotic activity of pentobarbital.

The fact that the disappearance of serum and brain pentobarbital was slower in TBA than in control rats, although the initial distribution of the drug given intravenously was not different in the two experimental groups, suggested the possibility that an impairment of drug metabolism was responsible for such an effect. It should be underlined that, whereas the hepatic function measured by means of the bromsulphalein clearance test was not significantly altered in tumor-bearing animals (unpublished data from this laboratory), studies carried out "*in vitro*" established that the liver homogenate ($9000 \times \mathbf{g}$) of rats and mice bearing different types of tumors metabolize zoxazolamine, aniline, amidopyrine and *p*-nitro-anisol to a lower extent than the respective controls. The enzymatic reactions which are impaired in TBA involve therefore aromatic and alkyl hydroxylation, N- and O-demethylation. The impairment of drug metabolism was evident also when the microsomal liver fraction ($105{,}000 \times \mathbf{g}$) was used as the enzymatic source and when the enzymatic activity was expressed per unit of weight of liver proteins. Therefore, the impaired metabolism was not related to the known decrease of protein synthesis present in the liver of TBA relative to controls [9] or to the presence of metastases in the hepatic tissue (a series of histologic examinations in fact revealed the absence of any metastatic tumor in the liver). The impairment of drug metabolism present in TBA may be further increased by a typical inhibitor of the liver microsomal enzymes such as SKF 525A and it may be overcome by an inducing agent (stimulator of liver microsomal enzymes) such as phenobarbital. The ratio between control and tumor-bearing rats of liver zoxazolamine metabolism was shifted from more than 3 to less than 2 by the inducing effect of phenobarbital.

The reasons for the impaired drug metabolism in TBA animals are not yet fully understood. Kato [22] has reported that the concentration of P450 and of NADPH cytochrome reductase was decreased in liver microsomes of TBA. In this paper preliminary data indicate that the administration of serum obtained from TBA decreases liver microsomal activity in control rats. Wilson [23] has shown that the impairment of drug metabolism present in animals bearing a transplantable pituitary mammotropic tumor was due to an increased serum level of somatotropin, one of the pituitary hormones produced by the tumor. It remains to be established whether there is also an increased hormonal level in our TBA and whether this may be responsible for the decrease of P450.

Whatever is the essential biochemical defect, it is evident that the damage is reversible as shown by the "inducibility" of liver microsomal enzymes in TBA and by the observation that the drug metabolism returns towards normal values after surgical removal of the tumor.

The findings here reported, i.e. impaired liver microsomal enzymes responsible for a lower metabolism of the drug and therefore for higher serum and tissue concentration, and ultimately for an increased drug activity, does not exclude that additional factors may play a role. For instance the low level of plasma proteins of TBA [24] may change the protein binding of drugs affecting therefore the level of free drug able to penetrate into brain or into other tissues.

The fact that the liver mass, in respect to the body weight, is increased in TBA vs controls [9, unpublished results from this laboratory] tends to compensate "*in vivo*" the reduced activity of microsomal enzymes. The decrease of the adipose tissue previously shown [25] may affect the storage of lipid soluble drugs and in this way change the serum and tissue levels.

The findings here reported have definite implications in the experimental chemotherapy. Several antitumoral drugs, including cyclophosphamide [26], procarbazine [27], 6 methylthiopurine [28], have been reported to be metabolized by liver microsomal enzymes. The fact that a given tumor may impair to a different degree the liver microsomal enzymes of the host could represent one of the factors explaining different sensitivity of various tumors to a given antitumoral agent. This effect may

also explain why there may be a different toxicity to a given drug in TBA versus control animals. Furthermore the importance of the impairment of drug metabolism induced by the presence of a tumor may be magnified in experiments where drug combinations are given, considering that several antitumoral drugs are also strong inhibitors [29] or activators [unpublished results] of liver microsomal enzymes.

Finally these studies could have also important implications at the clinical level, if these results were confirmed in tumor-bearing patients while considering the number of drugs given during an antitumoral therapy [30].

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REFERENCES

1. W. Kalow, Individual variation in drug metabolism as cause of drug toxicity. In *Drugs and Enzymes* (Edited by B. B. Brodie and J. R. Gillette), pp. 245–255. Pergamon Press, Oxford (1965).
2. E. F. McLuen and J. R. Fouts, The effect of obstructive jaundice on drug metabolism in rabbits. *J. Pharmacol. exp. Ther.* **131,** 7 (1961).
3. J. R. Fouts, R. L. Dixon and R. W. Shultice, The metabolism of drugs by regenerating liver. *Biochem. Pharmacol.* **7,** 265 (1961).
4. R. Kato, A. Takanaka, A. Takahashi and K. Onoda, Species differences in the alteration of drug metabolizing activities of liver microsomes by thyroxine treatment. *Jap. J. Pharmacol.* **19,** 5 (1969).
5. B. B. Brodie, J. R. Gillette and B. N. LaDu, Enzymatic metabolism of drugs and other foreign compounds. *Ann. Rev. Biochem.* **27,** 427 (1958).
6. J. R. Gillette, Oxidation by microsomal enzymes. In *Progress in Drug Research,* (Edited by E. Jucker), Vol. 6, p. 13. Birkhauser Verlag, Basel (1963).
7. J. R. Gillette, Biochemistry of drug oxidation and reduction by enzymes in hepatic endoplasmic reticulum. In *Advances in Pharmacology* (Edited by S. Garattini and P. A. Shore), Vol. 4, p. 219. Academic Press, New York (1966).
8. R. Kato, A. Takanaka and T. Oshima, Drug metabolism in tumor bearing rats. II. "*In vivo*" metabolisms and effects of drugs in tumor bearing rats. *Jap. J. Pharmacol.* **18,** 245 (1968).
9. J. T. Wilson, An investigation of the decrease in the metabolism of hexobarbital, aminopyrine and p-nitrobenzoic acid by liver from rats bearing a pituitary mammotropic tumor. *J. Pharmacol. exp. Ther.* **160,** 179, (1968).
10. G. Franchi and R. Rosso, Metabolic fate of zoxazolamine in tumor-bearing rats. *Biochem. Pharmacol.* **18,** 236 (1969).
11. R. Rosso, E. Dolfini and M. G. Donelli, Prolonged effect of pentobarbital in tumor-bearing rats. *Europ. J. Cancer* **4,** 133 (1968).
12. R. Rosso, E. Dolfini and G. Franchi, Metabolism of amphetamine in tumor-bearing rats. *Biochem. Pharmacol.* **17,** 633 (1968).
13. R. Kato, A. Takanaka, A. Takahashi and K. Onoda, Drug metabolism in tumor bearing rats. I. Activities of NADPH-linked electron transport and drug-metabolizing enzyme systems in liver microsomes of tumor-bearing rats. *Jap. J. Pharmacol.* **18,** 224 (1968).
14. J. T. Wilson, The effect of a pituitary mammotropic tumor on hepatic microsomal drug metabolism in the rat. *Biochem. Pharmacol.* **17,** 1449 (1968).
15. H. S. Reinhold, Quantitative evaluation of the radiosensitivity of cells of a transplantable rhabdomyosarcoma in the rat. *Europ. J. Cancer* **2,** 33 (1966).
16. M. R. Juchau, R. L. Cram, G. L. Plaa and J. R. Fouts, The induction of benzopyrene hydroxylase in the isolated perfused liver. *Biochem. Pharmacol.* **14,** 473 (1965).
17. B. B. Brodie, J. J. Burns, L. C. Mark, P. A. Lief, E. Bernstein and E. M. Papper, The fate of pentobarbital in man and dog and a method for its estimation in biological material. *J. Pharmacol. exp. Ther.* **109,** 26 (1953).
18. R. J. Fouts, Some *in vitro* assay conditions that affect detection and quantitation of phenobarbital-induced increases in hepatic microsomal drug-metabolizing enzyme activity. *Toxicol. appl. Pharmacol.* **16,** 48 (1970).

19. D. GILBERT and L. GOLDBERG, Liver response tests. 3. Liver enlargement and stimulation of microsomal processing-enzyme activity. *Food Cosmet. Toxicol.* **3,** 417 (1965).
20. O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193,** 265 (1951).
21. R. KATO, A. TAKANAKA and K. ONODA, Individual differences in the effect of drugs in relation to the tissue concentration of drugs. *Jap. J. Pharmacol.* **19,** 260 (1969).
22. R. KATO, A. TAKANAKA and A. TAKAHASHI, Activities of NADPH-linked electron transport system in tumor-bearing rats. *Gann* **59,** 83 (1968).
23. J. T. WILSON, Identification of somatotropin in a mixture of somatotropin, adrenocorticotropin hormone and prolactin which decrease liver drug metabolism in the rat. *Biochem. Pharmacol.* **18,** 2029 (1969).
24. S. GARATTINI, C. MOR and B. MURELLI, Comportamento delle proteine e delle lipoproteine seriche nel ratto portatore di carcinosarcoma di Walker. *G. ital. Chemioter.* **3,** 68 (1956).
25. A. BIZZI, S. GARATTINI and A. GUAITANI, Mobilization of plasma free-fatty acids during the growth of various experimental tumors. *Europ. J. Cancer* **4,** 117 (1968).
26. N. BROCK and H. J. HOHORST, Ueber die Aktivierung von Cyclophosphamide *in vivo* und *in vitro*. *Arzeneimittel-Forsch.* **13,** 1021 (1963).
27. H. AEBI, M. BAGGIOLINI, M. H. BICKEL and F. S. MESSIHA, Untersuchung über die N-Demethylierung des Cytostaticums Ibenzmethyzin (Natulan). *Helv. physiol. pharmacol. Acta* **24,** 1 (1966).
28. P. MAZEL, J. F. HENDERSON and J. AXELROD, S-Demethylation by microsomal enzymes. *J. Pharmacol. exp. Ther.* **143,** 1 (1964).
29. M. G. DONELLI, G. FRANCHI and R. ROSSO, The effect of cytotoxic agents on drug metabolism. *Europ. J. Cancer* **6,** 125 (1970).
30. D. P. RALL, Pharmacologic aspects of selective chemotherapy of leukemia and Burkitt's tumor. Combination chemotherapy: advertent and inadvertent. *Cancer Res.* **27,** 2650 (1967).

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Letter to the Editor

Selective Chemotherapy of Cancer Metastases with Triton WR 1339*

G. FRANCHI and S. GARATTINI
Istituto di Ricerche Farmacologiche "Mario Negri", Via Eritrea 62, 20157 *Milan, Italy*

TRITON WR 1339 (TWR), a formaldehyde polymer of polyoxyethylen ether of octylphenol, showed a marked inhibitory effect on cancer dissemination and metastases in various experimental conditions without affecting the growth of the "primary" tumour [1]. The mechanism responsible for this effect of TWR is still largely unknown although several alternative explanations have been suggested [2]. In any case, it is remarkable that TWR, in acute or chronic treatment, does not induce the characteristic toxic signs due to an inhibition of proliferation of normal cells, such as leukopenia, decrease of spleen weight and gastrointestinal lesions, common to most of the antitumoral drugs utilized in cancer chemotherapy.

It was therefore of interest to compare TWR and a well-known alkylating agent, cyclophosphamide, as far as their capacity to inhibit metastasis formation.

Female and male C57Bl/10 J/Sel inbred mice were transplanted intramuscularly (i.m.) in the right posterior leg with 0·1 ml of Lewis lung carcinoma [3, 4] (kindly supplied by Dr. K. Karrer, Wien) homogenate 1 : 50 in buffer phosphate, pH 6·9. Mice transplanted with Lewis lung carcinoma, a tumour which is isogenic for the strain of mice utilized and which regularly metastatizes in the lung in all animals, were divided into several groups.

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TWR was given by intraperitoneal injection at the dose of 50, 200 or 500 mg/kg beginning 3 days after tumour implantation, every day till the animals were sacrificed. Cyclophosphamide was also given by intraperitoneal injection at the dose of 32, 65 or 127 mg/kg on the days 3 and 4, 14 and 15, after the tumour implantation. The control group was treated with saline.

All the animals were sacrificed 25 days after tumour implantation and the tumour mass present in the leg was dissected and weighed. The lung of each mouse was fixed in a 40% formaldehyde solution. The metastatic nodules on the surface of the lungs were then counted and measured. The volume of each nodule was calculated according to the formula $\frac{4}{3}\pi a^3$ where a is the semi-axis of each nodule measured on a millimetric scale. The weight of lung metastatic tissue was calculated assuming that the density of the tumour is equal to one.

The results reported in Table 1 show that, as the dose of cyclophosphamide increases, both the "primary" and the metastatic tumour are significantly inhibited. On the contrary, TWR decreases the weight of metastases occurring in the lungs without reducing the weight of the tumour transplanted in the leg. By plotting the weight of the "primary" tumour against the weight of metastases for individual animals a direct correlation could be established between these two parameters for cyclophosphamide, but not for TWR.

It may be suggested, therefore, that the antimetastatic effect of cyclophosphamide may be partially dependent on a reduction of the cell

Table 1. *Effect of cyclophosphamide and Triton WR* 1339 *on "primary" tumour and lung metastases (Lewis lung carcinoma implanted intramuscularly in C57Bl/10 J/Sel mice)*

Treatment	Dose (mg/kg/ip)	No. of mice with tumour	Mice with metastases (%)	Av. "primary" tumour weight (g ± S.E.)	Av. weight of metastases (mg ± S.E.)
Saline	—	96	100	5·52 ± 0·15	26·5 ± 2·7
Cyclophosphamide	32	12	100	5·87 ± 0·15	9·1 ± 1·4*
	65	12	83	4·47 ± 0·20†	5·3 ± 1·1*
	127	12	12	1·08 ± 0·30*	0·1 ± 0·09*
Triton WR 1339	50	26	80	5·74 ± 0·20	1·2 ± 0·3*
	200	13	46	4·81 ± 0·33	0·7 ± 0·2*
	500	12	46	4·86 ± 0·24	1·8 ± 0·7*

Statistical analysis: *t* Student test; $*p < 0{\cdot}01$, $\dagger p < 0{\cdot}05$.
Regression lines between weight of the primary tumour and the weight of lung metastases.
Controls $y = 5{\cdot}15 + 0{\cdot}017x$; $r = 0{\cdot}331$ ($p < 0{\cdot}01$).
Cyclophosphamide $y = 2{\cdot}560 + 0{\cdot}267x$; $r = 0{\cdot}634$ ($p < 0{\cdot}01$).
Triton WR 1339 no correlation.

proliferation in tumour, while the action of TWR must be found on one of the steps ranging from the dissemination to the implantation of cancer cells in the lung. It should be recalled that TWR does not inhibit metastases which are already established in the lung (2).

TWR may be considered, therefore, as the first example of a chemical agent "selectively" acting on cancer metastases. The availability of this type of drug, devoid of general effects on cell proliferation and yet able to affect metastases, may have therapeutical significance.

REFERENCES

1. R. Rosso, M. G. Donelli, G. Franchi and S. Garattini, Effect of Triton WR 1339 on cancer dissemination and metastases. *Europ. J. Cancer* **5,** 77 (1969).
2. G. Franchi, L. Morasca, I. Reyers-Degli-Innocenti and S. Garattini, Triton WR 1339 (TWR), an inhibitor of cancer dissemination and metastases, *Europ. J. Cancer* **7,** 533 (1971).
3. K. Karrer, S. R. Humphreys and A. Goldin, An experimental model for studying factors which influence metastasis of malignant tumors. *Int. J. Cancer* **2,** 213 (1967).
4. H. Wexler, Accurate identification of experimental pulmonary metastases. *J. nat. Cancer Inst.* **36,** 641 (1966).

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Letter to the Editor

The Effect of 1-(Morpholinomethyl)-4-Phtalimido-Piperidindione-2,6 and Drostanolone Propionate on the Plasma Prolactin Concentration of Oestrone-Treated Orchidectomized R-Amsterdam Rats*

A. A. VAN DER GUGTEN

Biological Department, Antoni Van Leeuwenhoek-Huis, The Netherlands Cancer Institute, Amsterdam, The Netherlands

PROLACTIN has been implied as an important factor controlling growth of the 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced mammary tumours [1].

Drostanolone propionate (D.P.) (2α-methyldihydrotestosterone-propionate) and the cyclic imide 1-(morpholinomethyl)-4-phtalimido-piperidindione-2,6 (C.G.603.) have been reported to inhibit growth of the DMBA-induced mammary tumours in Sprague–Dawley rats [2]. It was thought possible that this effect was mediated through a decrease in prolactin secretion by the pituitary gland. The purpose of this study was to test this hypothesis by investigating whether these drugs are capable of reducing experimentally increased plasma prolactin levels.

Twenty orchidectomized rats (R-Amsterdam) were treated by weekly implantation of a 2 mg oestrone–cholesterol pellet (1 : 9 w/w) given between ages 16 and 26 weeks. The increased prolactin secretion was then maintained on a high level by the addition of oestrone in the drinking water (2 mg/l.).

The experiment was started (day 1) on 27 weeks of age. The animals (weighing 251·5 g ±21·0 g) were divided into three groups:

Group A 6 control animals.

Group B 7 animals, each fed with C.G.603., 88 mg in 1 ml of a 1% carboxymethyl cellulose (CMC) solution, 5 × weekly.

Group C 7 animals, each fed with C.G.603., 88 mg in 1 ml of 1% CMC solution, 5 × weekly and treated with 1 mg D.P. in 0·1 ml peanut oil i.m. 3 × weekly.

Prolactin levels were assayed by radioimmunoassay as described by Kwa *et al.* [3].

The results are summarized in Table 1.

They demonstrate that the cyclic imide significantly decreases the plasma prolactin level, and suggest that the combination of C.G.603. and D.P. has a more pronounced inhibitory effect than C.G.603. alone. These results are consistent with the possibility mentioned above that the tumour-inhibiting effect of these drugs is mediated through a decrease in prolactin secretion by the pituitary gland.

Accepted 5 April 1971.

*The chemicals used have been supplied by Chemie, Grünenthal GmbH, Stolberg/Rheinland, Germany.

Table 1. *Plasma prolactin concentration ng/ml* (± *S.D.*)

	Day 1	1 week	2 weeks	3 weeks
Group A	4270 ± 1290	5547 ± 1390	4316 ± 1339	4377 ± 782
Group B	3772 ± 1162	3269 ± 1134 $P<0{\cdot}01$	2668 ± 1140† $P<0{\cdot}05$	2468 ± 1062‡ $P<0{\cdot}01$
Group C	4046 ± 1609	2559 ± 664* $P<0{\cdot}01$	1322 ± 656 $P<0{\cdot}01$	1396 ± 522 $P<0{\cdot}001$

*4 animals (3 killed accidentally during tube feeding).
†6 animals (1 killed accidentally during tube feeding).
‡5 animals (1 killed accidentally during tube feeding).

The experimental groups are compared with the control group (A's). (Student's *t*-test).

N.B. The chemicals used have been submitted by Chemie, Grünenthal GmbH, Stolberg/Rheinland, Germany.

REFERENCES

1. O. H. Pearson, O. Llerena, L. Llerena, A. Molina and Th. Butler, Prolactin-dependent rat mammary cancer: A model for man? *Trans. Ass. Amer. Physicians*, **LXXXII,** 225 (1969).
2. H. Mückter, E. Frankus and E. Moré, Experimental investigations with 1-(morpholinomethyl)-4-phtalimido-piperidindione-2,6 and drostanolone propionate in dimethylbenzanthracene-induced tumors of Sprague–Dawley rats. *Cancer Res.* **30,** 430 (1970).
3. H. G. Kwa, A. A. van der Gugten and F. Verhofstad, Radioimmunoassay of rat prolactin preparations isolated from the "granular fraction" of pituitary tumour transplants and from normal pituitary glands. *Europ. J. Cancer* **5,** 559 (1969).

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Announcement

XIth International Cancer Congress

The Eleventh International Cancer Congress organized under the auspices of the UICC will be centred in Florence (October 20–26, 1974).

Following the opening ceremony at the Palazzo Vecchio, the first two days will be devoted to special sessions organized mainly on a unidisciplinary basis, in Florence and in nearby towns (Montecatini, Lucca, Siena and Perugia).

During the following days ordinary sessions of the Congress will be held in Florence itself.

The programme comprises multidisciplinary symposia in the mornings and workshops or presentations of proffered papers in the afternoons.

A memorial lecture will be delivered at the closing ceremony.

Advanced courses in clinical oncology are scheduled to follow the Congress.

The projection of scientific films and the display of scientific and commercial exhibits are planned.

Forms for registration and hotel accommodation as well as information on travel, tours and special events will be provided in the 2nd circular.

Families and guests accompanying members must be registered as associate members.

English, French and Italian will be the official languages of the Congress with simultaneous interpretation at most sessions.

DATES

Opening Ceremony: October 20 (Sunday afternoon) **Special Sessions:** October 21 and 22 (Monday and Tuesday) **Ordinary Sessions:** October 23 to 26 (Wednesday to Saturday) **Closing Ceremony:** October 26 (Saturday) **Advanced Courses:** October 28 and 29 (Monday and Tuesday)

Circular **2** will be issued in October 1972.

For information write to:

XI INTERNATIONAL CANCER CONGRESS
Istituto Nazionale per lo Studio e la Cura dei Tumori
20133 MILANO - Via G. Venezian 1 - Tel. 23.90

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Pietro Bucalossi

Secretary General
Umberto Veronesi

Europ. J. Cancer Vol. 7, p. i. Pergamon Press 1971. Printed in Great Britain.

The European Journal of Cancer is now ending the first six years of its existence. During this crucial initial period, the Editor was assisted in his task by a very able and devoted group of advisors represented by the members of the Editorial Advisory Board, who contributed their time and competence, and are responsible in great part for the successful development and establishment of the Journal. From 328 pages in the first volume, it has now grown to approximately 600 pages in volume 6. For the selection of manuscripts, the Editor has relied heavily on the judgement of the founder members, the Editorial Advisory Board and a vast group of distinguished referees, whose names appear elsewhere in the Journal. The importance of the contribution of these disinterested and selfless representatives of the scientific community is admired and gratefully acknowledged.

The seventh year of the Journal begins under favourable auspices. The number of manuscripts sent to the Editor is increasing, but more important, the quality of the manuscripts is also steadily improving. The Editorial Board has been modified, as it should be from time to time, in order to allow other opinions and attitudes to influence the contents of the Journal. The Editor wishes to express his thanks to all who helped him, and he hopes that they will continue to assist him in the future, as they did in the past. Succeeding to the first Board, the new Editorial Board will also provide guidance and leadership. We look forward to the future with confidence.

H. J. TAGNON

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Papers to be Published

K. MUSSHOFF, H. SCHMIDT-VOLLMER, H. RENEMANN and D. MERTON.
The reticulum cell sarcoma as an oncologic model for the formation and spreading, for stage classification and therapy indication of malignant lymphoma. (Accepted 26 March 1971.)

S. SAEZ.
Adrenal function in cancer: relation to the evolution. (Accepted 3 February 1971.)

D. G. R. BLAIR, C. P. ENG and J. F. MORGAN.
Nucleic acids and acid-soluble derivatives released by isoteric solutions of cryoprotective agents from Ehrlich-Lettré ascites carcinoma and mouse spleen cells. (Accepted 22 February 1971.)

W. S. BULLOUGH and J. DEOL.
Chalone-induced mitotic inhibition in the Hewitt keratinising epidermal carcinoma of the mouse. (Accepted 27 January 1971.)

K. HOSHIMO and C. DER LIN.
Induction of hyperplasia in mouse salivary gland isografts by the hyperplasia-inducing factor and isoproterenol or hormones. (Accepted 3 March 1971.)

G. PEDIO and J. R. RÜTTNER.
Cell-free transmission of a plasmocytoma to various strains of adult mice. (Accepted 3 March 1971.)

J. SZEPSENWOL.
Intracranial tumors in mice of two different strains maintained on fat-enriched diets. (Accepted 21 May 1971.)

B. SHOHAT and H. JOSHUA.
The effect of withaferin A on plant cells. (Accepted 17 May 1971.)

L. RUMI, C. PASQUALINI and S. K. RABASA.
Growth of Sarcoma 180 in splenectomized mice bearing diffusion chambers containing spleen or tumor cells. (Accepted 1 June 1971.)

W. J. BANKS, M. BHATNAGAR and J. F. MORGAN.
Comparative ultra-structural studies of tumorigenic and non-tumorigenic cell cultures derived from the 6C3HED mouse ascites tumor. (Accepted 5 April 1971.)

D. OTH, M. DONNER and C. BURG.
Measurement of the antitumoral immune reactions against a strain specific chemically-induced sarcoma in syngeneic and F_1 hybrid mice. (Accepted 17 May 1971.)

B. VAN DER WERF-MESSING.
Carcinoma of the bladder treated by preoperative irradiation followed by cystectomy. (Accepted 10 May 1971.)

A. A. VAN DER GUGTEN. (Letter to the Editor.)
The effect of 1-(morpholinomethyl)-4-phtalimide-piperidindione-2,6 and drostanolone propionate on the plasma prolactin concentration of oestrone-treated orchidectomized R-Amsterdam rats. (Accepted 5 April 1971.)

CO-OPERATIVE GROUP FOR CLINICAL TRIALS IN LUNG CANCER (E.O.R.T.C.) (Letter to the Editor.) Preliminary clinical screening with 6-aminochrysene in lung cancer. (Accepted 4 March 1971.)

J. L. AMIEL and M. BÉRARDET.
Effects of active specific and non-specific immunotherapy on E ♂ G2 leukemia according to the route of administration and combination of immunological stimulants. (Accepted 12 March 1971.)

B. J. HORTON and J. R. SABINE.
Metabolic controls in precancerous liver: defective control of cholesterol synthesis in rats fed N-2 fluoroenylacetamide. (Accepted 27 April 1971.)

H. DIPPLE and T. A. SLADE.
Structure and activity in chemical carcinogenesis. Studies of variously substituted 7-bromomethylbenz(a)anthracenes. (Accepted 10 May 1971.)

R. ROSSO, M. G. DONELLI, G. FRANCHI and S. GARATTINI.
Impairment of drug metabolism in tumor-bearing animals. (Accepted 21 April 1971.)

G. FRANCHI, L. MORASCA, I. REYERS-DEGLI-INNOCENTI and S. GARATTINI.
Triton WR 1339 (TWR), an inhibitor of cancer dissemination and metastases. (Accepted 17 May 1971.)

B. BOERYD, P. M. LUNDIN and K. NORRBY.
Tumour growth after intravenous, intraperitoneal and subcutaneous injection of syngeneic monodispersed tumour-cell suspension. (Accepted 17 May 1971.)

ROGER BASSLEER and CLAUDE DESAIVE.
Contribution to the study of the Ehrlich ascites tumor cells. A cytological and cytochemical analysis of the effects of sarcolysine. (Accepted 18 March 1971.)

JEAN KLASTERSKY.
Sensitivity to antibiotics of Gram-negative pathogens isolated from hospitalized patients having disseminated malignant disease. (Accepted 19 January 1971.)

WILLY F. PIESSENS, RODOLPHE HEIMANN, NICOLE LEGROS and JEAN-CLAUDE HEUSON.
Effects of Bacillus Calmette-Guérin (BCG) infection on residual disease of the rat mammary tumor after ovariectomy. (Accepted 3 March 1971.)

L. N. OWEN.
Serial transplantation of canine lymphocytic leukaemia. (Accepted 9 June 1971.)

J. J. ROBERTS, T. P. BRENT and A. R. CRATHORN.
Evidence for the inactivation and repair of the mammalian DNA template after alkylation by mustard gas and half mustard gas. (Accepted 21 June 1971.)

NAOMI K. COHN and KELLY H. CLIFTON.
Aspects of the biology and radiation response of cloned C3H mouse mammary carcinoma cells *in vitro* and *in vivo*. (Accepted 21 June 1971.)

P. M. INGLETON.
Acute effects of hepatocarcinogens on mouse-liver microsomal antigens. (Accepted 30 June 1971.)

G. FRANCHI and B. GARATTINI (Letter to the Editor).
Selective chemotherapy of cancer metastases with triton WR 1339. (Accepted 30 June 1971.)

B. BROWNSEY, E. H. D. CAMERON, K. GRIFFITHS, E. N. GLEAVE, A. P. M. FOREST and H. CAMPBELL.
Plasma dehydroepiandrosterone sulphate levels in patients with benign and malignant breast disease. (Accepted 5 July 1971.)

SABINE VON KLEIST, EKKEHARD COLLATZ and PIERRE BURTIN.
Studies of circulating antibodies in colon-cancer patients sera and their corresponding antibodies. (Accepted 30 June 1971.)

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L. TERENIUS.

Parallelism between oestrogen binding capacity and hormone responsiveness of mammary tumours in GR/A mice.

H. G. KWA, A. A. VAN DER GUGTEN, M. SALA and F. VERHOFSTAD.

Prolactin levels in plasma of mice with primary oestrone-induced pituitary tumours, hypophyseal grafts under the kidney capsule and of rats with hypophisycal grafts under the kidney capsule, or treated with chloorpromazine, with or without oestrone supplementation.

H. G. KWA, A. A. VERSTRAETEN and M. G. M. SCHEIJDE-BAKKER.

Isolation of prolactin from the "granular fraction" of pituitary tumour transplants. Improved method for the isolation of mouse and rat prolactin for radioimmunossay.

E. LEGRAND and J. F. DUPLAN.

Chromosome abnormalities in AKR/T1ALD leukaemias.

L. WIEST.

The effect of diethylnitrosamine on the distribution of cell classes in the parenchyma of the liver of newborn rats.

A. PERIN, A. SESSA, G. SCALABRINO, A. ARNABOLDI and E. CIARANFI.

Studies on the anti-tumour activity of aliphatic aldehydes—V. Preferential inhibition of protein synthesis in normal or neoplastic tissues in relation to molecular structure.

Europ. J. Cancer Vol. 7, pp. i–iv. Pergamon Press 1971. Printed in Great Britain

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